Article

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LncRNA MIAT Modulates LPS-Induced Acute Kidney Injury via BECN1-Dependent Autophagy by Interacting with PTBP1

Min Xu[1], Yanyan Zhou[1,*]

[1]Department of Critical Care Medicine, The Second Xiangya Hospital, Central South University, 410000 Changsha, Hunan, China
*Correspondence: iamzhouyanyan@csu.edu.cn; zxlacademic@126.com (Yanyan Zhou)
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Background: Autophagy plays critical adaptive and nonadaptive roles in the pathogenesis of Sepsis-associated acute kidney injury (Sepsis-AKI). However, it remains unknown whether myocardial infarction associated transcript (MIAT) is involved in the process of autophagy in Sepsis-AKI. This study aimed to explore the exact association between MIAT1 and Beclin 1 (BECN1)-mediated autophagy in Sepsis-AKI *in vitro*.
Methods: HK-2 (human renal tubular epithelial cell line) cells were stimulated by lipopolysaccharide (LPS) to construct a septic kidney injury cell model *in vitro*. The relative expression changes of genes or proteins in clinical samples and cells were examined by quantitative real-time polymerase chain reaction (qRT-PCR) or Western blot. Cell survival was detected by cell counting kit-8 (CCK-8) and flow cytometry analysis. The production of inflammatory mediators was determined using Enzyme-linked immunosorbent assay (ELISA) and qRT-PCR assays. The interlinked relationship between polypyrimidine tract-binding protein 1 (PTBP1) and MIAT or BECN1 was validated by RNA immunoprecipitation (RIP) and RNA pull-down detections.
Results: The expression of MIAT was up-regulated in Sepsis-AKI patients and LPS-stimulated HK-2 cells. Down-regulation of MIAT strikingly lightened LPS-induced cell apoptosis and inflammation, but enhanced cell viability. Evidenced by mechanistic experiments, MIAT silencing was confirmed to activate BECN1-mediated cell autophagy by interacting with PTBP1. Furthermore, the elimination of BECN1 remarkably reversed the antiapoptotic and anti-inflammatory roles mediated by MIAT silencing.
Conclusions: In summary, the experimental data reinforced that MIAT downregulation attenuated LPS-stimulated renal cell inflammatory injury by promoting BECN1-mediated autophagy activation through binding to PTBP1, providing some new insights into the function and mechanism of MIAT in Sepsis-associated acute kidney injury (Sepsis-AKI).

Keywords: sepsis; acute kidney injury; PTBP1; MIAT; BECN1; autophagy

Introduction

Sepsis is caused by the uncontrolled response of the host to infection, which is one of the main causes of death in patients with acute and critical illness [1]. During the development and occurrence of sepsis, the kidney is one of the earliest damaged organs. Statistically, approximately two thirds of patients with sepsis can develop acute kidney injury (AKI), clinically known as Sepsis-associated acute kidney injury (Sepsis-AKI), with a corresponding mortality rate to 70% [2,3]. Although some studies have been conducted, the immune inflammatory response, microcirculation disorders, and abnormal energy metabolism are all related to the pathogenesis of Sepsis-AKI [4]. However, the exact mechanism by which Sepsis-AKI is carried out remains elusive. Currently, there is no effective strategy for the early diagnosis and treatment of Sepsis-AKI. Therefore, the search for new biomarkers with higher sensitivity and specificity is a hot topic of clinical attention.

Long noncoding RNA (lncRNA), more than 200 nt in length, is one class of RNA transcripts widely expressed in organisms without protein-coding function. A large amount of literature has revealed the diverse regulatory mechanisms of lncRNA in messenger RNA (mRNA) degradation and translation, such as gene imprinting, splicing regulation, and chromatin remodeling [5–7]. Existing references have highlighted the critical role of lncRNA in Sepsis-AKI. Wang *et al.* [8] discovered that the small nucleolar RNA host gene 5 (SNHG5) was abnormally overexpressed in serum samples from patients with sepsis, and silencing of SNHG5 greatly promoted cell survival and decreased inflammatory reactions triggered by lipopolysaccharide (LPS) by regulating miR-374a-3p/TLR4/nuclear factor kappa-B (NF-κB) pathway. Similarly, the lncRNA CRNED1 increased in mouse kidney tissues after LPS stimulation and was closely correlated with severe pathological changes, including edema, rupture, and necrosis [9]. The myocardial infarction associated transcript (MIAT) is a long spliced noncoding RNA transcript located on chromosome

(chr) 22q12.1. A previous investigation showed that the alternation of the MIAT-related gene locus has been reported to be associated with a susceptibility to myocardial infarction [10]. MIAT and its targets such as miR-122, -150, -155, -182, -197, -375, -608 play an important role in sepsis [11]. Furthermore, MIAT has also identified as high expression in septic rat model and cell model, and was involved in the development of Sepsis-AKI by regulating apoptotic-related cysteine-aspartic proteases (Caspase)-8 [12]. However, the understanding of the exact mechanism and function of MIAT in Sepsis-AKI is still limited.

Autophagy is a highly conserved mechanism of cell degradation and circulation, which is responsible for resisting changes in the various conditions of the cellular stress environment and maintaining cell homeostasis, and plays an important role in regulating cell survival [13,14]. Numerous studies have implicated that autophagy plays critical adaptive and nonadaptive roles in the pathogenesis of many kinds of diseases through affecting a variety of cellular processes, including inflammation, immunity, and cell death [15,16]. In the septic mouse model, autophagy activation has a protective role against multiple organ injuries, such as lung, liver, heart, and kidney [17,18]. For example, Zhang *et al*. [19] revealed that SIRT6-mediated autophagy activation greatly alleviated Sepsis-AKI by reducing cell apoptosis and inflammatory cytokine production. However, it remains unknown whether MIAT is involved in the process of autophagy in Sepsis-AKI.

Polypyrimidine tract-binding protein 1 (PTBP1), an RNA-binding protein, is closely related to the development of inflammatory diseases including sepsis by regulating posttranscriptional events [20,21]. Furthermore, cumulative evidence showed that PTBP1 is involved in the regulation of autophagy in many disorders [20,22]. However, it is unclear whether PTBP1 plays a role in Sepsis-AKI.

In this study, the expression of MIAT in blood samples of patients with sepsis were examined. Subsequently, the LPS-stimulated HK-2 (human renal tubular epithelial cell line) septic cell model was conducted to further investigate the potential correlation between MIAT and autophagy [23–25] and the potential role of PTBP1 in Sepsis-AKI. The data from this study could offer new insights into the pathogenesis of Sepsis-AKI.

Methods

Collection of Clinical Samples

This is a non-interventional study. The patients' information and blood samples collected were approved by the Ethics Committee of the Second Xiangya Hospital, Central South University (NO.2017078). 30 blood samples from Sepsis-AKI patients and 30 blood samples from healthy control were obtained. Mononuclear cells (MNCs) were collected by density centrifugation by using Ficoll-Paque (TBD Sciences, Shanghai, China). Sepsis-AKI was diagnosed based on urinary tract infection (UTI) in combination with two or more syndromes of systemic inflammatory response syndrome [4]. Their privacy was protected without adding additional risks and financial burdens. Each patient has signed a written informed consent prior to this study. The collection of clinical samples and data was conducted in accordance with the Declaration of Helsinki. Each serum samples were obtained by centrifuging at 5000 g for 5 min for subsequent detections.

Cell Culture and Treatment

The HK-2 (human renal tubular epithelial cell line) cells used in these experiments were acquired from the Chinese Academy of Sciences (#SCSP-659, Cell Bank, Shanghai, China). Mycoplasma test result was negative. Short tandem repeat (STR) identification results showed that Amelogenin: X, Y; CSF1PO: 13; D13S317: 9; D16S539: 11,12; D5S818: 12; D7S820: 10,11; THO1: 9; TPOX: 8,9; VWA: 17,18. The STR results showed no multiple alleles and no significant cell cross contamination and it completely matched with HK-2 cells in ATCC database. HK-2 cells were cultured in Dulbecco's modified Eagle medium (#11995040, DEME; Thermo Fisher Scientific, Waltham, MA, USA) containing 10% fetal bovine serum (FBS; #12106C, Sigma-Aldrich, Waltham, MO, USA), 1% penicillin/streptomycin (#C0222, Biyuntian, Shanghai, China) under 37 °C and 5% CO_2 conditions (Thermo Fisher Scientific, Waltham, MA, USA). For cell stimulation, HK-2 cells were exposed to an LPS solution (#L5293, Sigma-Aldrich, St. Louis, MO, USA) with designed concentrations (0, 2.5, 5, 10 µg/mL) for 24 h.

Cell Transfection

Small interfering RNAs (siRNAs) targeting MIAT, PTBP1, Beclin1 (BECN1) (si-MIAT, si-PTBP1, si-BECN1) and mock control (si-NC) were generated from Genepharma (Shanghai, China). si-NC: 5′-CAGCAGGCACGACTGTGGACACGAA-3′, si-MIAT: 5′-CCAGGCUCCUUUAAACCAATT-3′, si-PTBP1: 5′-GCAGUUCUUAGCUCAUAUATT-3′ and si-BECN1: 5′-UGUAUCUUCGAUU CAAAGCGA-3′. For cell transfection, HK-2 cells at the density of 1×10^5 cells/well were inoculated into a 96 well plate. Lipofectamine 3000 (L3000150, Invitrogen, Carlsbad, CA, USA) was applied to transfect the aforementioned siRNAs into HK-2 cells. At post-transfection for 48 h, the efficiency of transfection was detected by quantitative real-time polymerase chain reaction (qRT-PCR).

Cell Viability Assay

Cell counting Kit-8 (CCK-8; KGA317, KeyGEN, Nanjing, China) was applied to analyze cell viability changes after indicated treatment for 24 h. In summary, HK-2 cells (5×10^3 cells) in each group were collected and seeded in a 96-well plate and cultured for 24 h. Sub-

sequently, CCK-8 regent (10 µL per well) was added and incubated for another 4 h. The optical density (OD) values at 450 nm were recorded by a microplate reader (Bio-Rad, Hercules, CA, USA). The cell viability (%) = OD (LPS)–OD (Blank)/OD (Control)–OD (Blank) × 100%. OD (LPS) means the OD 450 value of different concentration of LPS group; OD (Control) means the OD 450 value of control group with cells; OD (Blank) means the OD 450 value of blank group without cells.

Apoptosis Assay

The Annexin V-FITC/PI staining kit (V13242, Thermo Fisher Scientific, Waltham, MA, USA) was acquired to examine the cell apoptotic rate of HK-2 cells following different treatment. After stimulation, HK-2 cells were digested by trypsin and washed twice with PBS solution. Then, HK-2 cells were incubated in mixed reactions consisting of Annexin V-FITC (5 µL) and propidium iodide (PI, 10 µL) and for 10 min away from illumination. Apoptotic HK-2 cells were analyzed by flow cytometry (BD, San Jose, CA, USA).

Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

MNC samples including 30 Sepsis-AKI patients and 30 healthy control and HK-2 cells were obtained to extract total RNA samples by TRIzol reagent (#15596026CN, Invitrogen, Carlsbad, CA, USA). Subsequently, the cDNA template was generated by employing the Transcriptor First Strand cDNA Synthesis Kit (#04897030001, Wolcavi, Beijing, China). The PrimeScript RT Master Mix kit (RR036Q, TaKaRa, Shiga, Japan) was then carried out for qRT-PCR analysis on the ABI 7500 system (version 7.2, Thermo Fisher Scientific, Waltham, MA, USA). Relative RNA levels were quantified using $2^{-\Delta\Delta Ct}$ method [26]. The internal control was glyceraldehyde 3-phosphate dehydrogenase (GAPDH). The primer information was shown as follows: MIAT (Forward) 5′-TCCCATTCCCGGAAGCTAGA-3 and (Reverse) 5′-GAGGCATGAAATCACCCCCA-3′; PTBP1 (Forward) 5′-GGACGGCATTGTCCCAGAT-3′ and (Reverse) 5′-TTGGTGACCTTCCCAAAGGG-3′; BECN1 (Forward) 5′-GAGAACCTCAGCCGAAGACT-3 and (Reverse) 5′-CCTCTAGTGCCAGCTCCTTT-3′; tumor necrosis factor-α (TNF-α) (Forward) 5′-CCCAGGGACCTCTCTAA-3 and (Reverse), 5′-TGGGTACAGGCCCTCTGAT-3′; interleukin (IL)-1β (Forward) 5′-CGATGCACCTGTACGATAC-3′ and (Reverse) 5′-TCTTCAACACGCAGGACAG-3′; IL-6 (Forward) 5′-CCTTCCAAAGATGGCTGAAA-3 and (Reverse) 5′-CAGGGGTGGTTATTGCATCT-3′; light chain 3 (LC3): (Forward) 5′-GGTCCAGTTGTGCCTTTATTGA-3′, (Reverse) 5′-GTGTGTGGGTTGTGTACGTCG-3′; p62 (Forward) 5′-CTAGGCATCGAGGTTGACATT-3′, (Reverse) 5′-CTTGGCTGAGTACCACTCTTATC-3′; GAPDH: (Forward) 5′-AGGTCGGAGTCAACGGATTT-3′, (Reverse) 5′-TGACGGTGCCATGGAATTTG-3′; U6: (Forward) 5′-CTCGCTTCGGCAGCACA-3′, (Reverse) 5′-AACGCTTCACGAATTTGCGT-3′.

mRNA Stability Detection

To examine the stability of BECN1 mRNA, si-MIAT or co-transfected with si-MIAT and si-PTBP1 were transfected into HK-2 cells. Another 48 h cultured after transfection, cells were digested by trypsin and added actinomycin D (SBR00013, 5 µg/mL, Sigma-Aldrich, St. Louis, MO, USA) for 0, 3, 6 h. The total RNA was then obtained by TRIzol and examined by qRT-PCR.

Subcellular Fractionation

The isolation of RNAs from the cytoplasm and nucleus was performed by using NE-PER reagent (#78835, Thermo Fisher Scientific, Waltham, MA, USA). The purified RNAs were then allowed for further analysis using the qRT-PCR assay and normalized to U6 and GAPDH, respectively.

Western Blot Assay

In the present experiments, antibodies including Bcl-2 (#ab32124, 1:000, Abcam, Waltham, MA, USA), Bax (#ab32503, 1:5000, Abcam, Waltham, MA, USA), microtubule-associated protein 1 light chain 3B (LC3B) (#ab192890, 1:2000, Abcam, Waltham, MA, USA), p62 (#ab109012, 1:10000, Abcam, Waltham, MA, USA), BECN1 (#ab210498, 1:1000, Abcam, Waltham, MA, USA), PTBP1 (#ab133734, 1:10000, Abcam, Waltham, MA, USA), pro-caspase 3 (#ab32150, 1:000, Abcam, Waltham, MA, USA), cleaved caspase 3 (#ab2302, 1:200, Abcam, Waltham, MA, USA) and GAPDH (#ab181602, 1:10000, Abcam, Waltham, MA, USA) were used. In summary, RIPA buffer (Cell Signal Technology, Boston, MA, USA) was applied to lyse the HK-2 cells with different treatment. After that, total proteins were exacted and the BCA method was applied for measuring concentration (Beyotime Biotechnology, Shanghai, China). To separate proteins of different molecular weights, 20 µg proteins were loaded onto 10% gel for SDS-PAGE and then moved onto PVDF membranes (Millipore, Billerica, MA, USA). After the membranes were blocked with milk, washed, incubated with primary antibodies, washed, incubated with secondary antibody (horseradish peroxidase-conjugated affinity purefied goat anti rabbit immunoglobulin G (goat anti-rabbit IgG H&L), #ab6721, 1:5000, Abcam), and washed, enhanced chemiluminescence (ECL) solution was added to the membrane to visualize the bands. The bands' intensity was identified by ImageJ software.

ELISA Measurement

The supernatant of HK-2 cells was collected after different treatments for detection by Enzyme-linked immunosorbent assay (ELISA). ELISA kits of cytokines in-

cluding TNF-α (#ab181421), IL-6 (#ab178013), IL-1β (#ab214025) were acquired from Abcam (Waltham, MA, USA). The contents of T cytokines were examined by referring to the corresponding manuscript protocol.

RNA Immunoprecipitation (RIP) Assay

The interlinked relation about PTBP1 and MIAT or BECN1 was validated by the RIP assay. The experimental procedure was conducted following the manuscript protocol of the EZ-Magna RIP kit (#17-295, Millipore, Billerica, MA, USA). In summary, after the indicated treatment, HK-2 cells were collected and lysed in RIP buffer. The cell extracts were incubated with pretreated magnetic beads conjugated with anti-IgG (#ab172730, 1:1200, Abcam, Waltham, MA, USA) or anti-PTBP1(#ab93807, 1:200, Abcam, Waltham, MA, USA) for 24 h at 4 °C. Subsequently, the immunoprecipitated RNAs were isolated and incubated with Proteinase K for 30 min at 55 °C. The RNA was then obtained and detected by qRT-PCR.

RNA Pull Down

The Pierce Magnetic RNA-Protein Pull-Down Kit (#20164, Thermo Scientific, Waltham, MA, USA) was utilized to verify the direct interaction between PTBP1 and MIAT or BECN1. MIAT/BECN1 RNA was purified by the QIAGEN company (Dusseldorf, Germany). MIAT/BECN1 detected or antisense was labeled using a biotin RNA labeling mixture (AM7150, Ambion, Austin, TX, USA) and then bito-RNA-beads complex was obtained from the biotin RNA labeling mixture incubating with streptavidin magnetic beads. The cell lysate was then incubated with the complex for 1 h at 4 °C. Then, the proteins pulled from the beads were washed and extracted. Finally, the PTBP1 protein was analyzed by western blot.

Data Analysis

The experiments were carried out independently at least 3 times. The results were expressed as mean plus or minus the standard deviation (SD). The calculations were analyzed by Statistical Package for the Social Sciences (SPSS) software (version 22.0, SPSS Inc., Chicago, IL, USA). *T*-test was used to examine the differences between two groups, analysis of variance (ANOVA) and a Bonferroni *post hoc* test was used to examine the differences between multiple groups, and Person correlation was used to analyze the correlation between the expression of MIAT and Src. Two-sided *p* less than 0.05 was considered statistically significant. GraphPad Prism 5 software (GraphPad Software, Inc., La Jolla, CA, USA) was applied to generate the diagrams.

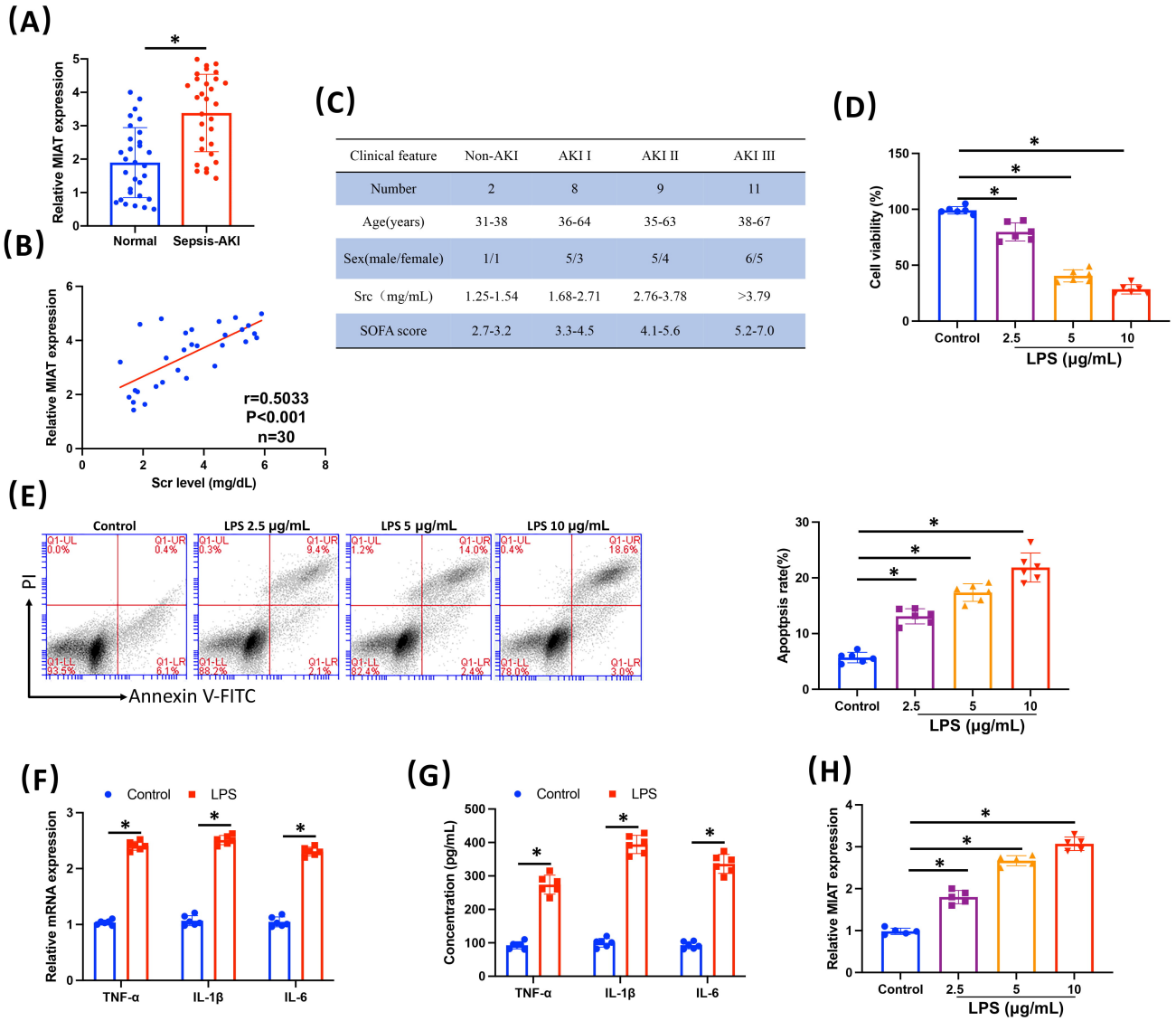
Results

LncRNA MIAT was Up-Regulated in Sepsis-AKI Patients and LPS-Stimulated HK-2 Cells

First, the level of lncRNA MIAT was analyzed in Sepsis-AKI patients by the qRT-PCR assay. The level of MIAT transcripts in serum samples obtained from Sepsis-AKI was higher than those of healthy controls ($p < 0.05$) (Fig. 1A). The relationship between MIAT expression level and serum creatinine (Src) was then analyzed. The results indicate that the higher level of MIAT, the higher value of Src (Fig. 1B). Scr increases with increasing MIAT expression level in most patients. Scr is a key marker of renal injury, which suggests that the higher the MIAT expression level, the greater the severity of renal injury. The clinical features of patients were showed in Fig. 1C. HK-2 cells were then cultured and stimulated with designed concentrations of LPS (0, 2.5, 5, 10 µg/mL) for 24 h. The viability of HK-2 cells treated with LPS was reduced in a concentration-dependent manner ($p < 0.05$) (Fig. 1D). Furthermore, the rate of apoptosis of HK-2 cells treated with LPS was increased in a concentration-dependent manner ($p < 0.05$) (Fig. 1E). Additionally, the mRNA levels of TNF-α, IL-1β and IL-6 were higher in the LPS treatment group (LPS 5 µg/mL for 24 h) (Fig. 1F), while the concentration in the cell supernatant of TNF-α, IL-1β and IL-6 were higher in the LPS treatment group (LPS 5 µg/mL for 24 h) (Fig. 1G). All the results indicated that LPS treatment of HK-2 cells resulted in inhibited cell viability, increased apoptosis, and promoted the release of cytokines. It can well simulate the pathological process of Sepsis-AKI. As expected, MIAT also increased significantly by LPS treating in HK2 cells (Fig. 1H). This evidence suggested that LncRNA MIAT was up-regulated in clinical samples and *in vitro* models.

Inhibition of MIAT Decreased Inflammatory Injury Caused by LPS Treatment in HK-2 Cells

Considering that MIAT may involve in promoting the process of Sepsis-AKI. HK-2 cells transfected with si-MIAT was conducted to confirm whether inhibition of MIAT could attenuate LPS-triggered inflammatory injury in HK-2 cells. HK-2 cells were transfected with si-NC or si-MIAT for 48 h, and then exposed to LPS stimulation. LPS stimulation did not increase MIAT expression after si-MIAT transfection in HK-2 cells ($p < 0.05$) (Fig. 2A). MIAT inhibition can attenuate the inhibitory effect of LPS-induced cell viability ($p < 0.05$) (Fig. 2B). Furthermore, MIAT inhibition can attenuate the promotion effect of LPS-induced cell apoptosis ($p < 0.01$) (Fig. 2C). The changes in apoptosis-related proteins were consistent with the apoptosis detected by flow cytometry. MIAT inhibition can reduce the level of LPS-induced pro-apoptotic proteins (Bax, cleaved caspase 3) and increase the level of the anti-apoptotic protein (B-cell lymphoma-2 (Bcl-2)) ($p < 0.05$)



Clinical feature	Non-AKI	AKI I	AKI II	AKI III
Number	2	8	9	11
Age(years)	31-38	36-64	35-63	38-67
Sex(male/female)	1/1	5/3	5/4	6/5
Src（mg/mL）	1.25-1.54	1.68-2.71	2.76-3.78	>3.79
SOFA score	2.7-3.2	3.3-4.5	4.1-5.6	5.2-7.0

Fig. 1. Long noncoding RNA (LncRNA) MIAT was up-regulated in HK-2 (human renal tubular epithelial cell line) cells stimulated by Sepsis-AKI and LPS. (A) The transcript level in serum samples obtained from Sepsis-AKI or healthy controls was determined by the qRT-PCR. n = 30. (B) Relationship between MIAT and Src expression level in serum samples obtained from Sepsis-AKI patients. n = 30. (C) The clinical features of patients. (D) HK-2 cells were stimulated with LPS (0, 2.5, 5, 10 μg/mL) for 24 h, cell viability was quantified by CCK-8 analysis. n = 6. (E) Cell apoptosis was detected by flow cytometry. n = 6. (F) The release of inflammatory mediators including TNF-α, IL-1β and IL-6 were tested by qRT-PCR assays. (G) The release of inflammatory mediators including TNF-α, IL-1β and IL-6 were tested by ELISA assays. n = 6. (H) The transcript level was detected by qRT-PCR assay. n = 6. Each experiment was independently repeated in triple. All data were presented as Mean $\pm$ standard deviation (SD). *$p < 0.05$. MIAT, myocardial infarction associated transcript; Sepsis-AKI, Sepsis-associated acute kidney injury; AKI, acute kidney injury; LPS, lipopolysaccharide; qRT-PCR, quantitative real-time polymerase chain reaction; Src, serum creatinine; CCK-8, cell counting kit-8; ELISA, Enzyme-linked immunosorbent assay; TNF-α, tumor necrosis factor-α; IL, interleukin.

(Fig. 2D). Furthermore, MIAT inhibition can attenuate the mRNA level of TNF-α, IL-1β and IL-6 stimulated by LPS ($p < 0.01$) and also attenuate the concentration of TNF-α, IL-1β and IL-6 stimulated by LPS ($p < 0.001$) (Fig. 2E,F).

MIAT Silencing Activated BECN1-Mediated Autophagy

In the model of septic mice, autophagy activation has a protective role against multiple organ injuries, including AKI [17]. The activation of the Beclin-1 autophagy initiation factor has been reported to have the potential to attenuate inflammation injury in sepsis [27]. To identify the relationship of MIAT and BECN1 in Sepsis-AKI,

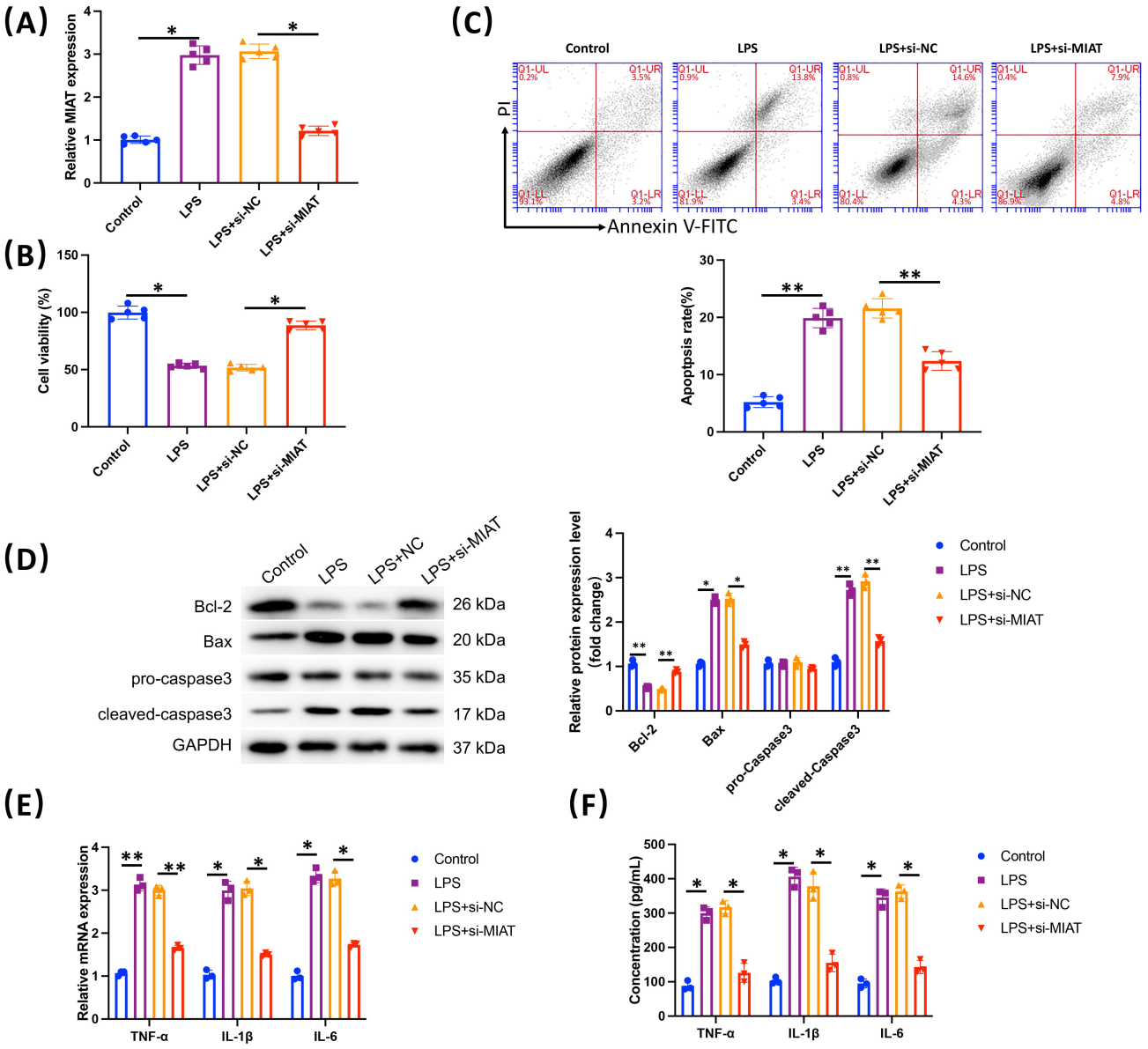


Fig. 2. MIAT inhibition attenuated the LPS-triggered inflammatory injury in HK-2 cells. HK-2 cells were transfected with si-NC or si-MIAT for 48 h, and then exposed to LPS stimulation. (A) The expression change of MIAT was evaluated by qRT-PCR. n = 6. (B) Cell viability was calculated using the CCK-8 assay. n = 6. (C) Cell apoptosis was analyzed using a flow cytometric assay. n = 6. (D) Bax, Bcl-2, and cleaved-cysteine-aspartic proteases (caspase) 3 were tested by western blot. n = 3. (E) The mRNA levels of TNF-α, IL-1β and IL-6 were identified by qRT-PCR assays. n = 3. (F) The concertation of TNF-α, IL-1β and IL-6 were identified by ELISA assays. n = 3. Each experiment was independently repeated in triple. All data were shown as Mean ± SD. *$p < 0.05$, **$p < 0.01$. GAPDH, glyceraldehyde 3-phosphate dehydrogenase; mRNA, messenger RNA; SD, standard deviation; Bcl-2, B-cell lymphoma-2.

si-BECN1 and si-BECN1+si-MIAT were transfected into HK-2 cells. Inhibition of BECN1 significantly downregulated BECN1, while silencing MIAT could partially reverse the effects of si-BECN1 ($p < 0.05$) (Fig. 3A). Next, we found that inhibition of BECN1 significantly decreased LC3B mRNA levels and increased p62 levels, while silencing MIAT could partially reverse the effects of si-BECN1 ($p < 0.05$) (Fig. 3B,C). Autophagy-related proteins including BECN1, LC3B, and p62 were further quantified by western blot. As expected, inhibition of BECN1 significantly decreased BECN1 and LC3B protein levels and increased p62 levels, while silencing MIAT partially reversed the effects of si-BECN1 ($p < 0.05$) (Fig. 3D). Furthermore, immunofluorescence detection of LC3B was also used to determine the role of BECN1, consistent with previous changes in RNA level and protein level (Fig. 3E).

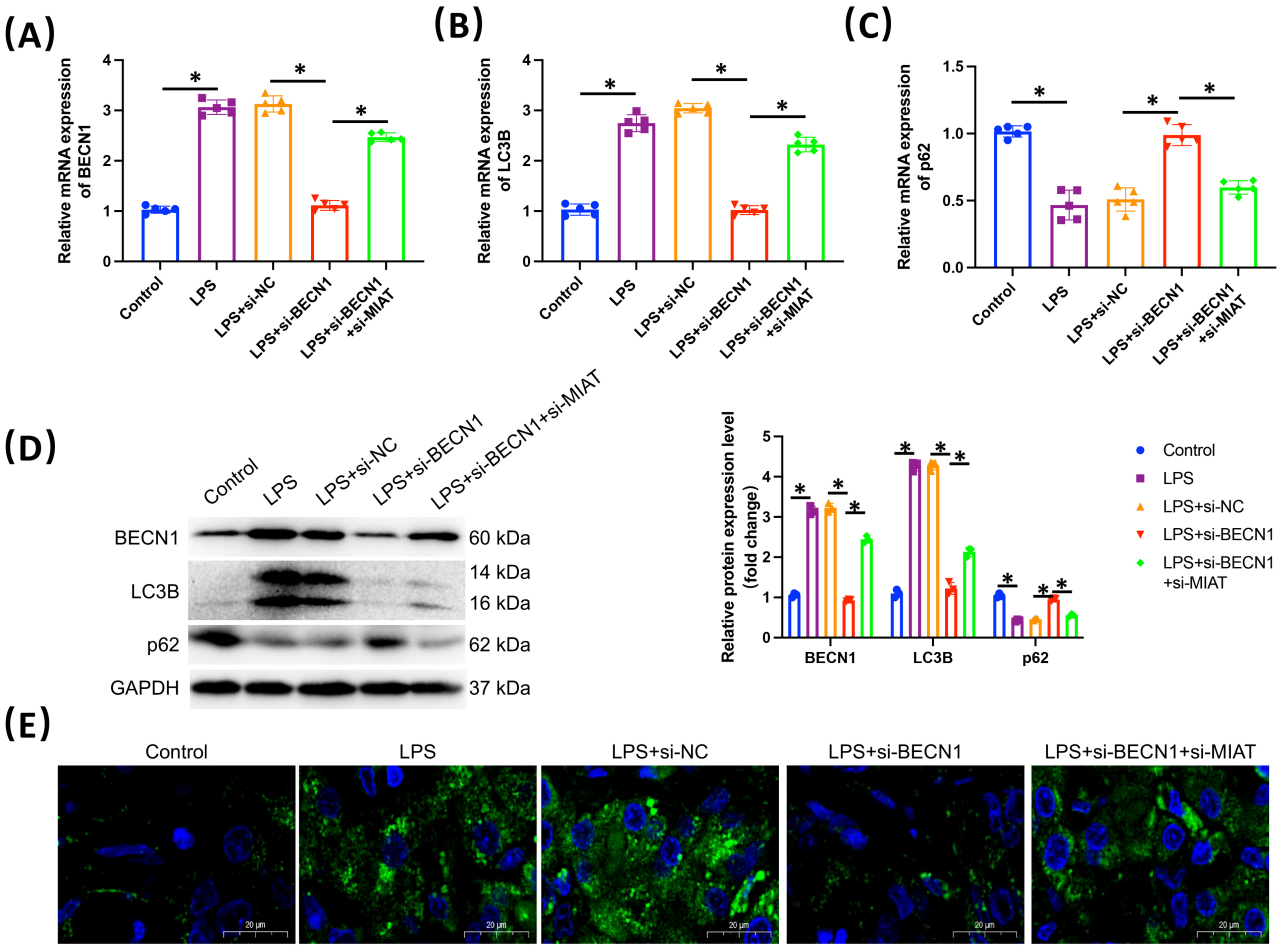


Fig. 3. MIAT silencing activated Beclin 1 (BECN1)-mediated autophagy. HK-2 cells were transfected with si-NC, si-BECN1 and si-BECN1+si-MIAT, respectively. After 48 h, cells were cultured in LPS-contained medium for 24 h. (A–C) Autophagy-related transcripts, including BECN1, p62, and LC3B were measured using a qRT-PCR assay. n = 6. (D) The autophagy-related proteins were further quantified by a Western blot assay. n = 3. Each experiment was independently repeated in triple. (E) LC3B was determined by immunofluorescence. All data were shown as Mean ± SD. *$p < 0.05$. LC3B, microtubule-associated protein 1 light chain 3B.

MIAT Induced BECN1 mRNA Decay by Interacting with PTBP1

It is reported that PTBP1 could regulate various biological processes by interacting with lncRNAs. Beclin-1 plays a protective role in sepsis, activating the Beclin-1 signaling pathway or upregulating Beclin-1 may have important therapeutic potential [28,29]. Furthermore, we explored whether MIAT could regulate BECN1 mRNA stability by interacting with PTBP1. We first clarified the distribution of MIAT in the nucleus and cytoplasm, and found that MIAT located in the nucleus more than in the cytoplasm (Fig. 4A). The interlinked relationship between BECN1 or MIAT and PTBP1 was determined by RIP and RNA pull-down assays. The data supported that MIAT and BECN1 can bind to PTBP1 ($p < 0.01$) (Fig. 4B,C). Silencing MIAT or PTBP1 significantly increased BECN1 mRNA and protein levels. MIAT and BECN1 are co located with PTBP1 ($p < 0.01$) (Fig. 4D–F). After administration by actinomycin D, the stability of BECN1 mRNA was measured by qRT-PCR. The data supported that silencing MIAT or PTBP1 significantly increases the stability of BECN1 mRNA (Fig. 4G). All the results suggested that MIAT could induce BECN1 mRNA decay by interacting with PTBP1.

MIAT Inhibition Relieved LPS-Triggered Inflammatory Injury by Activating BECN1-Dependent Autophagy

Inhibition of autophagy by si-BECN1 increased the LPS-induced cell viability reduction effect, while silencing MIAT significantly relieved LPS-induced cell injury ($p < 0.01$) (Fig. 5A). Inhibition of autophagy by si-BECN1 increased the LPS-induced apoptosis ($p < 0.05$), while silencing MIAT significantly relieved LPS-induced cell apoptosis ($p < 0.05$) (Fig. 5B). Furthermore, inhibition of autophagy decreased the level of Bcl-2 and increased the level of Bax and caspase 3, while silencing MIAT could reverse the ef-

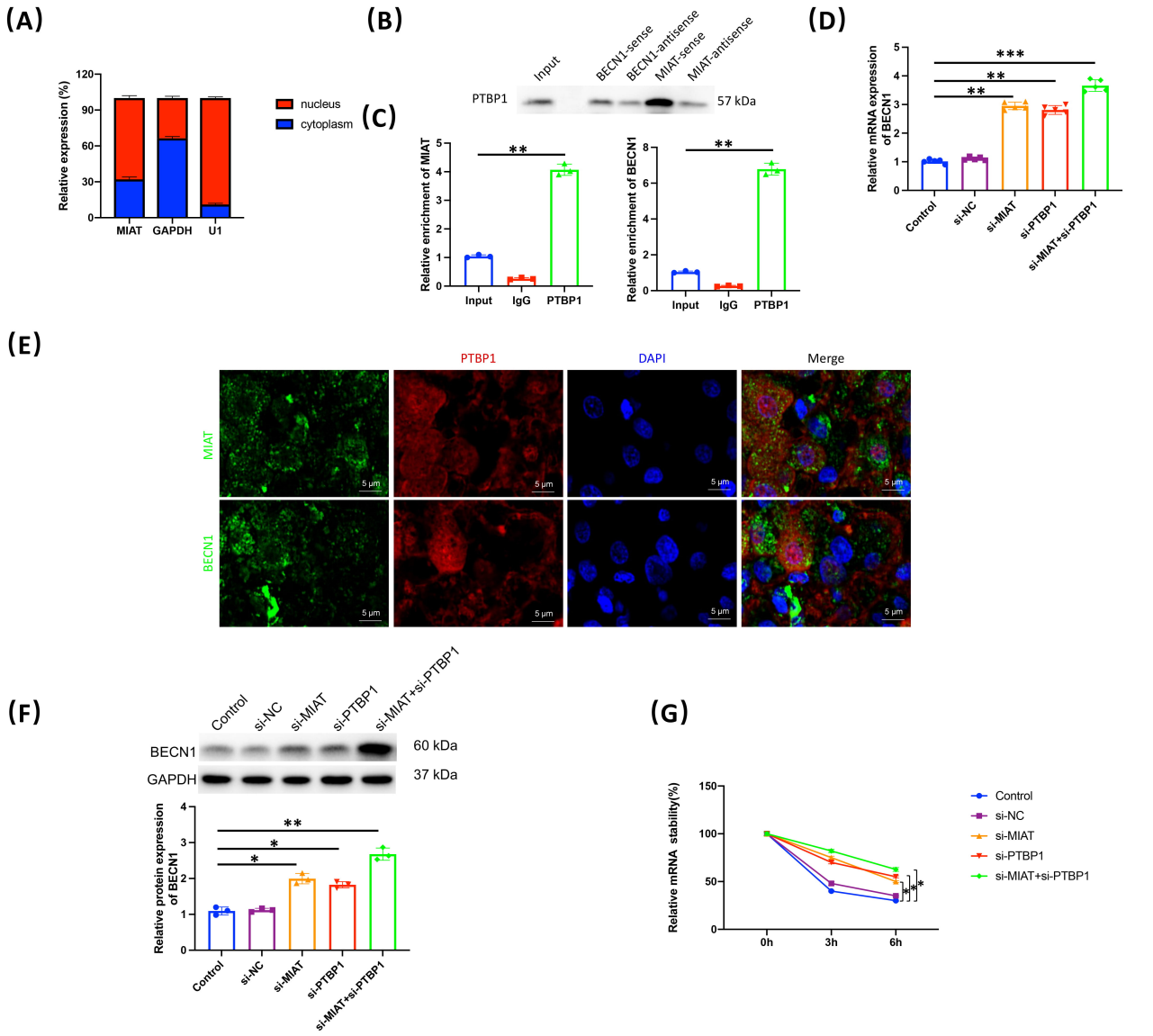


Fig. 4. MIAT silencing inhibited BECN1 mRNA decay by interacting with PTBP1. (A) The distribution of MIAT in the nucleus and cytoplasm was identified using the qRT-PCR assay. n = 6. (B,C) The interlinked relationship between BECN1 or MIAT and polypyrimidine tract-binding protein 1 (PTBP1) was evidenced by RIP and RNA pull down assays. HK-2 cells were transfected with si-NC, si-MIAT, and si-MIAT+si-PTBP1, respectively. (D) BECN1 mRNA level was calculated by qRT-PCR (n = 6). (E) MIAT, BECN1 and PTBP1 were determined by fluorescence in situ hybridization immunofluorescence. (F) BECN1 protein level was calculated by Western blot assays (n = 3). (G) After administration with actinomycin D, the stability of BECN1 mRNA was assessed by the qRT-PCR assay. Each experiment was independently repeated in triple. All data were shown as Mean ± SD. *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$. IgG, immunoglobulin G.

fect in the expression of apoptosis-related proteins caused by inhibition of autophagy ($p < 0.05$) (Fig. 5C). Furthermore, inhibition of autophagy can up-regulate the mRNA level of TNF-α, IL-1β, and IL-6, while si-MIAT can reverse this trend ($p < 0.05$) (Fig. 5D). Additionally, inhibition of autophagy can up-regulate the concentration of TNF-α, IL-1β, and IL-6, while si-MIAT can reverse this trend ($p < 0.05$) (Fig. 5E). All of these findings indicated that MIAT inhibition relieved LPS-triggered inflammatory injury by activating BECN1-dependent autophagy.

Discussion

Sepsis is a fatal disease that often leads to multiple organ failure and shock. AKI is a common complication of sepsis in critically ill patients, usually requiring renal replacement therapy. There is increasing evidence that AKI is due to a complex interplay between immune mechanisms, activation of the inflammatory cascade, and disturbed coagulation pathways [30,31]. Patients with Sepsis-AKI, especially those with more serious injury, have a significantly

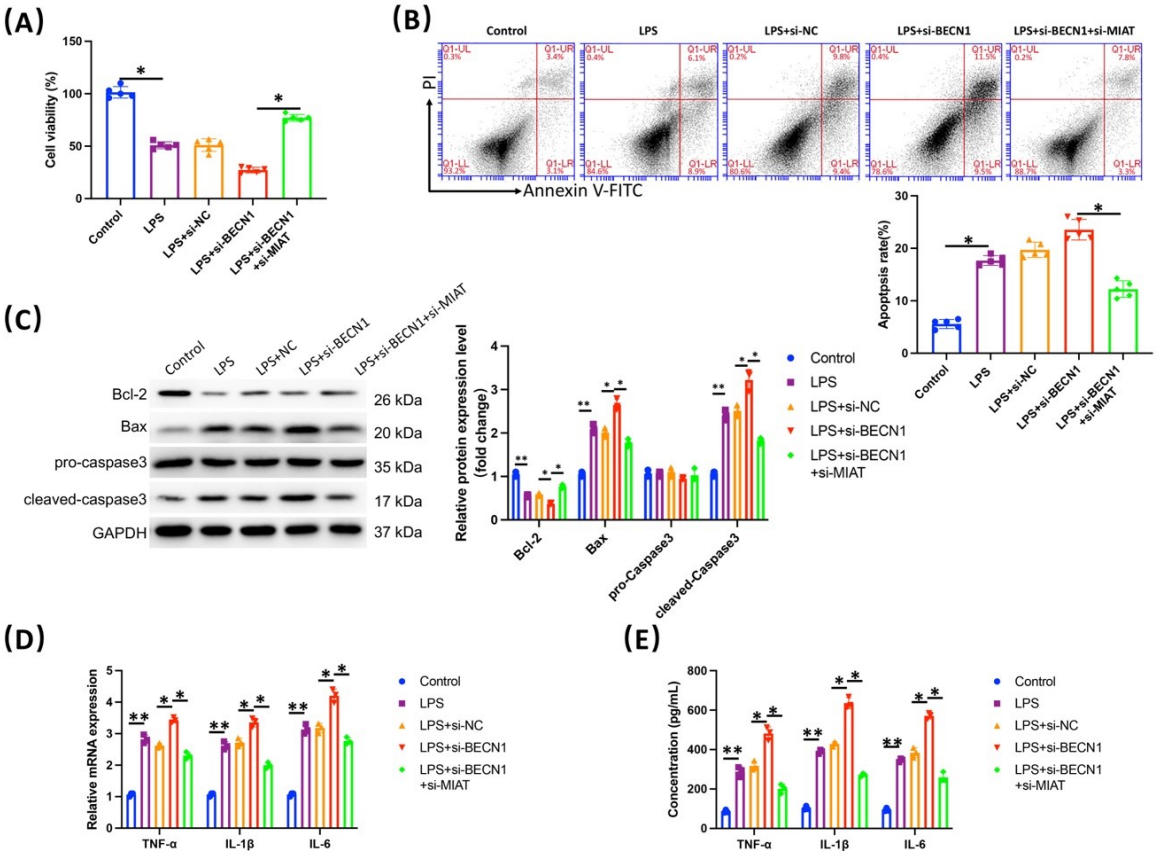


Fig. 5. MIAT inhibition relieved LPS-triggered inflammatory injury by activating BECN1-dependent autophagy. HK-2 cells were transfected with si-NC, si-BECN1 and si-BECN1+si-MIAT, respectively. After 48 h, cells were cultured in LPS-contained medium for 24 h. (A) Cell viability was verified using the CCK-8 assay. n = 6. (B) The change in cell apoptotic rate was tested by flow cytometry analysis. n = 6. (C) Bax, Bcl-2, and cleaved-caspase 3 levels were determined using a Western blot assay. n = 3. (D) The levels of TNF-α, IL-1β, IL-6 were measured by qRT-PCR assay. (E) The levels of TNF-α, IL-1β, IL-6 were measured by ELISA assays. n = 6. Each experiment was independently repeated in triple. All data were shown as Mean $\pm$ SD. *$p < 0.05$, **$p < 0.01$.

higher risk of death [32]. Inhibition of the inflammatory response and apoptosis of the Sepsis-AKI mouse model can effectively protect renal function and promote its recovery [33]. In this study, we found that the down-regulation of MIAT attenuated LPS-stimulated renal cell inflammatory injury by promoting BECN1-mediated autophagy activation through binding to PTBP1, providing some new information of MIAT in Sepsis-AKI.

Long noncoding RNAs (lncRNAs), a subset of nonprotein-coding RNAs (ncRNAs), exhibit crucial roles in the process of many kinds of human diseases. Recent evidence demonstrates the regulatory role of Lnc MALAT1 in inflammatory processes, and its role in the promotion of sepsis has become the focus of current research [34]. Another study has shown that Lnc cancer susceptibility candidate 2 (CASC2) is involved in some important human diseases, including sepsis, and the lower the expression of CASC2, the more severe the AKI injury. CASC2 can serve as a potential target for treating Sepsis-AKI by inhibition miR-155/NF-κB pathway-mediated inflammation [35]. MIAT, a new disease-related lncRNA, was recently found to be abnormally expressed in so many diseases including myocardial infarction, ischemic stroke, diabetic complications, and cancer, etc. and played a role in promoting disease development [36]. There are several studies on MIAT in sepsis. MIAT may bind to miR-29a to participate in Sepsis-AKI [12]. Another study found the potential therapeutic role of MIAT and miR-330-5p in myocardial injury by LPS treatment [37]. Inhibition of MIAT protects against MI-induced cardiac dysfunction through crosstalk with miR-10a-5p/EGR2 [38]. In this study, we also found that inhibition of MIAT down-regulation attenuated LPS-stimulated renal cell inflammatory injury, inhibition of MIAT is a potential therapeutic strategy for Sepsis-AKI.

Autophagy is the main intracellular degradation system. However, autophagy is a dynamic circulatory system that involves the regulation of many human diseases, especially inflammatory diseases [39]. It is reported that basal autophagy in the kidney is also crucial for the normal

homeostasis of proximal tubule. The absence of key autophagic proteins alters renal function, increases p62 levels, and induces oxidative stress damage [40]. Beclin 1 plays a key role in the autophagy regulation. Furthermore, Beclin 1 is also related in the regulation of endocytosis, apoptosis, and phagocytosis, etc. [41]. Induction of autophagy through SIRT1-mediated deacetylation of Beclin1 can effectively alleviate kidney damage both *in vivo* models and *in vitro* models of Sepsis-AKI and may be a potential strategy for future treatment [42]. Acetylated p53 was more likely to bind to Beclin1 and accelerate its ubiquitination mediated degradation. Therefore, deacetylated p53-mediated autophagy up-regulation can attenuate sepsis AKI [43]. The results of this study showed that MIAT silencing activated BECN1-mediated autophagy, suggesting that activation autophagy may serve as a new treatment option.

Most lncRNAs require interaction with one or more RNA-binding proteins (RBPs) to participate in a plethora of cellular functions [44]. PTBP1, a well-known RBP, plays biological roles after binding to lncRNA [45]. There is no report about the relationship of PTBP1 and MIAT. For the first time, we found that MIAT could induce BECN1 mRNA decay by interacting with PTBP1. There are already many studies about the PTBP1 could regulate autophagy through different mechanisms [46,47]. In this study, we found activation of BECN1-dependent autophagy is related to reducing LPS-triggered inflammatory injury by MIAT interacting with PTBP1 to induce BECN1 mRNA decay.

Conclusions

In summary, the experimental data reinforced that MIAT downregulation attenuated LPS-stimulated renal cell inflammatory injury by promoting BECN1-mediated autophagy activation through binding to PTBP1, providing some new insights into the function and mechanism of MIAT in Sepsis-AKI.

Availability of Data and Materials

The data sets used or analyzed during the current study are available from the corresponding author upon reasonable request.

Author Contributions

MX: Conceptualization, Methodology, Writing-Original draft preparation, Data curation, Visualization, Investigation; YZ: Conceptualization, Methodology, Writing-Reviewing and Editing, Supervision, Validation. Both authors read and approved the final manuscript. Both authors have participated sufficiently in the work and agreed to be accountable for all aspects of the work.

Ethics Approval and Consent to Participate

This is a non-interventional study. The patients' information and blood samples collected were approved by the Ethics Committee of the Second Xiangya Hospital, Central South University (NO.2017078). Their privacy was protected without adding additional risks and financial burdens. Each patient has signed a written informed consent before the study.

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Conflict of Interest

The authors declare no conflict of interest.

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