



Research article

Circular RNA expression in the lungs of a mouse model of sepsis induced by cecal ligation and puncture

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ABSTRACT

Circular RNAs (circRNAs) are novel endogenous RNAs with vital roles in the pathology of various diseases. However, their role in sepsis-induced lung injury is unknown. In this study, high-throughput gene sequencing was used to analyze the expression profiles of circRNAs in lung specimens of mice grouped by acute lung injury induced by cecal ligation and puncture (CLP) and sham. To identify differentially expressed circRNAs, the left lungs of sham ($n = 3$) and CLP ($n = 3$) mice were used for high-throughput sequencing. A total of 919 circRNAs were identified. Of these, 38 circRNAs showed significantly different expression levels between the groups ($P < 0.05$, fold change ≥ 2). The levels of 20 circRNAs were up-regulated and those of 18 others were down-regulated. In bioinformatics analysis of the source genes of these circRNAs, the genes were closely associated with the inflammatory response (e.g., the TGF- β , MAPK, Fc gamma R-mediated phagocytic, and VEGF pathways). Eight circRNAs with large intergroup differences, small intragroup differences, and high expression were selected for further validation by qRT-PCR. Two of the eight were significantly different. These two circRNAs were annotated with circRNA/miRNA interaction information downloaded from the TargetScan and miRanda databases and visualized. Our results provide novel insights into the roles of circRNAs in sepsis-induced acute lung injury.

1. Introduction

Sepsis is a dysfunction of the host response caused by infection that involves multiple organs and systems in the body, and it leads to organ failure and death. It is one of the main causes of death in intensive care units worldwide [1]. The incidence of sepsis in developed countries is as high as 100 cases per 100,000 people, and approximately 2% of hospitalized patients suffer from sepsis upon admission [1]. A Chinese epidemiological study found that 12.6% of deaths in 2015 were sepsis-related, and calculated the standardized mortality rate to be 66.7 cases per 100,000 people based on census data [2].

Many factors can lead to sepsis, including bacterial, viral, and fungal infections. Among the variety of complications are: shock, acute respiratory distress syndrome (ARDS), deep vein thrombosis, stress ulcers, metabolic acidosis, diffuse intravascular coagulation, and multiple organ failure. ARDS is a common complication of sepsis and the most serious phase of sepsis-induced lung injury. According to epidemiological

statistics, patients with ARDS from any cause account for 10.4% of intensive care unit admissions [3]. There have been advances in sepsis treatment: timely and appropriate antibiotic treatment, fluid resuscitation, vascular compression, drug administration as needed, and supportive treatment of depleted organs [4]. Despite this progress, the mortality rate of ARDS remains high and there is a lack of specific diagnostic markers for this condition [3]. Therefore, early diagnosis and treatment of sepsis-induced lung injury is particularly important.

CircRNAs are novel RNAs that have been extensively studied. Recent developments in sequencing show that circRNAs exist in all living organisms. They are abundantly expressed in eukaryotes, highly conserved, and expressed in specific cells or at certain developmental stages [5]. CircRNAs function biologically as miRNA sponges, translation templates, regulators of gene transcription, and they interact with RNA-binding proteins. These characteristics make circRNAs a good target for potentially new diagnostic and therapeutic agents [6].

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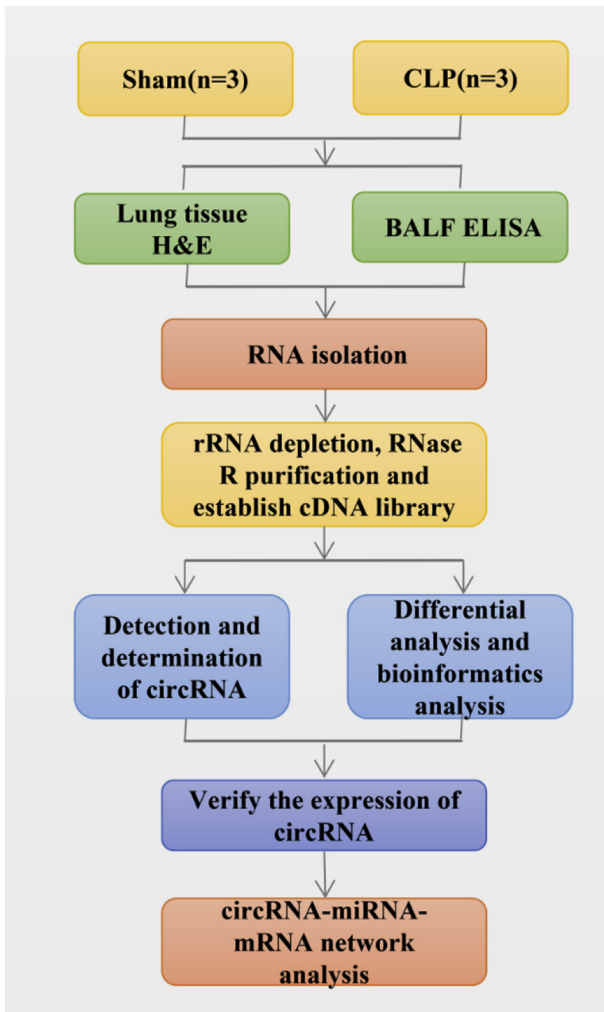
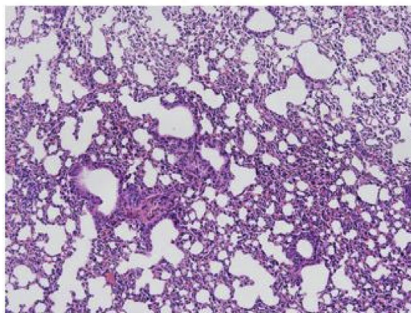


Figure 1. Flow chart.

Research on circRNAs in lung diseases has increased significantly since 2017 [7]. Wan et al. used a lipopolysaccharide-induced ARDS rat model to analyze lung circRNA expression profiles, and they identified 395 up-regulated and 562 down-regulated circRNAs [8]. In addition, microarray analysis of lung specimens of mice with pulmonary hypertension revealed 23 up-regulated and 41 down-regulated circRNAs [9]. These studies indicate that circRNAs are abundantly expressed in the lungs and may be involved in the pathogenesis of lung diseases.

A



B

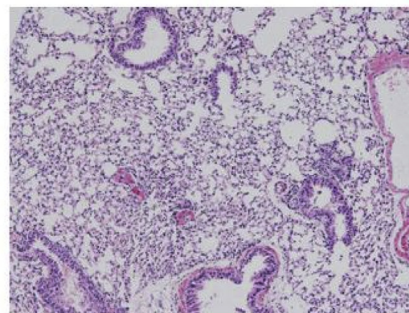


Figure 2. Mouse lung tissue HE staining (x100). (A) is the CLP group, and (B) is the Sham group. It can be seen that the CLP group has alveolar expansion and collapse relative to the Sham group, the alveolar space is widened, extensive vascular congestion, and a large number of inflammatory cells infiltrate.

This is an exploratory study of the expression profiles and functions of circRNAs in lung injury induced by sepsis. We performed high-throughput sequencing of lung specimens from control mice and mice with sepsis induced by CLP and identified differentially expressed circRNAs. Subsequently, we used quantitative reverse transcription polymerase chain reactions (qRT-PCR) to assess the potential of circRNA as a biomarker for pulmonary injury in sepsis.

2. Materials and methods

2.1. Animals

Ethical approval was obtained from the animal core facility of Nanjing Medical University. Experimental protocols were approved by the Nanjing Medical University Animal Care and Use Committee (IACUC-1702009). Specific pathogen-free adult C57BL/6 mice obtained from the Nanjing Medical University Animal Center weighing 20 ± 3 g were maintained for one week under controlled temperature and humidity with 12-h-light/12-h-dark cycles with access to standard laboratory food and water ad libitum. The study was approved by the Inspection of the Institutional Animal Care and Use Committee of Affiliated Children's Hospital of Nanjing Medical University.

2.2. Sepsis induced by cecal ligation and puncture and specimen collection in mice

Thirty mice were randomly and evenly divided into a sham and cecal ligation and puncture (CLP) group. Sepsis was induced by CLP as described by Rittirsch and colleagues [10]. In brief, midline laparotomy was performed under general anesthesia, and the cecum was exposed. To avert bowel obstruction, the distal half of the cecum was ligated to the ileocecal valve and then the center of the distal cecum was punctured with a 21-gauge needle. Cecal contents were manually expelled from the punctured cecum into the abdominal cavity. After returning the cecum into the abdomen, 1 mL of saline was subcutaneous injected as fluid resuscitation, thereby establishing a clinically-analogous sepsis mouse model. The abdomen was sutured using a single-layer technique. Sham mice were treated similarly, except that CLP was omitted. After 24 h, both groups of mice were euthanized, and the lungs were harvested.

2.3. Histological analysis

The right lower lobes of lungs of all surviving mice were harvested and fixed in 4% paraformaldehyde for 24 h, embedded in paraffin, and sectioned serially to generate 5- μ m sagittal sections. The sections were stained with hematoxylin and eosin and then examined under a light microscope.

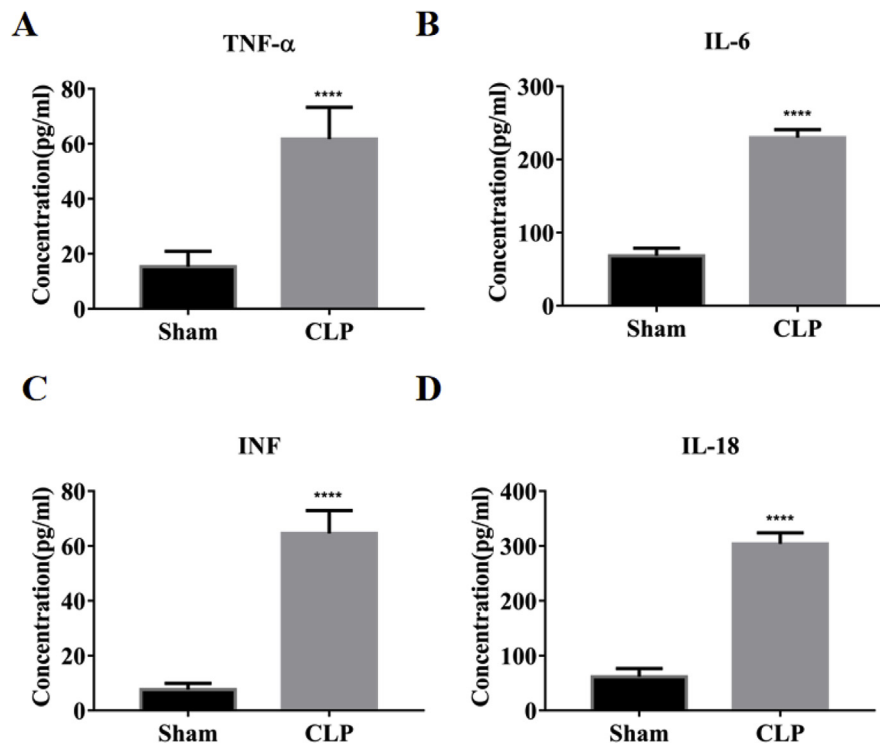


Figure 3. ELISA was used to detect the content of high level expression of inflammatory cytokines (A-D stand for TNF α , IL-6, INF and IL-18) in alveolar lavage fluid of CLP group and Sham group. The results showed that compared with the Sham group, the content of inflammatory cytokines in the CLP group was significantly increased, and the difference was statistically significant ($P < 0.05$).

2.4. Enzyme-linked immunosorbent assays

Mice were euthanized immediately 24 h after surgery and alveolar lavage fluid was collected from Sham and CLP mice. ELISA (Enzyme-linked immunosorbent assay) was used to measure the levels of the inflammatory cytokines TNF α , IL-6/18, and INF.

2.5. Sequencing analysis of circRNAs

To identify differentially expressed circRNAs, the left lungs of sham ($n = 3$) and CLP ($n = 3$) mice were used for high-throughput sequencing (Nanjing Decode Genomics Center, Nanjing, China) (Figure 1). Total RNA was extracted and ribosomal RNA was removed. A strand-specific RNA library was constructed. RNAs were digested into 200–500 bp fragments and then used as templates to synthesize cDNAs in the presence of a six-base random primer, dNTPs, RNase H (Thermo Scientific, USA), DNA polymerase I (New England Biolabs Inc., USA), and buffer (Thermo Scientific, USA). The PCR products were purified using the QiaQuick PCR kit (QIAGEN, GER) and end-repaired, followed by A-tail addition and sequencing-linker ligation. Fragment sizes were determined by agarose gel electrophoresis and then PCR amplification was performed. The sequenced library was used for Sanger sequencing by adopting a double-ended sequencing strategy.

Based on the TopHat comparison results, HTSeq software was used to compare the number of reads mapped to each gene by adopting default parameters, and the results were used to calculate the expression level of each gene. To identify differentially expressed genes, further analysis of different specimens (groups) was carried out using edgeR software (<http://www.bioconductor.org/packages/release/bioc/html/edgeR.html>).

2.6. Bioinformatics analysis

Gene Ontology (GO) term enrichment analysis was performed using the source genes of the differentially expressed circRNAs, and their functions were analyzed by cell composition, molecular function, and biological process. Differentially expressed circRNAs were subjected to GO term enrichment analysis (<http://geneontology.org/>) and Kyoto Encyclopedia of Genes and Genomes (KEGG) pathway enrichment analysis (http://kobas.cbi.pku.edu.cn/anno_iden.php).

2.7. Quantitative reverse transcription polymerase chain reaction analysis of circRNAs

Lung specimens obtained from mice of all surviving sham and CLP groups were used for quantitative reverse transcription. Total RNA was isolated and reverse transcribed for qRT-PCR analysis. Divergent primers were used to amplify selected circRNAs instead of the more commonly used convergent primers. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. We used SYBR for qRT-PCR to evaluate the results. The appearance of a single peak in the melting curve indicated primer specificity. The relative expression levels of circRNAs were analyzed using the $2^{-\Delta\Delta CT}$ method.

2.8. Prediction of circRNA-miRNA-mRNA network

Potential miRNA targets of circRNA were predicted by TargetScan and miRanda databases, and their possible target genes were predicted by key network nodes of miRNA. The circRNA-miRNA-RNA network was visualized using Cytoscape software.

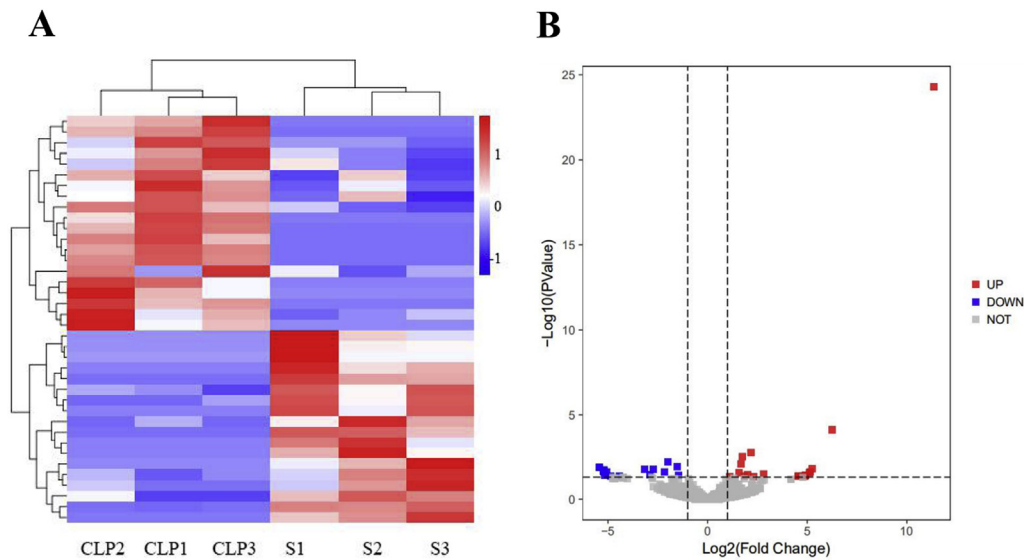


Figure 4. Differentially expressed circRNA. (A) Cluster maps, each row representing a gene, each column representing a sample, red being the up-regulated gene, and blue being the down-regulated gene. (B) Volcano map, the abscissa is the logarithm of the difference multiple, the ordinate is the P value after the logarithm, the red and blue dots represent the differentially expressed genes, the red is the up-regulated gene, and the blue is the down-regulated Gene.

2.9. Statistical analysis

Data analysis was performed using GraphPad Prism 7 software. The relative levels of differentially expressed circRNAs in the lungs of mice of the sham and CLP groups were compared by two-tailed *t*-tests with *P*-values less than 0.05 considered statistically significant.

3. Results

3.1. Animal model

3.1.1. General conditions of mice

Compared to the sham group, mice of the CLP group were apathetic, exhibited decreased activity, had increased eyelid secretions and partial bowel adhesions. Necropsy revealed intestinal adhesions, and in some mice, cecal necrosis. The first mouse died 12 h after the operation, and the number of deaths increased at 18 h. Five mice in the CLP group died 24 h after surgery for a mortality rate of 33%, however, none died in the sham group.

3.1.2. Histopathology and expression of inflammatory factors in mouse lung

Lung injury was more extensive (obvious alveolar expansion and collapse, increased alveolar septa, and vascular congestion) in the CLP group as visualized in hematoxylin and eosin-stained sections (Figure 2), which indicated successful establishment of lung injury. Compared to sham mice, the levels of inflammatory factors were significantly elevated in alveolar lavage fluid of CLP mice (Figure 3), which suggested an inflammatory response in the lungs.

3.2. High-throughput sequencing analysis

In high-throughput sequencing analysis, 919 circRNAs were identified in the lungs and there were differences in circRNA expression between the groups. Of the 38 circRNAs with the most significant differences in expression, 20 were up-regulated and 18 were down-regulated in mice of the CLP group (fold change >2.0 and *P* < 0.05, Figure 4, Table 1).

3.3. GO term and KEGG pathway enrichment analyses

The most common functions of the source genes of circRNAs were related to intracellular organelles, enzyme binding, and key metabolic processes. In the KEGG analysis, the most abundant signaling pathways of the source genes of circRNAs were the TGF- β , MAPK, Fc gamma R-mediated phagocytic, and VEGF pathways (Figure 5).

3.4. Validation of circRNAs

Lungs were harvested from ten mice in the CLP group and 15 in the sham group. The eight circRNAs in the CLP group with the most significant changes in expression in high-throughput sequencing analysis were selected; six were up-regulated (e.g., circRbm39, circMyrip, circEfnb2, circVav3, circPde5a, circLamp3) and two were down-regulated (e.g., circKlf7, circWdr49). These circRNAs were visualized using circPrimer software and validated by qRT-PCR using GAPDH as a reference. The expression of circEfnb2 in the CLP group was increased and that of circWdr49 was decreased compared to sham mice (*P* < 0.05) (Figure 6).

3.5. Sepsis-associated circRNA-targeted miRNA prediction

CircRNAs can act as miRNA sponges by binding to miRNAs, thereby inhibiting their function. The miRNA targets of circEfnb2 and circWdr49 circRNAs were predicted using TargetScan and miRanda databases, and a network diagram of circRNA-miRNA-mRNA interactions was generated. This network map shows the top four miRNAs that were potentially bound to the circRNAs and the five genes that were most likely targeted by each miRNA (Figure 7).

4. Discussion

Sepsis is a worldwide public health problem. In the United States, it is costly in terms of economics [11] and lives lost [12]. The treatment of sepsis has improved greatly with improving knowledge, however, the mortality rate remains high [13]. Studies have shown that the pathogenesis of sepsis may be related to a variety of conditions: an imbalance

Table 1. 38 Over- and under-expressed circRNAs derived from CLP group.

CircRNA	Host Gene Name	logFC	P-Value
Over-expressed			
circRNA1_intergenic	Intergenic	11.344617	5.19E-25
circRNA2_intergenic	Intergenic	6.23833863	7.77E-05
circRNA3_Efnb2	Efnb2	2.162477644	0.001781864
circRNA4_Pde5a	Pde5a	1.734672467	0.003124856
circRNA6_Tmbim6	Tmbim6	1.68003467	0.008488878
circRNA10_Rbm39	Rbm39	5.235563062	0.015207939
circRNA19_Rbm33	Rbm33	5.140947063	0.024557832
circRNA21_Myrip	Myrip	5.113310317	0.026455875
circRNA22_Gab1	Gab1	1.588893296	0.027676594
circRNA23_Oaz2	Oaz2	2.804161962	0.033612924
circRNA24_Vav3	Vav3	2.001977157	0.034678511
circRNA27_Arhgap26	Arhgap26	4.922274582	0.038537623
circRNA29_Tbc1d5	Tbc1d5	4.923555958	0.040619526
circRNA30_Man2a1	Man2a1	4.560705188	0.041042545
circRNA31_Esco1	Esco1	4.561630113	0.041897814
circRNA33_Tc2n	Tc2n	1.85105662	0.042644542
circRNA34_Camk2d	Camk2d	4.835375706	0.042780127
circRNA36_Hip1r	Hip1r	4.863802671	0.043542265
circRNA37_Lamp3	Lamp3	1.11621848	0.04821158
circRNA38_intergenic	intergenic	2.303708892	0.048370411
Down-expressed			
circRNA5_Epb4112	Epb4112	-1.989679384	0.006118574
circRNA7_Wdr49	Wdr49	-1.529111641	0.012572686
circRNA8_Phf20	Phf20	-5.435069809	0.012897742
circRNA9_intergenic	intergenic	-5.442591322	0.013743156
circRNA11_Zfp644	Zfp644	-3.16608411	0.017530848
circRNA12_Dtnb	Dtnb	-2.723202195	0.01805221
circRNA13_Sptan1	Sptan1	-5.22058555	0.01855269
circRNA14_intergenic	intergenic	-5.212015266	0.019090543
circRNA15_Ikzf1	Ikzf1	-5.237078297	0.01968517
circRNA16_Zbbx	Zbbx	-5.221045782	0.021668413
circRNA17_Ssbp2	Ssbp2	-5.195944163	0.024383668
circRNA18_Klf7	Klf7	-5.06557941	0.024479505
circRNA20_intergenic	intergenic	-2.156868993	0.025773824
circRNA25_Arhgef28	Arhgef28	-2.912239129	0.035381568
circRNA26_Utrn	Utrn	-1.467162408	0.037876046
circRNA28_Arhgef28	Arhgef28	-5.156309144	0.039941185
circRNA32_Dlc1	Dlc1	-4.868932713	0.042492594
circRNA35_Rnf4	Rnf4	-4.42719952	0.042839895

in the inflammatory response; immune dysfunction; coagulopathy; autophagy; and mitochondrial damage [14]. Genetic differences play an important role in the pathophysiology of certain diseases [15]. Establishing the relationship between genetic polymorphisms and sepsis-induced lung injury will improve our understanding of the mechanisms of sepsis.

The CLP model used in this study is the most widely used animal model for sepsis. As the gold standard in sepsis research, the CLP model has numerous advantages over other animal models. It conforms to the natural process of human infection, displays similar hemodynamic changes, and induces different degrees of sepsis severity [10].

Taken collectively, our findings of poor health, bowel adhesions, alveolar damage, and the presence of TNF α indicate the presence of a significant pulmonary inflammatory response. All of which strongly points to the successful establishment of an animal model of lung injury induced by sepsis.

An increasing number of studies have shown that circRNAs have important biological functions. MiRNAs are non-coding small RNAs that can regulate the translation of mRNAs by transcription of the

corresponding miRNA response elements [16]. Recent studies have demonstrated that some circRNAs contain miRNA response elements, which suggest that they may compete for miRNA binding sites as competing endogenous RNAs (ceRNAs), thereby affecting miRNA activity [17, 18]. CircRNA may also serve as a potential biomarker for sepsis. This paper presents good evidence that non-coding RNA may serve as a new biomarker and therapeutic target for sepsis and septic shock [19].

Previous studies have demonstrated that the functions of circRNAs are related to their host genes [15]. In this study, target genes were subjected to GO term and KEGG pathway analyses. Among the KEGG pathways identified in this study, TGF- β , MAPK, and JAK-STAT signaling pathways participate in the regulation of sepsis. The most abundant signaling pathways for gene enrichment are the TGF- β and MAPK pathways. The TGF- β signaling pathway is involved in many cellular processes, including cell growth, cell differentiation, and apoptosis. A previous study found that TGF- β 1, which can initiate an inflammatory cascade, is elevated during sepsis and is involved in its inflammatory process [20]. In addition, the MAPK pathway has important roles in physiological processes such as cell growth, proliferation, apoptosis, and

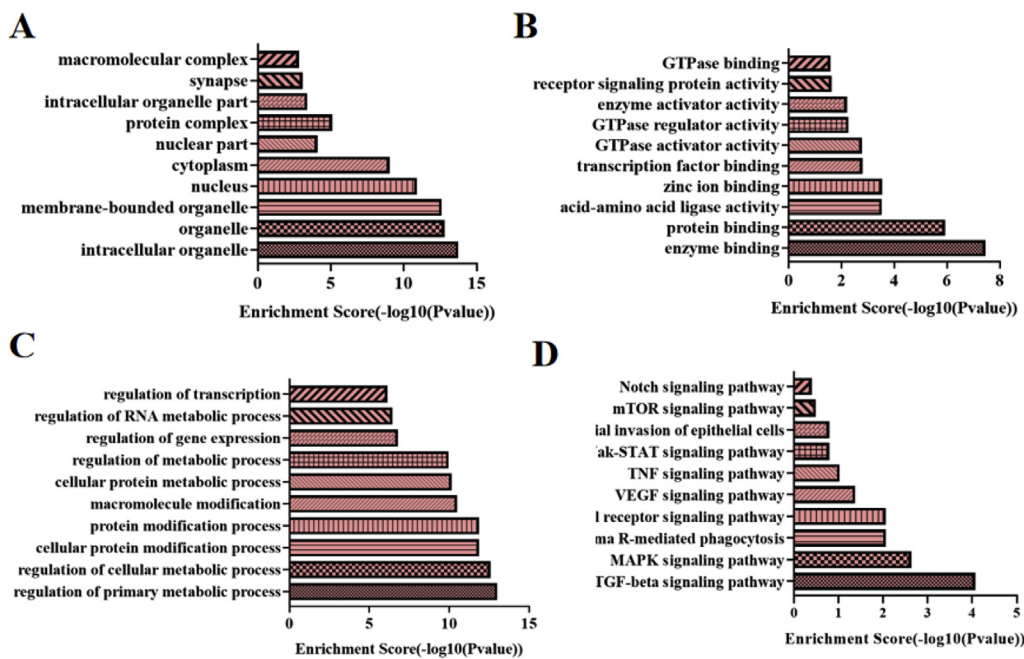


Figure 5. GO analysis and KEGG analysis of differentially expressed circRNA. (A) GO analysis of cellular components; (B) GO analysis of molecular function; (C) GO analysis of biological processes; (D) Pathway for differential expression of circRNA-derived genes.

differentiation. An important member of this pathway is p38 MAPK; blocking the p38 MAPK signaling pathway can alter the pathology of acute lung injury by regulating inflammation, apoptosis, and endothelial cell permeability [21].

In this study, two circRNAs (circEfnb2 and circWdr49) with significant intergroup differences in expression were identified. Up-regulated circEfnb2 and down-regulated circWdr49 were predicted to negatively regulate the function of mmu-miR-340-5p and mmu-miR-34b-5p. The circRNA-miRNA interaction network suggests that there is a binding site for miR-34b-5p on circWdr49. This is interesting because previous studies have shown that miR-34b-5p may have a role in acute lung injury by acting on the granule protein precursor progranulin (PGRN) [22]. PGRN is a secreted growth factor expressed in epithelial cells and macrophages, and it has protective roles in wound healing, inflammation, and apoptosis. In addition, the concentration of PGRN in bronchoalveolar

lavage fluid of mice with acute lung injury is decreased, and PGRN can significantly reduce lipopolysaccharide-induced pulmonary inflammation [23]. Accordingly, inhibiting miR-34b-5p may promote PGRN expression, thereby reducing inflammation and apoptosis in acute lung injury [22]. These findings support the mechanism of action of circRNAs in lung injury induced by sepsis.

There are some limitations in the study that must be noted. Research in this area is still exploratory, further study are needed to uncover the roles of specific targets and regulatory mechanism. Those findings will have scientific significance since function at the cellular level remains unclear. Additional studies of the downstream pathway for circRNA need to be conducted and at the same time screening for biomarkers is needed in clinical blood samples.

In conclusion, we conducted a preliminary study that profiles circRNA expression patterns in sepsis-induced acute lung injury. Our

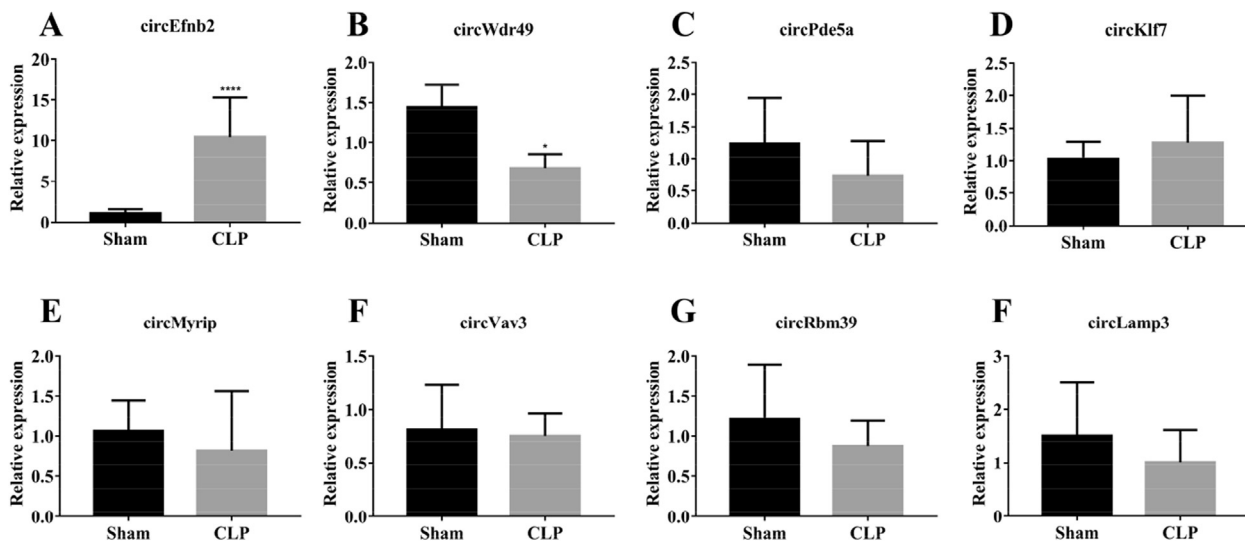


Figure 6. A-F, Histogram of relative expression levels of 8 circRNAs (circEfnb2, circWdr49, circPde5a, circKlf7, circMyrip, circVav3, circRbm39 and circLamp3) in the CLP and Sham groups. The expression levels of the up-regulated circEfnb2 and the down-regulated circWdr49 in the CLP group were significantly different than those in the Sham group ($P < 0.05$), and the other 6 were not statistically significant.

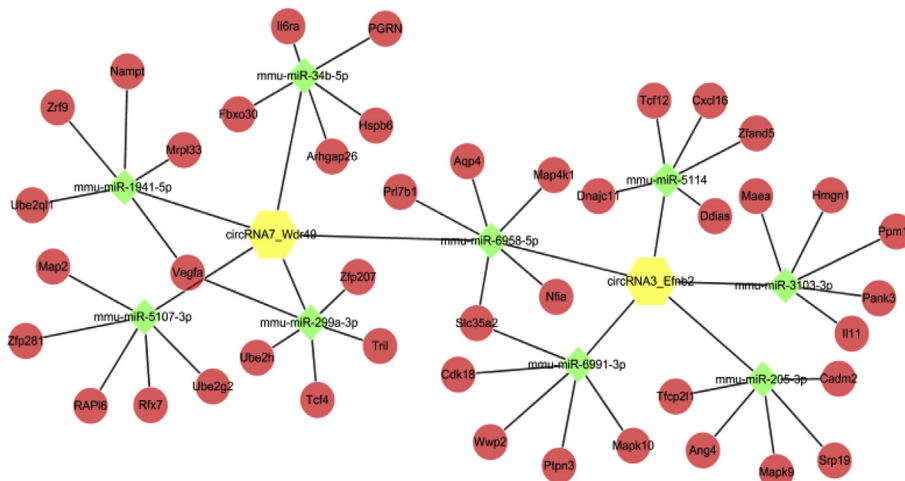


Figure 7. CircRNA-miRNA-mRNA interaction map. The figure consists of the 2 circRNAs, the top 5 miRNAs that potentially bind to the circRNA and the 5 most likely target genes to every miRNAs. Yellow represents circRNA, green represents miRNA, and red represents mRNA.

findings indicate that circRNAs may have key roles in the pathology of sepsis-induced lung injury. These results can be used as the basis for functional confirmation studies in the future. While still exploratory, we think this research will lead to additional mechanistic studies of circRNA that builds on the knowledge we have gained.

Declarations

Author contribution statement

H. Miao and J. Li: Conceived and designed the experiments.
J. Gu and J. Wu: Analyzed and interpreted the data.
C. Yuan: Analyzed and interpreted the data; Wrote the paper.
J. Yin and M. Zhang: Performed the experiments.

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Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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