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MiR-201-5p alleviates lipopolysaccharide-induced renal cell dysfunction by targeting NOTCH3

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Abstract. – OBJECTIVE: Lipopolysaccharide (LPS)-induced inflammation and dysfunction in the kidney may be the major risk factors for subsequent acute kidney injury (AKI). Previous studies have reported that up-regulation of notch receptor 3 (NOTCH3) expression is accompanied with renal epithelium and podocyte damage. Herein, we aimed to investigate whether NOTCH3 was involved in lipopolysaccharide (LPS)-induced AKI and renal cell dysfunction.

MATERIALS AND METHODS: Septic mice were established using LPS (20 mg/kg) intraperitoneally. mRNA and protein expression in the kidney and renal cell was performed by reverse transcription-quantitative polymerase chain reaction (RT-qPCR) and Western blotting, respectively. Cell counting kit-8 (CCK8) and flow cytometry were used to measure cell viability and apoptosis, respectively. Bioinformatics algorithm and Luciferase reporter gene assay were performed to validate whether NOTCH3 was a direct target of miR-201-5p.

RESULTS: Up-regulation of NOTCH3 and down-regulation of miR-201-5p were observed in the kidney of LPS-induced septic mice. LPS-stimulated TCMK-1 and MPC5 cells led to an increase in NOTCH3 and a decrease in miR-201-5p expression levels. Bioinformatics algorithm and experimental measurements validated that NOTCH3 was a direct target of miR-201-5p. Overexpression of miR-201-5p protected against LPS-induced renal cell growth inhibition, apoptosis and inflammatory response via the suppression of toll-like receptor 4 (TLR4)/NOTCH3 signaling pathway.

CONCLUSIONS: The novel role of miR-201-5p via the inhibition of LPS-activated TLR4/NOTCH3 might provide a potential therapeutic strategy for the treatment of LPS-induced AKI.

Key Words:

MicroRNA, Post-transcriptional regulatory mechanism, NOTCH3, Sepsis, Renal cell dysfunction.

Abbreviations

LPS, Lipopolysaccharide; AKI, acute kidney injury; miRNAs, microRNAs; ICU, intensive care units; GFR, glomerular filtration rate; TLR4, toll-like receptor 4; NF-κB, nuclear factor-kappa B; NLRP3, NOD-like receptor family pyrin domain containing 3; JAK, Janus protein tyrosine kinase; STAT, signal transducers and activators of transcription; I/R, ischemia/reperfusion; CSL, [CBF-1(C-promoter binding protein-1), Su(H) (Suppressor of Hairless), Lag1]; UTRs, untranslated regions; ELISA, enzyme-linked immunosorbent assay; TNF-α, tumor necrosis factor-α, IL, interleukin; BUN, blood urea nitrogen; Cys C, Serum cystatin C; β2-MG, β2-microglobulin; LDH, lactate dehydrogenase; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; RIPA, radio immunoprecipitation assay.

Introduction

Sepsis is one of the most common causes leading to AKI in intensive care units (ICU), accounting for 17% of patients in ICU[1]. Approximately 50% of AKI patients develop into renal failure, which increases the mortality rate of AKI patients during hospitalization[1]. Sepsis-induced AKI is closely associated with the elevated production of LPS from gram-negative bacteria[2]. LPS has the ability to decrease glomerular filtration rate (GFR) and rapid loss of renal function[2]. Inflammation-related signaling pathways, including TLR4/nuclear factor-kappa B (NF-κB), NOD-like receptor family pyrin domain containing 3 (NLRP3) inflammasome and Janus protein tyrosine kinase (JAK)/signal transducers and activators of transcription (STAT), play an important role in sepsis-induced AKI[3-5].

In mammals, NOTCH receptors consist of 4 members, NOTCH1-4, which have similar structure, consisting of a large extracellular, a small transmembrane and a transcription-activating intracellular domain[6]. During the over-activation of NOTCH signaling, the intracellular domain of NOTCH transfers into the nucleus and heterodimerizes with the DNA-binding transcription factor CSL [CBF-1(C-promoter binding protein-1),

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Su(H)(Suppressor of Hairless), Lag1] to form a nuclear transcription complex, which promotes the expression of downstream target genes[7]. Recently, NOTCH receptor signaling pathway has been reported participating in inflammatory response[7-10]. Notably, NF-κB-triggered over-activation of NOTCH3 sustains a pro-inflammatory environment in the kidney of ischemia/reperfusion (I/R) mouse model[6]. The expression level of NOTCH3 is increased in LPS-induced inflammatory response in RAW264.7 macrophage cells[11]. In addition, NOTCH3-derived inflammation contributes to AKI, chronic renal disease and renal fibrosis[6,12,13]. However, it is unclear whether NOTCH3 is involved in sepsis-induced AKI.

MicroRNAs (miRNAs) are small, non-coding and single-stranded RNAs (approximately 22 nucleotides) and have the ability to regulate gene transcription and translation by sequence-specific interaction with its 3'-untranslated regions (3'-UTRs)[14]. MiRNAs have emerged as a novel class of post-transcriptional regulators involving in a variety of biological processes, including sepsis-induced AKI[15-19]. Specifically, overexpression of miR-590-3p markedly improves survival outcomes in septic mice, significantly attenuates LPS-induced AKI and reverses LPS-induced podocyte growth inhibition and apoptosis[15]. Overexpressed miR-204 blocks LPS-induced cell injury and inflammatory response in rat mesangial cells[16]. In the present study, we revealed the up-regulation of NOTCH3 in the kidney of septic mice and identified that NOTCH3 was a direct target of miR-201-5p. In LPS-stimulated mouse podocyte and renal epithelial cell, an increase of NOTCH3 expression and cell apoptosis rate were attenuated by miR-201-5p gain-of-function. These findings suggested that miR-201-5p/NOTCH3 signaling was closely associated with sepsis-induced renal dysfunction, which might be a valuable therapeutic target for the treatment of AKI.

Materials and Methods

Animal Treatment

Male C57BL/6N (8-week-old, body weight, 20 ± 2 g) were purchased from Vital River Laboratories Co., Ltd (Beijing, China) and allowed to acclimate to the environment for 1 week. Septic mice were established by the intraperitoneal injection with LPS at the concentration of LPS (20 mg/kg). The mice were randomly divided into 2 groups as follows: the control (Con) group was injected with normal saline, and the LPS group was given 20 mg/kg of LPS intraperitoneally. The animal experiments were approved by the Ethics Committee of the Zhejiang Province People's Hospital (Hangzhou, China) and were performed in accordance with the Helsinki Declaration.

Cell Culture

Mouse renal epithelial cell line TCMK-1 was purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). Mouse podocytes (MPC5) were purchased from the National Infrastructure of Cell Line Resource (Serial number: 3111C0001CCC000230; Beijing, China). Cells were incubated in Dulbecco's Modified Eagle's Medium (DMEM; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and supplemented with 10% fetal bovine serum (FBS), 100 μg/mL streptomycin and 100 IU/mL penicillin (all from Sigma-Aldrich; Merck Millipore, Darmstadt, Germany).

Enzyme-Linked Immunosorbent Assay (ELISA)

The levels of tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β) and interleukin 6 (IL-6) in the serum of septic mice and in the supernatant of cultured TCMK-1 and MPC5 cells were measured by ELISA kit (Elabscience Biotechnology Co., Ltd, Wuhan, China). Blood urea nitrogen (BUN) was detected using an enzymatic kinetic method using commercial kit from Nanjing Jiancheng Biology Engineering Institute (Nanjing, China) with a SpectraMax M5 ELISA plate reader (Molecular Devices, LLC, Sunnyvale, CA, USA) according to the manufacturer's instructions. Serum cystatin C (Cys C) and β2-microglobulin (β2-MG) were measured using the RANDOX enzymatic assay (Randox Laboratories Limited, Crumlin, Antrim, UK). Lactate dehydrogenase (LDH) levels in the culture medium were measured by a commercial kit from Beyotime Institute of Biotechnology (Haimen, China).

RT-qPCR

Total RNA was extracted using miRNeasy Mini Kit (Qiagen, Inc., Valencia, CA, USA), according to the manufacturer's protocol. TaqMan® RT kit (Applied Biosystems; Thermo Fisher Scientific, Inc., Waltham, MA, USA) and TaqMan® MicroRNA assay (Applied Biosystems; Thermo

Fisher Scientific, Inc., Waltham, MA, USA) were used to perform RT-qPCR of miRNAs, according to the manufacturer's protocol. U6 small nuclear RNA was used as an endogenous control.

Moloney murine leukemia virus reverse transcriptase (Thermo Fisher Scientific, Inc., Waltham, MA, USA) and TaqMan® Universal PCR Master Mix (Thermo Fisher Scientific, Inc., Waltham, MA, USA) were used to perform RT-qPCR of NOTCH1-4 using the Applied Biosystems 7300 Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was served as internal control. The relative expression levels of miRNAs and NOTCH1-4 were calculated using the $2^{-\Delta\Delta Cq}$ method[20].

Western Blotting

Proteins were extracted with radio immunoprecipitation assay (RIPA) buffer (Beyotime Institute of Biotechnology, Haimen, China). Western blotting assay was determined as previously described[21]. The membranes were incubated with the primary antibody for NOTCH3 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA), cleaved-caspase-3 (Cell Signaling Technology, Inc., Danvers, MA, USA), cleaved-caspase-8 (Cell Signaling Technology, Inc., Danvers, MA, USA) and TLR4 (Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Subsequently, the membranes were incubated with an appropriate horseradish peroxidase (HRP)-conjugated secondary antibody (Santa Cruz Biotechnology, Inc., Dallas, TX, USA) at room temperature for 2 h and visualized with an enhanced chemiluminescence kit (Thermo Fisher Scientific, Inc., Waltham, MA, USA). Signals were analyzed with Quantity One® software version 4.5 (Bio Rad Laboratories, Inc., Hercules, CA, USA). Anti-β-actin (cat. no. sc-130065; 1: 2,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA) was used as the control antibody.

Luciferase Reporter Gene Assay

The miR-201-5p mimic (5'-UACUCAGUAAGGCAUUGUUCUU-3') and miR-NC (5'-UUGUGGUAUAGUCCUAUCCUCC-3') sequences were synthesized by Guangzhou RiboBio, Co., LTD (Guangzhou, China). TCMK-1 and MPC5 cells were transfected using Lipofectamine 2000 (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) at a final concentration of 100 nM. The cells were harvested for analysis after 48 h transfection. The wild-type (WT) and mutant-type (Mut) 3'-UTR of NOTCH3 were respectively inserted into the multiple cloning sites of the Luciferase expressing pMIR-REPORT vector (Ambion; Thermo Fisher Scientific, Inc., Waltham, MA, USA). For the Luciferase assay, TCMK-1 and MPC5 cells were seeded into 24-wells and co-transfected with Luciferase reporter vectors containing the WT or Mut 3'-UTR of NOTCH3, and miR-NC and miR-201-5p mimics sequences. The Luciferase activity was measured using a Luciferase reporter assay kit (Promega Corporation, Madison, WI, USA) according to the manufacturer's protocols.

CCK-8 Assay

In LPS-treated TCMK-1 and MPC5 cells after transfection with or without miR-201-5p mimics for 24 h, cell viability was detected using CCK-8 Cell Proliferation/Viability Assay Kit (Dojindo Molecular Technologies Inc., Kumamoto, Japan). Absorbance was recorded at 450 nm with a SpectraMax M5 ELISA plate reader (Molecular Devices, LLC, Sunnyvale, CA, USA) according to the manufacturer's instructions.

Flow Cytometry

In LPS-treated TCMK-1 and MPC5 cells after transfection with or without miR-201-5p mimics for 24 h, cell apoptosis was monitored using AnnexinV-FITC/PI apoptosis detection kit (Becton, Dickinson and Company, NJ, USA) according to the manufacturer's protocol. Apoptotic cell proportion was analyzed by flow cytometry (FACScan, BD Biosciences, San Jose, CA, USA) and calculated by CELL Quest 3.0 software (BD Biosciences, San Jose, CA, USA).

Statistical Analysis

Data were presented as the mean ± standard error of the mean. Statistical analysis was performed using SPSS software version 19.0 (IBM Corp., Armonk, NY, USA) and GraphPad Prism Version 7.0 (GraphPad Software, Inc., La Jolla, CA, USA). Student's *t*-test was used to analyze the differences between two groups. Differences between multiple groups were analyzed by one-way analysis of variance (ANOVA), followed by a post-hoc Tukey test. $p < 0.05$ was considered to indicate a statistically significant difference.

Results

Activated Inflammatory Response, AKI and Up-Regulated NOTCH3 Were Observed In Septic Mice

Previous studies have been reported that LPS triggers an increase in the levels of inflammatory cytokines, such as TNF-α, IL-1β and IL-6, in serum[22,23]. Consistent with these findings, acute systemic inflammation was found in our study, reflecting that the promotion of TNF-α, IL-1β and IL-6 levels in serum was validated in LPS-administrated mice at the concentration of 20 mg/kg for 12 h treatment (Figure 1A). LPS is widely used to induce an ideal animal model for exploring the pathogenesis of AKI in septic mice[2]. Our data supported the role of LPS in the development of AKI in septic mice, which was proved by the increase in AKI biomarkers, including BUN, serum Cys C and β2-MG (Figure 1B). To investigate the pathogenesis of LPS-caused AKI in septic mice, we focused on NOTCH receptor family members. The RT-PCR analysis demonstrated that LPS stimulation resulted in a significant up-regulation of NOTCH1 (1.6-fold) and NOTCH3 (4.2-fold) mRNA levels in the kidney of septic mice compared with that of in the control group (Figure 1C). However, the mRNA levels of NOTCH2 and NOTCH4 had no evident change in response to LPS administration (Figure 1C). Furthermore, we found that the protein expression of NOTCH3 was significantly increased in the kidney of LPS-treated mice compared with that in the control group (Figure 1D).

The Expression of MiRNAs Targeted to NOTCH3

To further explore the biological functions of miRNAs in LPS-induced AKI by modulating NOTCH3 expression, bioinformatics algorithm was executed by Targetscan (www.targetscan.org) and miRDB (www.mirdb.org) to predict the potential miRNAs, which could target modulation of NOTCH3 expression via binding with its 3'-UTRs. Prediction algorithm showed that a total of 27 miRNAs were filtrated by both Targetscan and miRDB. Among these miRNAs, 14 miRNAs and 3 miRNAs were significantly down-regulated and up-regulated, respectively, in the kidney of LPS-treated mice as compared to that in the control group (Table I). Otherwise, 10 miRNAs had no significant difference in the two groups

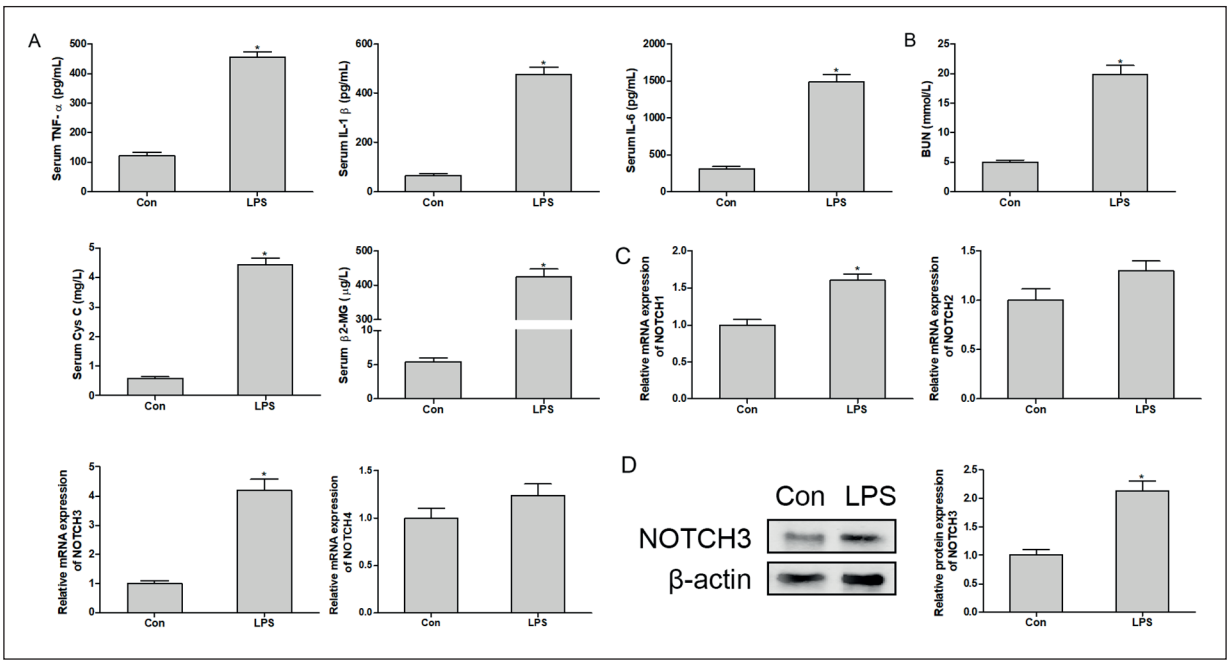

Figure 1. Activated inflammatory response, AKI and up-regulated NOTCH3 were observed in septic mice. The levels of inflammatory cytokines, such as TNF-α, IL-1β and IL-6, in serum were measured using ELISA assays 12 h after LPS injection (**A**). Serum AKI biomarkers, including BUN, serum Cys C and β2-MG, were measured 12 h after LPS injection (**B**). NOTCH1-4 mRNA levels was measured by RT-qPCR in the kidney 12 h after LPS injection (**C**). The protein levels of NOTCH3 were measured in the kidney using Western blotting assay 12 h after LPS injection (**D**). * $p < 0.05$, compared with NC group. Con, Control group; LPS, lipopolysaccharide.

Table I. MiRNAs expression in the kidney of LPS-treated mice.

	Relative expression	|Log2 (fold change)|	*p*-value
mmu-miR-6987-3p	Down	0.67	0.031
mmu-miR-466l-3p	Down	0.89	0.008
mmu-miR-6925-5p	Down	1.11	< 0.001
mmu-miR-6916-5p	Down	1.45	< 0.001
mmu-miR-6942-5p	Down	1.77	< 0.001
mmu-miR-6987-5p	Down	0.93	< 0.001
mmu-miR-201-5p	Down	3.44	< 0.001
mmu-miR-7030-5p	Up	0.23	0.178
mmu-miR-7076-5p	Up	0.44	0.345
mmu-miR-7075-5p	Up	0.56	0.136
mmu-miR-3083-5p	Up	0.67	0.089
mmu-miR-1231-3p	Down	0.99	< 0.001
mmu-miR-7212-5p	Down	1.34	< 0.001
mmu-miR-7117-5p	Down	0.17	0.678
mmu-miR-6946-5p	Down	1.85	< 0.001
mmu-miR-881-5p	Down	1.94	< 0.001
mmu-miR-759	Down	0.88	< 0.001
mmu-miR-150-3p	Up	2.34	< 0.001
mmu-miR-880-3p	Down	1.45	< 0.001
mmu-miR-7681-3p	Down	1.22	< 0.001
mmu-miR-6335	Down	0.45	0.138
mmu-miR-3087-3p	Up	0.77	0.015
mmu-miR-3104-5p	Up	0.78	0.009
mmu-miR-6940-5p	Up	0.34	0.234
mmu-miR-7648-3p	Down	0.12	0.467
mmu-miR-762	Down	0.09	0.763
mmu-miR-6971-5p	Down	0.13	0.318

(Table I). According to the $|Log_2$ (fold change), miR-201-5p was the maximum value. Therefore, we focused on miR-201-5p *in vitro* experiments.

LPS Regulated NOTCH3 and MiR-201-5p Expression In Vitro

To further elucidate the roles of NOTCH3 and miR-201-5p, we detected the expression levels of NOTCH3 and miR-201-5p in LPS-stimulated TCMK-1 and MPC5 cells. Consistent with the results *in vivo*, both mRNA and protein expression levels of NOTCH3 were significantly elevated in a concentration-dependent manner of LPS-stimulated TCMK-1 and MPC5 cells compared with that in the control group (Figure 2A and 2B). In addition, miR-201-5p was significantly reduced in a concentration-dependent manner of LPS-stimulated TCMK-1 and MPC5 cells compared with that in the control group (Figure 2C).

NOTCH3 Was a Direct Target of MiR-201-5p

Bioinformatics algorithm revealed that two conserved binding regions in the 3'-UTR of NOTCH3 had the ability to complementary pairing with miR-201-5p (Figure 3A). Next, to further elucidate whether miR-201-5p directly targeted NOTCH3, Luciferase reporter assays were performed in TCMK-1 and MPC5 cells. NOTCH3 WT or Mut plasmid was constructed and co-transfected with miR-NC or miR-201-5p mimics into TCMK-1 and MPC5 cells. The data indicated that transfection of miR-201-5p mimics resulted in the reduction of Luciferase activity in TCMK-1 and MPC5 cells containing NOTCH3 WT plasmid, while the Luciferase activity showed no significant change in TCMK-1 and MPC5 cells containing NOTCH3 Mut plasmid (Figure 3B). After transfection with miR-201-5p mimics into TCMK-1 and MPC5 cells, NOTCH3 mRNA and protein expression were significantly lower than that in the control group (Figure 3C and 3D). Collectively, these data uncovered that miR-201-5p could directly target NOTCH3 in TCMK-1 and MPC5 cells.

Overexpression of MiR-201-5p Attenuated LPS-Induced Renal Cell Damage

To determine the roles of miR-201-5p on LPS-induced renal cell damage, we first mea-

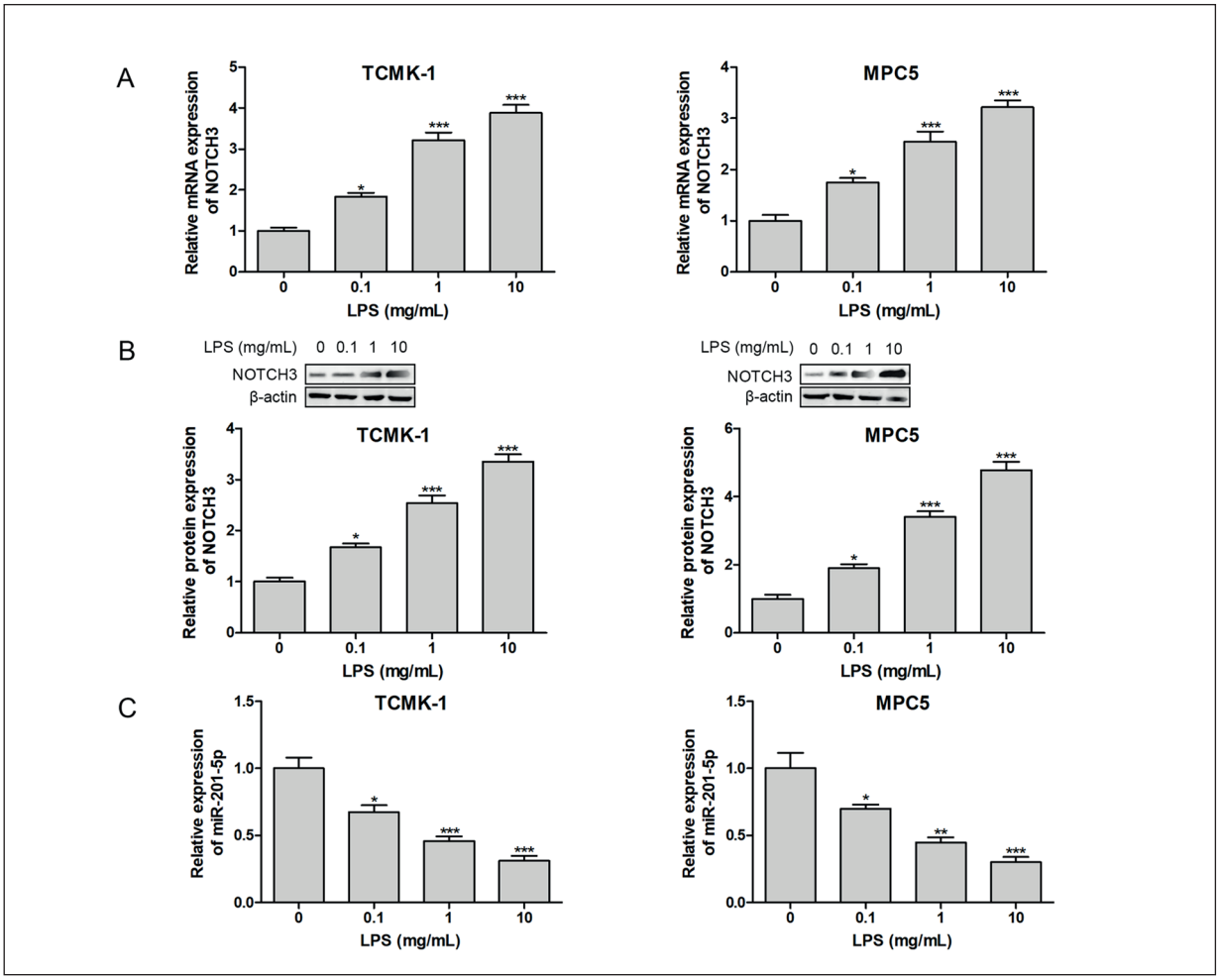

Figure 2. LPS regulated NOTCH3 and miR-201-5p expression *in vitro*. After TCMK-1 and MPC5 cells treatment with LPS at the concentration of 0, 0.1, 1 and 10 mg/mL for 24 h, the mRNA (**A**) and protein (**B**) expression levels NOTCH3 were performed by RT-qPCR and Western blotting, respectively; miR-201-5p levels were measured by RT-qPCR (**C**). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, compared with control group.

sured cell viability using CCK8 assay after transfection with miR-201-5p mimics into TCMK-1 and MPC5 cells in the presence of LPS (1 mg/mL). We found that cell growth rate was significantly inhibited in LPS-stimulated TCMK-1 and MPC5 cells, while transfection with miR-201-5p mimics markedly reversed LPS-induced growth inhibition in TCMK-1 and MPC5 cells (Figure 4A). Previous report indicates that LDH as a general marker of cellular damage is increased in LPS-induced inflammatory cell model[24]. Similarly, we also found an increased in LDH production in the culture medium in LPS-stimulated TCMK-1 and MPC5 cells. However, overexpression of miR-201-5p reversed the up-regulation of LDH production in LPS-treated TCMK-1 and MPC5 cells (Figure 4B). These findings suggested that overexpression of miR-201-5p had a protective effect against cytotoxicity caused by LPS. Moreover, apoptotic cell proportion and the protein expression of cleaved-caspase3 and cleaved-caspase8 were significantly increased in LPS-treated TCMK-1 and MPC5 cells compared with the control group. In contrast to these, overexpression of miR-201-5p blocked LPS-induced cell apoptosis via the down-regulation of cleaved-caspase3 and cleaved-caspase8 protein expression in TCMK-1 and MPC5 cells (Figure 4C, 4D and 4E).

Overexpression of MiR-201-5p Alleviated LPS-Induced Inflammation In Vitro

Consistent with our results in LPS-induced septic mice, LPS stimulation significantly accel-

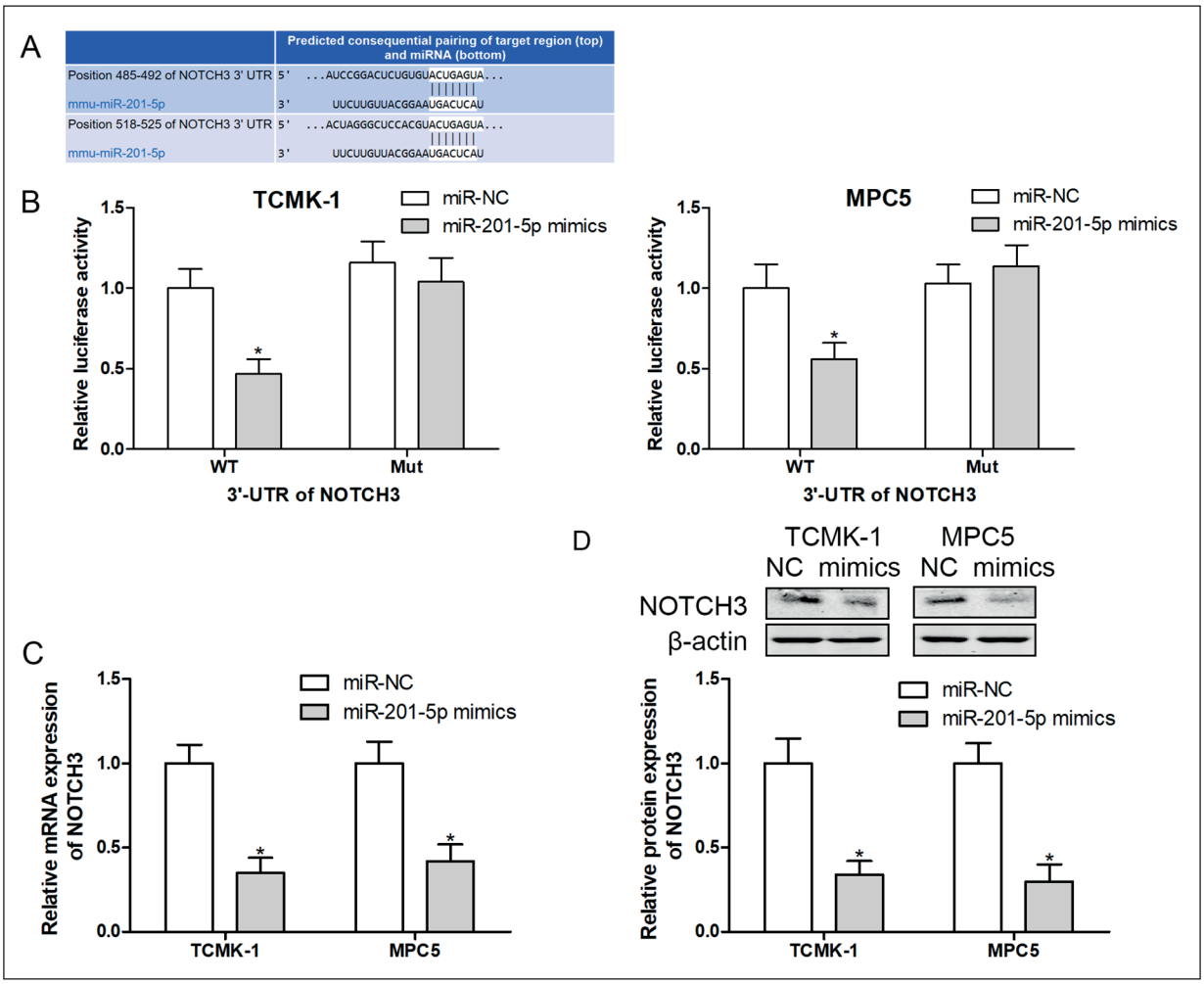

Figure 3. NOTCH3 was a direct target of miR-201-5p. Targetscan and miRDB were used to predict the conserved binding regions between NOTCH3 and miR-201-5p (**A**). Luciferase reporter assays were performed in TCMK-1 and MPC5 cells (**B**). After transfection with miR-201-5p mimics into TCMK-1 and MPC5 cells, the mRNA (**C**) and protein (**D**) expression levels NOTCH3 were performed by RT-qPCR and Western blotting, respectively. * $p < 0.05$, compared with control group.

erated the secretion of inflammatory cytokines, including TNF-α, IL-1β and IL-6, in TCMK-1 and MPC5 cells (Figure 5A and 5B). Of note, transfection with miR-201-5p into TCMK-1 and MPC5 cells could retard inflammatory cytokines release (Figure 5A and 5B). TLR4 as a canonical receptor for LPS mediates inflammatory response via recruitment and activation of downstream mediators TNF receptor-associated factor 6 (TRAF6) and NF-κB[25]. Recently, NOTCH3 as a downstream mediator of TLR4 involves in LPS-induced cellular damage[11]. In our study, we also found that the protein expression of NOTCH3 and TLR4 was augmented in LPS-treated TCMK-1 and MPC5 cells, while miR-201-5p gain-of-function significantly suppressed LPS-induced expression of NOTCH3 and TLR4 (Figure 5C).

Discussion

The present work demonstrated that up-regulation of NOTCH3 and down-regulation of miR-201-5p were observed in the kidney of LPS-induced septic mice and LPS-stimulated TCMK-1 and MPC5 cells. Bioinformatics algorithm and experimental measurements validated that

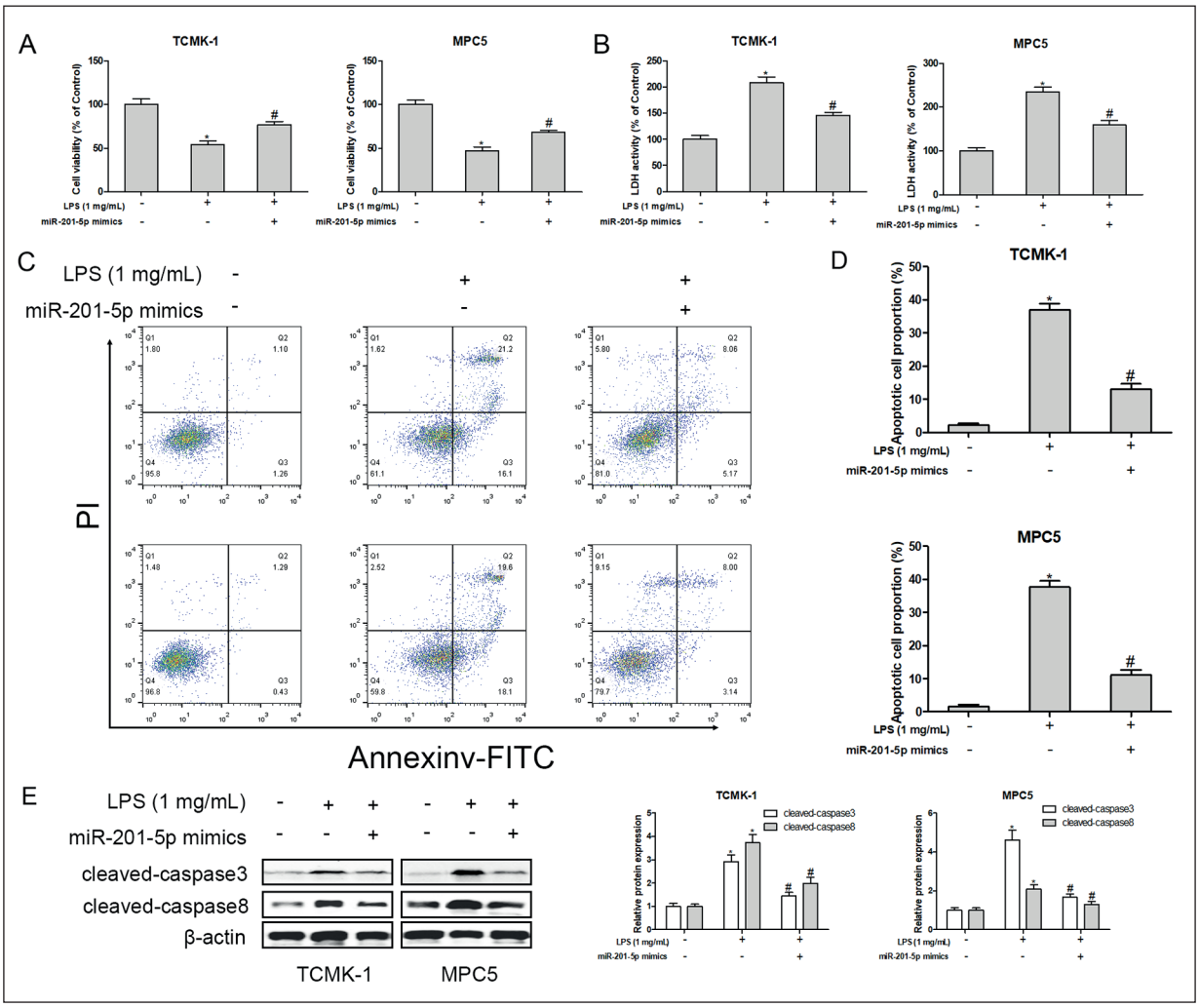

Figure 4. Overexpression of miR-201-5p attenuated LPS-induced renal cell damage. In LPS-treated TCMK-1 and MPC5 cells after transfection with or without miR-201-5p mimics, cell viability was measured using CCK8 assay (**A**); LDH activity in the supernatant (**B**); Annexin V-FITC/PI double staining was used to measure cell apoptosis (**C** and **D**); the protein expression levels of cleaved-caspase3 and cleaved-caspase8 were measured by Western blotting (**E**). * $p < 0.05$, compared with control group; # $p < 0.05$, compared with LPS treatment group.

NOTCH3 was a direct target of miR-201-5p. Overexpression of miR-201-5p showed a protective effect against LPS-induced renal cell growth inhibition, apoptosis and inflammatory response via the suppression of TLR4/NOTCH3 signaling pathway (Figure 6).

There are a growing number of studies focusing on kidney diseases, such as renal fibrosis, glomerulonephritis and AKI[6,26-28]. In mouse models of AKI, sustained over-activation of NOTCH signaling has been described[27-29]. For example, the protein expression levels of NOTCH ligand Delta-like 1 and NOTCH1 intracellular domain are enhanced in the kidney of cisplatin-treated mice, and over-activation of NOTCH1 signaling is recapitulated in cisplatin-treated human renal epithelial cell line (HK-2)[29]. In addition, ischemia-induced AKI pathogenesis may be associated with up-regulation of NOTCH3 expression and NOTCH target genes[27]. NOTCH2 signaling involves in renal I/R injury-associated inflammation and apoptosis in a rat model[28]. Xiong et al[11] showed that the up-regulation of NOTCH3 in RAW 264.7 macrophages might be associated with LPS-induced AKI. However, the functions of NOTCH3 in LPS-induced AKI and renal cell dysfunction are barely reported.

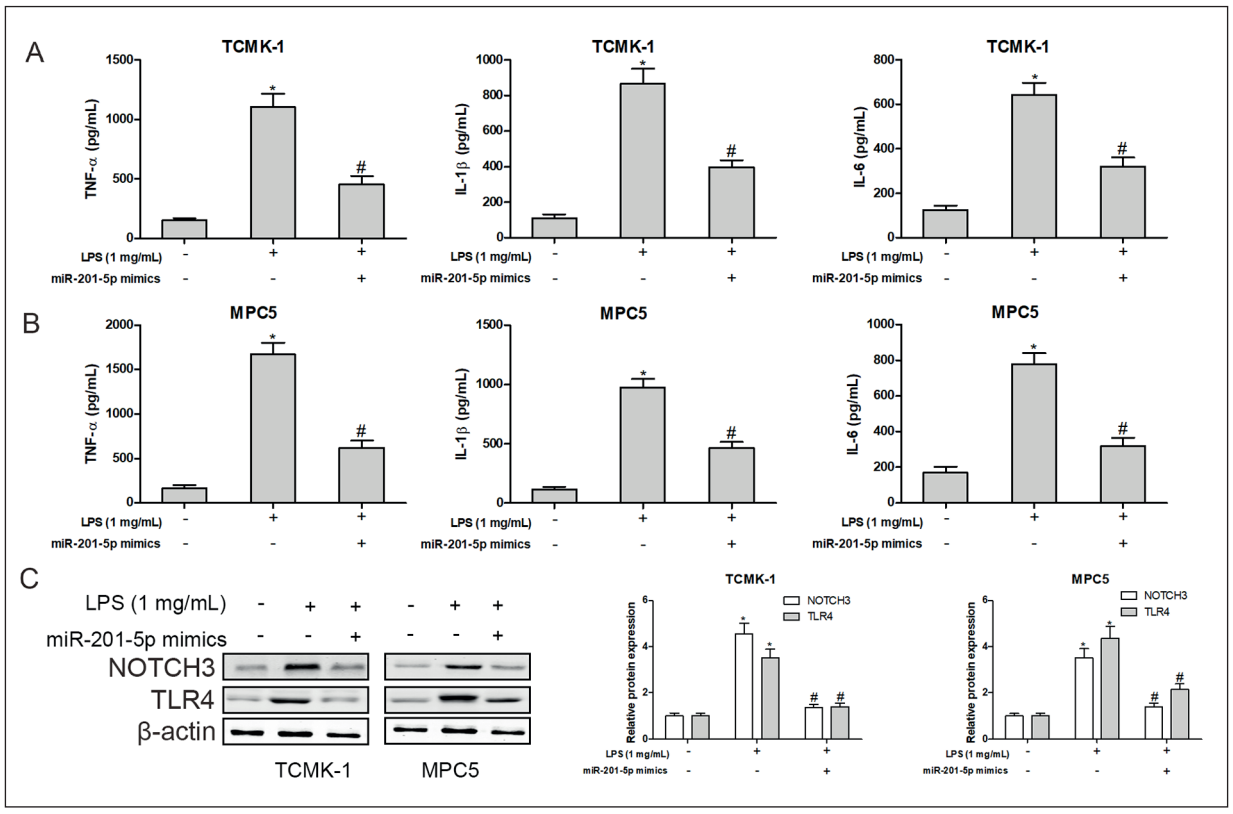

Figure 5. Overexpression of miR-201-5p alleviated LPS-induced inflammation *in vitro*. In LPS-treated TCMK-1 and MPC5 cells after transfection with or without miR-201-5p mimics, inflammatory cytokines, such as TNF-α, IL-1β and IL-6, were measured in the supernatant of TCMK-1 (**A**) and MPC5 (**B**) cells; the protein expression levels of TLR4 and NOTCH3 were measured by Western blotting (**C**). * $p < 0.05$, compared with control group; # $p < 0.05$, compared with LPS treatment group.

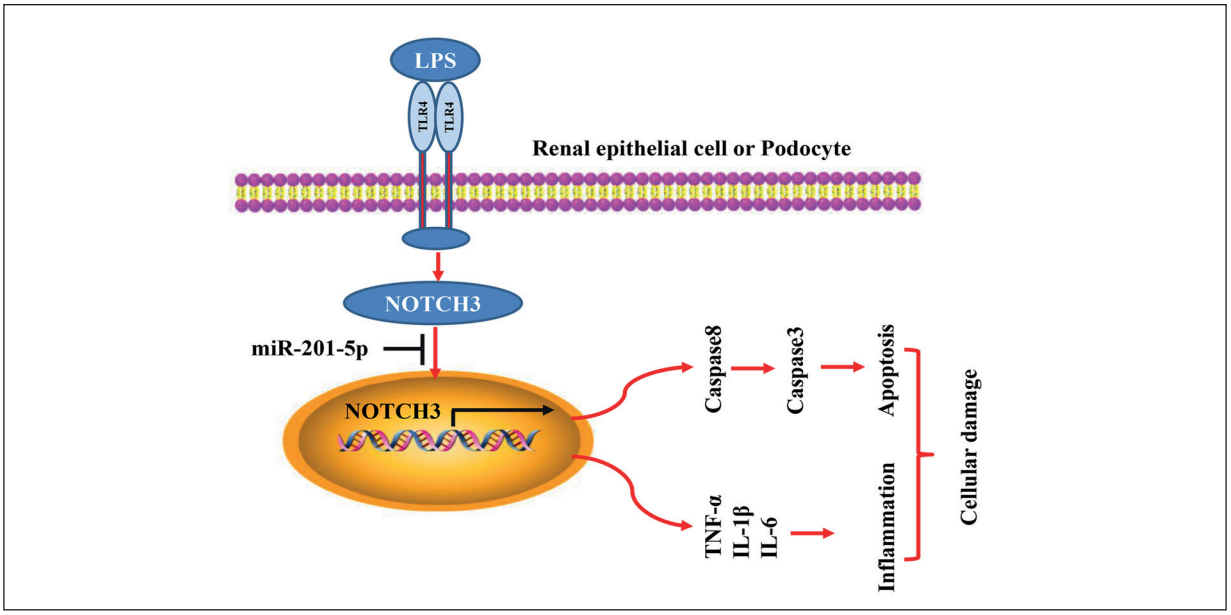

Figure 6. A molecular mechanism diagram for how the miR-201-5p suppressed LPS-induced inflammation and renal cell dysfunction by modulating TLR4/NOTCH3 signaling pathway.

In patients with lupus nephritis or focal segmental glomerulosclerosis, NOTCH3 expression is emerged in the podocyte, but not in normal kidney[13]. NOTCH3 knockout in mice protects against nephrotoxic sheep serum-induced crescentic glomeruli and fibrin deposits and reduces renal inflammation[13]. Over-activation of NOTCH3 in the injured epithelium sustains a pro-inflammatory environment leading to a rapid deterioration of renal function and structure in the mouse model with I/R and unilateral ureteral obstruction[6]. These findings suggest that over-activation of NOTCH3 and inflammation in podocyte and renal endothelial cell may contribute to AKI. Endothelial cells and podocytes play a key role in sepsis-induced AKI via the regulation of inflammatory cell infiltration and GFR[15,30]. Therefore, LPS-induced podocyte and renal endothelial cell damage may be the main cause of AKI. In our study, we found that LPS-induced AKI in septic mice and cell growth inhibition, the promotion LDH production and apoptotic cell proportion occurred synchronously with the up-regulation of NOTCH3 expression *in vivo* and *in vitro*. More importantly, molecular mechanism analysis revealed that NOTCH3 was a direct target of miR-201-5p, transfection with miR-201-5p mimics into TCMK-1 and MPC5 cells significantly repressed the mRNA and protein expression of NOTCH3. Overexpression of miR-201-5p had the ability to block LPS-induced cell death via obstructing LPS-activated TLR4/NOTCH3 signaling *in vitro*.

Differentially expressed miR-201-5p is closely related to the pathogenesis of polycystic ovary syndrome and spinal cord injury[31,32]. Bioinformatic analysis revealed that miR-201-5p is predicted to target a large set of genes, including 2749 transcripts and containing a total of 3357 sites [Targetscan (www.targetscan.org)], with different functions[31,32]. In the present study, we found that miR-201-5p was down-regulated in LPS-treated mice and TCMK-1 and MPC5 cells, while transfection of miR-201-5p mimics into TCMK-1 and MPC5 cells protected against LPS-induced inflammatory response, cell growth inhibition, increase in LDH production and apoptotic cell proportion. Moreover, miR-201-5p as post-transcriptional regulator targeted the inhibition of LPS-activated TLR4/NOTCH3 signaling, suggesting that miR-201-5p had the function of repressing LPS-induced renal cell dysfunction by targeting TLR4/NOTCH3 signaling.

Conclusions

Taken together, miR-201-5p protected against LPS-induced renal cell dysfunction by attenuation of inflammatory responses and cell apoptosis through the suppression of TLR4-mediated NOTCH3 activation. The novel role of miR-201-5p might provide a potential therapeutic strategy for the treatment of LPS-induced AKI.

Conflict of Interest

The Authors declare that they have no conflict of interests.

Ethics Approval

The animal experiments were approved by the Ethics Committee of the Zhejiang Province People's Hospital (Hangzhou, China) and were performed in accordance with Helsinki Declaration.

Authors' Contribution

Study design: YSY and WC; literature research, data acquisition and data analysis: YSY, MF, YXY and WC; manuscript preparation and manuscript editing: YSY, MF, YXY and WC; manuscript review: YSY, MF, YXY and WC; cell experiments: YSY and MF; animal experiments: YXY and WC; final approval of the version to be published: YSY, MF, YXY and WC.

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