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GAS5 promotes podocyte injury in sepsis by inhibiting PTEN expression

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Abstract. – OBJECTIVE: To investigate the potential role of long noncoding RNA (lncRNA) growth arrest specific transcript 5 (GAS5) in sepsis-induced podocyte injury and its underlying mechanism.

MATERIALS AND METHODS: The sepsis model was established by lipopolysaccharide (LPS) induction in podocytes. The expression levels of Nephrin and GAS5 were detected by quantitative Real-time polymerase chain reaction (qRT-PCR) after LPS induction in podocytes for 12 h, 24 h and 36 h, respectively. Western blot was used to detect the expression level of Nephrin in sepsis-induced podocytes. The mRNA expressions of GAS5 and Nephrin in podocytes were detected after transfection of GAS5 siRNA. Albumin influx in podocytes after GAS5 knockdown was detected by Transwell assay. Western blot was used to detect the protein expression of Snail in sepsis after GAS5 knockdown. The target gene of GAS5 was predicted by bioinformatics analysis. QRT-PCR and Western blot were used to detect the protein and mRNA levels of PTEN (phosphatase and tensin homolog deleted on chromosome ten). Nephrin expression and the albumin inflow after PTEN knockdown were then measured. The expression of PI3K/AKT/GSK3β was also detected after GAS5 was downregulated while PTEN was upregulated.

RESULTS: LPS stimulation downregulated the mRNA expression of Nephrin in podocytes and achieved the lowest level at 24 h. The protein expression change of Nephrin was consistent with its mRNA expression. In the septic state, the albumin influx of podocytes remarkably increased, but the function of podocyte barrier was weakened. Besides, GAS5 expression decreased in a time-dependent manner in LPS-induced podocytes. After GAS5 knockdown by siRNA, Nephrin expression and the function of podocyte barrier were significantly reduced. Snail expression was also upregulated in septic state, and GAS5 knockdown increased the expressions of phosphorylated Snail and PI3K/AKT/GSK3β. After knockdown of GAS5, the mRNA and protein levels of PTEN significantly decreased, which was contract to the expression of Snail. However, overexpression of PTEN could reverse the promotive effect of GAS5 on PI3K/AKT activation.

CONCLUSIONS: GAS5 expression decreased in sepsis-induced podocyte injury, and GAS5 was involved in regulating sepsis-induced podocyte injury by reducing PTEN expression.

Key Words:
Sepsis, Podocyte injury, GAS5, PTEN.

Introduction

Sepsis is a systemic inflammatory response syndrome caused by infection, leading to renal ischemia, decreased renal perfusion and renal dysfunction. A variety of complex pathophysiological mechanisms, such as cell injury, apoptosis, oxidative stress, mitochondrial dysfunction and inflammatory response in sepsis, are involved in the occurrence of renal injury[1,2]. Numerous studies[3,4] have shown that podocyte injury is significantly associated with sepsis and can induce proteinuria. Lipopolysaccharide (LPS) is reported to induce nuclear factor-kappa B (NF-kB) activation in podocytes and then promotes the release of inflammatory factors[5]. Podocytes are terminal differentiated visceral epithelial cells located outside the basement membrane of the glomerulus. They are the last line of defense that constitutes the glomerular filtration barrier. As an important part of the podal fissure membrane, Nephrin is closely related to the podocyte injury. Abnormal expression of Nephrin directly affects the structure of the hiatus membrane and causes the loss of urinary protein[6]. Since podocytes are not renewable and lack of repair ability, podocyte injury is a key event in the oc-

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currence and progress of glomerular diseases. Adrenocortical hormone, immunosuppressant, and anticoagulant therapies, are the current treatments of proteinuria or the protective approaches of podocytes. However, their clinical effects are far away from satisfactory. Therefore, it is very important to find diagnostic biomarkers and therapeutic targets for septic kidney injury. Long noncoding RNA (lncRNA) is a class of RNA with more than 200 nt in length and could not encode protein. Transcriptional products become mature lncRNAs after splicing or non-splicing, with or without polylysine tails. LncRNAs are located in the nucleus or cytoplasm with complex and changeable functions. Compared with miRNAs, expressions of lncRNAs are featured with cell specificity, tissue specificity and developmental stage specificity[7]. LncRNAs are important regulators in the development of many biological events, such as cell cycle, DNA methylation, histone modification, transcription and transcriptional regulation[8]. Previous studies have proved that lncRNAs are involved in the occurrence and development of many diseases, including kidney diseases[9-12]. It is also reported that lncRNA promotes the epithelial-mesenchymal transition (EMT) process of podocytes by regulating expressions of vimentin and Nephrin in diabetic nephropathy[13]. In addition, lncRNA01619 acts as a ceRNA to regulate miR-27a / FOXO1 axis and leads to ER stress and podocyte injury[14]. Moreover, the close relation to the metabolic changes of podocytes allows lncRNA to be an important link in the pathogenesis of kidney diseases[15]. PTEN (phosphatase and tensin homolog deleted on chromosome ten) is a negative regulator of the PI3K/AKT signaling pathway. A large number of studies have shown that PTEN plays a protective role in podocyte injury in diabetic nephropathy, while PTEN deletion stimulates the occurrence of proteinuria[16, 17]. Additionally, PTEN deficiency promotes cytoskeletal remodeling and then accelerates the progression of diabetic nephropathy[18]. It is reported[19] that microRNAs-21 and microRNAs-128 participate in septic podocyte injury by regulating PTEN expression in LPS-induced podocytes. Growth arrest specific transcript 5 (GAS5) derived from the 5'-terminal oligopyrimidine gene is mainly involved in the regulation of cell growth and proliferation[20]. GAS5 exerts a variety of biological function, such as induction/inhibition of apoptosis, cell cycle arrest, etc. However, the role of GAS5 in podocytes has not been studied yet.

Materials and Methods

Cell Culture

Conditional immortalized podocyte line was cultured in Roswell Park Memorial Institute-1640 (RPMI-1640) medium (HyClone, South Logan, UT, USA) containing 10% fetal bovine serum (FBS) (Gibco, Rockville, MD, USA) and 2-10×10^4 U/L recombinant mouse gamma interferon in 5% CO_2 at 37°C. After cell culture for 3-5 days, cells were maintained in RPMI-1640 medium containing 5% FBS. Mature podocytes were treated with 10 μg/mL LPS.

Transfection

Appropriate amount of cells were seeded in 6-well plates and cultured overnight. Transfection plasmids were diluted in 150 μL of serum-free medium and the transfection reagent Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) was also diluted in 150 μL of serum-free medium. After incubation for 5 minutes at room temperature, the above two reagents were mixed together and incubated again for 15 minutes at room temperature before adding to the 6-well plates. After incubation at 37°C for 5 h, the medium was replaced with serum-containing medium.

RNA Extraction And Quantitative Real-Time Polymerase Chain Reaction (qRT-PCR)

Cells were washed with phosphate-buffered saline (PBS) buffer and then lysed with TRIzol (Invitrogen, Carlsbad, CA, USA). After maintenance at room temperature for 5 minutes, the lysate was collected into RNase-free Eppendorf (EP) tubes, and chloroform was added to each tube (the ratio of chloroform to TRIzol was 1:5). Subsequently, the RNA was sequentially separated using isopropanol and 75% ethanol, and centrifuged for extracting total RNA. Reverse transcription PCR (RT-PCR) was performed using PrimeScript RT kit (TaKaRa, Otsu, Shiga, Japan). The mRNA expression of each gene was detected by qRT-PCR. GAPDH (glyceraldehyde 3-phosphate dehydrogenase) was selected as an internal reference. The experiment was independently repeated for 3 times. The primers were as follows: Nephrin (F: 5'-AAGACGAGGAGGAACTGAAC-3', R: 5'-CAAATCGGACAACAAGACG-3'), GAS5 (F: 5'-CTTCTGGGCTCAAGTGATCCT-3', 5'-TTGTGCCATGAGACTCCATCAG-3'), PTEN (F: 5'-GCAGAAAGACTTGAAGGCGTA-3', 5'-TTGGCGGTGTCATAATGTCT-3'), GAPDH

(F: 5'-AGGTCGGTGTGAACGGATTTG-3', 5'-TGTAGACCATGTAGTTGAGGTCA-3').

Western Blot

Total protein was extracted and loaded in equal amounts. After being separated by sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), the proteins were transferred to membranes, which were then blocked with 5% skim milk for 1 hour. These membranes were incubated with anti-Nephrin, anti-Snail, anti-PTEN, anti-PI3K, anti-p-PI3K, anti-AKT, anti-p-AKT, anti-GSK3β, anti-p-GSK3β and GAPDH (Cell Signaling Technology, Danvers, MA, USA) overnight at 4°C. After being washed with 1×TBST (Tris-buffered saline and Tween 20) (Beyotime, Shanghai, China) for 5 times (6 min for each time), the secondary antibody (Goat anti-rabbit IgG: 1:3000) was used to incubate the membrane for 2 h at room temperature. After washing with 1×TBST for 1 min, the chemiluminescent substrate kit (Thermo Fisher Scientific, Waltham, MA, USA) was used for protein band exposure. A multicolor fluorescent gel imaging system was used for analyzing the blot results.

Transwell Assay

Podocytes were seeded into the upper layer of the Transwell chamber pre-coated with type I collagen. 12 days later, the podocytes were serum starved overnight and received LPS induction or transfection. After that, the podocytes were washed with phosphate- buffered saline (PBS) (Beyotime, Shanghai, China) containing 1 mmol/L $CaCl_2$ and $MgCl_2$. Serum-free medium was added to the upper chamber, and a complete medium containing 5% FBS was added to the lower chamber. After incubation at 37°C for 4-6 hours, totally 30 μL of medium in the chamber were transferred to a dedicated 96-well plate. A microplate reader was used to analyze the fluorescence absorbance at the wavelength of 490 nm. The experiment was independently repeated for 3 times.

Statistical Analysis

The SPSS (Statistical Product and Service Solutions) 22.0 statistical software (IBM, Armonk, NY, USA) was used for data analysis. All data were expressed as ($\bar{x} \pm s$). Independent sample *t*-test was performed to compare the data of two groups, and the comparison of multiple groups was performed by one-way ANOVA, followed by post-hoc test (Least Significant Difference). $p<0.05$ was considered statistically significant.

Results

GAS5 was Downregulated in Podocytes During Sepsis

A sepsis model was established in podocytes by induction of 10 μg/mL LPS. QRT-PCR results showed that the mRNA expression of Nephrin is significantly downregulated with the prolongation of LPS induction, reaching to the lowest level at 24 h (Figure 1A). In addition, the protein expression of Nephrin also significantly decreased 24 h after LPS stimulation (Figure 1B). Transwell assay showed an increased albumin influx after LPS treatment, suggesting the disrupted podocyte barrier function (Figure 1C). Similarly, the expression level of GAS5 was also downregulated with the prolongation of LPS induction (Figure 1D). Above results suggested that GAS5 might be involved in the process of sepsis-induced podocyte injury.

Decreased GAS5 Promoted the Podocyte Injury

To investigate the specific role of differentially expressed GAS5 in septic podocyte injury, GAS5 was knocked down in podocytes (Figure 2A). GAS5 knockdown remarkably inhibited the expression of Nephrin in podocytes, indicating that downregulated GAS5 might mediate the podocyte injury (Figure 2B). We examined changes in albumin influx after knockdown of GAS5, further confirming the potential role of GAS5 in podocyte injury (Figure 2C). Knockdown of GAS5 remarkably upregulated Snail expression in the sepsis state (Figure 2D). Studies[21] have shown that Snail could inhibit the expression of Nephrin in the transcriptional level. In order to verify whether GAS5 could regulate the expression of Nephrin, we examined the expression level of Snail after downregulating GAS5 expression. The results showed that protein level of Snail significantly increases (Figure 2E), suggesting that abnormally upregulated Snail might be an important element involving in GAS5-regulated podocyte injury.

Downregulated GAS5 Inhibited the Expression of PTEN

Researches[22] have shown that PTEN has an important protective effect in sepsis-induced renal injury. Our bioinformatics analysis also indicated that the GAS5 promoter region might regulate PTEN expression (Figure 3A). In addition, we examined the expression level of PTEN in

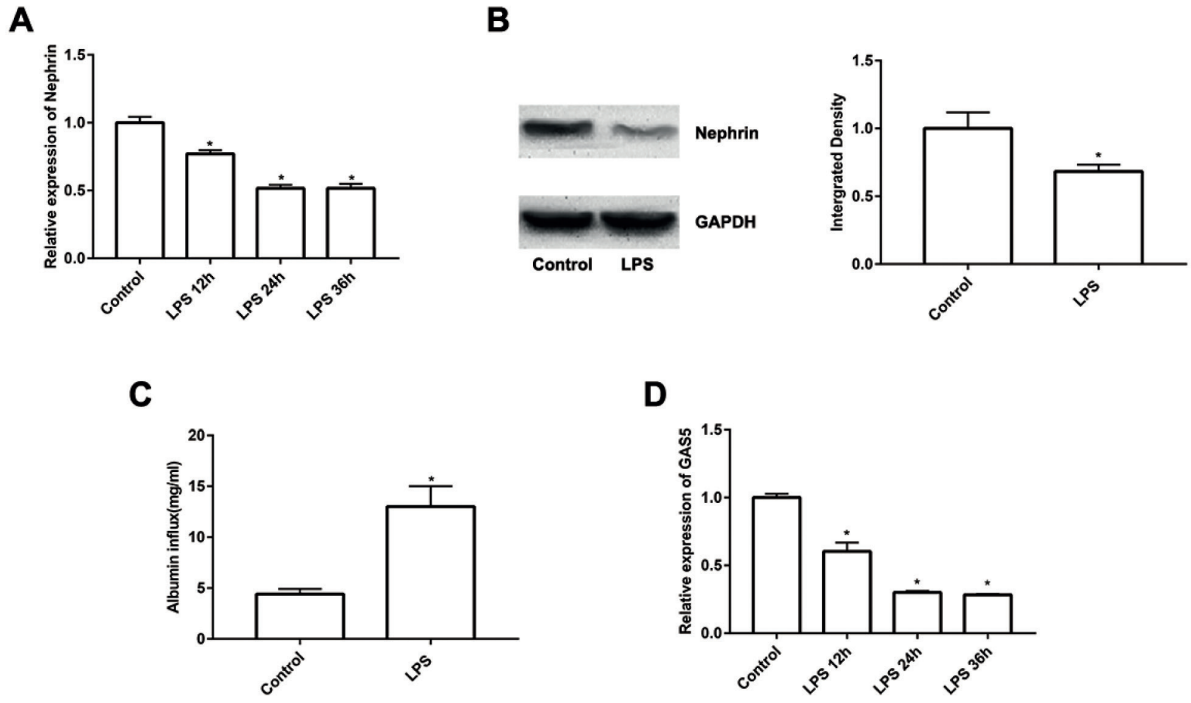

Figure 1. GAS5 was downregulated in podocytes during sepsis. ***A,*** In LPS-induced sepsis model, the expression of Nephrin in podocytes decreased and reached the lowest level at 24 h. ***B,*** LPS induction reduced the protein level of Nephrin. ***C,*** Podocyte barrier function was impaired in sepsis model. ***D,*** LPS inhibited the GAS5 expression.

LPS-treated podocytes. The results showed that PTEN expression is significantly downregulated (Figure 3B). Based on the opposite expression trend of GAS5 and PTEN in LPS-induced cells, we detected the PTEN expression in cells with GAS5 knockdown. The results also exhibited a remarkable decrease of PTEN, further verifying that GAS5 could regulate PTEN expression (Figure 3C, 3D). In addition, knockdown of PTEN downregulated the expression of Nephrin and upregulated the expression of albumin influx (Figure 3E, 3F, 3G). These results demonstrated that LPS downregulates Nephrin expression by inhibiting the PTEN expression, thus increasing podocyte albumin influx and finally leading to podocyte injury.

GAS5 Regulated PI3K/AKT Pathway Through Mediating PTEN

PTEN has been reported to exert specific phosphatase activity and negatively regulates the PI3K/AKT/GSK3β pathway[23]. After downregulation of GAS5, expressions of p-PI3K, p-AKT and p-GSK3β significantly increased, while no significant differences were found in expressions of PI3K/AKT/GSK3β (Figure 4A). To validate the role of PTEN in the regulation of PI3K/AKT/GSK3β expression by GAS5, podocytes were co-transfected with overexpression plasmid of PTEN and si-GAS5. Increased p-PI3K, p-AKT and p-GSK3β were remarkably downregulated after co-transfection. However, expressions of PI3K/AKT/GSK3β did not alter (Figure 4B). PTEN significantly reversed the activation of the PI3K/AKT pathway caused by GAS5 knockdown, further confirming that GAS5 regulated the PI3K/AKT pathway by targeting PTEN.

Discussion

Sepsis is a life-threatening organ dysfunction caused by the dysfunctional response to infection, it's the disease stage of which is characterized by severe sepsis and septic shock[24]. Kidney injury is one of the most common complications in the development of sepsis, and its severity is significantly associated with the high mortality rate of sepsis[25]. Sepsis-induced kidney injury has a high mortality rate[26]. Therefore, clarifying the pathoge-

nesis of renal injury in sepsis and searching for the possible diagnosis and treatment of sepsis renal injury are of great importance. In the present study, LPS induction downregulated the expression of Nephrin in podocytes and the podocyte barrier function, indicating of the successful construction of sepsis podocyte injury model. LncRNA has no protein-encoding ability and lacks a significant open reading frame. In recent years, researches on lncRNA have rapidly progressed, especially in the field of oncology. However, the functions of most lncRNAs in the kidney remain to be fully elucidated. Previous works[27, 28] have shown that lncRNA can be differentially expressed in the renal innate cells under disease conditions. It is reported that lncRNA plays an important regulatory role in the occurrence of many kidney diseases, including diabetic nephropathy, renal inflammation and fibrosis, renal transplant rejection, renal cell carcinoma, acute kidney injury, etc.[29]. For example, lncRNA PVT1 promotes LPS-induced HK-2 cells by regulating TNF-α and JNK/NF-κB pathways[30]. Besides, lncRNA MALAT1/miR-146a/NF-κB pathway also plays a key role in LPS-induced kidney injury[31]. LncRNA-GAS5, a growth-inhibiting specific transcript-5, shows an important role in cell growth, cell cycle arrest and apoptosis[32]. Additionally, GAS5 has been shown to play a significant regulatory role in stromal stem cell differentiation[32]. However, the role of GAS5 in septic kidney injury has not yet been elucidated. We found that the expression level of GAS5 is significantly downregulated after LPS stimulation of podocytes for 24 h. Knockdown of GAS5 markedly enhanced the podocyte injury, suggesting that inhibition of GAS5 in septic kidney injury might damage podocytes. PTEN is often considered as a negative regulator of PI3K/AKT signaling pathway. It antagonizes PI3K by its dephosphorylation to form PIP2 at the 3'-carboxyl site, thereby achieving negative regulation of the PI3K/AKT pathway[33]. We predicted the presence of PTEN binding sites in the promoter sequence of GAS5 by bioinformatics analysis. At the same time, PTEN was downregulated after GAS5 knockdown, while stimulated PI3K/AKT activation. Interestingly, overexpression of PTEN reversed the activation of PI3K/AKT pathway by GAS5 knockdown, further suggesting the regulatory effect of GAS5 on PI3K/AKT pathway depends on PTEN. Phosphorylated GSK3β has been reported to regulate the expression of Snail[34]. In the present study, the expression of Snail significantly increased after GAS5 knockdown. Consistent with previous studies, Snail inhibited the expression of Nephrin by binding to Nephrin E-Box sequence, suggesting that the pro-

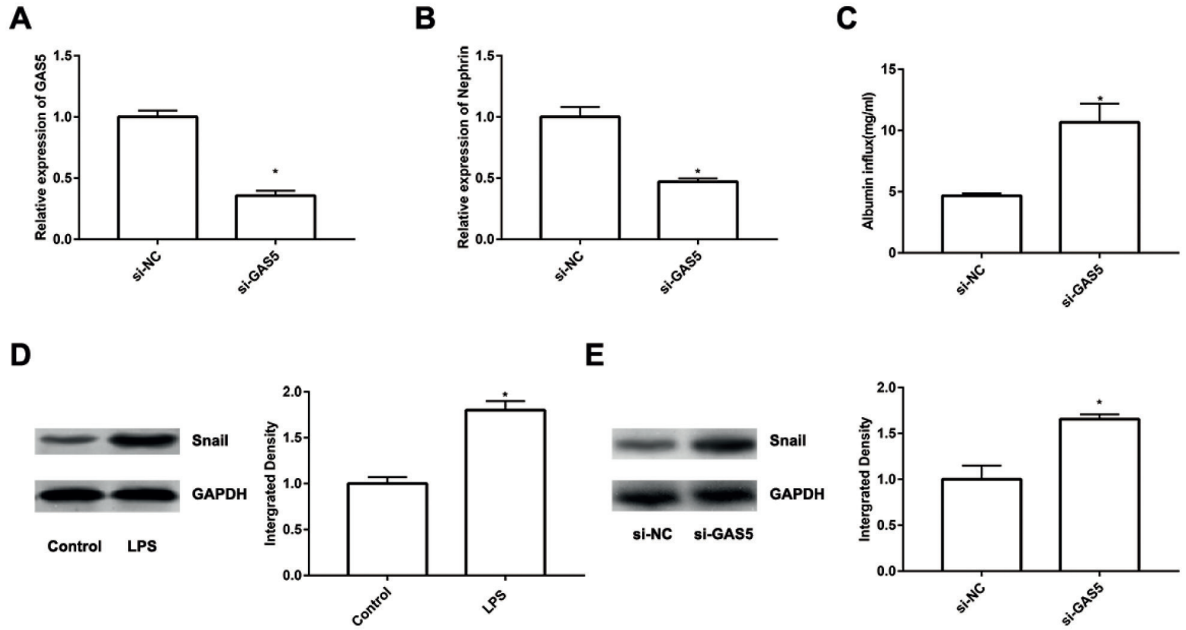

Figure 2. Decreased GAS5 promoted the podocyte injury. ***A,*** GAS5 expression was knocked down by siRNA transfection. ***B,*** Downregulated GAS5 inhibited Nephrin expression. ***C,*** Downregulated GAS5 damaged podocyte barrier function. ***D,*** LPS promoted Snail expression. ***E,*** Downregulated GAS5 promoted Snail expression.

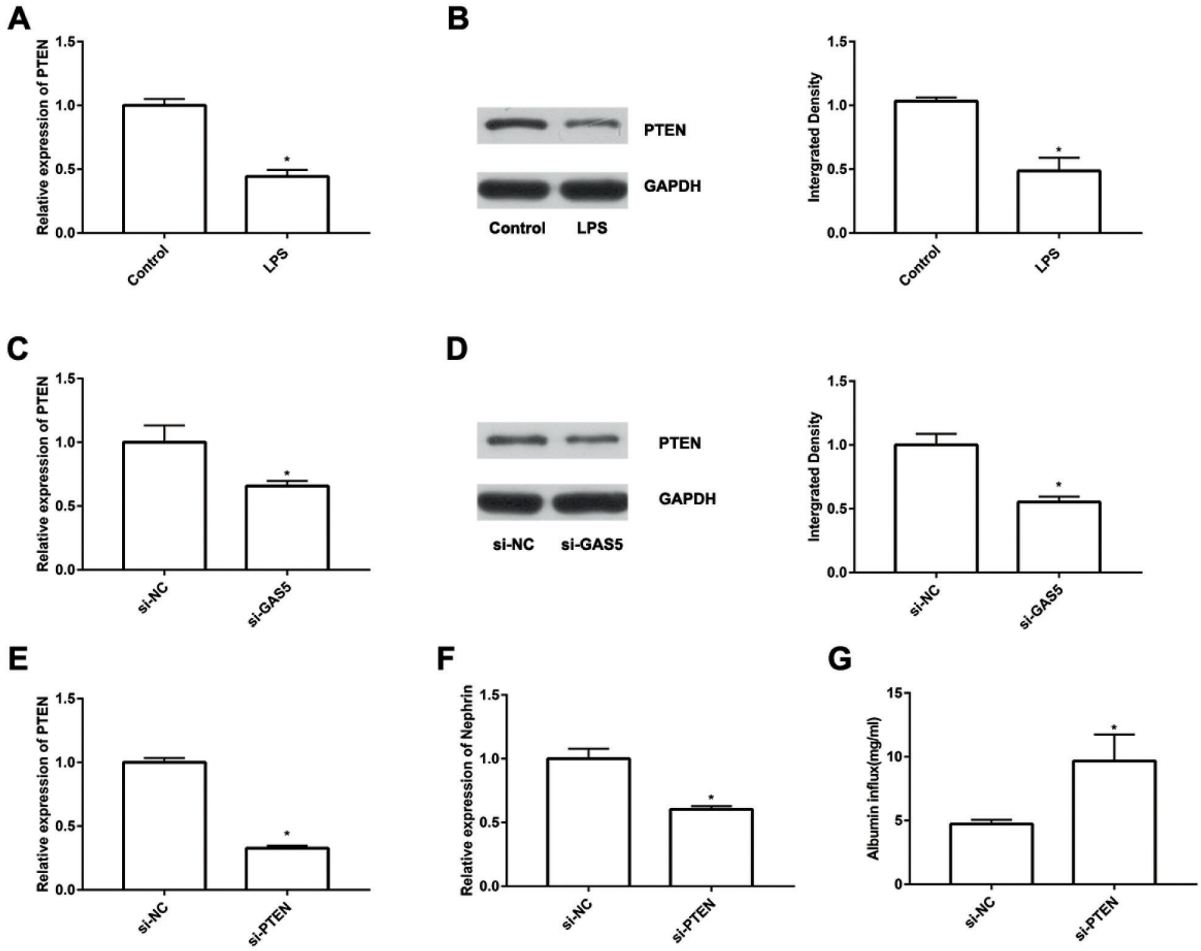

Figure 3. Downregulated GAS5 inhibited the expression of PTEN. ***A, B,*** Bioinformatics analysis predicted that GAS5 might regulate PTEN. The RNA and protein expressions of PTEN decreased in the model constructed by LPS. ***C, D,*** Downregulated GAS5 inhibited the RNA and protein expressions of PTEN. ***E,*** PTEN expression was knocked down by siRNA transfection. ***F,*** Downregulated PTEN inhibited Nephrin expression. ***G,*** Downregulated PTEN increased albumin influx.

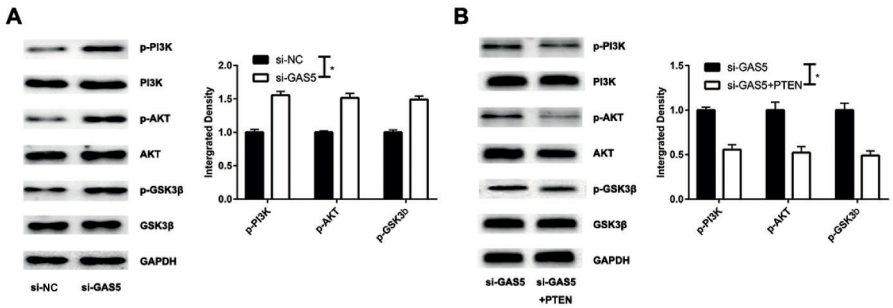

Figure 4. GAS5 regulated PI3K/AKT pathway through PTEN. ***A,*** Downregulated GAS5 activated the PI3K/AKT pathway. ***B,*** Overexpression of PTEN reversed the effect of GAS5 on PI3K/AKT pathway.

cess of sepsis-induced podocyte injury might be related to the upregulation of Snail expression[19]. In conclusion, GAS5 inhibited PTEN expression and led to sepsis-induced podocyte injury.

Conclusions

We found that GAS5 was downregulated in the podocyte injury model of sepsis. GAS5 regu-

lates the activation of PI3K/AKT/GSK3β and the expression of Snail through targeting PTEN, thus participating in sepsis-induced podocyte injury.

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Conflict of Interest

The Authors declare that they have no conflict of interest.

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