Long non-coding RNA PVT1 regulates LPS-induced acute kidney injury in an *in vitro* model of HK-2 cells by modulating the miR-27a-3p/OXSR1 axis

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Received October 23, 2019; Accepted Ferbruary 18, 2020

DOI: 10.3892/etm.2022.11490

Abstract. Sepsis is a severe inflammatory disease caused by infection that can lead to multiple organ failure. Acute kidney injury (AKI) is considered to be a major cause of septic mortality in infected organs. Previous studies have revealed that non-coding RNAs are involved in AKI, but the underlying mechanisms are mostly unknown. The present study aimed to explore the role of long non-coding RNA plasmacytoma variant translocation gene 1 (lncRNA PVT1) in lipopolysaccharide (LPS)-induced acute kidney injury and the underlying mechanism. In the present study, reverse transcription-quantitative PCR analysis indicated that, in HK-2 cells treated with LPS, the mRNA expression levels of lncRNA PVT1 and oxidative stress responsive kinase 1 (OXSR1) were upregulated, and the expression of microRNA (miR)-27a-3p was downregulated. Furthermore, LPS treatment could promote the secretion of tumor necrosis factor (TNF)-α and interleukin (IL)-6, inhibit cell proliferation and induce apoptosis, which was rescued by PVT1 knockdown. Dual-luciferase reporter assay, RIP assay and pull-down assay results demonstrated that miR-27a-3p may be a target miR of PVT1, and that OXSR1 is the target gene of miR-27a-3p. Moreover, it was found that miR-27a-3p overexpression decreased the secretion of TNF-α and IL-6, promoted cell proliferation and inhibited apoptosis in LPS-treated HK-2 cells, which could be reversed by OXSR1 overexpression. Therefore, the present results indicated that lncRNA PVT1 regulated inflammatory cytokine secretion, cell proliferation and apoptosis by targeting miR-27a-3p, and modulating OXSR1 expression in LPS-induced HK-2 cells.

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Key words: acute kidney injury, sepsis, long non-coding RNA plasmacytoma variant translocation gene 1, microRNA-27a-3p, oxidative stress responsive kinase 1

Introduction

Sepsis is caused by a detrimental inflammatory response to infection, and is associated with a high mortality rate of >40% (1). In particular, acute kidney injury (AKI) caused by sepsis accounts for ~50% of all AKI cases (2). A previous study revealed that various factors are related to sepsis-induced AKI, including non-coding RNAs (3).

Whole-genome transcriptome sequencing analysis showed that encoded protein genes account for ~2% of human transcripts, while other transcripts are mostly non-protein-coding genes (4,5). Previous studies have focused on the important role of long non-coding RNAs (lncRNAs) in molecular function (6), and >50,000 human lncRNAs have been identified (7).

The transcript of the human gene lncRNA plasmacytoma variant translocation gene 1 (lncRNA PVT1) is located in chromosome 8q24, which is widely recognized as a cancer-associated region (8). PVT1 is involved in human diseases, and plays a crucial role in the development and progression of various cancer types, such as gallbladder cancer (9), leukemia (10), hepatocellular cancer (11-13), breast cancer (14) and ovarian cancer (15). In addition, it has been revealed that PVT1 plays a key role in a variety of pathological conditions, including sepsis (16-18).

lncRNA PVT1 is upregulated in tissues of septic models, and regulates sepsis-induced heart inflammation and cardiac function by interacting with microRNA (miRNA/miR)-143 and the mitogen-activated protein kinase (MAPK)/NF-κB pathway (17). Furthermore, PVT1 aggravated lipopolysaccharide (LPS) induced myocardial injury via miR-29a/HMGB1 axis (18). PVT1 also promotes cell proliferation, invasion and epithelial-mesenchymal transition by downregulating miR-16-5p in renal cell carcinoma *in vitro* (19). Moreover, it has been shown that PVT1 promotes the cell cycle and apoptosis of cervical cancer cells by activating the NF-κB pathway (20). lncRNA PVT1 downregulation is also involved in the increased rate of apoptosis and reduced proliferation of acute lymphoblastic leukemia cells (21). Therefore, these

studies suggest that lncRNA PVT1 generally acts as an oncogene in a variety of cancer types.

miRNAs are widely studied due to their essential role in development, tissue-specific expression and close relationship to human diseases (22). miR-27a is abnormally upregulated in several types of cancer and has been identified as an oncogene during tumorigenesis (23-26). Furthermore, miR-27a-3p has been reported to participate in the pathological processes of various diseases, including the immune response and inflammatory response (27-29). Among these disease types, miR-27a-3p is downregulated in sepsis (30). It has also been shown that miR-27a-3p is associated with inflammation and exerts an anti-inflammatory effect in sepsis (30). However, the underlying mechanisms remain unknown.

Therefore, the aims of the present study were to investigate the regulation of lncRNA PVT1 and miR-27a-3p in sepsis of AKI, and to identify the downstream mechanisms. Thus, the present results may facilitate the development of novel treatments for sepsis and related inflammation.

Materials and methods

Cell culture and transfection. The HK-2 cell line was purchased from the American Type Culture Collection, and cultured in DMEM (Gibco; Thermo Fisher Scientific, Inc.) with 10% FBS (Gibco; Thermo Fisher Scientific, Inc.), 100 IU/ml penicillin and 100 mg/ml streptomycin (Beijing Solarbio Science & Technology Co., Ltd.). Cells were grown and maintained at 37˚C in a 5% CO_2 humidified incubator. At ~60% confluence, HK-2 cells were treated with different concentrations (0, 0.1, 2, 5 and 10 μg/ml) of LPS (Sigma-Aldrich; Merck KGaA) at 37˚C for 8 h, and then 5 μg/ml was selected to generate a sepsis cell model based on previously reported research (16).

miR-27a-3p mimic (miR-27a-3p), oxidative stress responsive kinase 1 (OXSR1) overexpression vector (OXSR1), PVT1 overexpression vector (pcDNA-PVT1) and their negative controls (NC mimic or empty pcDNA3.1 vector) were purchased from Shanghai GenePhama Co., Ltd. Small interfering (si)-PVT1, anti-miR-27a-3p and their NCs (si-NC and anti-NC) were also purchased from Shanghai GenePhama Co., Ltd. The sequences of siRNA, miR mimic, inhibitor and NC were as follows: si-PVT1, 5'-GCUUGGAGGCUGAGG AGUUTT-3'; miR-27a-3p mimic, 5'-UUCACAGUGGCUAA GUUCCGC-3'; miR-27a-3p inhibitor, 5'-GCGGAACTTAG CCACTGTGAA-3'; si-NC, 5'-UUCUCCGAACGUGUCA-3'; miRNA mimic NC, 5'-UUUGUACUACACAAAAGU ACUG-3'; and miRNA inhibitor NC, 5'-CAGUACUUUUGUG UAGUACAAA-3'. Cell were seeded in 6-well plates and cultured to 70% confluence. siRNAs (50 nM), miR mimics (50 nM), miR inhibitors (100 nM) and plasmids (5 μg) were transfected into HK-2 cells using Lipofectamine® 3000 (Thermo Fisher Scientific, Inc.). After transfection for 48 h, the cells were collected for further experiments.

Reverse transcription-quantitative PCR (RT-qPCR). Total RNA was extracted from HK-2 cells using TRIzol® reagent (Invitrogen; Thermo Fisher Scientific, Inc.). cDNAs of lncRNA and mRNA were generated using M-MLV reverse transcriptase (Invitrogen; Thermo Fisher Scientific, Inc.) at 42˚C for 1 h. A TaqMan miRNA RT kit (Thermo Fisher Scientific, Inc.) was used for the RT-qPCR of miR-27a-3p (RT was performed at 16˚C for 30 min, 42˚C for 30 min and 85˚C for 5 min; qPCR was performed as follows: initial denaturation at 95˚C for 10 min; hold at 55˚C for 2 min and 72˚C for 2 min; followed by 12 cycles of 95˚C for 15 sec and 60˚C for 4 min). TaqMan Universal Master mix II (Thermo Fisher Scientific, Inc.) was used for the qPCR of lncRNA and mRNA (initial denaturation at 95˚C for 10 min followed by 40 cycles of 95˚C for 15 sec and 60˚C for 1 min). U6 and 18s rRNA were used for normalization. All data were calculated with the $2^{-\Delta\Delta Cq}$ method (31). The following primer sequences were used: PVT1 forward, 5'-TGA GAACTGTCCTTACGTGACC-3' and reverse, 5'-AGAGCACC AAGACTGGCTCT-3'; miR-27a-3p forward, 5'-GCGCGTTCA CAGTGGCTAAG-3' and reverse, 5'-AGTGCAGGGTCCGAG GTATT-3'; 18S rRNA forward, 5'-GGCCCTGTAATTGGAAT GAGTC-3' and reverse, 5'-CCAAGATCCAACTACGA GCTT-3'; and U6 forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATT TGCGT-3'.

ELISA. The levels of TNF-α and IL-6 were measured by ELISA using the cell culture medium. The cell culture medium was centrifuged at 1,000 x g for 10 min at 4˚C, and the supernatant was collected. Commercially available ELISA kits for TNF-α and IL-6 (cat. nos. 550610 and 550799, respectively; BD Biosciences) were used according to the manufacturer's instructions. Results were read at an optical density of 450 nm using a Spectra Max Plus plate reader (Molecular Devices LLC). Measurements were performed in triplicate, and P-values were computed using two-tailed Student's t-tests.

Cell Counting Kit-8 (CCK-8) assay. HK-2 cells were seeded into 96-well plates at a density of 10,000 cells/well. After 12 h of incubation at 37˚C, cells were transfected for 24 h in the presence of 0-10 μg/ml LPS and then washed three times with PBS. Then, 10 μl/well CCK-8 solution (Sigma-Aldrich; Merck KGaA) was pipetted into each well, and the plate was incubated for 1.5 h at 37˚C. Absorbance was measured at 450 nm using a microplate reader (Model 550; Bio-Rad Laboratories, Inc.).

Cell apoptosis. HK-2 were seeded in 96-well plates at a density of 10,000 cells/well for 12 h. The cells were transfected as described above. Then, the cells were prepared into single-cell suspension and double-stained with 5 μl FITC-Annexin V and 5 μl propidium iodide at room temperature for 15 min in the dark using the FITC-Annexin V apoptosis detection kit (BD Biosciences). The staining was followed by analysis using a flow cytometer (FACScan; BD Biosciences) equipped with Cell Quest software (version 5.1; BD Biosciences). The apoptosis rate was calculated as the percentage of early + late apoptotic cells.

Western blotting. Total protein was extracted by RIPA buffer (Cell Signaling Technology, Inc.). The concentration of protein was measured with a bicinchoninic acid (BCA) protein assay kit (Bio-Rad Laboratories, Inc.). Equal quantities of proteins (20 μg/lane) were separated on 10% SDS-PAGE gels and transferred to PVDF membranes. The membranes were blocked with 5% non-fat milk in 1X Tris-buffered saline and 0.1% Tween-20 at room temperature for 1 h. Membranes were

then incubated overnight at 4˚C with primary antibodies for Bcl-2 (1:1,000; cat. no. ab59348; Abcam), Bax (1:1,000; cat. no. ab32503; Abcam), cleaved caspase-3 (C-caspase-3; 1:1,000; cat. no. ab49822; Abcam), OXSR1 (1:1,000; cat. no. ab97694; Abcam), phosphorylated (p)-inhibitor of κB (IκBα; 1:1,000; cat. no. 2859; Cell Signaling Technology), total-IκBα (1:1,000; cat. no. 4812; Cell Signaling Technology) p-p65 (1:1,000; cat. no. 3033; Cell Signaling Technology), total-p65 (1:1,000; cat. no. 8242; Cell Signaling Technology) and GAPDH (1:5,000; cat. no. ab9485; Abcam). The membranes were then probed with appropriate secondary antibodies at room temperature for 2 h [horseradish peroxidase-conjugated goat anti-rabbit IgG H&L (1:5,000; cat. no. ab205718; Abcam)]. The target protein levels were detected using enhanced chemiluminescence reagents (Pierce; Thermo Fisher Scientific, Inc.) and analyzed with Image J (version 1.8.0; National Institutes of Health).

Dual-luciferase reporter assay. The putative binding sites between miR-27a-3p and PVT1 or OXSR1 were predicted by StarBase 2.0 (http://starbase.sysu.edu.cn/starbase2/) and TargetScanHuman 7.2 (http://www.targetscan.org/), respectively. The fragments from the 3' untranslated regions (3'UTR) of PVT1 or OXSR1, containing the predicted miR-27a-3p binding site, were synthesized and cloned into the *Xho*I and *Not*I sites of a psiCHECK-2 vector (Promega Corporation) to form the reporter vectors PVT1-wild-type (wt) or OXSR1-wt. The corresponding mutants (mut) were constructed by mutating the miR-27a-3p seed region binding site, and were referred to as the reporter vectors PVT1-mut or OXSR1-mut. Then, miR-27a-3p mimic or NC mimic (50 nM) were co-transfected with the reporter vectors (5 μg) containing either the targeting sequences or the corresponding mutants using Nanofectin transfection reagent (Shanghai ExCell Biology, Inc.). After transfection for 48 h, luciferase activity was determined using a Dual-Luciferase Assay Kit (GeneCopoeia) and detected by multimode detector (Beckman Coulter, Inc.). Firefly luciferase activity was used for normalization.

RNA immunoprecipitation (RIP) assay. RIP was performed using an EZ-Magna RIP RNA-Binding protein immunoprecipitation kit (EMD Millipore). Cells stably transfected with NC or miR-27a-3p were lysed by RIP lysis buffer, and 100 μl cell lysates was incubated with RIP buffer containing magnetic beads conjugated with human anti-argonaute 2 antibody (1:500; cat. no. 04-642; EMD Millipore) or normal mouse IgG (1:500; cat. no. 12-371; EMD Millipore), which was the negative control, at 4˚C overnight. Then, proteinase K buffer was used to digest the protein from samples and the immunoprecipitated RNA was extracted using TRIzol reagent (Invitrogen). Purified RNA was analyzed by RT-qPCR to detect the expression levels of lncRNA PVT1 and OXSR1 in the precipitates as aforementioned. The primer sequences for OXSR1 were 5'-AAAGACGTTTGTTGGCACCC-3' (forward) and 5'-GCC CCTGTGGCTAGTTCAAT-3' (reverse).

RNA pull-down assay. Probe miR-27a-3p (50 nM) was biotinylated by a biotinylation kit (Thermo Fisher Scientific, Inc.) and transfected into HK-2 cells with Lipofectamine 3000. After transfection for 48 h, cells were lysed and incubated with Dynabeads M-280 Streptavidin (10 mg/ml; Invitrogen; Thermo Fisher Scientific, Inc.) at 25˚C for 1 h. The complexes were isolated using streptavidin agarose beads (Invitrogen; Thermo Fisher Scientific, Inc.). The bound RNAs were detected using RT-qPCR as aforementioned.

Statistical analysis. All experiments were performed in triplicate. Data are presented as the mean ± SD. Results were analyzed using two-tailed Student's t-test for two groups, and one-way ANOVA for multiple groups followed by Tukey's test. All statistical analyses were performed using SPSS 19.0 (SPSS, Inc.). $P<0.05$ was considered to indicate a statistically significant difference.

Results

lncRNA PVT1 expression is upregulated, while miR-27a-3p is downregulated in LPS-induced HK-2 cells. To investigate the roles of lncRNA PVT1 and miR-27a-3p in septic AKI progression *in vitro*, HK-2 cells were selected for septic AKI model construction by LPS stimulation. To detect cell viability after LPS treatment, cells were incubated with LPS at various concentrations (0, 0.1, 2, 5 and 10 μg/ml) for 8 h, and it was found that low cell viability was negatively associated with enhanced concentrations of LPS (Fig. 1A). Based on the significant difference with the control group (0 μg/ml), 5 μg/ml LPS administration was selected for further experiments. Moreover, the mRNA expression levels of PVT1 and miR-27a-3p were examined by RT-qPCR, and it was demonstrated that PVT1 was significantly increased in LPS-treated cells, while the opposite was observed for miR-27a-3p (Fig. 1B and C). Therefore, the present results indicated that LPS-induced HK-2 cells exhibited enhanced expression of PVT1 and downregulated expression of miR-27a-3p.

PVT1 knockdown decreases inflammatory cytokines secretion, promotes cell survival and inhibits apoptosis in LPS-treated HK-2 cells. To further investigate the effect of lncRNA PVT1 on LPS-treated HK-2 cells, loss-of-function assays were performed. The transfection efficiency of si-PVT1 was detected, and it was found that this transfection significantly suppressed PVT1 expression (Fig. S1A). Moreover, it was identified that si-PVT1 significantly blocked the promotive effect of LPS on PVT1 expression compared with the LPS+scramble group (Fig. 2A). Subsequently, the levels of the inflammatory cytokines TNF-α and IL-6 were detected. After silencing PVT1 expression, the secretion levels of these immune factors were significantly reduced compared with the LPS+scramble group (Fig. 2B and C). Furthermore, it was found that cell viability was significantly increased after PVT1 knockdown (Fig. 2D). Then, flow cytometry was used to assess the apoptotic rate, and it was identified that the number of apoptotic cells decreased significantly after PVT1 silencing (Fig. 2E). Moreover, the expression of the apoptosis inhibitor Bcl-2 was significantly increased, while the protein expression levels of the apoptotic factors Bax and C-caspase-3 were significantly decreased in the LPS+si-PVT1 group (Fig. 2F). Collectively, the present results suggested that knockdown of lncRNA PVT1 in LPS-induced HK-2 cells decreased TNF-α and IL-6 secretion, promoted cell activity and inhibited apoptosis.

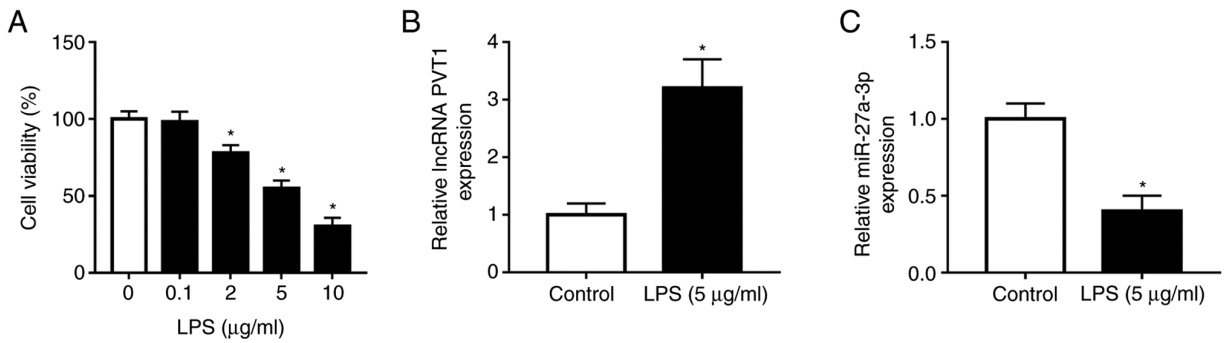

Figure 1. lncRNA PVT1 expression is upregulated in LPS-induced HK-2 cells. (A) Cell Counting Kit-8 assay was used to assess the viability of LPS-induced HK-2 cells. (B) RT-qPCR assay was used to detect the mRNA expression level of PVT1 in LPS-induced (5 μg/ml) HK-2 cells and untreated cells (control). (C) mRNA expression level of miR-27a-3p in LPS-induced (5 μg/ml) HK-2 cells and untreated cells (control) were determined by RT-qPCR. *P<0.05 vs. 0 μg/ml. LPS, lipopolysaccharide; RT-qPCR, reverse transcription-quantitative PCR; PVT1, plasmacytoma variant translocation gene 1; lncRNA, long non-coding RNA; miR, microRNA

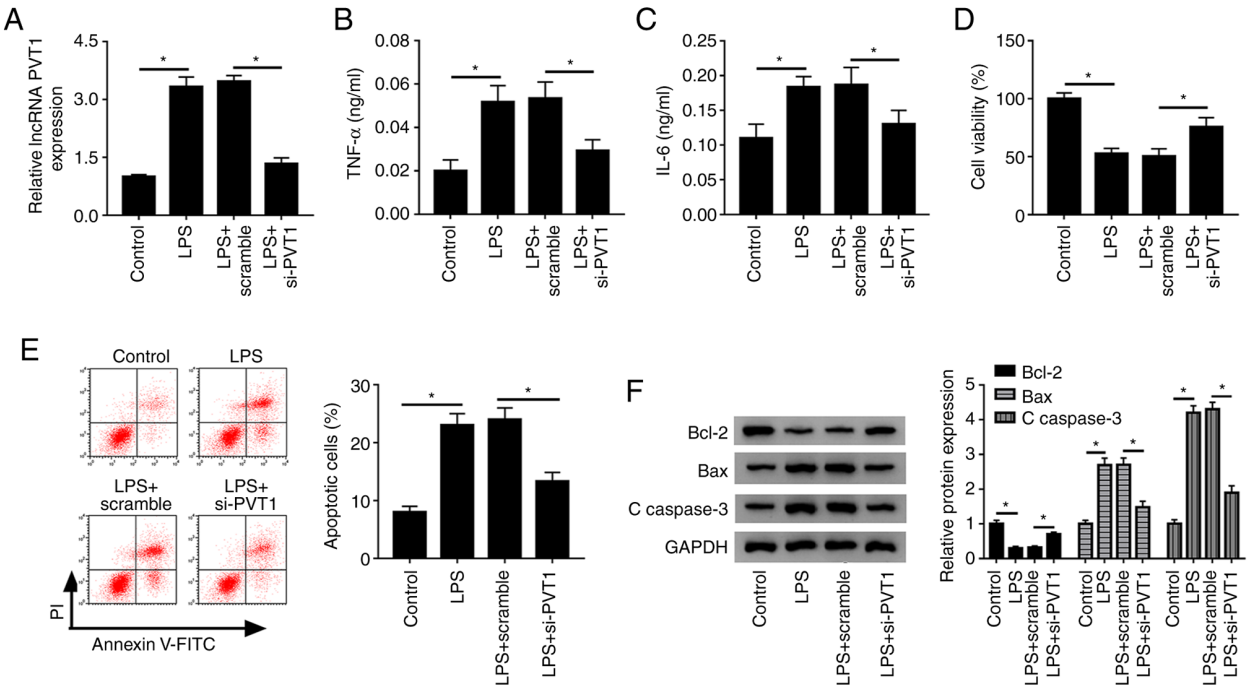

Figure 2. PVT1 knockdown alleviates inflammation, promotes cell survival and inhibits apoptosis. (A) Relative expression of lncRNA PVT1 in control, LPS, LPS+scramble and LPS+si-PVT1 groups were detected in HK-2 cells by reverse transcription-quantitative PCR. Levels of the inflammatory cytokines (B) TNF-α and (C) IL-6 were measured by ELISA. (D) Cell viability and (E) apoptotic rates of control, LPS, LPS+scramble and LPS+si-PVT1 groups were assessed with Cell Counting Kit-8 assay and flow cytometry, respectively. (F) Protein expression levels of Bcl-2, Bax and C-caspase 3 was measured in each group using western blotting. *P<0.05. C-, cleaved; LPS, lipopolysaccharide; PVT1, plasmacytoma variant translocation gene 1; lncRNA, long non-coding RNA; miR, microRNA; si, small interfering RNA; TNF-α, tumor necrosis factor-α; IL, interleukin; PI, propidium iodide

miR-27a-3p overexpression decreases inflammatory cytokines secretion, promotes cell survival and inhibits apoptosis in HK-2 cells after LPS treatment. Next, the present study investigated the role of miR-27a-3p in LPS-induced HK-2 cells. It was demonstrated that miR-27a-3p overexpression was successful after transfection with miR-27a-3p mimic (Fig. S1C). When LPS-induced cells were overexpressed with miR-27a-3p, it was identified that miR-27a-3p expression was significantly increased (Fig. 3A). Moreover, the secretion of TNF-α and IL-6 were significantly decreased after miR-27a-3p mimic transfection (Fig. 3B and C). It was also demonstrated that miR-27a-3 overexpression increased cell viability (Fig. 3D), and the number of apoptotic cells was significantly decreased (Fig. 3E). Furthermore, after miR-27a-3p overexpression, the expression of the apoptosis inhibitor Bcl-2 was promoted, while the expression levels of the apoptotic pathway proteins Bax and C-caspase-3 were significantly decreased (Fig. 3F). Thus, the present results indicated that overexpression of miR-27a-3p in LPS-induced HK-2 cells reduced inflammation, promoted cell activity and inhibited apoptosis, which was similar to PVT1 knockdown.

miR-27a-3p is a target miRNA of PVT1. The relationship between PVT1 and miR-27a-3p was predicted with StarBase, and it was identified that miR-27a-3p contained complementary sequences with PVT1 (Fig. 4A). Then, dual-luciferase

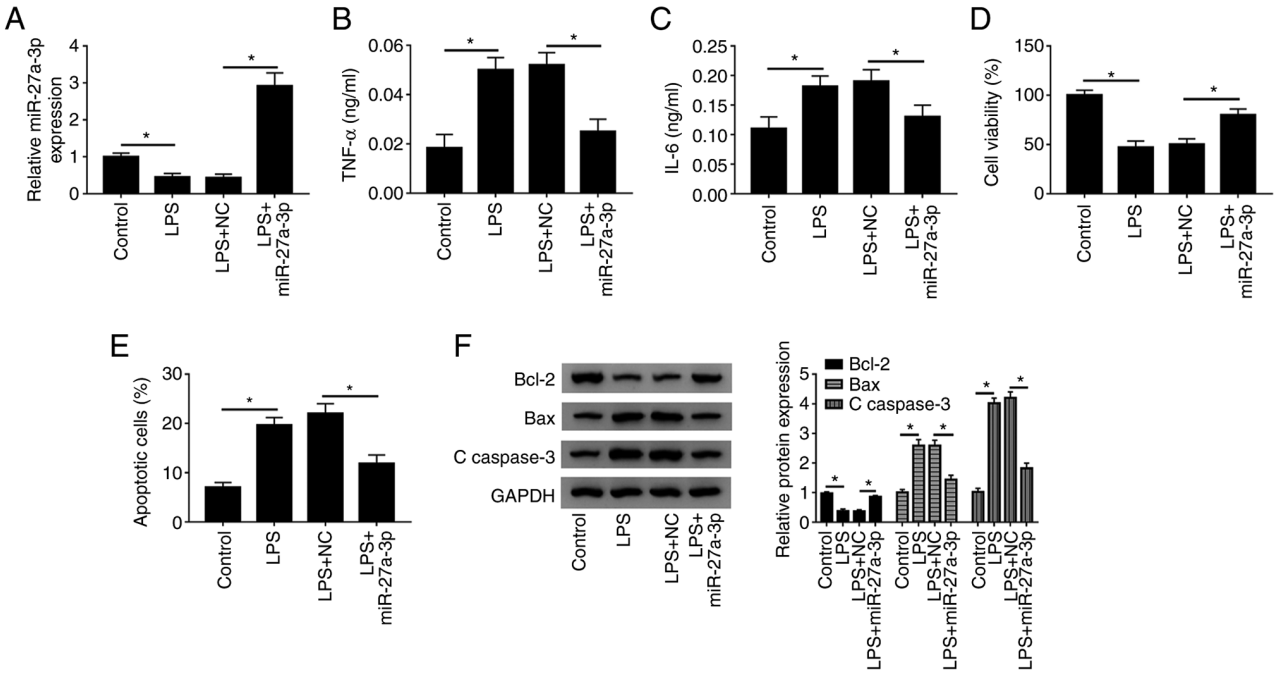

Figure 3. Effect of miR-27a-3p overexpression is similar to that of PVT1 knockdown in HK-2 cells. (A) Relative expression of miR-27a-3p in control, LPS, LPS+NC and LPS+miR-27a-3p groups was detected by reverse transcription-quantitative PCR. Secretion levels of (B) TNF-α and (C) IL-6 were measured by ELISA. (D) Cell viability and (E) apoptotic rates of control, LPS, LPS+NC and LPS+miR-27a-3p groups were detected by Cell Counting Kit-8 analysis and flow cytometry, respectively. (F) Protein expression levels of Bcl-2, Bax and C-caspase3 were measured in each group using western blotting. *P<0.05. C-, cleaved; LPS, lipopolysaccharide; PVT1, plasmacytoma variant translocation gene 1; lncRNA, long non-coding RNA; miR, microRNA; TNF-α, tumor necrosis factor-α; IL, interleukin; NC, negative control.

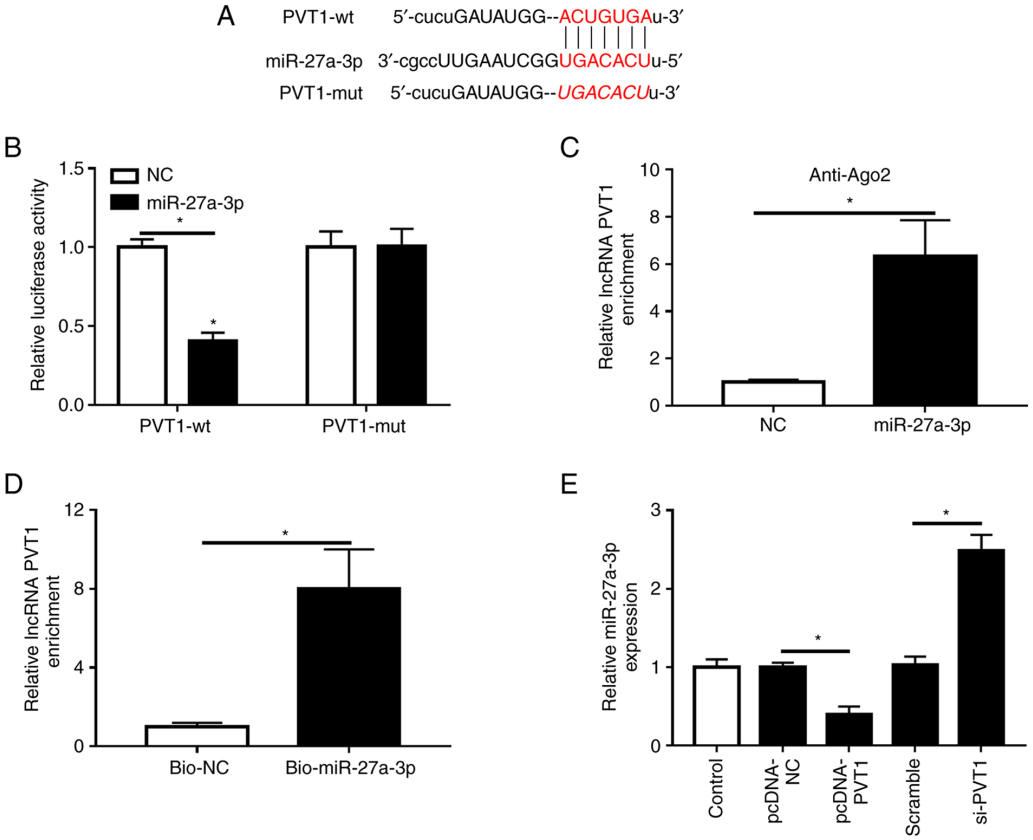

Figure 4. miR-27a-3p is the target miRNA of PVT1. (A) StarBase predicted that miR-27a-3p was a potential miRNA of PVT1. (B) Luciferase reporter assay was performed to assess the relationship between miR-27a-3p and PVT1. (C) Enrichment of PVT1 was detected by RT-qPCR after incubation with Anti-Ago2 in RIP assay. (D) Enrichment of PVT1 was detected by RT-qPCR after transfection with Bio-NC or Bio-miR-27a-3p in RNA pull down assay. (E) Relative expression of miR-27a-3p after PVT1 overexpression or silencing was detected in HK-2 cells by RT-qPCR. *P<0.05. PVT1, plasmacytoma variant translocation gene 1; lncRNA, long non-coding RNA; miR/miRNA, microRNA; si, small interfering RNA; NC, negative control; wt, wild-type; mut, mutant; Ago2, argonaute 2; Bio, biotinylated.

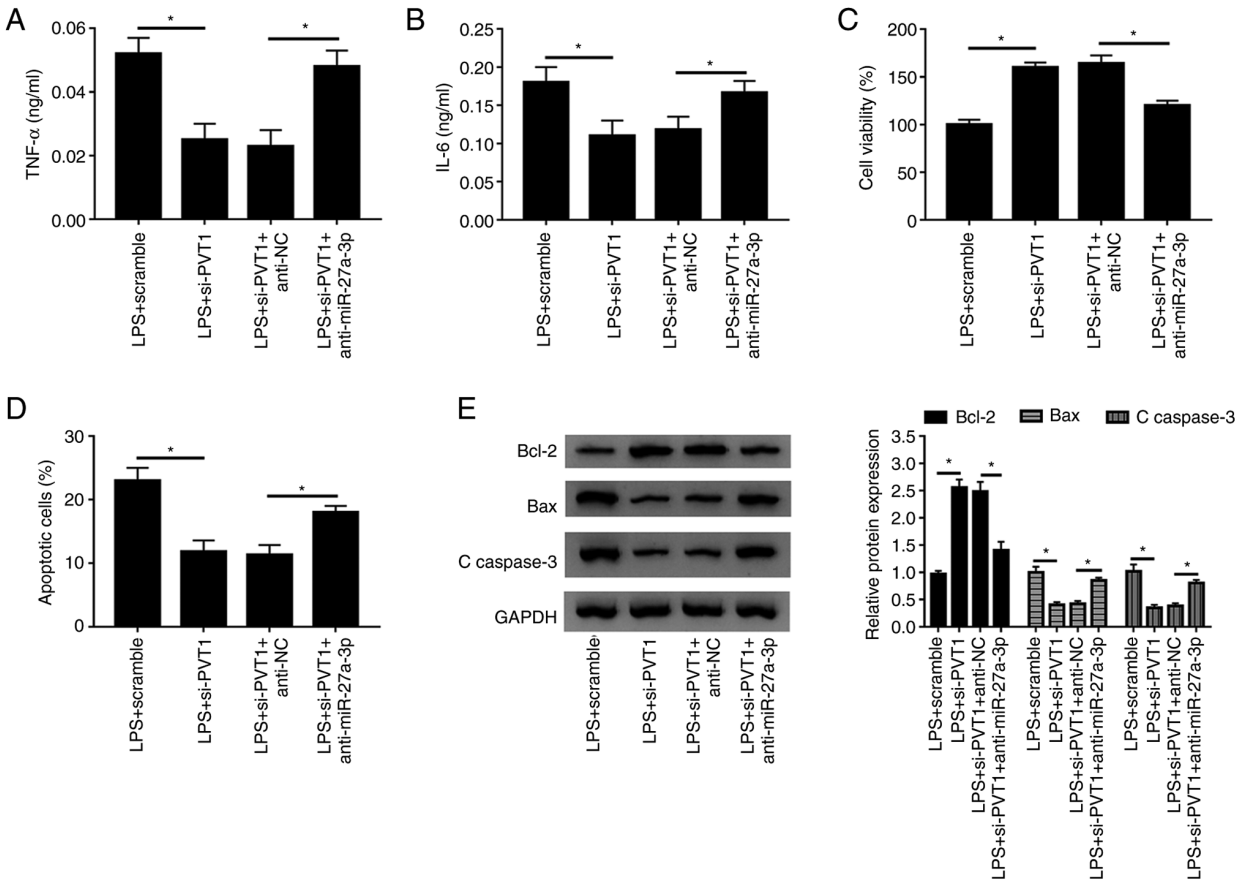

Figure 5. Decreasing the expression of miR-27a-3p slows the effect of PVT1 knockdown. Levels of (A) TNF-α and (B) IL-6 of LPS+scramble, LPS+si-PVT1, LPS+si-PVT1+anti-NC and LPS+si-PVT1+anti-miR-27a-3p groups were detected in HK-2 cells by ELISA. (C) Cell viability and (D) apoptotic rates of the groups were detected by Cell Counting Kit-8 and flow cytometry, respectively. (E) Protein expression levels of Bcl-2, Bax and C-caspase 3 were measured in each group using western blotting. *P<0.05. C-, cleaved; LPS, lipopolysaccharide; PVT1, plasmacytoma variant translocation gene 1; miR, microRNA; si, small interfering RNA; TNF-α, tumor necrosis factor-α; IL, interleukin; NC, negative control.

reporter vectors were constructed, and miR-27a-3p mimic or miR-NC were co-transfected with PVT1-wt or PVT1-mut in HK-2 cells. It was found that miR-27a-3p, but not miR-NC, reduced the luciferase activity of PVT1-wt, but did not affect that of PVT1-mut (Fig. 4B). Furthermore, RIP and pull-down experiment results demonstrated that PVT1 directly targets miR-27a-3p in HK-2 cells (Fig. 4C and D). It was also identified that transfection of pcDNA-PVT1 significantly upregulated the expression of PVT1, thus exhibiting a successful overexpression efficiency (Fig. S1B). The present results suggested that miR-27a-3p may be a target miRNA of PVT1. Furthermore, when PVT1 was overexpressed in HK-2 cells, the expression of miR-27a-3p was significantly decreased; however, when PVT1 was knocked down, the expression of miR-27a-3p was significantly increased (Fig. 4E).

miR-27a-3p silencing reverses the effect of PVT1 knockdown in LPS-treated HK-2 cells. To further examine the functional relationship between PVT1 and miR-27a-3p, si-PVT1 and anti-miR-27a-3p were co-transfected into HK-2 cells as the experimental group. The transfection efficiency of anti-miR-27a-3p was determined, and it was found that anti-miR-27a-3p significantly inhibited miR-27a-3p expression (Fig. S1D). It was also identified that the secretion of the inflammatory cytokines TNF-α and IL-6 were elevated in LPS-induced HK-2 cells co-transfected with si-PVT1 and anti -miR-27a-3p (Fig. 5A and B). Moreover, a significant decrease in cell activity was detected using a CCK-8 assay (Fig. 5C), and flow cytometry results demonstrated that the number of apoptotic cells was significantly increased after miR-27a-3p silencing (Fig. 5D). Furthermore, the expression of the apoptosis inhibitor Bcl-2 was significantly decreased, and the expression levels of Bax and C-caspase-3 were significantly elevated after miR-27a-3p silencing (Fig. 5E). Therefore, the results indicated that miR-27a-3p knockdown in cells attenuated the effects of si-PVT1 transfection, triggered inflammatory responses and accelerated apoptosis.

OXSR1 is the target gene of miR-27a-3p. TargetScan identified that OXSR1 was a potential target of miR-27a-3p (Fig. 6A). In the present study, miR-27a-3p or miR-NC, along with OXSR1-wt or OXSR1-mut were co-transfected into HK-2 cells. Luciferase reporter assay results demonstrated that overexpression of miR-27a-3p reduced the luciferase activity of cells with OXSR1-wt, but not with OXSR1-mut (Fig. 6B). Furthermore, RIP experiments indicated that miR-27a-3p directly targeted OXSR1 in HK-2 cells (Fig. 6C). The present findings suggested that overexpression of miR-27a-3p significantly decreased OXSR1 expression, while anti-miR-27a-3p significantly increased OXSR1 expression in HK-2 cells (Fig. 6D).

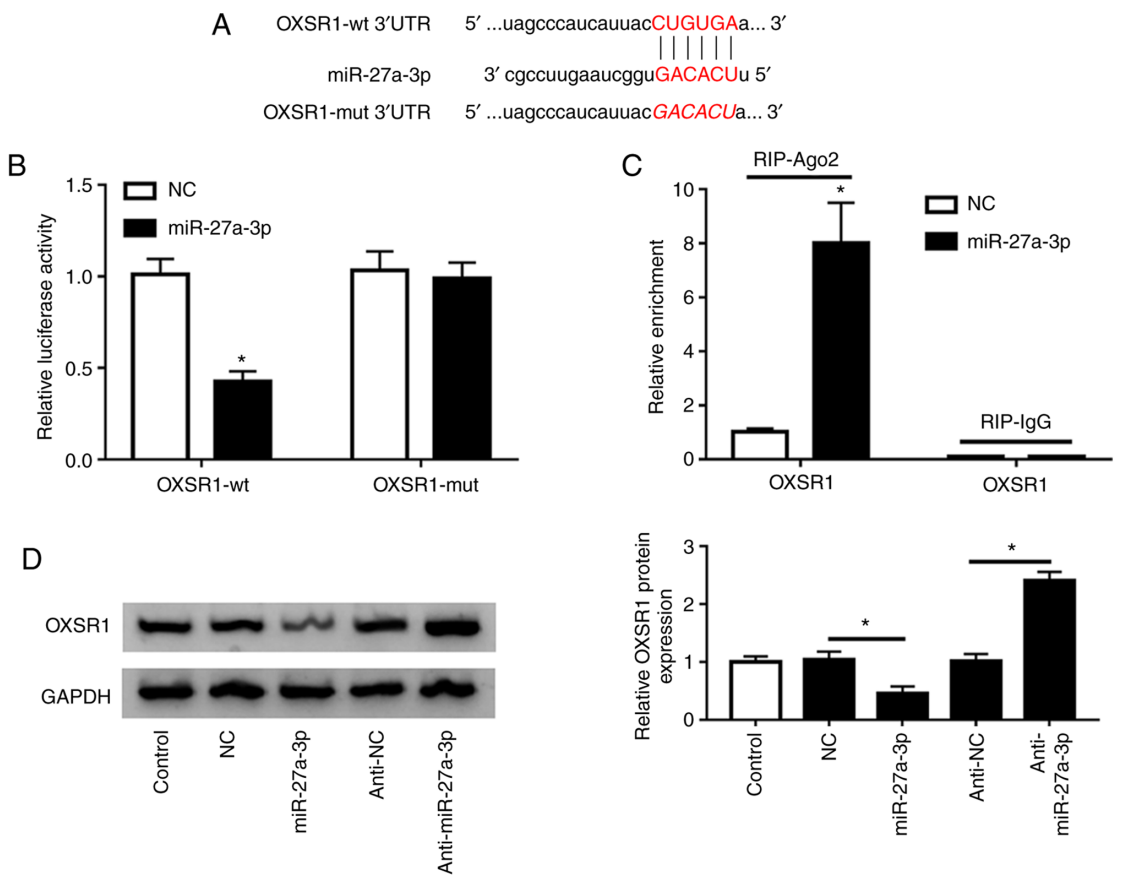

Figure 6. OXSR1 is a target gene of miR-27a-3p. (A) OXSR1 was predicted by TargetScan as a potential target for miR-27a-3p. (B) Luciferase reporter assay was conducted to assess the interaction between miR-27a-3p and OXSR1. (C) RNA immunoprecipitation assay was used to detect enrichment relative to input, and to identify the relationship between miR-27a-3p and OXSR1. (D) Protein expression of OXSR1 after transfection with miR-27a-3p or anti-miR-27a-3p was measured in HK-2 cells using western blotting. *P<0.05 vs. NC or as indicated. miR, microRNA; NC, negative control; wt, wild-type; mut, mutant; OXSR1, oxidative stress responsive kinase 1; 3'UTR, 3'untranslated region; Ago2, argonaute 2.

Replenishment of OXSR1 reverses the effect of miR-27a-3p overexpression. The present study examined the expression of OXSR1 in HK-2 cells by western blotting, and found that the protein expression of OXSR1 was significantly increased after LPS treatment (Fig. 7A). Furthermore, western blot analysis suggested that the protein expression of OXSR1 was significantly increased in HK-2 cells transfected with OXSR1 (Fig. S1E). To assess the functional relationship between miR-27a-3p and OXSR1, miR-27a-3p, miR-27a-3p+vector or miR-27a-3p+OXSR1 were transfected into HK-2 cells. When miR-27a-3p was co-transfected with OXSR1, it was found that the secretion of TNF-α and IL-6 significantly increased (Fig. 7B and C). Moreover, a significant decrease in cell viability was detected (Fig. 7D), and the number of apoptotic cells was significantly increased in the miR-27a-3p+OXSR1 group (Fig. 7E). In addition, the expression of Bcl-2 was significantly decreased, and the expression levels of Bax and C-caspase-3 were significantly increased (Fig. 7F). Thus, the present results indicated that overexpression of OXSR1 reversed the effect of miR-27a-3p overexpression in LPS-treated HK-2 cells.

PVT1 regulates OXSR1 expression and NF-κB pathway activation via miR-27a-3p. To investigate the interactions between PVT1, miR-27a-3p and OXSR1, si-PVT1 and anti-miR-27a-3p were transfected into LPS-treated HK-2 cells. It was identified that the expression level of OXSR1 was significantly increased when miR-27a-3p and PVT1 were knocked down (Fig. 8A). Moreover, when the expression levels of miR-27a-3p and PVT1 were decreased, the expression levels of p-IκBα and p-p65 were significantly increased (Fig. 8B). Collectively, these findings suggested that PVT1 regulated OXSR1 expression and activation of the NF-κB pathway via miR-27a-3p.

Discussion

Previous studies have shown that AKI is a common symptom in patients with sepsis, with 26-50% of patients in developed nations with sepsis suffering from AKI, compared with 7-10% of patients with primary kidney disease-associated AKI (32-36). However, the exact mechanisms of AKI are not fully understood. Recently, clinical studies have shown that AKI is associated with increased mortality in patients with sepsis (37,38).

lncRNA PVT1 is abundant in several solid tumors, and its abnormal expression is associated with tumor metastasis and recurrence (39,40). A previous study has revealed that transient expression of PVT1 promotes cell proliferation and tumor formation in nude mice, whereas deletion of PVT1 in tumor cells reduces tumorigenicity (7). In small cell lung cancer (SCLC), PVT1 has been evaluated as a candidate

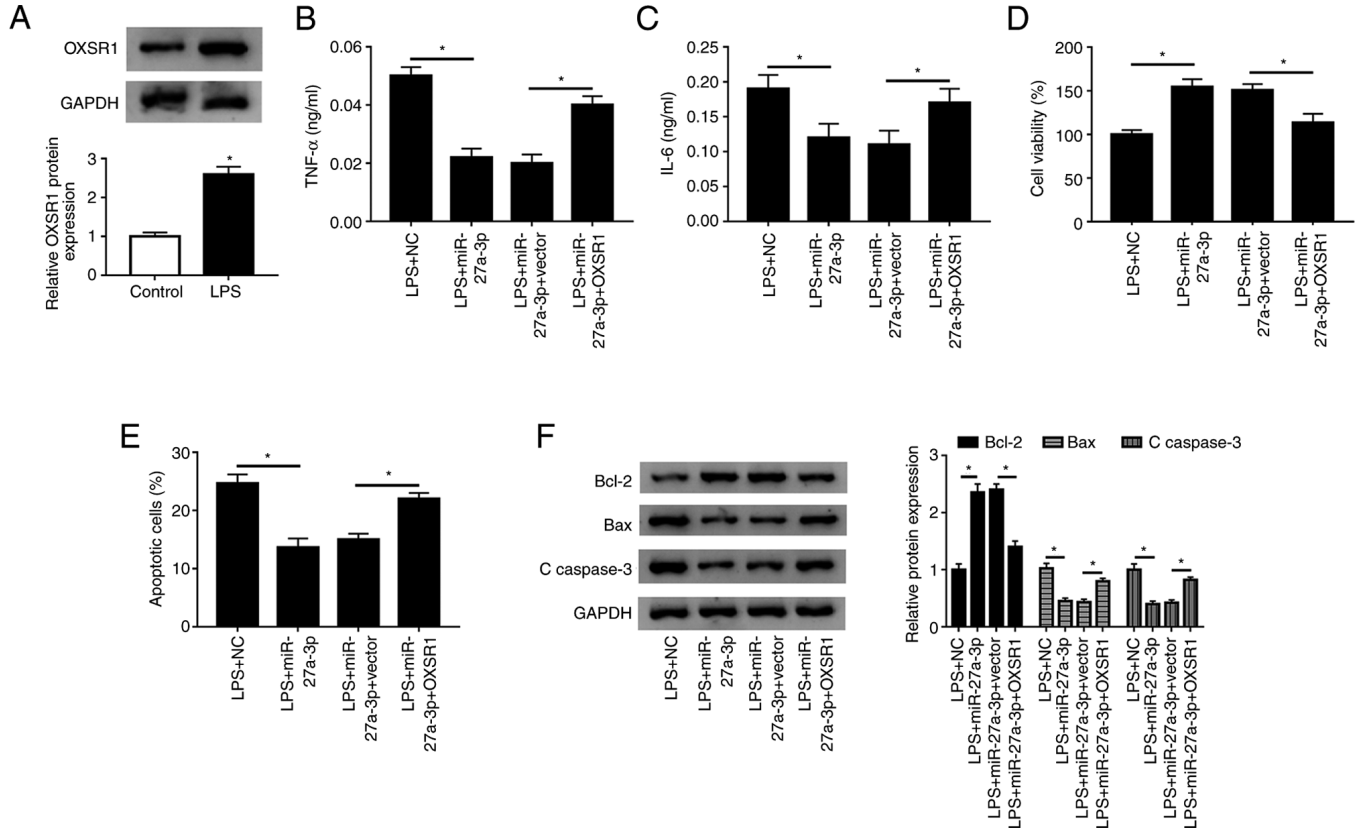

Figure 7. Overexpression of OXSR1 reverses the effect of miR-27a-3p overexpression. (A) Protein expression of OXSR1 was measured in HK-2 cells using western blotting after LPS treatment. Secretion levels of (B) TNF-α and (C) IL-6 in LPS+NC, LPS+miR-27a-3p, LPS+miR-27a-3p+vector and LPS+miR-27a-3p+OXSR1 groups were detected in HK-2 cells by ELISA. (D) Cell viability and (E) apoptotic rates of groups were detected by Cell Counting Kit-8 and flow cytometry, respectively. (F) Protein expression levels of Bcl-2, Bax and C-caspase 3 were measured in each group using western blotting. *P<0.05 vs. control or as indicated. C-, cleaved; LPS, lipopolysaccharide; PVT1, plasmacytoma variant translocation gene 1; miR, microRNA; TNF-α, tumor necrosis factor-α; IL, interleukin; NC, negative control; OXSR1, oxidative stress responsive kinase 1.

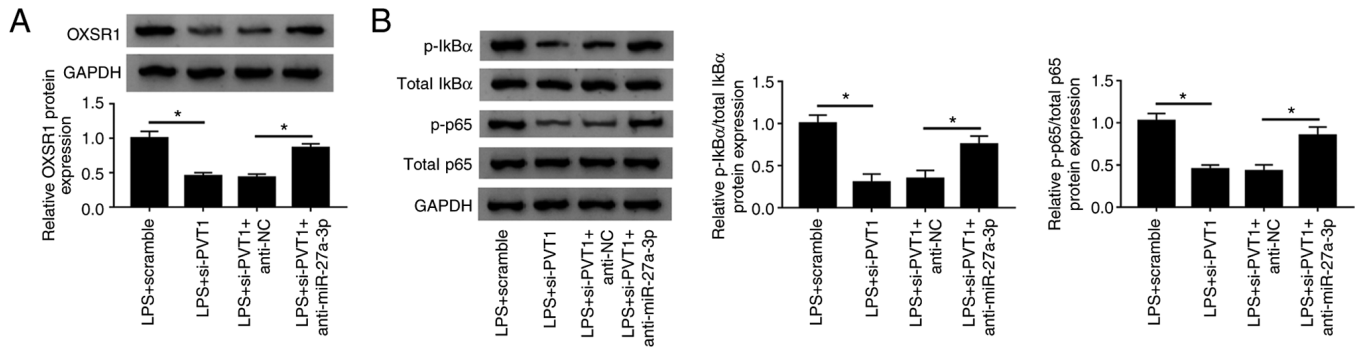

Figure 8. Long non-coding PVT1 regulates OXSR1 expression and NF-ϰB pathway activation via miR-27a-3p. HK-2 cells were subjected to LPS+scramble, LPS+si-PVT1, LPS+si-PVT1+anti-NC, and LPS+si-PVT1+anti-miR-27a-3p. (A) Western blot analysis was used to measure the protein expression of OXSR1 in each group. (B) Protein expression levels of p-IκBα and p-p65 of HK-2 cells were measured using western blotting. *P<0.05. NC, negative control; OXSR1, oxidative stress responsive kinase 1; p, phosphorylated; LPS, lipopolysaccharide; PVT1, plasmacytoma variant translocation gene 1; miR, microRNA; si, small interfering RNA; IκBα, inhibitor of κB.

biomarker for diagnosis and prognosis, as high expression of PVT1 is positively associated with the status of clinical stage, overall survival time, lymph node metastasis and distant metastasis in patients with SCLC (41). Moreover, PVT1 expression in paraffin-embedded tissues also indicates poor prognosis in nasopharyngeal carcinoma, and PVT1 knockdown may induce apoptosis (42). Although PVT1 has been shown to facilitate cell proliferation and inhibit apoptosis in various cancer types, the function of PVT1 in healthy tissues is unknown. A previous study reported that PVT1 was induced by LPS and PVT1 knockdown alleviated LPS induced myocardial injury s (18). In line with these studies, the present results suggested that the expression of lncRNA PVT1 was increased in HK-2 cells after LPS administration. Furthermore, it was found that knockdown of PVT1 could inhibit the secretion of inflammatory cytokines, promote cell survival and inhibit

apoptosis. Moreover, luciferase reporter assay, RIP assay and pull-down assay results indicated that miR-27a-3p may be a target miRNA of lncRNA PVT1. It was also demonstrated that after PVT1 knockdown, the expression of miR-27a-3p was significantly increased.

miRNAs are evolutionarily conserved, small non-coding RNAs 21-25 nucleotides in length that interact with short motifs in the 3'UTR of target mRNAs, leading to their transcript destabilization, translational inhibition or both (43). miRNAs are critical post-transcriptional regulators of the majority of human genes that can respond to physiological and pathological stress, with a primary function to maintain the systemic homeostatic balance (44). The present results suggested that miR-27a-3p overexpression inhibited TNF-α and IL-6 secretion, rescued cell proliferation and inhibited apoptosis. To further understand the regulatory mechanism of miR-27a-3p, OXSR1 was predicted to be a potential target gene of miR-27a-3p. Moreover, dual-luciferase reporter assay and RIP assay results identified that OXSR1 was a downstream target gene of miR-27a-3p. It was also found that OXSR1 expression was altered following transfection with the miR-27a-3p mimic or anti-miR-27a-3p.

OXSR1, a member of Ser/Thr kinase family, regulates downstream kinases in response to environmental stress, which is critical for its phosphatase activity and tumor suppressor function (45,46). It has been reported that OXSR1 and the inflammation marker IL-6 are significantly increased in serum specimens from patients with diabetes undergoing hemodialysis compared with healthy participants (47). Qin *et al* (48) showed that overexpression of OXSR1 increases the expression of Bcl-2, but suppresses Bax and C-caspase-3 expression levels. Moreover, it was hypothesized that this effect may be involved in the regulation of p38/MAPK/NF-κB signaling pathway, and the upregulated protein expression levels of p-p38 and p-p65 (48).

NF-κB is a critical nuclear transcription factor from different Rel family proteins, including p50, p65, p52 and RelB (49). p65 is one of the most common subtypes localized in the cytoplasm, and can be translocated into the nucleus by binding to the promoter region of the target gene, regulating the expression of various genes encoding pain and inflammatory mediators (50). IκBα combines with NF-κB proteins to form a complex, which cannot enter the nucleus (50). After IκBα is phosphorylated, NF-κB proteins are released (50). In the present study, it was found that PVT1 knockdown significantly decreased the ratios of p-p65/total p65 and p-IκBα/total IκBα, and silencing miR-27a-3p reversed these effects. Therefore, the present results indicated that PVT1 knockdown inhibited the NF-κB signaling pathway by regulating miR-27a-3p.

In conclusion, the present results suggested that lncRNA PVT1 regulated OXSR1 expression and activation of the NF-κB pathway via miR-27a-3p to respond to AKI inflammation.

Acknowledgements

Not applicable.

Funding

No funding was received.

Availability of data and materials

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

Authors' contributions

QY, QS and PJ conceived and designed the present study. QS and PJ collected the data and performed the experiments. QS performed the data analysis and interpretation. QY and QS were involved in the preparation of manuscript. QY and QS confirm the authenticity of all the raw data. All authors read and approved the final manuscript.

Ethics approval and consent to participate

Not applicable.

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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