Article

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MiR-342-5p Alleviates Inflammatory Responses in Sepsis-Induced AKI by Targeting PFN1

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Background: The development of several human diseases has been linked to microRNA-342-5p (miR-342-5p). However, its specific function in the inflammatory reactions associated with sepsis-triggered acute kidney injury (AKI) remains unexplored. This study aims to delve into the role of miR-342-5p in the progression of sepsis-induced AKI.

Methods: To understand the underlying mechanism of miR-342-5p in sepsis, we established sepsis models through induction with lipopolysaccharide (LPS). The cell proliferation and apoptosis were assessed using the 5-ethynyl-2′-deoxyuridine (EdU) method and flow cytometry, respectively. Bioinformatics analysis was employed to predict the downstream binding target of miR-342-5p, and their interaction was confirmed through a dual-luciferase reporter gene assay. The rescue tests were conducted to further explore the action pathway of miR-342-5p.

Results: The administration of LPS significantly decreased the HK2 cell proliferation ($p < 0.05$), and increased cell apoptosis. Additionally, there was a notable downregulation in miR-342-5p expression accompanied by an elevated level of inflammatory factor ($p < 0.05$). Furthermore, the overexpression of miR-342-5p effectively attenuated the LPS-induced inflammatory response in HK2 cells ($p < 0.05$). Profilin-1 (PFN1) emerged as a pivotal player in sepsis pathogenesis, serving as a critical downstream target of miR-342-5p. Interestingly, rescue experiments demonstrated that the stimulatory effects of miR-342-5p mimics were counteracted by the overexpression of PFN1 in sepsis.

Conclusions: MiR-342-5p alleviates inflammatory responses in sepsis by targeting PFN1.

Keywords: miR-342-5p; inflammation; LPS; PFN1; sepsis

Introduction

Sepsis, characterized by abnormalities in physiological, pathological, and biochemical processes, is triggered by infections [1]. As a significant public health concern [2,3], severe sepsis contributes to organ failure by escalating oxidative stress and inflammatory responses [4]. Sepsis represents the leading cause of AKI, accounting for over 50% of cases, with a higher mortality rate up to 70%. Nevertheless, the pathophysiological mechanisms underlying sepsis-induced acute kidney injury (AKI) remain incompletely understood.

Accurately predicting the incidence and trends of sepsis-induced AKI has been challenging. According to the published results, an inflammatory response similar to sepsis-induced AKI can be triggered by lipopolysaccharide (LPS) in mice and cells [5–7]. The ideas of sepsis/inflammatory response induction, control, and effector mechanisms (including mediators) have been documented [8]. Inflammatory signals initiate the differentiation of M1 macrophages, a process known as polarization. An excessive or prolonged release of cytokines that promote inflammation, can activate monocyte/macrophage cell lines and enzymes called matrix metalloproteinases (or collagenases). Furthermore, cytokines with anti-inflammatory properties help in modulating the severity of sepsis, thereby influencing the development, multiplication, and isotype switching of B cells [9–11]. Unfortunately, the intricate regulatory mechanisms underlying sepsis-induced AKI remain largely unexplored and enigmatic.

Epigenetically, miRNAs regulate the transcriptional expression or inhibit the degradation of mRNAs [12]. This regulation occurs through a specific and direct interaction, where the 5′ end of the miRNA binds to a non-coding region at the 3′ end of the target mRNA, encompassing 6–8 nucleotides [13,14]. MiRNAs have been identified as controllers of multiple aspects of inflammation in many diseases [15–17]. For instance, research has demonstrated that miR-181a is abundantly expressed in exosomes originating from mesenchymal stem cells, thereby modulating the inflammatory response [18]. MiR-543-3p promotes the expression of anti-inflammatory genes and MDSC-regulating genes, including IL-10 [19]. Previous results have indicated that the downregulation of microRNA-342-5p (miR-342-5p) exacerbates neonatal bronchopulmonary dysplasia through the Raf1 regulator Spred-3 [20]. Furthermore,

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miR-342-5p suppresses the growth and development of osteosarcoma by regulating Wnt7b [21]. However, the mechanism of the impact of the miR-342-5p on the onset of sepsis is still unknown.

Profilin-1 (PFN1) serves as a crucial actin-binding protein that regulates the cellular processes such as migration and division. Mutation or deletion of PFN1 can also result in certain human diseases, making it a highly researched topic. PFN1 exhibits inhibitory effects on breast cancer cell proliferation and upregulates the PTEN gene, thereby suppressing the AKT pathway. Consequently, it leads to the elevation of p27 protein, which binds to the Cdk2 complex, inducing G1-phase cell cycle arrest and ultimately inhibiting cell proliferation [22]. In addition, PFN1 promotes vascular wall thickening and lumen diameter reduction by activating JAK2-STAT3, JNK, and p38-MAPK, ultimately leading to hypertension [23]. The role of the PFN1 in cancer is well established, but its involvement in sepsis remains relatively unexplored. This study aimed to investigate the relationship between miR-342-5p and PFN1 in LPS-treated HK2 cells, and evaluate their impact on the occurrence and progression of sepsis.

Materials and Methods

Cell Culture and Transfection

HK-2 cells, which are human proximal tubule epithelial cells, were acquired from SUNNCELL (SNL-165, Wuhan, China) and cultured in DMEM supplemented with 10% fetal bovine serum and 1% streptomycin/penicillin. The cells were incubated at 37 °C in a 5% CO_2 environment. The STR test confirmed the authenticity of the cells as HK2 and verified the absence of any cross-contamination. All cells have passed mycoplasma testing.

The cells underwent transfection using Lipofectamine® 2000 (Invitrogen, Carlsbad, CA, USA), which was diluted in a serum-free medium. The miR-342-5p mimic, miR-342-5p inhibitor, pcDNA3.1-(+)-PFN1 (pc-PFN1), and their respective controls (NC mimic, NC inhibitor, and pcDNA3.1-(+)) were obtained from GenePharma (GP-ZL-0495, Suzhou, China) and diluted to a concentration of 50 mol/L in a serum-free medium. After a 12-hour (h) transfection period, the status of the transfected cells was closely monitored. Subsequently, the RNA was extracted from the cells at 48 h post-transfection, and its quantity was precisely determined using quantitative reverse transcription polymerase chain reaction (qRT-PCR).

Detection of Content of TNF-α, IL-1β, and IL-6

Cells were initially seeded into a 96-well plate at a density of 1 million cells per mL and allowed to adhere for 24 h. Subsequently, they were exposed to various concentrations of LPS (0.1, 1 and 5 mg/L; 916374, Sigma, St. Louis, CA, USA) for 24 h. After this exposure, the supernatants were carefully removed, and the levels of tumor necrosis factor-alpha (TNF-α) (Abs551104T), interleukin-1 beta (IL-1β) (Abs551101), and interleukin-6 (IL-6) (Abs551103) were quantitatively assessed using specific enzyme-linked immunosorbent assay (ELISA) kits provided by Absin (Shanghai, China).

Table 1. Primers used for qRT-PCR.

Gene		Primer sequence (5′-3′)
miR-342-5p	Forward	ACTAGGGGTGCTATCTGTGA
	Reverse	GTGCAGGGTCCGAGGT
PFN1	Forward	CATCGTGGGCTACAAGGACTCG
	Reverse	CCAAGTGTCAGCCCATTCACGT
GAPDH	Forward	TATGATGATATCAAGAGGGTAGT
	Reverse	TGTATCCAAACTCATTGTCATAC
U6	Forward	GCTTCGGCAGCACATATACTAAAAT
	Reverse	CGCTTCACGAATTTGCGTGTCAT

qRT-PCR, quantitative reverse transcription polymerase chain reaction; *PFN1*, profilin-1; *miR-342-5p*, microRNA-342-5p.

qRT-PCR

RNA was extracted from cellular and tissue samples using TRIzol reagent (15596026, Thermo Fisher Scientific, Waltham, MA, USA), ensuring complete dissolution. Subsequently, reverse transcription was carried out with 10 µL of 5× PrimeScript® RT Master Mix (RR036A, Takara, Otsu, Japan). qRT-PCR was then executed using SYBR Premix Ex Taq™ (DRR041A, Takara, Otsu, Japan), facilitated by a Real-Time PCR System (StepOnePlus Real-Time PCR System, Thermo Fisher Scientific, Waltham, MA, USA). The primer sequences used can be found in Table 1. Relative gene expression was determined and normalized through the comparative $2^{-\Delta\Delta Ct}$ method.

5-Ethynyl-2′-Deoxyuridine (EdU) Assay

The cells were inoculated into a 96-well plate (8×10^3 cells/well). After 24 h, each well was added with 100 µL of 50 µM EdU solution (40284ES60, Yeasen, Shanghai, China) and cells were treated for 2 h. Then, the cells were fixed with 4% methanol and exposed to fluorescently labeled EdU for 30 minutes (min). Finally, the cells were stained with DAPI (40728ES03, Yeasen, Shanghai, China) and photographed in three randomly selected fields under an inverted fluorescence microscope (IX73, Olympus, Tokyo, Japan).

Flow Cytometry

After centrifugation, the cells were resuspended in 100 µL of propidium iodide (PI) (40744ES60, Yeasen, Shanghai, China) in the dark for 15 min. Subsequently, the apoptotic cells were distinguished using the Annexin V-FITC/PI Apoptosis Detection kit (40302ES50, Yeasen, Shanghai, China). Finally, flow cytometry was employed to analyze the cells.

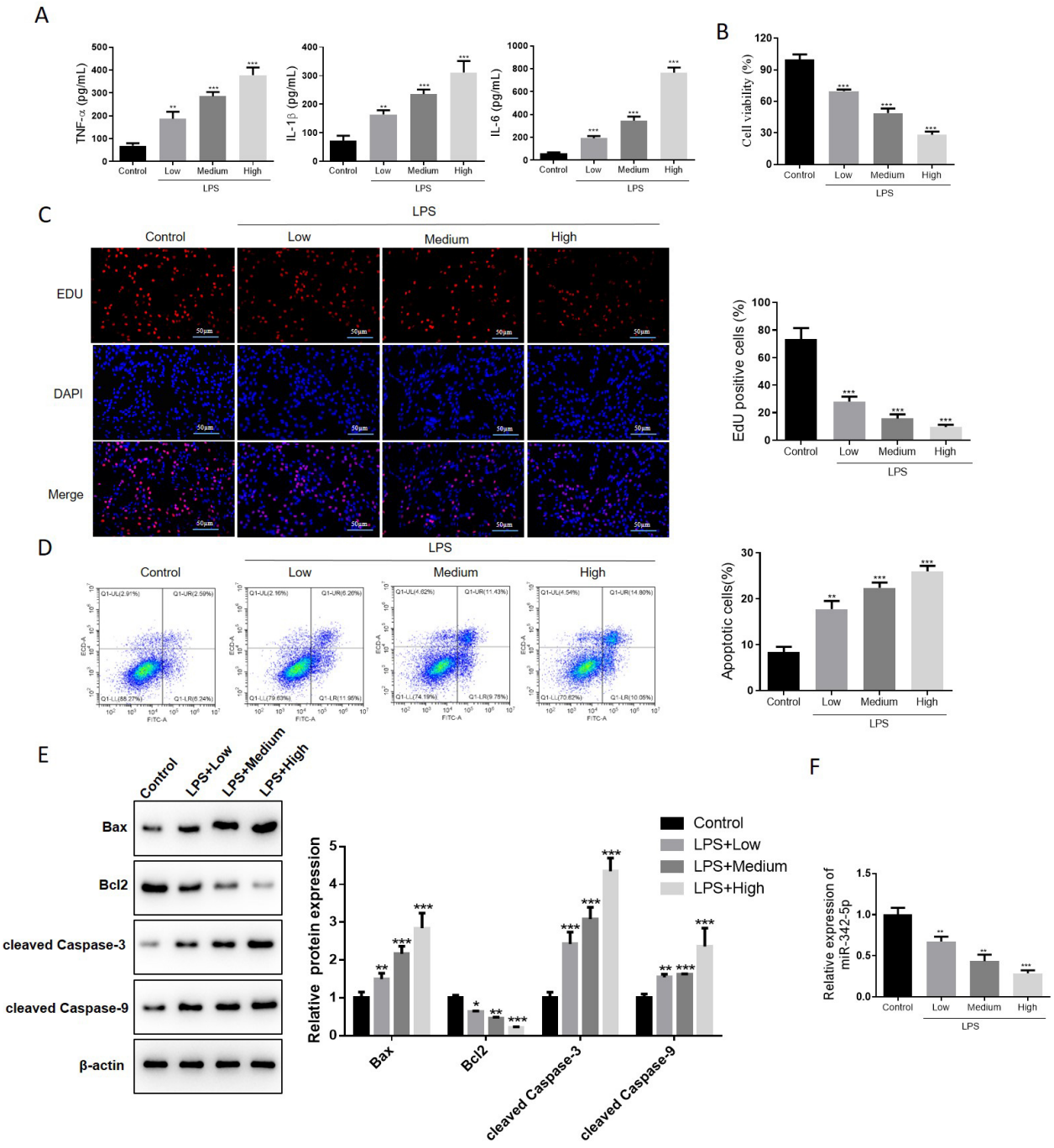

Fig. 1. The expression of miR-342-5p decreased in lipopolysaccharide (LPS)-treated HK2 cells. Cells were exposed to varying concentrations of LPS, and their responses were subsequently analyzed. (A) Enzyme-linked immunosorbent assay (ELISA) analysis was employed to assess the contents of tumor necrosis factor-alpha (TNF-α), interleukin-1 beta (IL-1β), and interleukin-6 (IL-6) inflammatory cytokines known to be involved in immune responses. (B,C) Cell viability was evaluated using both Cell Counting Kit-8 (CCK-8) and 5-ethynyl-2'-deoxyuridine (EdU) assays, which provide distinct insights into cell metabolic activity and proliferative capacity. (D) Flow cytometry was performed to quantitatively assess the apoptosis of cells treated with LPS, allowing for a precise measurement of apoptotic rates. (E) Western blot analysis was performed to measure the expression of proteins fundamental to apoptosis and cell death regulation, including BCL2-Associated X (Bax), B-cell lymphoma-2 (Bcl-2), cleaved caspase-3, and cleaved caspase-9. (F) qRT-PCR was employed to gauge the levels of miR-342-5p. *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared to the control group. Data are from three independent experiments.

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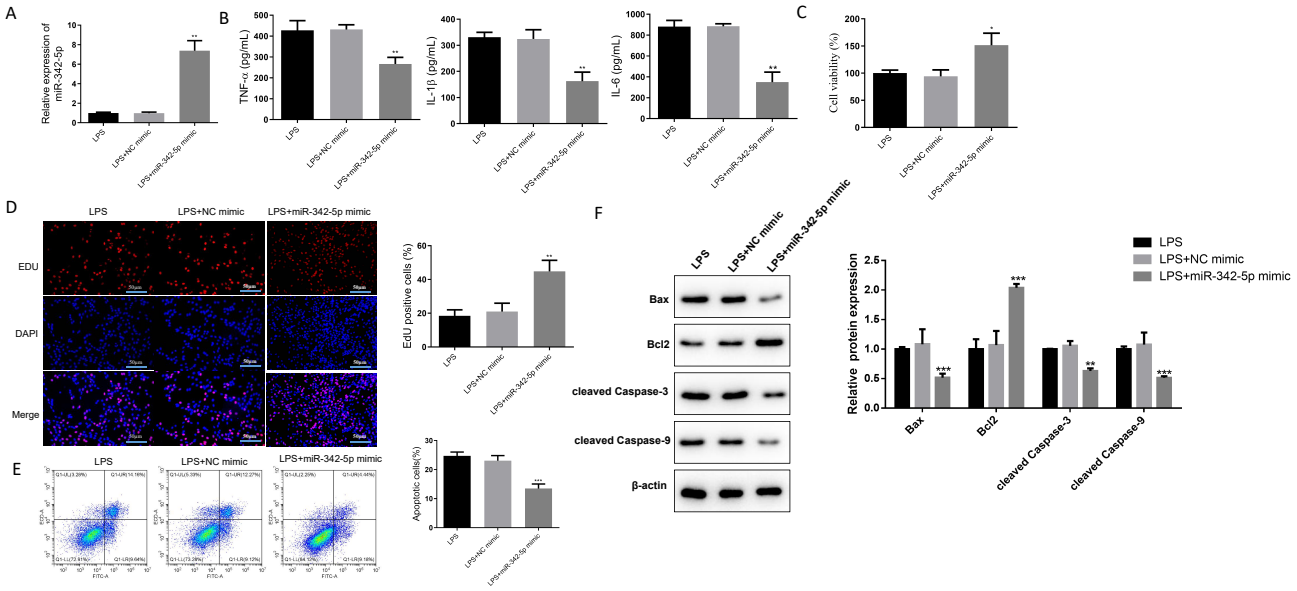

Fig. 2. Overexpression of miR-342-5p delayed LPS-induced inflammation in HK2 cells. (A) The efficiency of miR-342-5p overexpression was determined. (B) TNF-α, IL-1β, and IL-6 protein levels were significantly reduced, as assessed by ELISA. (C,D) Cell viability and proliferation were evaluated through CCK-8 and EdU assays, respectively (scale bar = 50 µm). (E) Apoptosis in LPS-treated cells was assessed using flow cytometry. (F) Expressions of Bax, Bcl-2, cleaved caspase-3, and cleaved caspase-9 were quantified via western blot analysis. All comparisons yield statistical significance (*$p < 0.05$, **$p < 0.01$, ***$p < 0.001$) relative to the LPS+NC mimic group, with data from three independent experiments.

Dual-Luciferase Reporter Gene Assay

To develop the reporter constructs, vectors containing either wild-type (PFN1-WT) or mutant (PFN1-MUT) versions of the PFN1 gene were engineered along with pGL3 vectors carrying WT or MUT sequences for miR-342-5p binding sites. A luciferase cassette in the psiCHECK-2 vector was transcribed upon mutation of the miR-342-5p binding site within the 3′ untranslated region (UTR) of PFN1. Subsequently, HK2 cells were transfected using Lipofectamine 2000 (11668019, Thermo Fisher Scientific, Waltham, MA, USA). After 48 h, the activity of Renilla luciferase was quantified using a dual-luciferase reporter gene assay kit sourced from Yeasen (11402ES60, Shanghai, China).

Western Blotting

The concentration of the extracted protein was determined using a Bicinchoninic Acid (BCA) kit (P0009, Beyotime, Shanghai, China). Following isolation, the purified protein was mixed with a sample buffer and subjected to denaturation by rigorous heat treatment at an elevated temperature for a precise duration of five min. Afterward, the protein samples were loaded onto a 10% SDS-PAGE gel (30 mg/well) and transferred to a PVDF membrane (88518, Millipore, Bedford, MA, USA). The membrane was blocked with BSA and incubated overnight at 4 °C with primary antibodies, including BCL2-Associated X (Bax) (1:2000, ab182733, Abcam, Boston, MA, USA), B-cell lymphoma-2 (Bcl-2) (1:2000, ab182858, Abcam, Boston, MA, USA), PFN1 (1:2000, ab124904, Abcam, Boston, MA, USA), β-actin (1:10,000, ab8226, Abcam, Boston, MA, USA), cleaved caspase-3 (1:2000, ab32042, Abcam, Boston, MA, USA), and cleaved caspase-9 (1:2000, sc-56077, Santa Cruz Biotechnology, Santa Cruz, CA, USA). The following day, the membrane was incubated for 1 h with HRP-conjugated anti-rabbit secondary antibodies (1:10,000, ab6721, Abcam, Boston, MA, USA). Finally, the gray values were analyzed and interpreted employing Quantity One software (version 4.62, Bio-Rad Laboratories, San Francisco, CA, USA).

Statistical Analysis

Each assay was replicated three times to ensure reproducibility and consistency. All the data were described as mean ± standard deviation and analyzed using one-way ANOVA and *t*-test. $p < 0.05$ was considered statistically significant.

Results

The Expression of MiR-342-5p Decreased in LPS-Treated HK2 Cells

The results in Fig. 1A revealed that the levels of pro-inflammatory cytokines were significantly elevated with the increasing concentration of LPS, indicating a significant trend ($p < 0.05$). Furthermore, Fig. 1B illustrates that LPS reduced cell viability in a concentration-dependent manner,

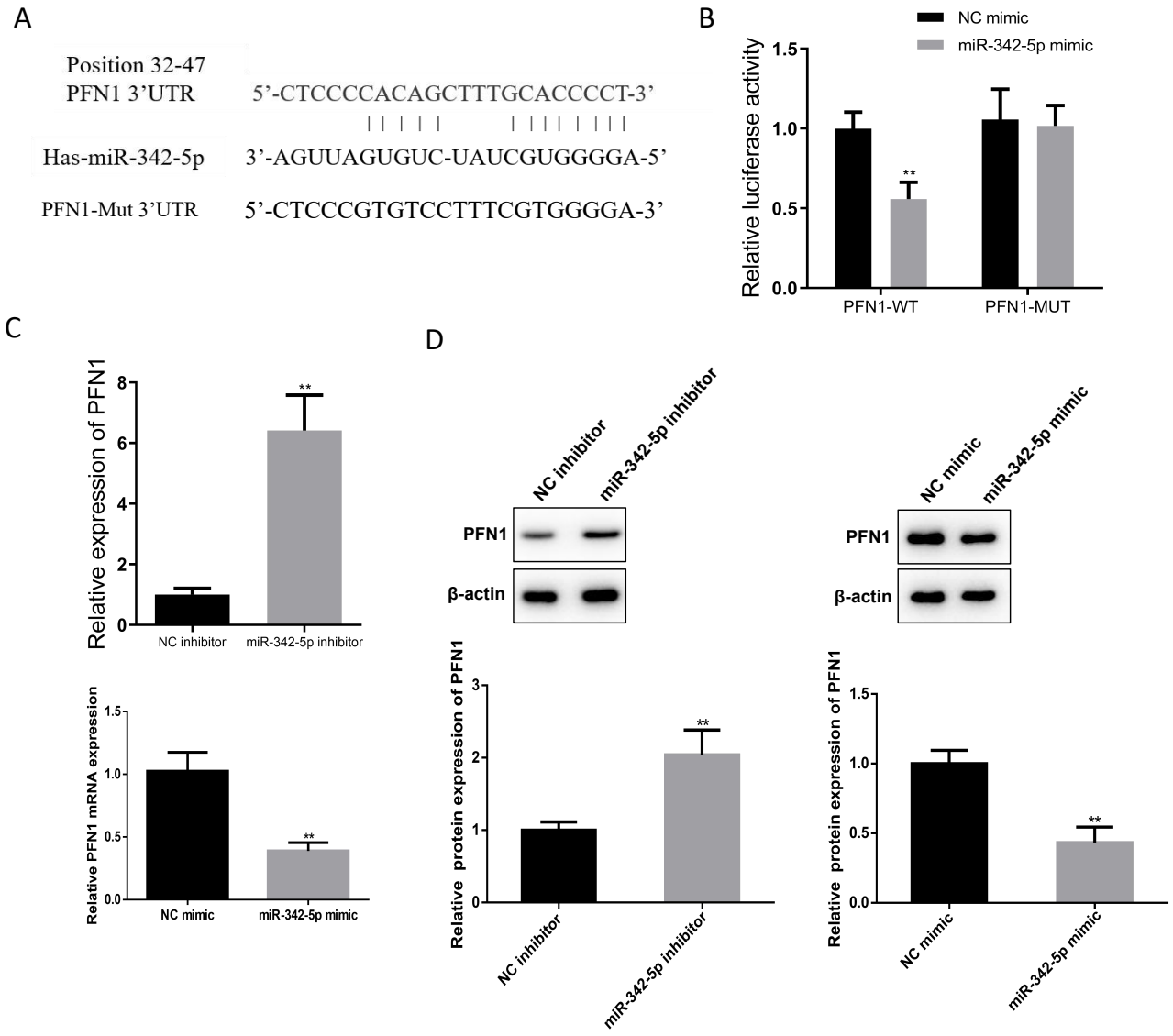

Fig. 3. PFN1 functioned as a primary target of miR-342-5p in LPS-treated HK2 cells. (A) Prediction of binding sites between miR-342-5p and PFN1 was accomplished utilizing TargetScan, ENCORI, and miRWalk bioinformatics tools. (B) A dual-luciferase reporter gene assay was conducted to elucidate the interplay between miR-342-5p and PFN1. A dual-luciferase reporter gene assay was conducted to investigate the relationship between miR-342-5p and PFN1. (C) Changes in the PFN1 mRNA level were assayed using qRT-PCR. (D) HK2 cells exposed to LPS were subsequently treated with either the miR-342-5p inhibitor or miR-342-5p mimic, followed by western blotting to assess PFN1 protein levels. Statistical significance was denoted as $^{**}p < 0.01$ compared to the negative control (NC) mimic or NC inhibitor, with data from three independent experiments.

with higher concentrations leading to greater decreases in cell viability ($p < 0.05$). EdU staining results revealed that LPS decreased the number of EdU-positive cells, indicating a reduction in cell proliferation ($p < 0.05$; Fig. 1C). Flow cytometry analysis in Fig. 1D further confirmed the induction of apoptosis in HK-2 cells by LPS, with a significant increase in apoptotic cells compared to the control group ($p < 0.05$). Finally, Fig. 1E shows that compared to the control group, Bax, cleaved caspase-3, and cleaved caspase-9 were upregulated, while Bcl-2 was downregulated in the LPS group, indicating alterations in apoptotic markers ($p < 0.05$). Intriguingly, Fig. 1F reveals a substantial decrease in miR-342-5p expression, which is known to play a role in inflammation, specifically in the LPS-treated group ($p < 0.05$).

Overexpression of MiR-342-5p Delayed LPS-Irritatived Inflammation in HK2 Cells

To investigate its function, miR-342-5p was overexpressed in LPS-treated HK2 cells (5 mg/L), and the transfection efficiency is displayed in Fig. 2A. Evaluation of the levels of TNF-α, IL-1β, and IL-6 revealed significant de-

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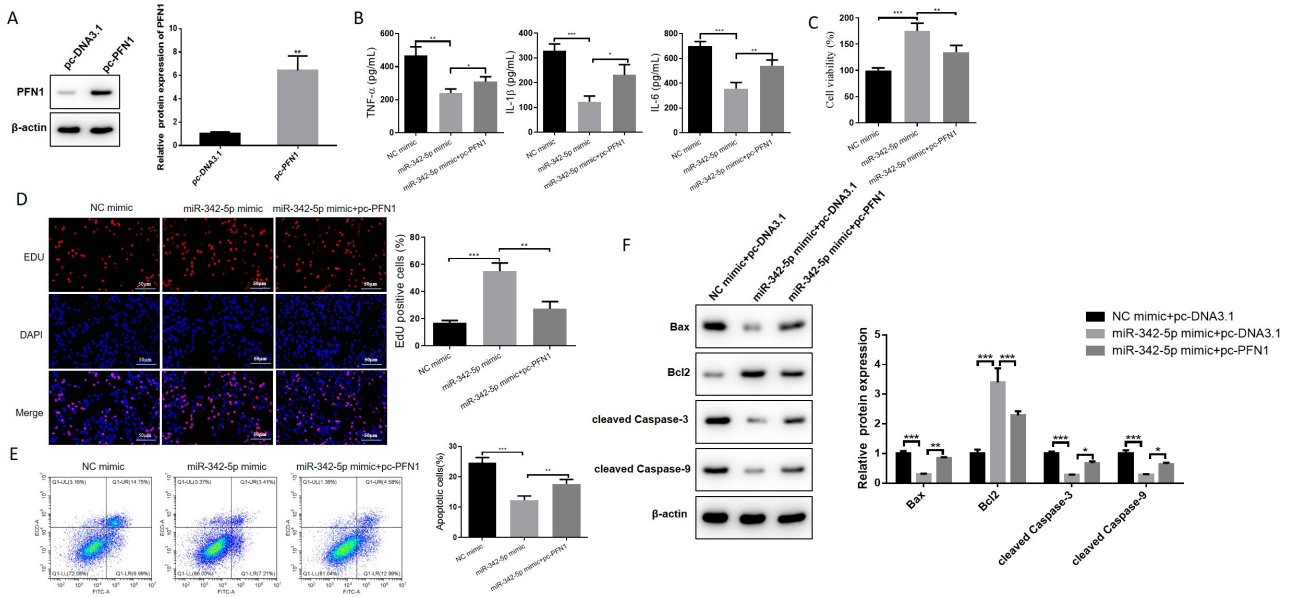

Fig. 4. Inflammation developed in LPS-treated HK2 cells through the miR-342-5p/PFN1 axis. (A) Protein expression of PFN1 was detected by western blot analysis, $^{**}p < 0.01$ versus pc-DNA3.1. (B) Expressions of TNF-α, IL-1β, and IL-6 were measured using ELISA. (C) Cell viability was assessed by CCK-8 analysis. (D) Proliferation ability was assessed by EdU analysis. (E) Apoptosis ability was quantified using flow cytometry. (F) Western blot analysis was employed to quantify the protein expression of Bax, Bcl-2, cleaved caspase-3, and cleaved caspase-9, scale bar = 50 μm, statistical significance was determined with $^{**}p < 0.01$ and $^{***}p < 0.001$ compared to NC mimic and $^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$ compared to miR-342-5p mimic, with data from three independent experiments.

creases in the LPS+miR-342-5p mimic group ($p < 0.05$; Fig. 2B). In contrast, the LPS+miR-342-5p mimic condition improved cell viability and increased the number of EdU-positive cells, indicating a boost in cellular proliferation ($p < 0.05$; Fig. 2C,D). The results of flow cytometry revealed that upregulation of miR-342-5p reduced the LPS-induced apoptosis ($p < 0.05$; Fig. 2E). Furthermore, the results showed that miR-342-5p upregulation led to a rise in Bcl-2 expression, whereas Bax, cleaved caspase-3, and cleaved caspase-9 were downregulated in the LPS+miR-342-5p mimic group ($p < 0.05$; Fig. 2F), suggesting a protective effect on cell survival.

PFN1 Functioned as a Primary Target of MiR-342-5p

To unravel the molecular mechanism underlying miR-342-5p's stimulatory effects on HK2 cell proliferation and survival, we employed TargetScan, ENCORI, and miRWalk to identify potential targets. It was determined that PFN1 was the key downstream target of miR-342-5p (Fig. 3A). To demonstrate their relationship, the 3' UTR of PFN1-WT or PFN1-MUT luciferase reporter vectors was created and delivered onto cells. The data demonstrated a significant decrease in luciferase activity upon miR-342-5p overexpression when the wild-type vector was used ($p < 0.05$), while no effect was observed with the mutant construct (Fig. 3B). Additionally, western blotting and qRT-PCR further validated the direct targeting of PFN1 by miR-342-5p, as the inhibitor of miR-342-5p led to an increase in both mRNA and protein levels of PFN1 in HK2 cells ($p < 0.05$; Fig. 3C,D), whereas overexpression of miR-342-5p resulted in a corresponding decrease.

Inflammation Developed in LPS-Treated HK2 Cells through the MiR-342-5p/PFN1 Axis

To determine whether PFN1 influences the regulatory role of miR-342-5p on LPS-treated HK2 cells, PFN1 was overexpressed using pc-DNA3.1 plasmids. The overexpression efficiency is depicted in Fig. 4A. MiR-342-5p mimic decreased the protein levels of TNF-α, IL-1, and IL-6, which were counteracted by overexpression of PFN1 ($p < 0.05$; Fig. 4B). As shown by the Cell Counting Kit-8 (CCK-8) assay, overexpression of PFN1 counteracted the pro-proliferation effects of miR-342-5p mimic. Similarly, overexpression of PFN1 reduced the number of EdU-positive cells ($p < 0.05$; Fig. 4C,D). Notably, the protective effect of miR-342-5p mimic against LPS-induced apoptosis in HK2 cells was abated by PFN1 overexpression, as evidenced by increased cell apoptosis ($p < 0.05$; Fig. 4E). According to western blotting, PFN1 overexpression partially reversed the anti-apoptotic influence of miR-342-5p ($p < 0.05$; Fig. 4F).

Discussion

LPS, a component of the outer membrane in Gram-negative bacteria, is renowned for triggering widespread inflammatory responses in the body. In scientific studies, a sepsis model is frequently utilized as a tool for exploring various molecular mechanisms, including metabolism, apoptosis, and inflammation LPS-induced sepsis represents a systemic inflammatory reaction that is closely associated with numerous cardiac diseases [24–26]. In recent years, the incidence of sepsis has been escalating, presenting a significant challenge to public health and the quality of life. To develop effective therapeutic strategies, it is imperative to delve deeper into the mechanisms underlying the onset and progression of sepsis. In this research, we meticulously engineered sepsis models using LPS as an inducer and our experimental outcomes confirmed the efficacy of this approach. Our research demonstrated a substantial increase in the secretion of key inflammatory cytokines, specifically IL-6, IL-1β, and TNF-α, from HK2 cells upon exposure to LPS. This compelling evidence not only confirmed the establishment of a biologically relevant sepsis mimic but also highlighted the pivotal role of these cytokines in mediating the cellular response during LPS-induced inflammation.

MiR-342-5p plays a crucial regulatory role in certain cancers and coronary artery disease, yet its influence on sepsis remains unexplored. Previous research has confirmed its capacity to restrain the proliferation, migration, and invasion of osteosarcoma by modulating Wnt7b [21]. The upregulation of miR-342-5p prompts the migration of endothelial cells, accompanied by a reduction in endothelial markers and an increase in mesenchymal markers, suggesting an augmentation in the endothelial-mesenchymal transition process [27]. The influence of miR-342-5p on the inflammatory response has been investigated. Notably, miR-342-5p exhibits an elevated expression level in atherosclerosis and displays a positive association with inflammatory factors. This observation implies a regulatory role for miR-342-5p in both atherosclerosis and cytokine secretion [28]. In this study, we observed that the level of miR-342-5p was reduced in HK2 cells treated with LPS. However, it was observed that the enforced expression of miR-342-5p exhibited a remarkable inhibitory effect on the LPS-induced increase in inflammatory cytokine secretion and apoptosis, while fostering an environment that favored cellular proliferation. This finding highlights the potential therapeutic implications of targeting miR-342-5p in modulating detrimental inflammatory responses and preserving cellular integrity under LPS stress.

Research to explore the downstream pathway of miR-342-5p is currently being conducted. In the present study, bioinformatics analysis predicted that PFN1 is one of the potential targets of miR-342-5p. PFN1, an actin-binding protein abundantly present in eukaryotic cells, primarily regulates the dynamic equilibrium between actin polymerization and depolymerization. In addition to this, it participates in transmembrane protein transport, activates the small G protein signaling pathway and regulates nuclear gene transcription, among other crucial pathological and physiological processes [29]. PFN1 efficiently promotes tumor progression and proliferation, including colorectal cancer [30], endometrial cancer [31], and breast cancer [32]. Moreover, PFN1 has a major impact on inflammation [31,33]. Our findings revealed that miR-342-3p inhibits the PFN1 mRNA level by directly targeting its 3' UTR. The upregulation of PFN1 exerts a counteracting influence, negating the anti-inflammatory impact of miR-342-5p in the context of sepsis. This highlights the intricate interplay between these molecules in regulating immune response dynamics during this critical condition.

Conclusions

In conclusion, miR-342-5p emerges as a pivotal player in the etiology and progression of sepsis, primarily through its significant modulation of PFN1 expression. This highlights the crucial regulatory function of microRNAs in governing the complex pathophysiological processes associated with this life-threatening infection. Our findings establish a theoretical foundation for the early detection of sepsis and offer a novel therapeutic approach for treating this condition.

Availability of Data and Materials

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

Author Contributions

YL designed the study and drafted the manuscript. ZC was responsible for the collection and analysis of the experimental data. YL and ZC revised the manuscript critically for important intellectual content. Both authors read and approved the final manuscript. Both authors have participated sufficiently in the work to take public responsibility for appropriate portions of the content and agreed to be accountable for all aspects of the work in ensuring that questions related to its accuracy or integrity.

Ethics Approval and Consent to Participate

Not applicable.

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Conflict of Interest

The authors declare no conflict of interest.

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