

RESEARCH ARTICLE

MALAT1 regulates PCT expression in sepsis patients through the miR-125b/STAT3 axis

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Abstract

Background: Procalcitonin (PCT) is an important marker in diagnosing sepsis. However, some other diseases can also cause an increase in PCT. PCT still has some limitations in the clinical application of diagnosing sepsis. Therefore, it is of great significance to clarify the regulatory mechanism of PCT expression in sepsis and provide new therapeutic targets for sepsis.

Methods: Blood samples from clinical patients were collected, and peripheral blood monocytes were isolated. Bioinformatics was performed to find the ceRNA regulatory network of STAT3/PCT. MALAT1 and miR-125b were detected by qRT-PCR. MALAT1 was located by fluorescence in situ hybridization (FISH) in U937 cells, and the regulatory relationship between MALAT1, miR-125b, and STAT3 was verified by double luciferase activity report and RNA pull-down assay. U937 cells were transfected with miR-125b, and the effects of the MALAT1/miR-125b/STAT3 pathway on gene and protein secretion levels of PCT were verified by qRT-PCR, western blot, and ELISA.

Results: In the serum of sepsis patients and lipopolysaccharide(LPS)-induced U937 cells, MALAT1, STAT3, and PCT gene expression levels were significantly increased, while miR-125b expression level was decreased. FISH results showed that the MALAT1 transcript was mainly located in the nucleus. The double luciferase activity report and RNA pull-down assay results suggested a targeted regulatory relationship between MALAT1, miR-125b, and STAT3. LPS-induced U937 cells transfection with MALAT1 siRNA decreased STAT3 protein expression and phosphorylation level and the expression of PCT. Co-transfection with miR-125b inhibitor effectively reversed this phenomenon.

Conclusions: MALAT1 could upregulate the expressions of STAT3 and PCT by targeted adsorption of miR-125b.

KEYWORDS

MALAT1, miR-125b, procalcitonin, sepsis, STAT3

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1 | INTRODUCTION

Sepsis is defined as a systemic inflammatory response syndrome at risk for body response disorders and lethal organ failure. Sepsis is considered the most common cause of death among hospitalized critically ill patients. Although sepsis treatment measures were highly improved, sepsis still causes more than 700,000 deaths in China.¹ Therefore, further clarifying pathogenesis, through early identification, and appropriate treatment, can improve the prognosis of patients with sepsis.

Procalcitonin (PCT) is the most widely used serum biomarker for sepsis clinical diagnosis and therapeutic applications. The serum PCT tests are negative in healthy individuals. However, in the case of sepsis caused by bacterial infection, the PCT synthesis was induced in all tissues rapidly, and the level of PCT increases rapidly between 2 and 6 h and peaks within 6–24 h after infection.^{2,3} Therefore, in the early stage of bacterial infection, detecting the levels of serum PCT can determine whether there is a bacterial infection. In addition, if patients respond to treatment, PCT levels will return to the normal range more quickly than C-reactive protein,⁴ and dynamic monitoring of PCT levels can help guide antibiotic management of sepsis.⁵ Although PCT has the characteristics of high sensitivity and rapid response, it can also lead to the increase of PCT in noninfectious inflammatory reactions and neuroendocrine tumors at specific sites. In other words, the regulation mechanism of PCT expression is still unclear. Therefore, clarifying the regulatory mechanism of PCT expression in the occurrence of sepsis is helpful to improve the timeliness and accuracy of PCT as a marker of sepsis diagnosis and is expected to discover new markers of sepsis in the process of research. It also provides a potential target for the treatment of sepsis.

Studies have shown that LPS can activate NF- κ B and STAT3 signaling, increasing PCT expression.^{6,7} JAK/STAT pathway is not only a common pathway for the signaling of multiple cytokines and growth factors⁸ but also one of the central signal transduction systems in cells during sepsis and plays an essential role in the occurrence and development of sepsis.⁹ MicroRNA(miRNA) is a class of noncoding small RNA that has been discovered in recent years and is involved in the regulation of gene expression at the post-transcriptional level. miRNA is involved in various physiological and pathological processes, including ecophysiological processes such as sepsis inflammation and immunosuppression.¹⁰ Many studies have found that miR-125b/STAT3 pathway is extensively involved in various physiological and pathological processes. For example, Xiao et al. found that miR-125b can inhibit the proliferation of osteosarcoma cells by regulating the MAPK-STAT3 signaling pathway in the study of osteosarcoma.¹¹ Zhang et al.¹² found that miR-125b in septic monocytes directly acts on STAT3 and participates in the regulation of PCT by detecting peripheral blood samples of patients with sepsis. To further understand the upstream regulation of miR-125b, we believe that long noncoding RNAs can act as competing endogenous RNA(ceRNA) of miR-125b to co-regulate PCT expression in sepsis. Combined with bioinformatics analysis, it was found that lncRNA

metastasis-associated lung adenocarcinoma transcript 1 (MALAT1) had potential binding sites with miR-125b. Studies on mouse cecal ligation puncture showed that MALAT1 competitively binding miR-125b regulates the P38 MAPK/NF κ B signaling pathway and further affecting sepsis-induced cardiac dysfunction.¹³ Liu et al.¹⁴ found in the study of diabetic retinopathy that MALAT1 enhances the expression of cadherin protein and promotes retinal neovascularization by binding miR-125b. In their research on neuroinflammation, Ma et al.¹⁵ found that MALAT1 may interact with miR-125b to inhibit neuronal apoptosis and inflammation and promote the growth of Alzheimer's Disease neurites.

On this basis, we believe that MALAT1 may act as the ceRNA of miR-125b and regulate the expression of STAT3, thereby affecting the expression of PCT in inflammatory cells. In this study, the regulation mechanisms of MALAT1 on miR-125b and downstream genes were investigated by establishing in vitro and in vivo models were clarified.

2 | PATIENTS AND METHODS

2.1 | Database screening and bioinformatics analysis

The calculation and analysis are done through miRDB (www.mirdb.org) and ENCORI (<https://rna.sysu.edu.cn/ENCORI/index.php>) database calculation and analysis with miRNAs binding sites to STAT3 were screened out. Through the RNA22 database (<https://cm.jefferson.edu/rna22/Precomputed/>), forecast the binding sites of microRNAs binding to lncRNA. lncAtlas website (<http://lncatlas.org.eu/>) was used to analyze lncRNA subcellular localization.

2.2 | Clinical sample collection and processing

University of Chinese Academy of Sciences, Hwamei Hospital approved this study (ethical number: NBEY-2019-083-01). The research protocol conformed with the latest edition of the Declaration of Helsinki. Before initiating this study, obtained written informed consent from all subjects or their first-degree relatives.

A total of 150 patients were enrolled, including 50 patients with sepsis (mean age 56.37 ± 14.53 years, 27 males and 23 females), 50 patients with septic shock (mean age 55.44 ± 14.98 years, 26 males and 24 females), and 50 healthy control groups (mean age 56.78 ± 15.12 years, 25 males, 25 women). Sepsis Patients are from EICU of Hwamei Hospital, University of Chinese Academy of Sciences, Ningbo, from May 2015 to May 2021. Peripheral blood samples were collected from all enrolled patients, and monocytes were isolated from peripheral blood. Inclusion criteria: (1) All patients with sepsis or septic shock met the sepsis 3.0 diagnostic criteria; (2) Have detailed and complete clinical follow-up data. 10 ml peripheral blood samples were collected from all enrolled individuals.

2.3 | Isolated peripheral blood mononuclear cells (PBMC)

PBMC were isolated from all 75 blood samples according to the human peripheral blood mononuclear cell isolation kit (CAT No. P8680, Solarbio).

2.4 | qRT-PCR

The expression levels of MALAT1, miR-125b, STAT3, and PCT genes in PBMC were detected. The RNA was extracted with Trizol reagent. The RNA was dried at room temperature for 10 min, then dissolved in RNase-free water, and stored at -80°C . Using an ultraviolet spectrophotometer determined the concentration and purity of RNA. Primers for MALAT1, miR-125b, STAT3, and PCT were designed and synthesized by Genewiz Company. Reverse transcription of RNA into cDNA was following instructions of PrimeScript RT and One Step PrimeScript miRNA cDNA Synthesis Kit. The reaction solution was taken for quantitative fluorescence PCR, and quantitative fluorescence PCR was performed according to the instructions of the SYBR[®] Premix Ex Taq[™] II kit. β -actin and U6 were used as reference genes for normalization. Each experiment was repeated three times. Primer design: MALAT1-F: 5'- AGG TAA AGC TTG AGA AGA T -3', MALAT1-R: 5'- GGA AGT AAT TCA AGA TCA A -3'; STAT3-Forward primer: 5'- CTT TGA GAC CGA GGT GTA TCA CC -3', STAT3-Reverse primer: 5'- GGT CAG CAT GTT GTA CCA CAG G -3'; PCT-F:5'- CTG CAC TGG TGC AGG ACT ATG T -3', PCT-R: 5'- CAG ATT ACC GCA CCG CTT AGA TC -3'; β -actin-F: 5'- AAA TCT GGC ACC ACA CCT TC -3'; β -actin-R: 5'- AAC GCT TCA CGA ATT TGC GT -3'; miR-125b-F: 5'- CCA GAT ACT GCG TAT GTG TG -3', miR-125b-R: 5'- GTC ACC TGA TCC CAT CTA AC -3'; U6-F: 5'- CTC GCT TCG GCA GCA CA -3', U6-R: 5'- AAC GCT TCA CGA ATT TGC GT -3'.

2.5 | Serum PCT level detection

PCT level in peripheral blood samples was detected by AQT90 FLEX Full-automatic fluorescence immunoassay analyzer (Radiometer). PCT level in the cell was detected by ELISA kit (cat no. PP790, Beyotime Biotechnology Co., LTD.)

2.6 | Cell culture and treatment

U937 cells were purchased from Zhejiang Ruyao Biotechnology Co., LTD. U937 cells were cultured on RPMI1640 (GIBCO, Life Technologies) medium containing 10% fetal bovine serum, penicillin (100 U/ml), and streptomycin (100 U/ml). All cells were incubated at 37°C in a 5% CO_2 incubator. The U937 + LPS group was U937 cells treated with 1 $\mu\text{g}/\text{mL}$ LPS (16 h), and the U937 group was non-LPS-treated U937 cells. MALAT1, miR-125b, STAT3, and PCT mRNA in U937 cells were detected by qRT-PCR (same method as above).

2.7 | Fluorescence in situ hybridization (FISH)

After RNA extraction from U937 cells, the MALAT1 fragment was amplified using Taq enzyme and added polyA tail by PCR. Amplified primer sequence: forward primer-1-CTG GGA GCA GAA AAC AGC AG, reverse primer-1-TGC CCC AAC ACT GAA CTA CA. forward primer-2-GCT GGA GTA ACT GGC ATG TG, reverse primer-2-ACC AAA GAC CTC GAC ACC AT. After electrophoresis, the gel was cut and recycled and the purified product added polyA was connected to the T vector. The positive clones were obtained by Blue-White Screening. The plasmid successfully inserted into the MALAT1 fragment was extracted. RNA probes containing Cy3-UTP markers were transcribed according to APEX[™] HyperScribe[™] T7 High Yield Cy3 RNA Labeling Kit and purified for subsequent FISH experiments.

U937 cells were taken and washed with cold PBS, then fixed with 4% paraformaldehyde at 4°C for 20min. After PBS cleaning 3 times, 0.5% Triton X-100 was added for transmembrane treatment for 5 min. After PBS cleaning, Cy3 -MALAT1 probe hybridization solution was added to the cell culture plate, and conducted overnight at 37°C . MALAT1 antagonist was used as the negative control. After the hybridization, the cells were washed three times with hybridization lotion (2 \times SSC) at 42°C . The nuclei were stained with 1 $\mu\text{g}/\text{mL}$ DAPI at room temperature for 10 min. After washing with PBS, an anti-fluorescence quenching agent was dropped to seal the nuclei. The results were captured by a fluorescence microscope (DM500, Leica).

2.8 | Dual-luciferase reporter

Double luciferase reporter gene detection: primers were synthesized for wild-type and mutant sequences of MALAT1 and STAT3. The double luciferase reporter plasmid was constructed using pIS0, with PRL-TK as an internal reference plasmid. 293T cells were transfected at 60%~80% fusion degree. miR-125b mimic and NC mimic 2.5 μL , with the final concentration of 100 nM, was commissioned to be synthesized by Genewiz Company. Lipo 2000 was used for transfection. Promega GloMax was used to measure the dual-luciferase reporting system combined with Promega. Relative luciferase activity was expressed as the ratio of firefly luciferase activity to Renilla luciferase activity.

Among them, wild-type MALAT1 amplified primer sequences: *ScaI*-F-5'-AAA GAG GCA CGA ACA CCT TCA GGG AC -3', *XbaI*-R-5'-TGC TCT AGA CCA CCA ATC CCA ACC GTA ACA G-3'; amplified primer sequence of mutant MALAT1: *ScaI*-F-5'-AAA GAG CTC GCA CGA ACA CCT AGT CCC T-3', *XbaI*-R-TGC TCT AGA CCA CCA ATC CCA ACC GTA ACA G-3'; wild-type STAT3 amplified primer sequences: *ScaI*-F-5'-AAA GAG GCA TGT CTG GTT GAG TTC TCA GGG A-3', *XbaI*-R-5'-TGC TCT AGA CCC TCT AGC TGT TCT GCC TCA C-3'; amplified primer sequence of mutant STAT3: *ScaI*-F-5'-AAA GAG GCA TGT GTG GTT GAG CTC AGG GA-3', *XbaI*-R-5'-TGC TCT AGA CCA CCA ATC CCA ACC GTA ACA G-3'.

2.9 | RNA pull-down

U937 cells were transfected with 50 nM biotinylated miR-125b-5p mimic or MUT miR-125b-5p (convergence 50%) using Lipofectamine 3000 (Invitrogen). Cells were harvested 24 h after transfection and lysed in lysis buffer. Cell lysates were incubated with washed streptavidin magnetic beads (Life Technologies) for 3 h. The beads were washed, and the mRNAs interacting with miRNA were extracted using Trizol reagent. The abundance of MALAT1 was analyzed by qPCR.

2.10 | Cell transfection

According to the known sequences of MALAT1, miR-125b, and STAT3 in NCBI, MALAT1, si-MALAT1 negative control sequence, si-LncRNA-MALAT1 sequence, miR-125b mimic negative control, miR-125b mimics, NC inhibitor (NC in) and miR-125b inhibitor (miR-125b in), STAT3 sequence, STAT3 negative control sequence, and si-STAT3 sequence was constructed by Shanghai Sangong Biological Company. STAT3 and MALAT1 overexpression plasmids were constructed by pCDH plasmids.

When the Cells were inoculated and cultured in 24-well plates when the cell confluence reached 50%–60% for transfection. U937 cell's confluence reached 50%–60% after being inoculated in 24-well plates, cells were grouped for transfection according to LipofectaminTM 2000 instructions. The first stage was divided into 5 groups: control group, LPS group, STAT3 group, si-NC2 group, and si-STAT3 group. The second stage was split into 7 groups: control group, LPS group, NC mimic, miR-125b mimic, NC in group, miR-125b in group, miR-125b in+si-STAT3 group. The third stage of LncRNA-MALAT1 expression intervention (divided into 6 groups): Control group, LPS group, LncRNA-MALAT1 group, si-NC1 group, si-LncRNA-MALAT1 group, transfected si-LncRNA-MALAT1 + miR-125b inhibitor group. Except for the control group, the above three-stage groups gave cells 100 ng/mL LPS stimulation after transfection. Cells from each group were collected 6 h after LPS treatment. qRT-PCR was used to detect the gene expression levels of lncRNA-MALAT1, miR-125b, STAT3, and PCT in the transfected cells. Western blot was used to detect the expression and phosphorylation levels of STAT3. PCT protein expression level was detected by ELISA kit.

2.11 | Western blot

Total protein was extracted from U937 cells by RIPA kit, and the protein concentration was determined by BCA kit (cat no. P0012, Beyotime Biology). Polyacrylamide gel electrophoresis was performed at 40 μ g/lane. After protein isolation, the protein was transferred to the PVDF membrane by wet transfer method and sealed with 5% skim milk at room temperature for 2 h. Primary antibody

STAT3(cat no. A19566, ABclonal) and p-STAT3 (cat no. AP0715, ABclonal) diluted at 1:1000 were added, respectively, and incubated at 4°C overnight. The PVDF membrane was 3 times washed by TBST, and HRP labeled sheep anti-rabbit IgG antibody was added to incubate at room temperature for 1 h. After rinsing with TBST, added ECL reaction solution and photographs were taken using a UVP gel imaging system. The relative protein expression level was calculated as the optical density value ratio of the target band to the inner reference band (β -actin). The experiment was repeated three times for each group.

2.12 | Statistics

All experimental data were expressed as (mean \pm SD) and repeated 3 times independently. GraphPad Prism 8.0 software was used for statistical analysis and mapping. T-test was used for comparison between the two groups. One-way ANOVA was performed for comparison between multiple groups. $p < 0.05$ was considered statistically significant.

3 | RESULTS

3.1 | Expression of miR-125b, STAT3, and PCT in sepsis

PBMC of healthy samples, sepsis patients, and septic shock patients were detected by qPCR. The results showed that compared with the PBMC of the healthy control group, the expression level of the miR-125b gene in the blood of sepsis patients and septic shock patients was significantly decreased, while MALAT1, STAT3, and PCT expression levels were significantly increased ($p < 0.05$, Figure 1A–D). The serum secretion level of PCT in patients with sepsis and septic shock was considerably higher than that in the healthy control group. The difference was statistically significant ($p < 0.05$, Figure 1E). The results in Figure 1F showed that after the occurrence of sepsis, the gene expression level of miR-125b decreased gradually within 5h, and reached the lowest value after 5h. The expression level of the PCT gene increased gradually within 7 h after the onset of sepsis (Figure 1G).

3.2 | LncRNA-MALAT1 probe preparation and FISH

Figure 2A shows the MALAT1 amplification bands verified by agarose gel electrophoresis, with sizes of 538 bp and 589 bp, respectively. Figure 2B shows the white colony of the target clone after the T vector was inserted into the plate culture. FISH test results showed the localization of MALAT1 RNA transcripts (many red fluorescence signals of MALAT1 in the nucleus) (Figure 2C).

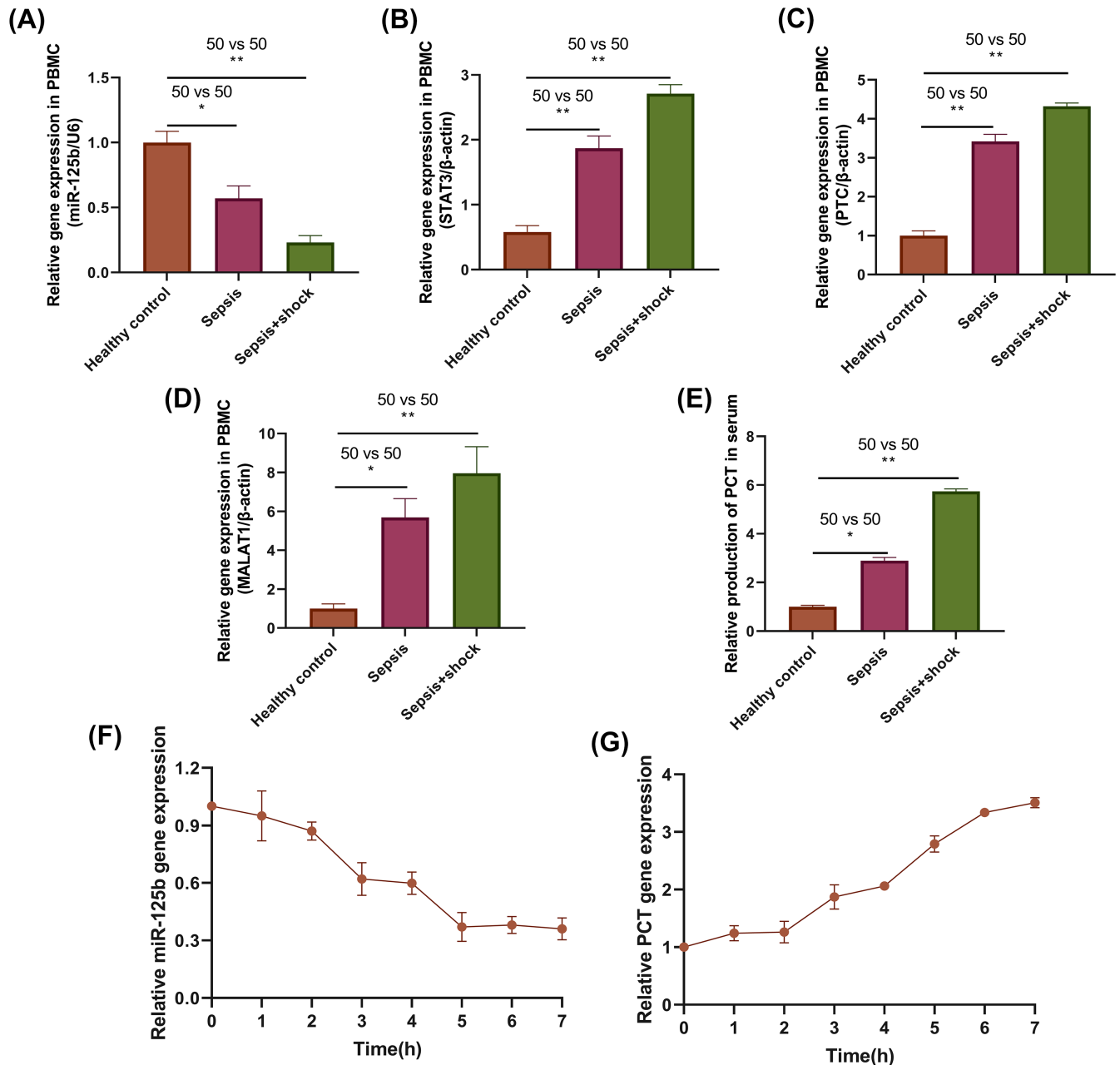


FIGURE 1 Expression of miR-125b, STAT3, and PCT in peripheral blood of patients with sepsis. (A) qPCR was used to detect the expression level of the miR-125b gene in serum. (B) qPCR was used to detect the expression level of the STAT3 gene in serum. (C) The expression level of the PCT gene was detected by qPCR. (D) The expression level of the MALAT1 gene in serum was detected by qPCR. (E) Detected the content of PCT in serum by automatic immunofluorescence analyzer Mini Vidas. (F) qPCR was used to detect miR-125b gene expression level in blood at an hourly rate of 0–7 h after sepsis. (G) qPCR was used to detect PCT gene expression level in blood every h within 0–7 h after sepsis. ** $p < 0.01$, * $p < 0.05$, compared with the healthy control group, the difference was statistically significant

3.3 | LncRNA-MALAT1/miR-125b/STAT3 interacted

The cells of the U937 group and U937+LPS group were detected by qPCR. The results showed that MALAT1 and STAT3 gene expression levels were significantly increased in the U937+LPS group compared with the U937 group, while the expression level of the miR-125b gene was inhibited, and the difference between the groups was

statistically significant (Figure 3A–C $p < 0.01$). Figure 3D shows the binding site and mutation site sequence of MALAT1 and miR-125b-5p. We observed a significant decrease in fluorescence intensity after co-transfection of wild-type MALAT1 with miR-125b-5p (compared with miR-NC co-transfection, $p < 0.05$). There was no significant change in fluorescence intensity after co-transfection of MALAT1 mutant with miR-125b (Figure 3E). Figure 3F shows the binding site and mutation site sequence of STAT3 and miR-125b. A

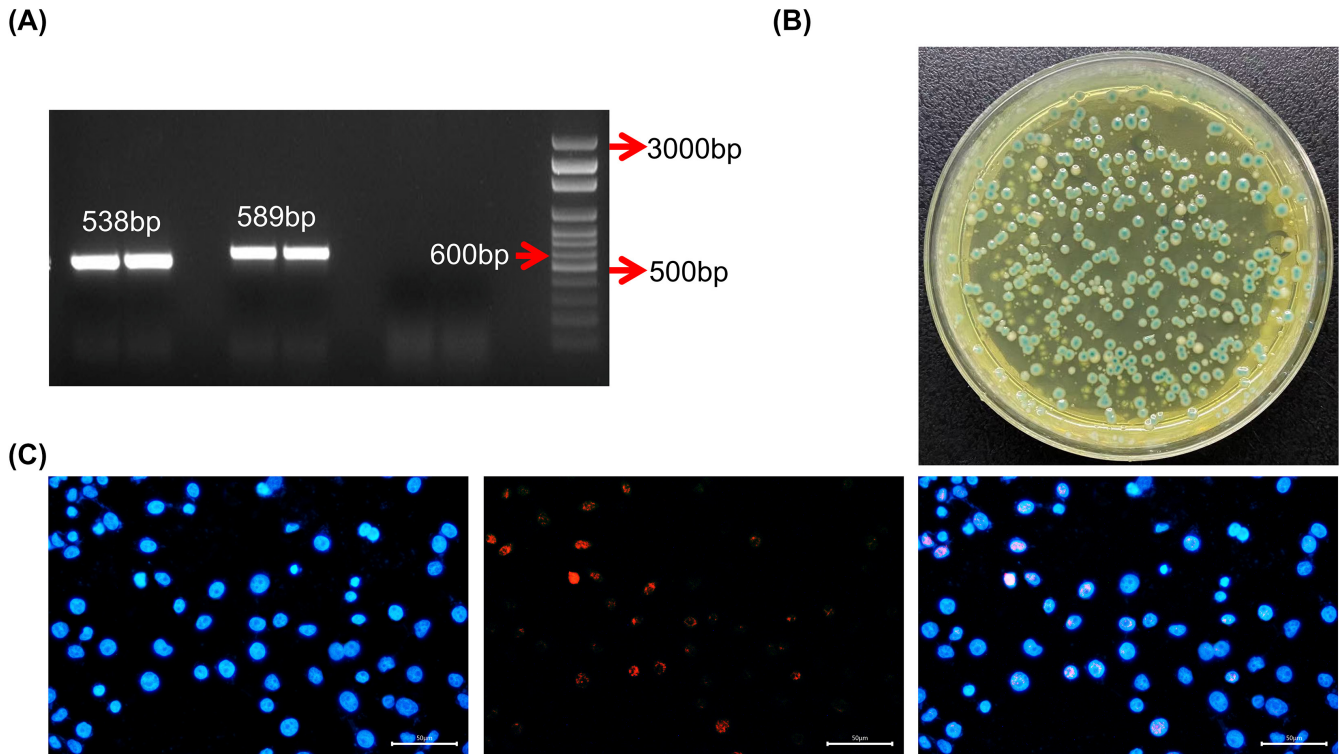


FIGURE 2 MALAT1 FISH test. (A) Agarose gel electrophoresis of PCR amplification band of MALAT1 gene. (B) X-gal plate was used for T vector cloning and screening, with white as the target colony. (C) FISH results were captured under a fluorescence microscope

significant decrease in fluorescence intensity was observed after co-transfection of wild-type STAT3 with miR-125b-5p (compared with miR-NC co-transfection, $p < 0.05$). However, there was no significant change in fluorescence intensity after co-transfection of STAT3 mutant with miR-125b (Figure 3G). After the adsorption of miR-125b by RNA pull-down assay, 2% agarose gel electrophoresis was performed (Figure 3H and 3I). The results showed that the amplification band of the MALAT1 gene was the brightest after miR-125b-5p probe adsorption. qPCR results also showed that the expression level of the MALAT1 gene in the miR-125b-5p probe group was significantly higher than that in the input group and the MUT probe group ($p < 0.05$).

3.4 | Impact of STAT3 on PCT

Cells were transfected with STAT3, si-NC, and si-STAT3 and studied based on a 100-ng/mL LPS model. qPCR results showed (Figure 4A–D) that transfection with STAT3, si-NC, and si-STAT3 did not notably affect the expression levels of MALAT1 and miR-125b genes ($p > 0.05$). Transfection of STAT3 significantly increased the expression levels of STAT3 and PCT genes, respectively ($p < 0.01$). However, Transfection with si-STAT3 significantly inhibited the expression levels of STAT3 and PCT genes. The western blot results showed that the expression and phosphorylation levels of STAT3 were significantly increased after transfection and LPS stimulation for 6 h (compared with the control group,

Figure 4E–G, $p < 0.01$). After si-STAT3 transfection, the expression and phosphorylation level of STAT3 protein were significantly downregulated ($p < 0.05$). The results showed that PCT was significantly upregulated after transfection with STAT3 (Figure 4H, $p < 0.01$) and decreased the PCT content after transfection with si-STAT3 ($p < 0.05$).

3.5 | miR-125b affects the expression of PCT through STAT3

Cells were transfected with NC mimic, miR-125b mimics, NC inhibitor, miR-125b in, and si-STAT3 and studied based on 100 ng/mL LPS model. qPCR results showed (Figure 5A–D) that transfection with miR-125b mimic did not significantly affect MALAT1 gene expression level but significantly inhibited STAT3 and PCT gene expression levels ($p < 0.01$). The co-transfection of miR-125b in and si-STAT3 could effectively reverse the abnormal elevation of STAT3 and PCT genes caused by inhibition of miR-125b expression. The western blot results showed significant inhibition of expression of STAT3 and phosphorylation of STAT3 after transfection with miR-125b mimic (Figure 5E–G, $p < 0.01$). The expression and phosphorylation levels of STAT3 were upregulated considerably after transfection with miR-125b ($p < 0.05$). This effect was reversed after simultaneous transfection with si-STAT3. The ELISA results showed that PCT was inhibited after transfection with miR-125b mimic (Figure 5H, $p < 0.01$). After transfection with miR-125b in,

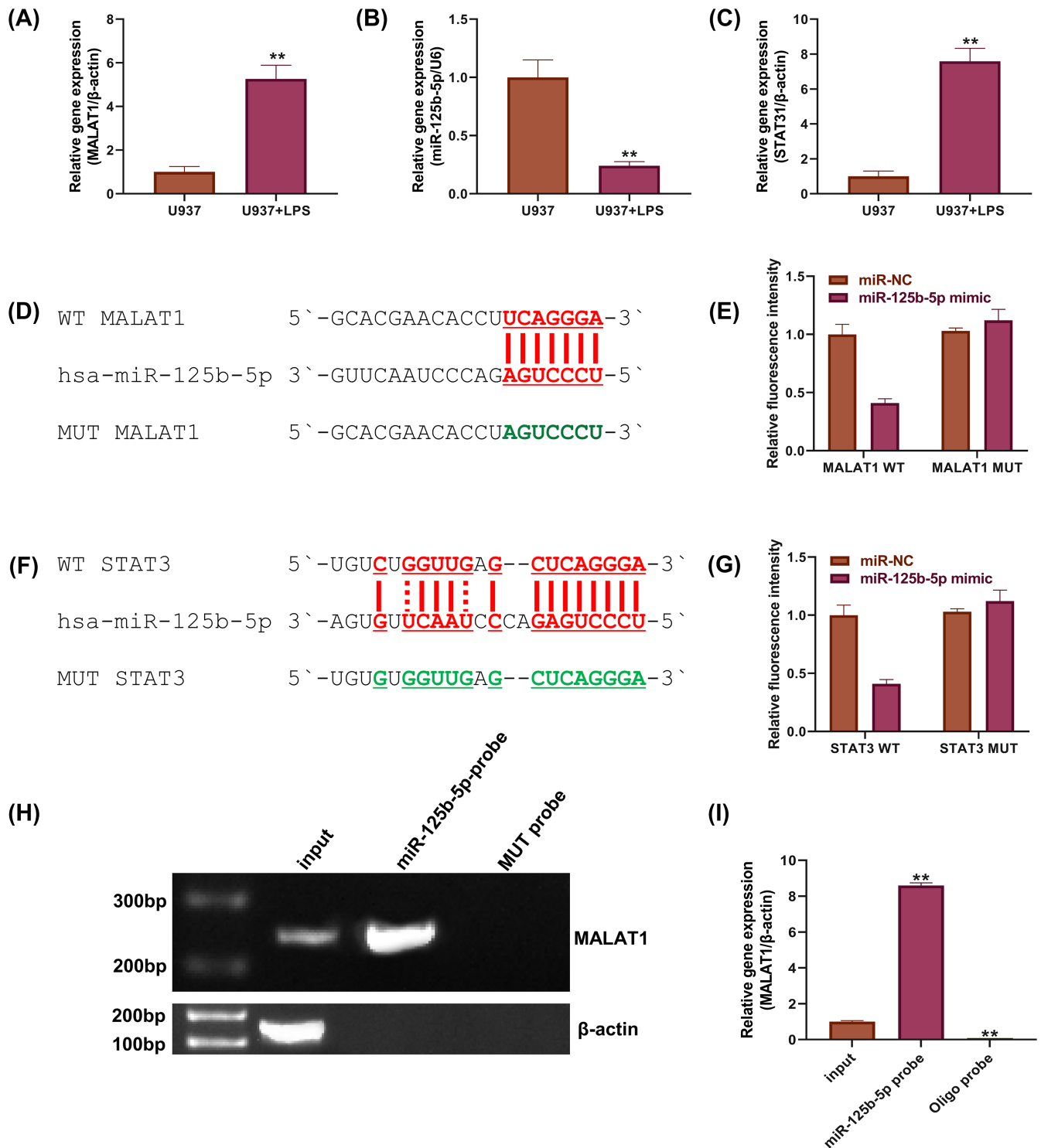


FIGURE 3 Verification of targeted binding relationships between MALAT1 and miR-125b, STAT3, and miR-125b. (A) MALAT1 gene expression was detected by qPCR. (B) The expression level of the miR-125b gene was detected by qPCR. (C) The expression level of the STAT3 gene was detected by qPCR. (D) The targeted binding site and mutation sequence of MALAT1 and miR-125b. (E) Dual luciferase reporter detection results. (F) The targeted binding site of STAT3 and miR-125b and mutation sequence of STAT3. (G) Dual luciferase reporter detection results. (H) After the RNA pull-down experiment, the MALAT1 gene was amplified by PCR and verified by agarose gel electrophoresis. (I) MALAT1 gene expression was detected by qPCR. ** $p < 0.01$, * $p < 0.05$, compared with U937 or the input group

PCT content was significantly upregulated ($p < 0.05$) but decreased after co-transfection with miR-125b in and si-STAT3 ($p < 0.05$). In brief, inhibition of miR-125b intensifies could promote an increase in

the expression and phosphorylation level of STAT3 in LPS-induced cells and the secretion of PCT by U937. Inhibition of the STAT3 gene effectively reversed this effect.

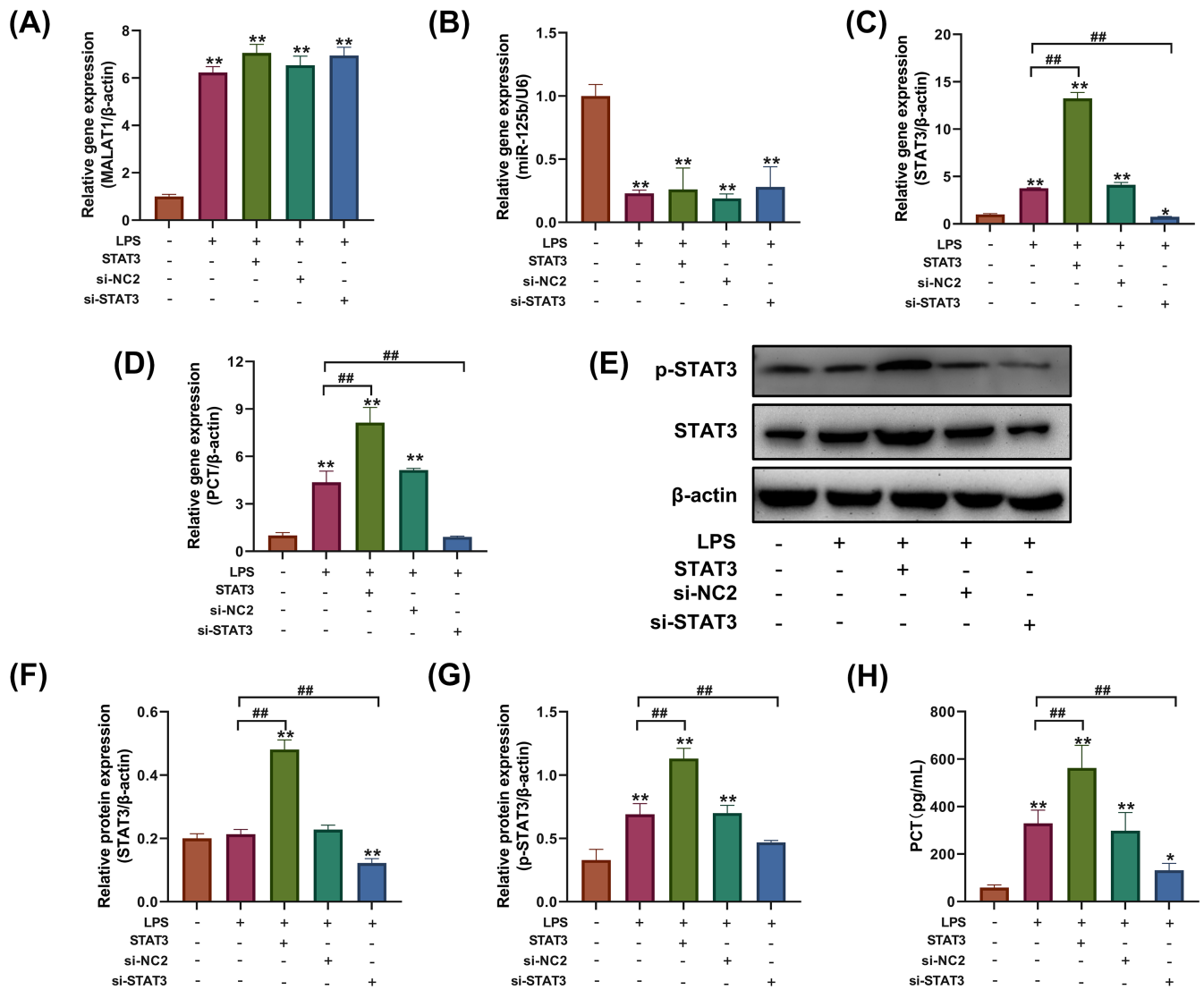


FIGURE 4 Effects of STAT3 on PCT content. (A) MALAT1 gene expression was detected by qPCR. (B) The expression level of the miR-125b gene was detected by qPCR. (C) The expression level of the STAT3 gene was detected by qPCR. (D) The expression level of the PCT gene was detected by qPCR. (E) The expression and phosphorylation levels of STAT3 were detected by western blot. (F-G) Western blot results were statistically quantified. (H) The content of PCT was detected by ELISA. ## $p < 0.01$, # $p < 0.05$, the two groups of wired were compared. ** $p < 0.01$, * $p < 0.05$, compared with the control group

3.6 | MALAT1 competitively binds to miR-125b to regulate the effect of STAT3 on PCT expression in sepsis

Cells were transfected with MALAT1, si-NC, si-MALAT1, and miR-125b inhibitor in and studied based on 100-ng/mL LPS model. qPCR results (Figure 6 A-D) showed that MALAT1 overexpression could inhibit the gene expression level of miR-125b but increased the gene expression levels of STAT3 and PCT. However, co-transfection of miR-125b in and si-MALAT1 could effectively reverse the inhibition of MALAT1 expression resulting in the inhibition of STAT3 and PCT genes. Western blot (Figure 6E-G) was used to detect STAT3 phosphorylation, and the results showed that the expression and phosphorylation levels of STAT3 were increased after overexpression of MALAT1 ($p < 0.01$). Significantly inhibited the expression of

STAT3 and phosphorylation STAT3 levels, after transfection with si-MALAT1 ($p < 0.05$). This effect was effectively reversed by simultaneous transfection of miR-125b in. ELISA determined the content of PCT in cells (Figure 6H), and ELISA the results showed that PCT content increased after overexpression of MALAT1 (compared with the LPS group, $p < 0.01$), and was significantly inhibited after transfection with si-MALAT1 ($p < 0.05$).

4 | DISCUSSION

In this study, the effect of STAT3 on the expression level of PCT that is gradually increased during systemic inflammation in sepsis patients is analyzed through a clinical study, which proved that high expression of STAT3 can upregulate the expression of PCT. This means

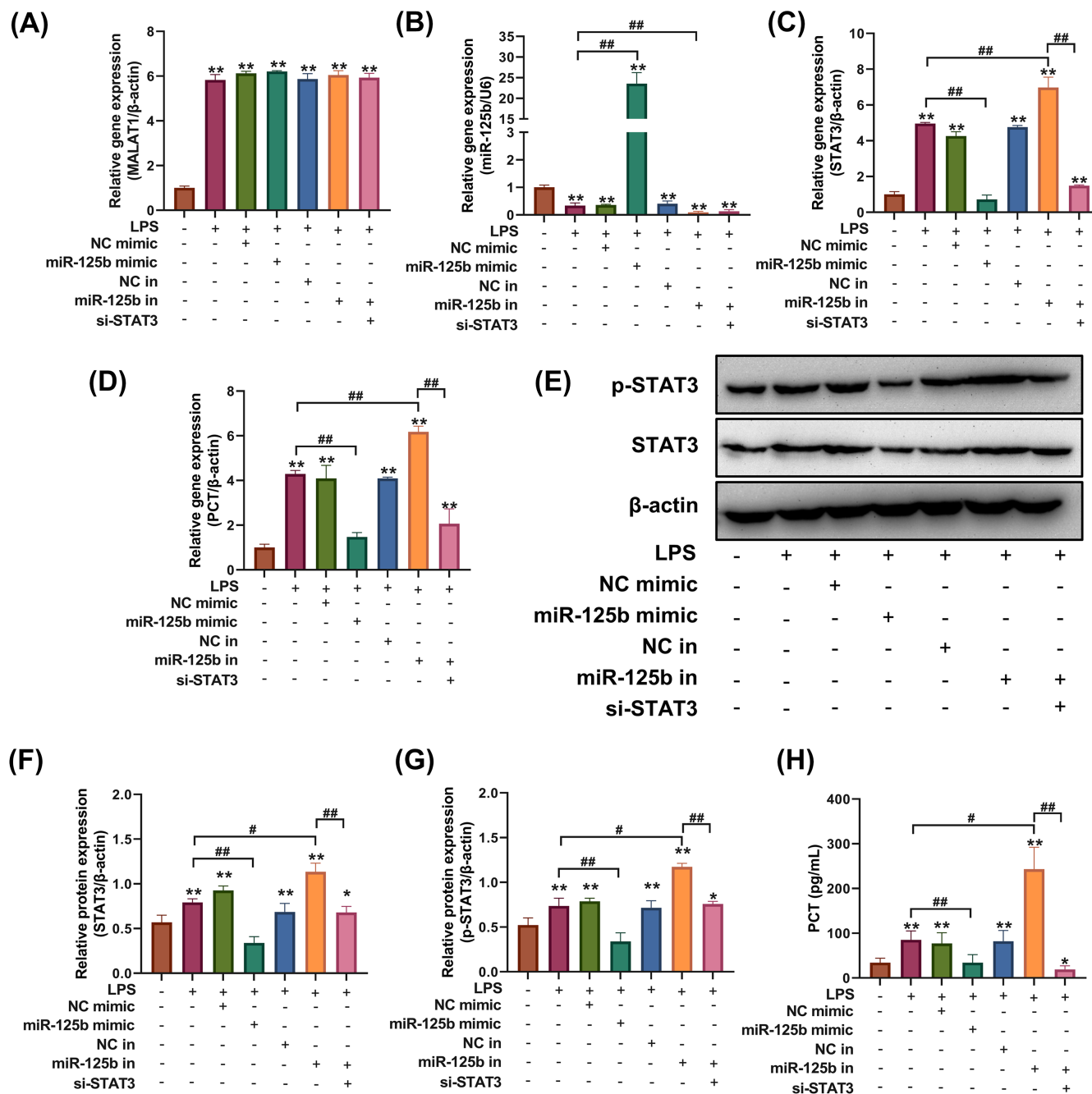


FIGURE 5 miR-125b affects the expression of PCT by STAT3. (A) MALAT1 gene expression was detected by qPCR. (B) The expression level of the miR-125b gene was detected by qPCR. (C) The expression level of the STAT3 gene was detected by qPCR. (D) The expression level of the PCT gene was detected by qPCR. (E) the expression and phosphorylation levels of STAT3 were detected by western blot. (F–G) Western blot results were statistically quantified. (H) The content of PCT was detected by ELISA. $^{##}p < 0.01$, $^{\#}p < 0.05$, the two groups of wired were compared. $^{**}p < 0.01$, $^{*}p < 0.05$, compared with the control group

that the expression of PCT may be affected by STAT3. Lei et al.¹⁶ also proposed the potential of STAT3 as a biomarker and therapeutic target for sepsis in future. Therefore, studies on PCT regulatory networks can map to ceRNA regulatory networks of STAT3. Our previous studies proved that miR-125b could reduce PCT release by binding STAT3 targeted.⁹

With the deepening of LncRNA research, the critical regulatory role of the interaction between LncRNA and miRNA has

been continuously explored in sepsis on the occurrence and development of sepsis inflammation and immune imbalance. The competing endogenous RNA (ceRNA) hypothesis is proposed as an essential post-transcriptional regulation mechanism. LncRNA, pseudogene, mRNA, and other endogenous RNAs contain some identical miRNA binding sites, which can competitively bind common miRNAs to weaken their inhibition of target genes, thereby improving the expression level of target genes and regulating

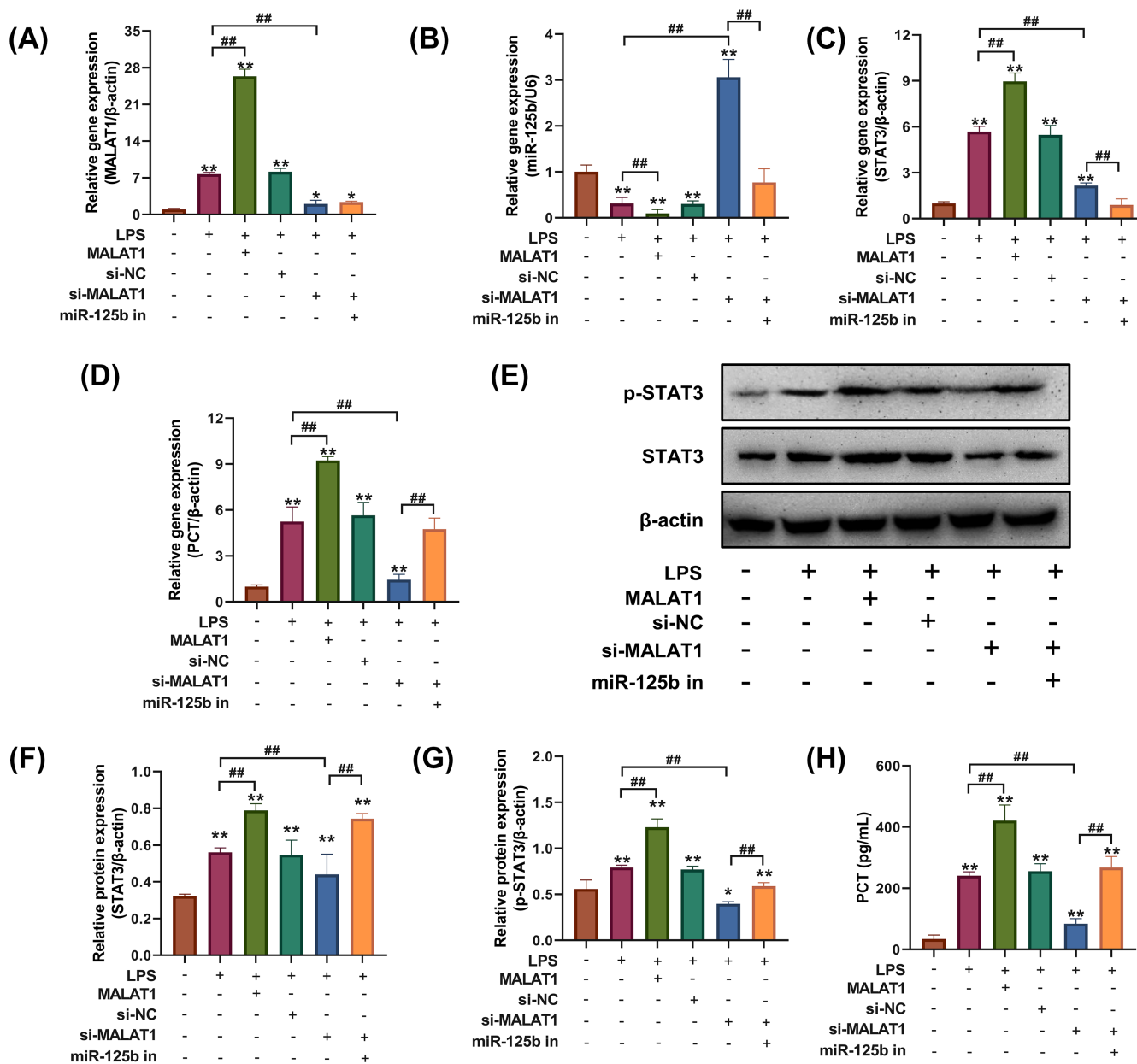


FIGURE 6 Effects of MALAT1, miR-125b, and STAT3 on the expression of PCT in sepsis. (A) MALAT1 gene expression was detected by qPCR. (B) The expression level of the miR-125b gene was detected by qPCR. (C) The expression level of the STAT3 gene was detected by qPCR. (D) The expression level of the PCT gene was detected by qPCR. (E) the expression and phosphorylation levels of STAT3 were detected by western blot. (F–G) Western blot results were statistically quantified. (H) The content of PCT was detected by ELISA. $^{##}p < 0.01$, $^{\#}p < 0.05$, the two groups of wired were compared. $^{**}p < 0.01$, $^{*}p < 0.05$, compared with the control group

cell life activities.¹⁷ This new hypothesis has gradually become a research hotspot and has been confirmed in various signaling pathways.

In sepsis studies, LncRNA GAS5 was found to aggravate myocardial inhibitory injury in mice through the microRNA-449B/HMGB1 axis and NF- κ B signaling pathway.¹⁸ LncRNA THRIL upregulates TNF- α expression through sponge adsorption of miR-19a after up-regulation in sepsis, and further damaging human bronchial epithelial cells.¹⁹ LncRNA NEAT1 regulates the NF- κ B pathway through competitive binding of miR-204 and affects septic-induced acute kidney injury.²⁰ Therefore, LncRNA regulates the expression of

miRNA through sponge adsorption is critical in sepsis. Consequently, we explored the mechanism of miR-125b/STAT3 on the expression of PCT in sepsis from the perspective of ceRNA.

Studies have shown that LncRNA-MALAT1 targets miR-125b to regulate inflammation or other pathogenesis in various diseases.²¹ For example, one experiment demonstrated that LncRNA-MALAT1 promotes neovascularization by mediating the miR-125b/VE-cadherin axis in an endothelial cell model of diabetic retinopathy.¹⁴ In Feng's study,²² the expression of LncRNA-MALAT1 and miR-125b in plasma of sepsis patients was negatively correlated, and the level of miR-125b was downregulated in sepsis patients, suggesting that

miR-125b is the target of LncRNA-MALAT1 in sepsis and plays an anti-inflammatory role in sepsis. Our experiment also observed a high expression level of MALAT1 in patients with sepsis and a higher expression level in patients with septic shock.

This study determined the targeted binding sites of MALAT1 and miR-125b by target binding prediction of MALAT1 and miR-125b through the miRDB database. Subsequently, luciferase constructs containing wild-type or mutant MALAT1 were generated and assayed to demonstrate that luciferase activity of wild-type MALAT1 was inhibited by miR-125b mimic. In contrast, luciferase activity of mutant MALAT1 was not affected. Therefore, MALAT1 has a targeted binding relationship with miR-125b. At the same time, we also combined the FISH experiment to locate MALAT1. During the FISH experiment, there were 4 spliceosomes of MALAT1, and we amplified them according to their conservative sites and designed 4 primers. The results showed that it could amplify 2 of them to obtain MALAT1 gene fragments in large quantities. Since FISH probes most domestic companies synthesized by designing oligonucleotide fragment markers with a size of dozens of nucleotides, there may be a problem of reduced specificity. Therefore, we extended the probe fragment to obtain a probe with higher specificity. FISH experiments were performed using two MALAT1 components labeled Cy3, and it is evident that MALAT1 was highly expressed mainly in the nucleus of U937 cells. This is the same as the location of MALAT1 in the study by Deepak Singh et al.²³ MALAT1 RNA is highly conserved in mammals, mainly located in the nuclear spot or SC35 domain, and the nuclear structure contains proteins and RNAs involved in pre-mRNA processing.^{24,25} This provides the basis for the binding of miR-125b-5p. In addition, an RNA pull-down assay was used to verify the binding relationship between MALAT1 and miR-125b, and sponge adsorption was confirmed.

To determine whether MALAT1/miR-125b/STAT3 regulates the expression level of PCT, we combined siRNA technology to achieve gene expression regulation through cell transfection technology. The results showed that transfection of MALAT1 siRNA could significantly inhibit the gene and protein expression levels of STAT3 and PCT. Therefore, we believe that MALAT1 regulates PCT expression through the ceRNA network. In addition, studies have proved that the central role of MALAT1 in sepsis is to cause inflammatory co-reactions and play a proinflammatory role. Downregulation of MALAT1 expression can inhibit the inflammatory response caused by sepsis, which has the potential to treat sepsis.²⁶ In conclusion, MALAT1 can upregulate STAT3 and PCT by inhibiting the expression of miR-125b, and MALAT1 has a proinflammatory effect on sepsis. Therefore, MALAT1 is expected to assist or even replace PCT as a new diagnostic marker for sepsis and may also be a potential target for sepsis treatment.

CONFLICTS OF INTEREST

The authors declare that they have no competing interests.

DATA AVAILABILITY STATEMENT

The data sets during the current study are available from the corresponding author on reasonable request.

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