Cellular and Molecular Biology

E-ISSN : 1165-158X / P-ISSN : 0145-5680

www.cellmolbiol.org

CMB Association Publisher

Circular RNA PARG adjusts miR-140-3p to influence progression in sepsis

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ARTICLE INFO

Original paper

Article history:

Received: February 04, 2023

Accepted: May 12, 2023

Published: May 31, 2023

Keywords:

Biomarker, circular RNA PARG, lipopolysaccharide, miR-140-3p, sepsis

ABSTRACT

Sepsis has been characterized as a frequent medical problem with high mortality and severe complication medical problem in the intensive care unit (ICU). Here, qRT-PCR was used to examine circRNA PARG expression levels in patients with sepsis and in human pulmonary microvascular endothelial cells (HPMEC). Lipopolysaccharide (LPS)-simulated HPMEC were hybridized using RNA-Fluorescence in situ hybridization to confirm the location of circRNA PARG and miR-140-3p. The biological role of downregulated circRNA PARGin cellular proliferation, apoptosis, and inflammatory and apoptosis responses was evaluated. performed A dual-luciferase reporter assay was performed to determine the relationship between the circRNA PARG with miR-140-3p. In this study,circRNA PARG aberrant expression was found, and the effects of circRNA PARG on lipopolysaccharide (LPS)-stimulated apoptosis of HPMEC cells were further investigated. Down-regulated circRNA PARG led to significant alleviation of LPS-simulated cell apoptosis via inhibition of inflammatory and apoptosis-related genes, while upregulated circRNA PARG exhibited the opposite effects. Further findings indicated that circRNA PARG positively modulated the relative level of miR-140-3p, which has been confirmed using the luciferase reporter assay. Upregulated circRNA PARG led to a reversal of LPS-simulated cells after transfection of miR-140-3p mimic. In general, a novel insight into understanding the important effects of circRNA PARG in sepsis is provided.

Doi: http://dx.doi.org/10.14715/cmb/2023.69.5.17

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Introduction

Sepsis is a potentially life-threatening disease and causes infection and systemic inflammatory reaction (1). Sepsis can result in multiple organ dysfunction and systemic inflammatory responses; the most obvious manifestation is acute respiratory distress syndrome (ARDS) (2,3). Critically ill patients are often admitted to intensive care units (ICUs) due to sepsis (4). The sepsis-associated mortality rate of patients with sepsis is as high as 40%. At present, a variety of key sepsis-related genes have been isolated and evaluated. Although some progress has been made in basic research, no effective method for sepsis treatment is available.

Recent research has revealed that noncoding RNAs are involved in various cell signaling pathways and participate in the progression of numerous diseases. These molecules include circular RNAs (circRNAs) (5,6), long noncoding RNAs (lncRNAs) (7), and microRNAs (miRNAs) (8). As a novel member of noncoding RNAs, circRNAs are abundant in eukaryotes. These RNAs exert a biological role in the progression of various diseases and cellular pathways (9). More importantly, the unique stability of circRNAs with cell- and tissue-specific expression in body fluids is considered a potential biomarker for sepsis. This type of RNA can modulate genes by affecting transcription levels and then be translated by adjusting RNA-binding miRNAs and proteins (10,11).

MiRNAs are also members of endogenous noncoding RNA molecules and the length is 18–23 nucleotides, which are widely expressed in serum and tissues. Through binding with specific base pairs on messenger RNA (mRNAs), the translation of mRNA is inhibited or degraded, resulting in a decrease in the target protein level, which further regulates the intercellular signaling pathways (12-14). Accumulating studies have shown that miRNAs are associated with various biological progression, including proliferation, apoptosis, mitochondrial dysfunction, immune response, and other pathological processes (15-17). The abnormal expression of miRNAs is common in diseases, so the application of miRNAs in the treatment of various diseases has attracted more and more attention (18,19). In sepsis, a study revealed that miR-140 could inhibit WNT11 expression and prohibit skeletal muscle lactate release in septic mice (20). miR-140 is a potential target of circRNA PATG during sepsis. Another study showed that miR-140 could regulate inflammatory factors and myeloperoxidase in lung A549 cells (21).

In this study, our findings investigated the aberrant expression of circRNA PARG found in the blood of septic patients, resulting in dysregulation of expression of the inflammatory and apoptosis-related genes that control the biology of cell progression. In addition, the potential functional mechanism of circRNA PARG is associated with understanding the pathogenesis of sepsis from the perspective of circRNA.

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Materials and Methods

Clinical samples

Septic patients (n = 80) and healthy volunteers (n = 80) were enrolled in the current study. The project was approved by the Ethics Committee of the First Affiliated Hospital of Gannan Medical College. All participants involved in this research provided informed consent.

Cell line and cell transient transfection

The human pulmonary microvascular endothelial cell (HPMEC) was obtained from ATCC and seeded in Dulbecco's modified Eagle’s medium (DMEM), supplemented with fetal bovine serum ([FBS]; 10%) and penicillin (1%). The cell plate was cultured at 37 °C with 5% CO_2. A concentration of 5 mg/ml LPS (Sigma, MO, USA) was prepared in phosphate-buffered saline (PBS), and a final concentration of 10 ug/ml LPS was injected into the medium. LPS was used to stimulate the HPMEC cells, which were further used to investigate the circRNA PARG role in sepsis.

HPMEC cells (1×10^5 cells) per well were cultured in a 6-well plate. When HPMEC cells grew to 70% confluence, cell transfection was performed. For silencing studies, small interfering RNAs targeting circRNA PARG (si-circRNA PARG) and miR-140-3p mimic have been acquired from GenePharma Company (Shanghai, China). Lipofectamine 2000 (Invitrogen) has been utilized for transient transfection. For overexpression studies, circRNA PARG has been constructed into the pcDNA 3.1 vector. Briefly, 50 nMsi-circRNA PARG, 50 nM miR-140-3p mimic, or 10 ug pcDNA-circRNA PARG was used for transfection. After 6 h of transfection, the cell culture medium was replaced with a serum-containing medium for an additional 24 or 48 h. The treated cells were collected for further experiments.

RT-qPCR assay

All RNA was isolated from the HPMECs by TRIzol (Invitrogen). Prepared RNA (500 ng) was directly reverse-transcribed into cDNA and then subjected to PCR. All primers used in the current study were obtained from Sangon Biotech (Shanghai). The internal controls for circRNA and miRNA were glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and U6, respectively. The reaction cycle was set to 40. All relative levels were assayed using the $2^{-\Delta\Delta CT}$ assay. All primers sequences see in Table 1.

circRNA PARG characterization

For actinomycin D (Sigma-Aldrich) treatment, 2 ug/ml reagent was added to HPMEC cells and then incubated for 0, 4, 8, 12, and 24 h. For RNase R (Epicentre Technologies, Madison, USA) treatment, 2 ug reagent was added to the HPMECs for 30 min. Later, total RNA was isolated, and the relative levels of the circular and linear subtypes were measured.

RNA-Fluorescence *in situ* hybridization

Transfected LPS-simulatedHPMECs were culture on cover slips and washed with cytoskeletal (CSK) buffer. The cells were later fixed with 4% paraformaldehyde in a 4 °C incubator for an additional 10 min. The hybridization liquid containing probe (DIGlabelled FAM circRNA PARG or Cy3-labelled miR-140-3p) was denatured at 76 °C for an additional 10 min. The hybrid solution was then dropped on a hanging plate, placed with the cover slide, sealed with rubber cement, and hybridized at 37 °C for 24 h in a wet cassette. After hybridization, the rubber and cover slide was discarded, and the hanging slide was washed with formamide. 4’,6-amidino-2-phenylindole (DAPI) solution was added to the slide, and the coverslip was placed on top and then incubated under a dark. Excessive fluid on the slide was discarded, and the slide was sealed with an antifade reagent seal.

CCK8 assay

CCK8 reagent (100 µL) was injected into the transfected LPS-simulated HPMECs at 0, 24, 48, and 72 h. After 12 h incubation, absorbance was examined at OD_{450} using a microplate reader (Bio-Rad, USA), and the inhibition of the rate proliferation was further calculated.

Colony forming assay

Transfected LPS-simulated HPMECs were cultured in a six-well plate. When the number of cells was greater than 50, the supernatant medium was removed, cells were washed with PBS, and the cells were then stained with 1 ml crystal violet (Sigma-Aldrich). A colony size of 50 µm was determined. All results were performed in triplicate.

Flow cytometry

The apoptosis rate was assayed using Annexin propidium iodide (PI) staining after which flow cytometry was performed. After 48 h, transfected HPMECs were harvested, washed, and centrifuged. A cell level of 1×10^5 in a 100 ul volume was suspended in binding buffer supplemented with 2 ul Annexin-V-FITC in the dark for 15 min. After washing, treated cells were immediately re-suspended in fresh binding buffer, and 1 ul PI was injected into the tube and incubated for 20 min. Cells were examined and analyzed by ModFit. Fl2-w and FL2-A were utilized, and conjoined cells were removed.

Dual-luciferase reporter system

Wild-type circRNA PARG, mutant circRNA PARG, and miR-140-3p were synthesized by Sangon Biotech

Table 1. Primer list.

Gene name	Primer name	Primer sequence
GAPDH	GAPDH-F(homo)	CACCCACTCCTCCACCTTTGA
	GAPDH-R(homo)	TCTCTCTTCCTCTTGTGCTCTTGC
U6	U6-F(homo)	CTCGCTTCGGCAGCACA
	U6-R(homo)	AACGCTTCACGAATTTGCGT
miR-140-3p	miR-140-3p-F	TGCGGCAGTGGTTTTACCCTATG
	miR-140-3p-R	CCAGTGCAGGGTCCGAGGT

(Shanghai, China) and then subcloned into pcDNA 3.1 vector. LPS-simulated HPMECs at a density of 3 × 10^4 per well were seeded in a 24-well plate and then co-transfected with miR-140-3p mimics, miR-NC with pcDNA 3.1 vector-wt-circRNA PARG, or pcDNA 3.1 vector-mut-circRNA PARG. After 48 h, luciferase activities were examined.

Protein extraction and western blotting

The treated HPMECs were collected and resuspended in a radioimmunoprecipitation (RIPA) lysis buffer (Beyotime, China). Each sample (10 ug) was loaded on a sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and immediately transferred into a polyvinylidene fluoride membrane (PVDF) membrane. Next, the membrane was blocked and incubated with the first antibodies (1:1000) at 4 °C for 12 h. On the following day, the treated membrane was incubated with the appropriate horseradish peroxidase (HRP)-labeled secondary antibodies (1:5000; Abcam) for an additional 1 h. Finally, the relative expression level was examined using an electrochemiluminescence (ECL) kit. The primary antibodies included anti-Bcl-2 antibody (#15071), anti-Bax antibody (#5023), anti-cleaved-caspase-3 antibody (#9661), annti-caspase-3 antibody (#9662), and actin (1:3000, #3700), which were obtained from Cell Signaling.

Statistical analysis

All results were obtained in triplicate from independent experiments and were examined by the SPSS software (SPSS, Chicago) and are expressed as the means ± standard deviation (SD). The calculation method was examined using the Student's t-test. $p < 0.05$ indicated statistical significance.

Results

The relative level of circRNA PARG is upregulated in sepsis

The relative level of circRNA PARG in septic patients (n = 80) was found to be upregulated. circRNA PARG showed an increase in expression level when compared with normal groups (n = 80; Figure 1A). The receiver operating characteristic curve (ROC curve; Figure 1B) confirmed similar results. Furthermore, the stability and localization of circRNA PARG increase during the progression of sepsis. After incubation with actinomycin D, circRNA PARG expression presented an unchanged level, and linear mRNA expression was reduced. It is suggested that circRNA PARG presented a stable expression level (Figure 1C). When incubated with RNase R treatment, circRNA PARG expression levels showed no changes, while linear mRNA levels decreased. The results suggest that circRNA PARG shows a stable characteristic. The localization of circRNA PARG in the cytoplasm was analyzed (Figure 1E).

Downregulation of circRNA PARG affects biological functions in LPS-simulated HPMEC cells

As HPMEC cells are a model for studying sepsis, the function of circRNA PARG in HPMEC cells was further detached. Small interfering RNA was applied to produce a circRNA PARG knockdown, and it was used to downregulate circRNA PARG levels in LPS-simulated HPMEC cells. The CCK8 assay was used to detect the expression of PARG. LPS was applied to simulate HPMEC cells for 24, 48, and 72 h after which the optimum circRNA PARG expression was measured. As shown in Figure 2A, circRNA PARG expression reached the highest level at 72 h and exhibited a significant difference compared with the control (p< 0.001). Therefore, LPS simulation at 72 h was performed for subsequent experiments. The colony-forming assay indicated that the number of colonies significantly decreased in LPS-simulated HPMEC cells compared with the control group (p<0.01). In contrast, circRNA PARG silence led to a significant increase in LPS-simulated colonies number (p<0.01; Figure 2B). Furthermore, the influence of circRNA PARG on HPMECcell apoptosis was detected. The apoptosis rate showed a significant increase in LPS-simulated HPMEC cells (p<0.001), while circRNA PARG silence caused a decrease in LPS-simulated apoptosis (p<0.01; Figure 2C).

Downregulated circRNA PARG alleviates LPS-simulated inflammatory and apoptosis responses

The waterfall-like inflammation cascade presents a critical factor in the pathogenic of ARDS. An ELISA assay was used to measure levels of cellular inflammation in cells. As shown in Figure 3A, the mRNA levels of IL-6, -8, and -1β and TNF-α exhibited a remarkable upregula-

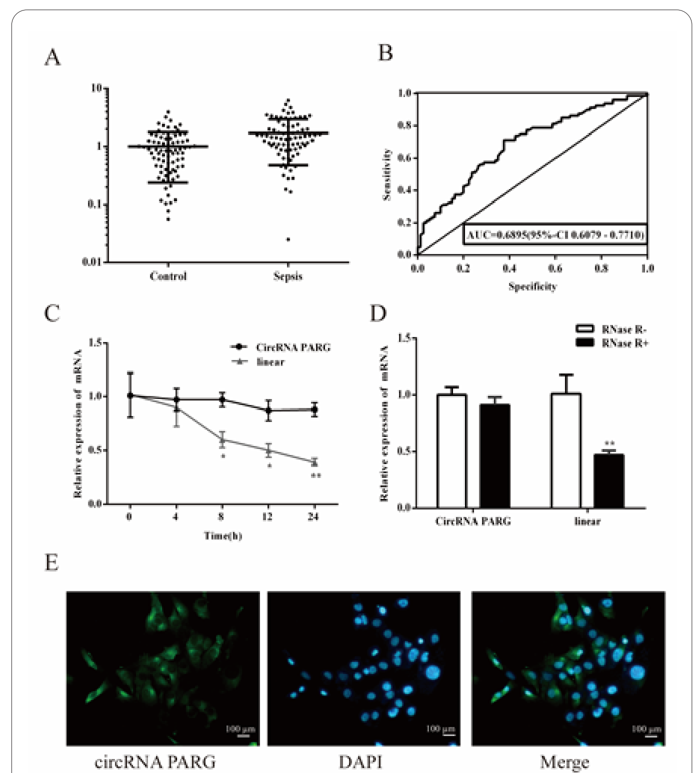

Figure 1. circRNApoly (ADP-ribose) glycohydrolase (PARG) was upregulated in the serum of sepsis patients. A. The expression level of circRNA PARG in the serum of septic patients (n = 80) and healthy people (n = 80) was measured by real-time reverse transcriptase polymerase chain reaction (qRT-PCR). B. C. The mRNA expression of circRNA PARG on human pulmonary microvascular endothelial cells (HPMECs) treated with actinomycin D. D. The mRNA expression of circRNA PARG on HPMEC cells treated with RNase R. E. The localization of circRNA PARG (left) and 4'-6-diamidino-2-phenylindole (DAPI) in the middle is presented, and the merged picture was provided (right). All results were exhibited as mean ± standard deviation (SD) for n = 3 in the triplicate experiments. *p<0.05 and **p<0.01, n = 3.

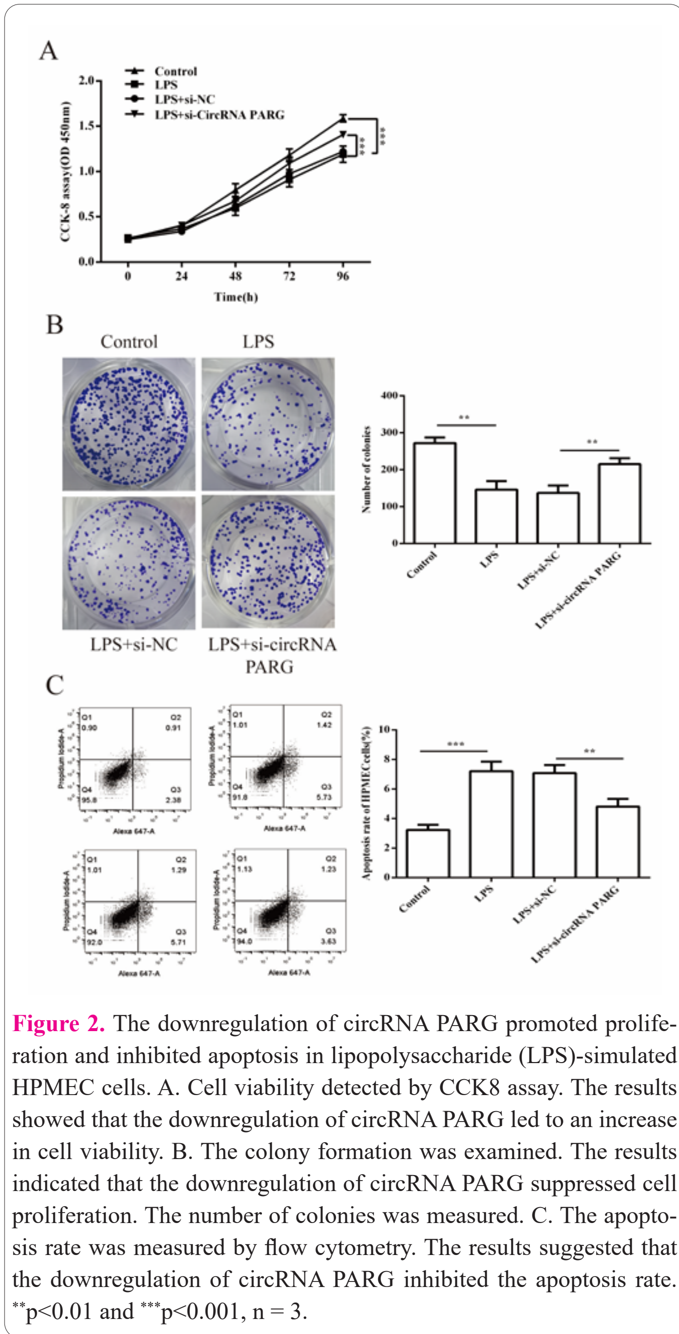

Figure 2. The downregulation of circRNA PARG promoted proliferation and inhibited apoptosis in lipopolysaccharide (LPS)-simulated HPMEC cells. A. Cell viability detected by CCK8 assay. The results showed that the downregulation of circRNA PARG led to an increase in cell viability. B. The colony formation was examined. The results indicated that the downregulation of circRNA PARG suppressed cell proliferation. The number of colonies was measured. C. The apoptosis rate was measured by flow cytometry. The results suggested that the downregulation of circRNA PARG inhibited the apoptosis rate. $^{**}p<0.01$ and $^{***}p<0.001$, n = 3.

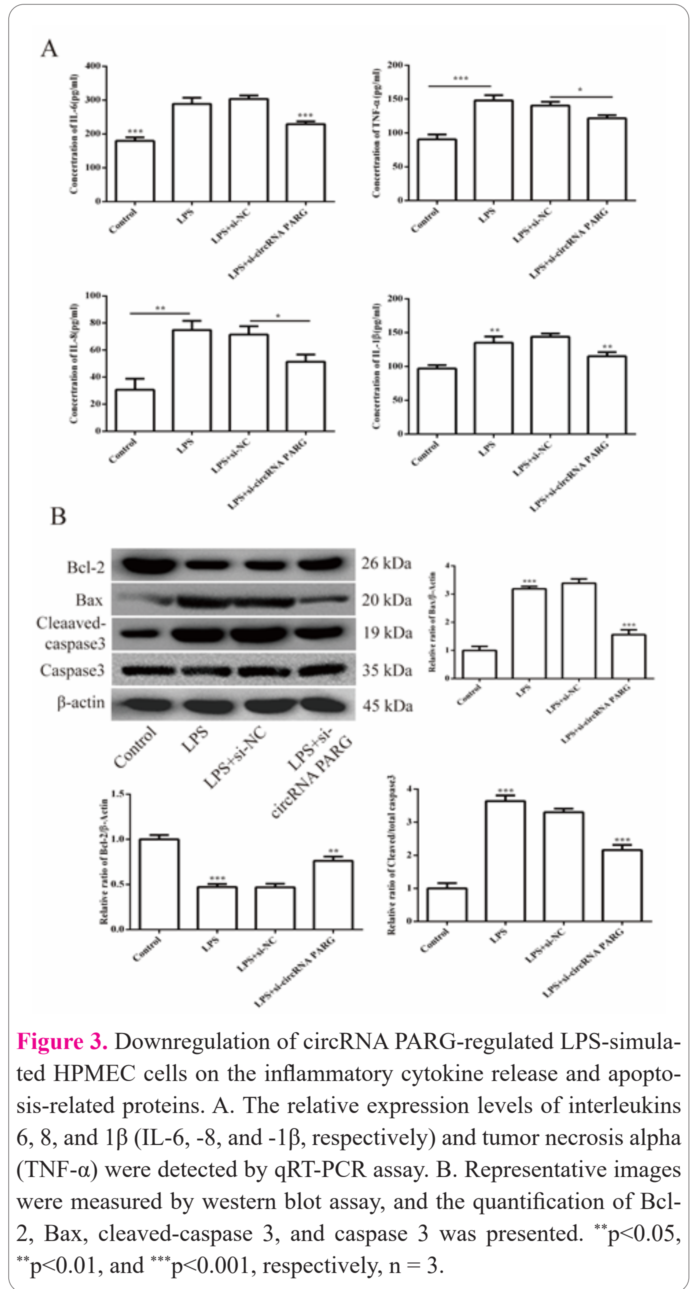

Figure 3. Downregulation of circRNA PARG-regulated LPS-simulated HPMEC cells on the inflammatory cytokine release and apoptosis-related proteins. A. The relative expression levels of interleukins 6, 8, and 1β (IL-6, -8, and -1β, respectively) and tumor necrosis alpha (TNF-α) were detected by qRT-PCR assay. B. Representative images were measured by western blot assay, and the quantification of Bcl-2, Bax, cleaved-caspase 3, and caspase 3 was presented. $^{**}p<0.05$, $^{**}p<0.01$, and $^{***}p<0.001$, respectively, n = 3.

tion in LPS-simulated HPMEC cells, whereas circRNA PARG silence caused a decrease in the LPS-simulated HPMEC expression when compared with the negative one ($p<0.001$, $p<0.05$).

Apoptosis-related genes were further investigated. As seen in Figure 3B, the relative level of Bax and cleaved caspase-3 exhibited remarkable upregulation in LPS-simulated cells ($p<0.001$); however, the protein expression of Bcl-2 decreased ($p<0.001$), while, the relative level of Bax and cleaved caspase-3 was downregulated after circRNA PARG was silenced in LPS-simulated cells when compared with the negative control ($p<0.001$). The relative level of Bcl-2 showed a significant increase ($p<0.01$).

Upregulated circRNA PARG reverses the miR-140-3p influence

To further investigate whether circRNA PARG exerts a biological effect through miR-140-3p, the relationship between circRNA PARG and miR-140-3p was examined using a dual-LUC activity system. Our data indicate that circRNA PARG could be a possible target of miR-140-3p and affect its expression ($p<0.01$; Figure 4A). Upregulated circRNA PARG led to an increase in the effects on proliferation and colony formation induced by miR-140-3p (Figure 4B and C). In contrast, circRNA PARG and miR-140-3p upregulation caused a reversal in apoptosis suppression by the miR-140-3p mimic ($p<0.01$; Figure 4D). Additionally, it was further confirmed that these RNAs all co-localized in the cytoplasm (Figure 4E). These results suggest that circRNA PARG and miR-140-3p had a role in LPS-simulated HPMEC cells.

Upregulated circRNA PARG and miR-140-3p function on LPS-simulated inflammatory and apoptosis responses

Apoptosis-related genes and inflammatory factors were also measured. As shown in Figure 5A, the relative level of IL-6, -8, -1β and TNF-α presented a significant downregulation in LPS-simulated HPMEC cells after transfection with miR-140-3p mimic when compared with the negative groups ($p<0.01$). In contrast, upregulated circRNA PARG led to a significant increase in the expression of these factors ($p<0.01$). Additionally, the levels of Bax and cleaved caspase-3 decreased in LPS-simulated cells transfected

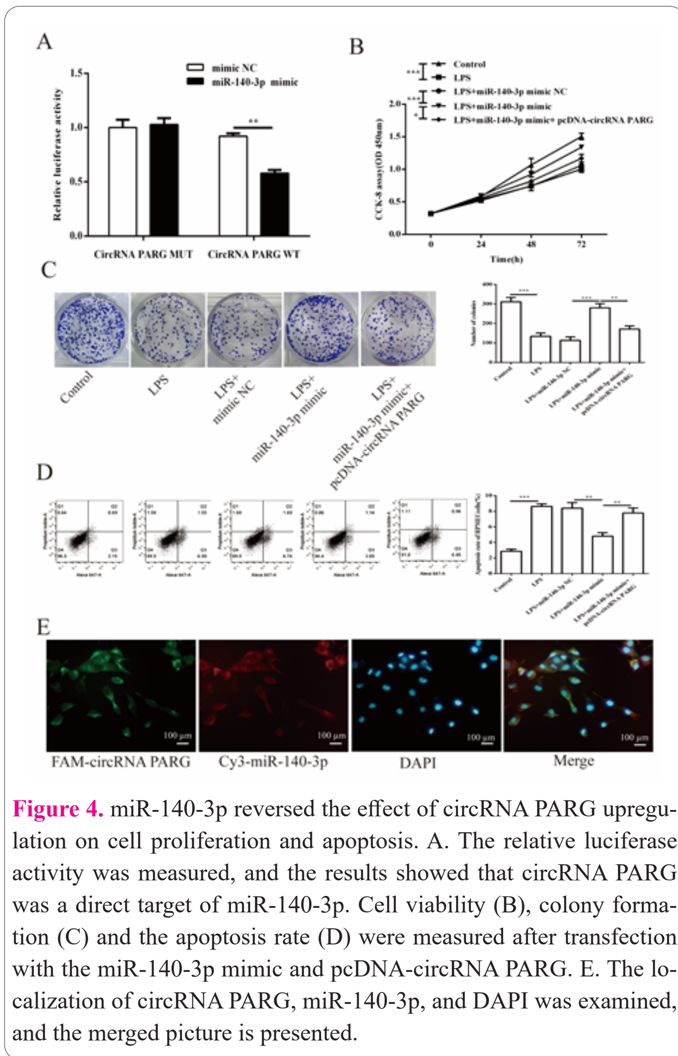

Figure 4. miR-140-3p reversed the effect of circRNA PARG upregulation on cell proliferation and apoptosis. A. The relative luciferase activity was measured, and the results showed that circRNA PARG was a direct target of miR-140-3p. Cell viability (B), colony formation (C) and the apoptosis rate (D) were measured after transfection with the miR-140-3p mimic and pcDNA-circRNA PARG. E. The localization of circRNA PARG, miR-140-3p, and DAPI was examined, and the merged picture is presented.

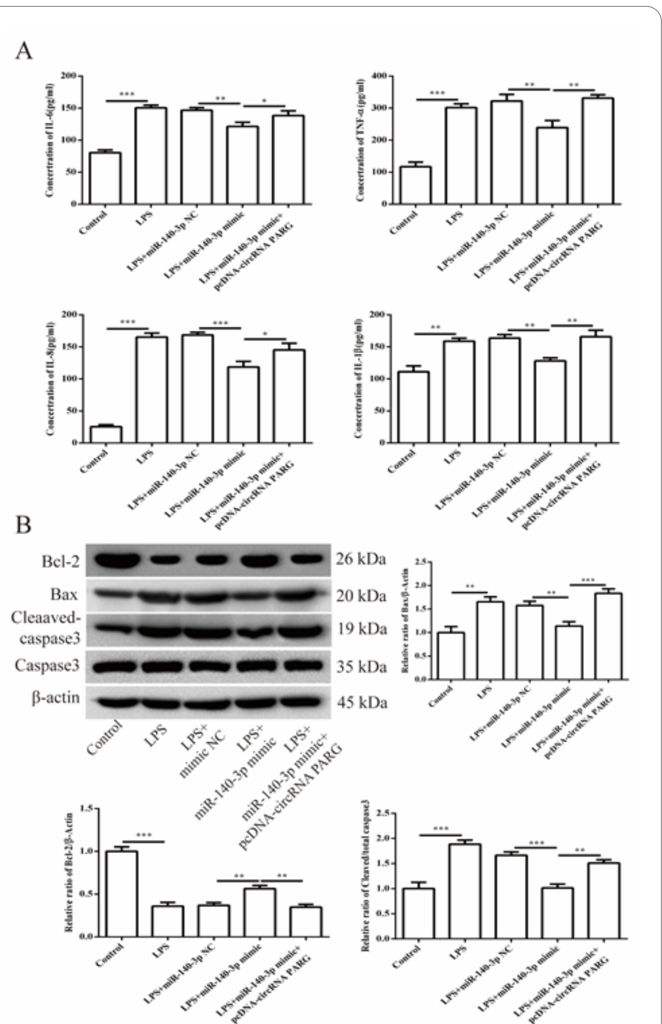

Figure 5. The upregulation of circRNA PARG modulated LPS-simulated HPMEC cells on the inflammatory cytokine release and apoptosis-related proteins. A. The relative expression levels of IL-6, -8, and -1β and TNF-α were measured by qRT-PCR assay. B. The relative protein levels of Bcl-2, Bax, cleaved-caspase 3, and caspase 3 are presented. $^{**}p < 0.05$, $^{**}p < 0.01$, and $^{***}p < 0.001$, respectively, n = 3.

with miR-140-3p mimic group (p<0.01), and the level of Bcl-2 increased (p<0.01). Interestingly, upregulated circRNA PARG caused a reversal in protein expression levels of apoptosis-related genes induced by the miR-140-3p mimic (Figure 5B).

Discussion

Sepsis is a serious infection-related issue in different types of organ dysfunction and is considered one of the causes of high morbidity and mortality rates in the ICU. So far, no effective treatment for sepsis has been found (22). The investigation of molecular mechanisms in sepsis progression could ameliorate diagnostic and therapeutic treatments, resulting in a reduced risk for sepsis patients. Noncoding RNAs have been demonstrated to be associated with the regulation of sepsis pathophysiology and could all be considered novel markers for sepsis patients (23-26).

Due to their properties, circRNAs present stable and less degradable characteristics and have been demonstrated with different diseases, including sepsis (27-29). For all these reasons, circRNAs may exert a critical function in sepsis and have been considered as novel biomarkers for sepsis treatment (30,31). However, the direct function that circRNAs might play in sepsis progression remains unknown because elucidation of the function of circRNAs has only become relevant in recent years. To date, several pieces of evidence have strongly implied that circRNA might play a key role as a molecular sponge to adjust the function of target miRNAs. For example, CircVMA21 attenuates sepsis by causing a reduction in inflammation and apoptosis in rats and HK2 cells via modulation of miRN-9-39 (32). CircPRKCI alleviates LPS-simulated HK2 cell injury by acting as a sponge for the miR-545 targetZEB2 gene (33). In this study, it was demonstrated for the first time that serum circRNA PARG levels were higher in septic patients when compared with healthy volunteers. Downregulated circRNA PARG prevented pulmonary microvascular endothelial cells from undergoing apoptosis.

Interestingly, circRNAs harbor the miRNA binding site, adjust miRNA levels, and regulate mRNA expression levels (10). miRNAs act as critical elements of cell maintenance (34,35). In this study, it was found that circRNA PARG directly targeted miR-140-3p, and both of them were localized in the cytoplasm. Upregulation of miR-140-3p in LPS-simulated HPMEC cells promoted proliferation and suppressed apoptosis, whereas upregulated circRNA PARG could reverse these effects. CircRNA PARG acts as a critical mediator of HPMEC cell progression and exhibits the activity of possible tumor inducer because it decreases proliferation and increases apoptosis via targeting miR-140-3p.

It was known that the critical progression in sepsis is the

cytokine storm, which influences and mediates the initial anti-inflammatory period by affecting inflammatory cytokine levels (36). These elements can strongly influence the development of sepsis (37-39). In this study, circRNA PARG led to a significant upregulation of the expression of TNF-α and IL-6, -8, and IL-1β. More importantly, circRNA PARG also modulated apoptosis-related genes, including Bcl-2, Bax, and cleaved-caspase3. Taken together, these findings suggest that the possible underlying activity for circRNA PARG is closely related to sepsis progression. This finding could provide a novel perspective on circRNAs as promising therapeutic targets for sepsis.

However, the interaction between circRNA PARG and miR-140 might be a part of the mechanism of circRNA PARG in sepsis. The target of miR-140 needs to be resolved in future experiments. Whether the circRNAPARG/miR-140 axis forms a central pathway in the regulation in inflammatory factors and apoptosis-related genes is still unclear.

The research indicates for the first time that circRNA PARG expression is highly upregulated in septic patients. Upregulated circRNA PARG led to the enhancement of proliferation and suppression of apoptosis. Functionally and mechanistically, circRNA PARG modulated cell progression by adjusting miR-140-3p levels and downregulation of inflammatory and apoptosis-related protein expression. It reveals the pro-inflammatory characteristics of circRNA PARG during sepsis progression.

Ethical recognition and consent to participation

Not Applicable.

Agree to published

All the authors agreed to be published.

Disclosure statement

The authors of this study declare no conflict of interest.

Fundings

Not Applicable.

Author contributions

Hongyi Zheng and Chaoyu Wu performed the majority of the research and wrote the manuscript. Xianghong Liu, Jinliang Peng and ZhengbiaoXue was involved in the field study, analyzed data and interpreted the data. Chaoyu Wu designed the study and revised the manuscript.provided valuable discussions.

Data availability statement

The data used to support the findings of this study are included within the article.

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