Rosiglitazone prevents acute pancreatitis through inhibiting microRNA-26a expression

YAN CHEN[1], WEI XIANG[1], XIANG LI[1], DAMING WANG[1] and CHUNYAN QIAN[2]

[1]Department of Emergency and [2]Drug Clinical Trial Institution, The First People's Hospital of Changzhou,
The Third Affiliated Hospital of Soochow University, Changzhou, Jiangsu 213003, P.R. China

Abstract. The aim of the present study was to investigate the regulatory effect of rosiglitazone on the progression of acute pancreatitis (AP) and pancreas injury, and the underlying mechanism. An AP rat model was established using caerulein and validated by detection of amylase, lipase, tumor necrosis factor-α (TNF-α), interleukin-6 (IL-6) and transforming growth factor-β (TGF-β) serum levels. Pancreatic injury was assessed by pathological examination. The expression levels of microRNA (miR)-26a in AP rats and AR42J cells were analyzed using reverse transcription-quantitative PCR (RT-qPCR). Luciferase reporter gene assay was applied for detecting whether miR-26a bound to the target gene phosphatase and tensin homolog (PTEN). The regulatory effect of rosiglitazone on the PI3K/AKT signaling pathway was analyzed by western blot analysis. Results demonstrated that establishment of an AP model was successful with severe pancreas injury and classic AP phenotypes observed in rats. Increased serum expression of amylase, lipase, TNF-α, IL-6 and TGF-β were observed in AP rats. Rosiglitazone pretreatment prevented AP progression through suppression of miR-26a expression via binding to and degrading PTEN. Western blot analysis demonstrated that rosiglitazone blocked the PI3K/AKT signaling pathway through PTEN. In conclusion, it was determined that rosiglitazone prevented AP by downregulating miR-26a via the PI3K/AKT signaling pathway.

Introduction

Acute pancreatitis (AP) is an inflammatory disease that affects the pancreas. The pathogenesis of AP is complex and involves multiple factors, such as hyperlipidemia, alcoholism and biliary diseases (1). The prognosis of mild AP is typically good, with a very low mortality rate; however, severe AP may cause serious consequences and have a poor prognosis (2). AP progression is associated with the activation of pancreatin, cytokines and chemokines (3,4). Therefore, it is of great clinical significance to explore the inflammation mechanism and to find a potential treatment that targets the inflammation process.

Rosiglitazone, currently the most effective thiazolidinedione drug, is principally used for the treatment of diabetes (5,6). Numerous studies have demonstrated that rosiglitazone can increase insulin sensitivity and decrease insulin resistance (5-7). However, in recent years, due to better understanding of peroxisome proliferator activated receptor (PPAR)γ and its ligands, rosiglitazone has been determined to have significant effects on the inflammatory response, cell differentiation and cell metabolism (8-10). Previous research suggested that rosiglitazone exhibits anti-inflammatory effects on osteoporosis, acute or chronic gastrointestinal diseases and other systemic inflammatory response syndromes (7,11-12).

MicroRNA (miRNA) is a non-coding, single-chain RNA (18-25 nucleotides in length), which can bind to the 3'-untranslated region (UTR) of target genes and suppress the translation or promote the degradation of genes (13). Although miRNA only accounts for ~1% of the human genome, it regulates ~60% protein expression (14). Present studies determined that miRNAs are involved in various cellular functions, such as proliferation, differentiation and the inflammation response (13,14). miRNA (miR)-26 is located in chromosome 19q14.12, which is closely associated with tumor development by regulation of tumor cell proliferation and apoptosis (15). In addition, miR-26a is involved in the allergic inflammatory reaction and the toll like receptor 4 (TLR-4)-mediated inflammatory response (16,17). However, the specific role of miR-26a in AP has not been fully elucidated.

The aim of the present study was to investigate the regulatory effect of rosiglitazone on the progression of AP and pancreas injury, and its underlying mechanism.

Materials and methods

Animal model. A total of 40 male Sprague Dawley rats (age, 6-8 weeks; weight, 180-200 g) were purchased from Model Animal Research Center of Nanjing University (Nanjing, China). The rats were housed in a temperature-controlled room

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Correspondence to: Dr Chunyan Qian, Drug Clinical Trial Institution, The First People's Hospital of Changzhou, The Third Affiliated Hospital of Soochow University, 185 Juqian Street, Changzhou, Jiangsu 213003, P.R. China
E-mail: cy95139@163.com

(21±2°C) with 60-70% relative humidity on a 12-h light: dark cycle (lights on at 06:00). All rats had free access to water and food. The rats of specific pathogen free level were randomly divided into three groups: The control group (n=10), the AP model group (n=15) and the rosiglitazone-treated group (n=15). AP model rats were anesthetized by intraperitoneal injection of pentobarbital sodium (30 mg/kg) then injected intraperitoneally with 50 μg/kg caerulein (MedChemExpress) five times at 1-h intervals. Rats in the control group were given the same volume (1 ml each time) of 0.9% NaCl. Rats in the rosiglitazone-treated group were administered with rosiglitazone (4 mg/kg, Sigma-Aldrich; Merck KGaA) by intraperitoneal injection 1 h before the first injection of caerulein. Peripheral blood was collected 6, 12 and 24 h after the last injection of caerulein then the rats were sacrificed for pancreatic tissues. Parts of the pancreatic tissues were stored in liquid nitrogen, whilst the remainder were fixed for histological analysis. All animals used in the experiment were obtained from the Model Animal Research Center of Nanjing University. This experiment was approved by Soochow University Ethics Committee (Soochow, China).

ELISA. Blood samples were maintained at room temperature for 20 min then centrifuged at 1,000 x g at 4°C for 15 min for serum sample preparation. Serum contents of amylase (cat. no. SL-B23234), lipase (cat. no. LM04412B), tumor necrosis factor-α (TNF-α; cat. no. QY-R2813), interleukin-6 (IL-6; cat. no. HS2102) and transforming growth factor-β (TGF-β; cat. no. FS-E6931) were determined using ELISA kits purchased from Guidechem. In brief, the standard solution was added to each well and incubated for 2 h at 20°C. Then the liquid was removed and an anti-biotin antibody was added for a 1-h incubation at 20°C. Each well was washed then horseradish peroxidase-labeled streptavidin work solution was added. Following a 1-h incubation at 20°C, substrates were added for color development in the dark. Termination solution was added 15-30 min later, prior to detection of optical density.

AP assessment in rats. Pancreas tissues were fixed in 10% neutral buffered formaldehyde for >24 h at room temperature. Tissue samples were embedded in paraffin blocks and sliced into 5 μm sections for hematoxylin and eosin (H&E) staining (hematoxylin, 5 min at room temperature and eosin, 3 min at room temperature). Histological analysis was performed using a light microscope (magnification, x400).

Histological scoring of pancreatic tissue was performed to grade the severity and extent of acinar edema (0, no edema; 1, inter lobular edema; 2, intralobular edema; and 3, inter acinar edema), inflammation [0, no inflammation; 1, inflammatory cells in ducts; 2, inflammatory cells in the parenchyma (<50% of the lobules); and 3, inflammatory cells in the parenchyma (>50% of the lobules)] and finally acinar cell necrosis (0, no necrosis; 1, <5% necrosis; 2, 5-20% necrosis; and 3, 20-50% necrosis).

Bioinformatic analysis. Bioinformatic analysis predicted that PTEN was the potential target gene of miR-26a (http://www.targetscan.org). In detail, rat was selected as species in the first search box and miR-26a-5p was put into the microRNA name search box. PTEN was found in the table of results.

Cell culture and transfection. Rat pancreatic AR42J cells (Type Culture Collection of the Chinese Academy of Sciences) were cultured in DMEM-F12 containing 20% fetal bovine serum (both Gibco; Thermo Fisher Scientific, Inc.), 100 U/ml penicillin and 100 g/l streptomycin and maintained in a 5% CO_2 incubator. The culture medium was changed every 2 days.

AR42J cells were inoculated into 24-well plates at $1x10^5$ cells/well. When the cell density reached 70-80%, miR-26a inhibitor (hsa-miR-26a-in; Hanbio Biotechnology Co., Ltd.), miR-26a mimics (hsa-miR-26a-mi; Hanbio Biotechnology Co., Ltd), phosphatase and tensin homolog (PTEN) small interfering (si)-RNA and related non-targeting control (NC) siRNA (both GenePharma) were transfected into cells using Lipofectamine® 2000 (Invitrogen; Thermo Fisher Scientific, Inc.) according to the manufacturer's instructions. The sequences were as follows: PTEN siRNA forward, 5'-AACCCACCACAGCUAGAACTT-3' and reverse, 5'-AAG UUCUAGCUGUGGUGGGTT-3' and NC siRNA forward, 5'-UUCUCCGAACGUGUCACGUTT-3' and reverse, 5'-ACG UGACACGUUCGGAGAATT-3'. Following incubation for 24-48 h, cells were collected for the following experiments.

In vitro AP model. One day prior to model establishment, AR42J cells were inoculated into 6-well plates with $1x10^5$/ml and treated with 10 nmol/l caerulein. Following caerulein treatment for 8 h at 37°C cells were then treated with 0, 0.01, 0.1 or 1 μM rosiglitazone for 24 h.

RNA extraction and reverse transcription-quantitative (RT-q) PCR. Total RNA from cells was extracted with TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), and then mixed with radioimmunoprecipitation assay (RIPA) lysis buffer (Beyotime Institute of Biotechnology). The same volume of chloroform was added into the mixture and centrifuged at 10,000 x g at 4°C for 5 min. The aqueous phase was collected into Eppendorf tubes. Following addition of 0.5 ml 99% isopropanol to the mixture, the Eppendorf tubes were rinsed with ethanol to remove the residue then samples were air dried at the room temperature. Total RNA was reverse transcribed into cDNA using Takara PrimeScript™ RT Master Mix kit (Takara Biotechnology Co., Ltd.). qPCR was performed using SYBR® Green Master Mix (Takara Bio, Inc.). The thermocycling conditions as: 40 cycles of 95°C for 30 sec, 95°C for 5 sec and 60°C for 31 sec. GAPDH served as the internal control for PTEN, while U6 served as the internal control for miR-204. Primers sequences were as follows: miR-26a-5p forward, 5'-GGATCCGCAGAAACTCCAGAGAGAAGGA-3' and reverse, 5'-AAGCTTGCCTTTAGCAGAAAGGAGGTT-3'; PTEN forward, 5'-GTTTACCGGCAGCATCAAAT-3' and reverse, 5'-CCCCCACTTTAGTGCACAGT-3'; GAPDH forward, 5'-CGGAGTCAACGGATTTGGTCGTAT-3' and reverse, 5'-AGCCTTCTCCATGGTGGTGAAGAC-3'; and U6 forward, 5'-CTCGCTTCGGCAGCACA-3' and reverse, 5'-AACGCTTCACGAATTTGCGT-3'. The $2^{-\Delta\Delta Cq}$ method was used to determine the relative expression levels (18). mRNA expression levels were normalized to GAPDH, whereas miRNA expression levels were normalized to U6.

Western blot analysis. Cells in the logarithmic growth phase were collected and digested by pancreatin, subsequently rinsed
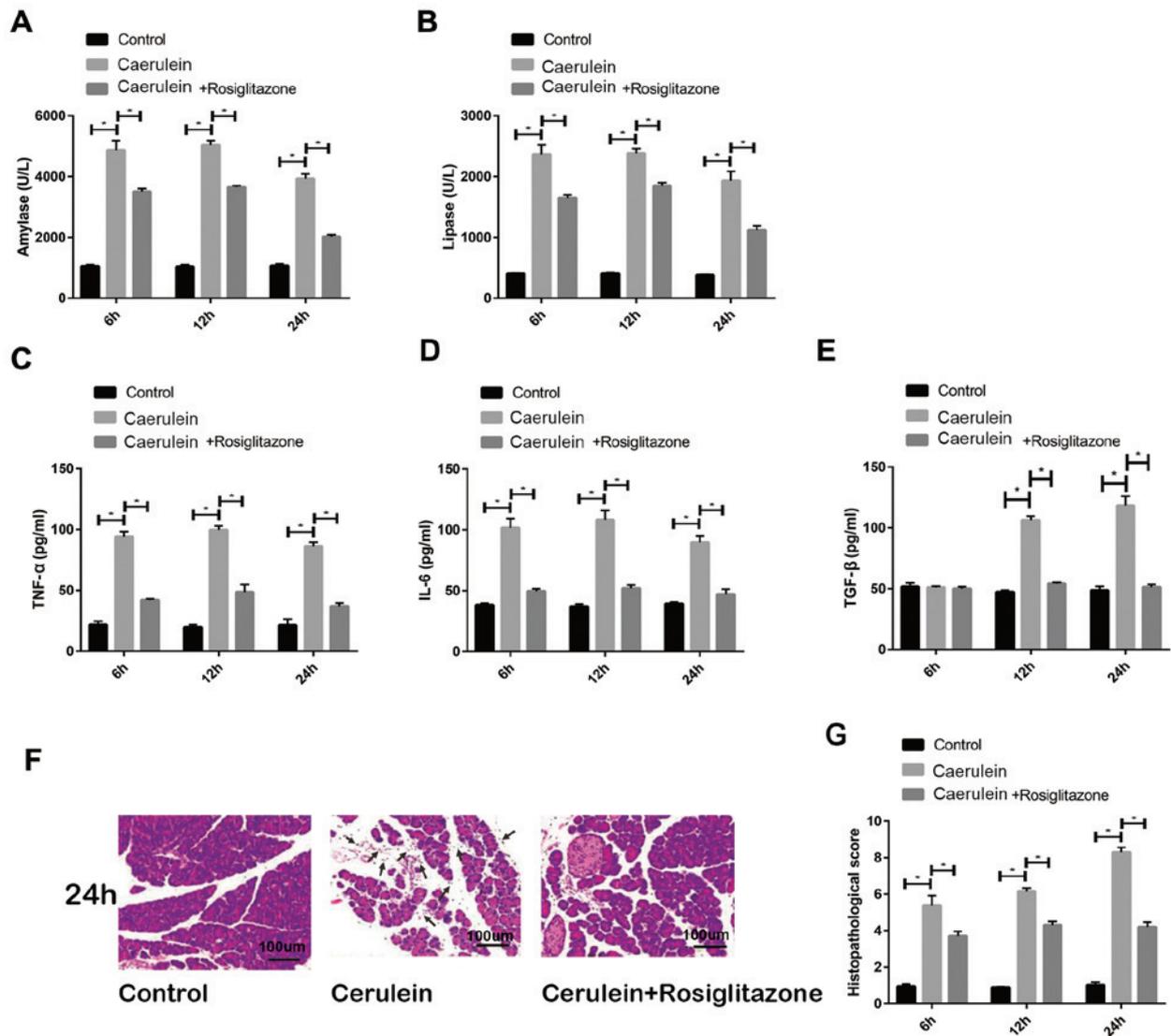
Figure 1. Rosiglitazone prevents the progression of AP. (A) Serum expression of amylase, (B) lipase, (C) TNF-α, (D) IL-6 and (E) TGF-β were detected following establishment of an AP model and pretreatment with rosiglitazone. (F) Representative H&E staining images of pancreas tissue in control, caerulein and rosiglitazone treatment groups, black arrow showed the infiltration of immune cells. (G) H&E staining immunohistochemical scores in the pancreas. Each experiment was repeated three times. *$P<0.05$. AP, acute pancreatitis; TNF-α, tumor necrosis factor-α; IL-6, interleukin-6; TGF-β, transforming growth factor-β; H&E, hematoxylin and eosin.

by PBS and fully lysed by RIPA buffer (Beyotime Institute of Biotechnology). Following oscillating incubation at 4℃ for 10 min, cells were decomposed using sonication at 20 kHz at 4℃ for 2 min then centrifuged at 5,000 x g for 10 min at 4℃ for supernatant preparation. Protein concentration was quantified using bicinchoninic acid protein assay (Pierce; Thermo Fisher Scientific, Inc.). Proteins (10 μg) were separated by 12% SDS-PAGE, followed by an electrophoretic transfer onto polyvinylidene fluoride membranes (EMD Millipore). Membranes were placed into 5% non-fat milk to block non-specific binding at 25℃ for 1 h. Membranes were then incubated with primary antibodies including PTEN (1:500; cat. no. ab32199; Abcam), phosphorylated (p)-PI3K (1:500; cat. no. ab182651; Abcam), PI3K (1:500; cat. no. ab151549; Abcam), p-AKT (1:500; cat. no. ab38449; Abcam), AKT (1:500; cat. no. ab8805; Abcam), GAPDH (1:500; cat. no. ab8245; Abcam) overnight at 4℃. Finally, the membranes were rinsed with PBS and incubated with goat horseradish peroxidase-conjugated

goat anti-rabbit IgG H&L secondary antibody (1:1,000; cat. no. ab7090; Abcam) at 25℃ for 1 h. Protein bands were visualized following a 3-min incubation with enhanced chemiluminescence reagent (Thermo Fisher Scientific, Inc.). Quantity One (version 4.0; Bio-Rad Laboratories, Inc.) was used for densitometric analysis.

Luciferase reporter gene assay. Cells were seeded and cultured into 24-well plates at a density of 5x10⁴ cells/well. The cells were transfected with Lipofectamine 2000 (Thermo Fisher Scientific, Inc.). The pGL3-PTEN-3'UTR wild-type or mutant plasmid (0.5 μg/ml; Hanbio Biotechnology Co., Ltd) was co-transfected with miR-26a mimic (0.2 μg/ml) or miR-26a NC (0.2 μg/ml) and pRL-TK *Renilla* plasmid (0.02 μg/ml; Promega Corporation) into the cells. Following incubation for 48 h at room temperature, cells were collected for analysis of the luciferase activities of both firefly and *Renilla* using a Dual Luciferase® Reporter Assay System (Promega Corporation).
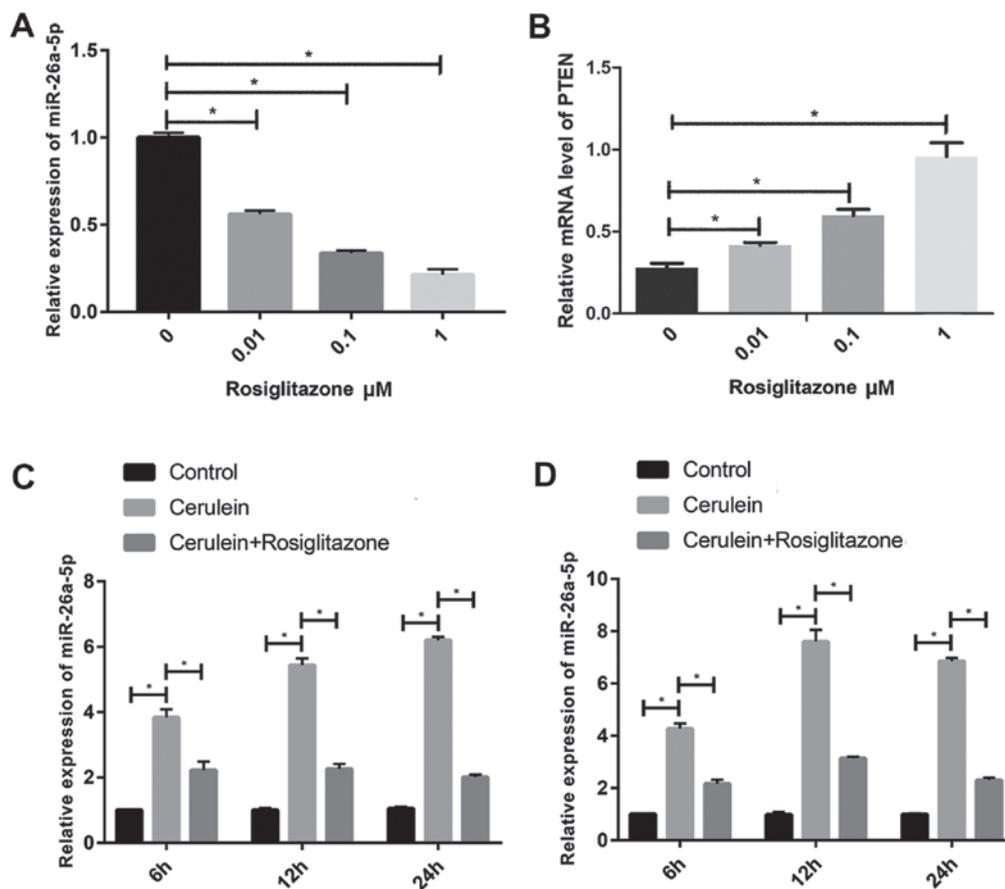
Figure 2. Rosiglitazone suppresses the expression level of miR-26a-5p *in vitro* and *in vivo*. (A) Expression level of miR-26a-5p following pretreatment with different concentrations of rosiglitazone analyzed by RT-qPCR. (B) mRNA level of PTEN following pretreatment with different concentrations of rosiglitazone analyzed by RT-qPCR. (C) Expression level of miR-26a-5p in the pancreatic tissues of the control group, the AP model group and the rosiglitazone-treated group. (D) Expression levels of miR-26a-5p in the serum of the control group, the AP model group and the rosiglitazone-treated group analyzed by qRT-PCR. Each experiment was repeated three times. *P<0.05. miR, microRNA; RT-qPCR, reverse transcription-quantitative PCR; PTEN, phosphatase and tensin homolog; AP, acute pancreatitis.

Firefly luciferase activity was normalized by comparing the activity levels to pRL-TK *Renilla*.

Statistical analysis. Statistical analysis was performed by Statistical Product and Service Solutions v.19.0 (IBM Corp.). All data were expressed as mean ± standard deviation. Statistical difference was assessed using two-tailed Student t-test for comparisons amongst two groups. Comparisons between multiple groups was performed using one-way analysis of variance test followed by Least Significant Difference post hoc test. P<0.05 was considered to indicate statistical significance.

Results

Rosiglitazone reduces serum levels of amylase and cytokines. Rats were intraperitoneally injected with caerulein for the establishment of an AP rat model. Results demonstrated that serum expressions of amylase, lipase, TNF-α, IL-6 and TGF-β were significantly increased in the AP model group compared with control group, whilst the levels decreased in the rosiglitazone-treated group (Fig. 1A-E). Pathological examination of the pancreas indicated that caerulein induced infiltration of immune cells and pancreas injury (Fig. 1F). Rosiglitazone

pretreatment remarkably and significantly reduced the level of pancreas injury at all timepoints compared with caerulein treatment (Fig. 1G).

Rosiglitazone suppresses miR-26a expression. AR42J cells were pretreated with different concentrations of rosiglitazone (0, 0.01, 0.1 and 1 μM). Results revealed that miR-26a expression in AR42J cells was significantly decreased in what appears to be a dose-dependent manner by rosiglitazone compared with the untreated group (Fig. 2A) whilst PTEN mRNA expression was significantly increased in what appears to be a dose-dependent manner compared with the untreated group (Fig. 2B). In addition, expression levels of miR-26a in pancreatic tissues and serum of rats was determined. miR-26a expression was significantly increased in the AP model group but decreased with rosiglitazone pretreatment compared with control group (Fig. 2C and D). Results indicated that rosiglitazone may regulate AP via miR-26a.

miR-26a regulates PTEN expression whilst PTEN has no effect on miR-26a. Bioinformatic analysis predicted that PTEN was the potential target gene of miR-26a (http://www.targetscan.org). Transfection efficacy of constructed plasmids was first verified by RT-qPCR as miR-26a mimics significantly
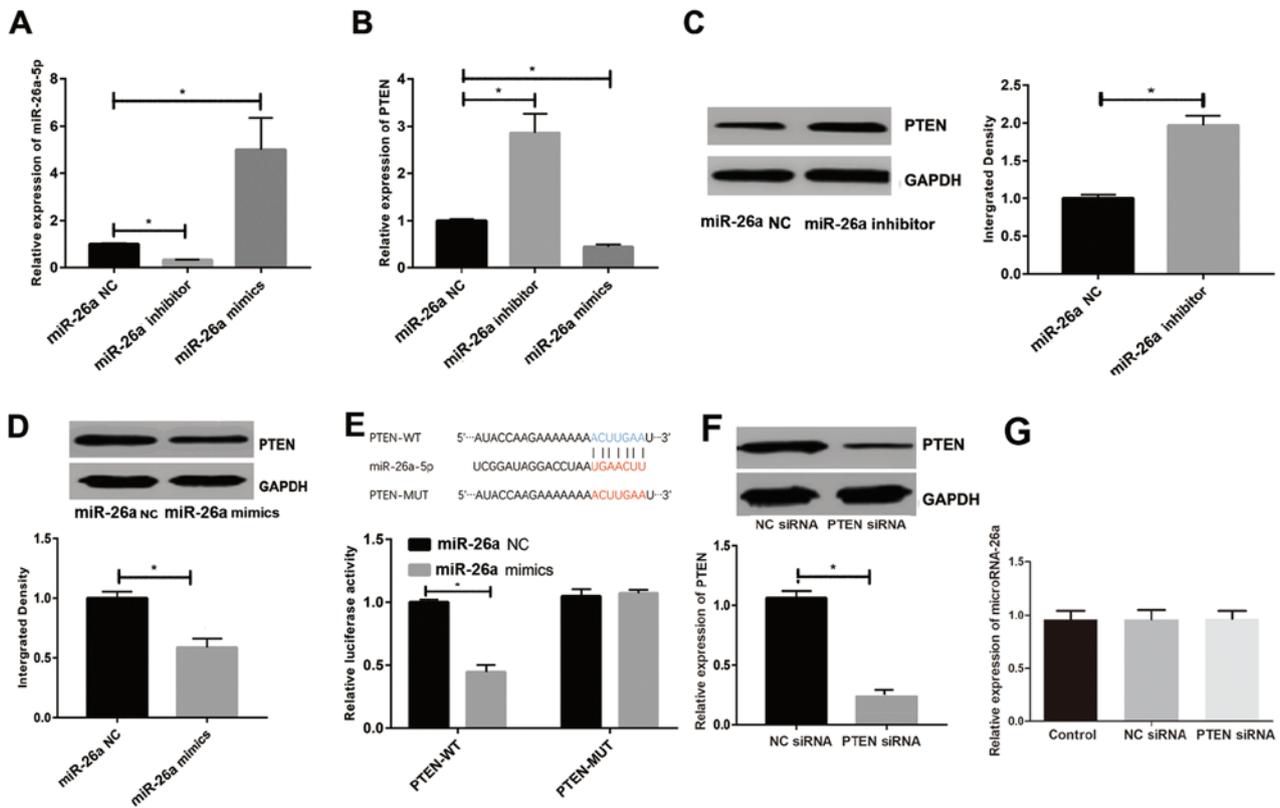
Figure 3. PTEN is the target gene of miR-26a. (A) Expression levels of miR-26a-5p following transfection with miR-26a mimic, miR-26a NC, inhibitor and mimics. (B) Overexpression of miR-26a suppressed the expression of PTEN. (C) Expression level of PTEN following miR-26a knockdown detected by western blot analysis. (D) Expression level of PTEN following miR-26a overexpression detected by western blot analysis. (E) miR-26a can target the 3'-UTR of PTEN with mutated sequences highlighted in blue and red. (F) Expression level of PTEN was decreased in cells transfected with si-PTEN. (G) Expression level of miR-26a following knockdown of PTEN. Each experiment was repeated three times. $^{*}P<0.05$. PTEN, phosphatase and tensin homolog; miR, microRNA; NC, non-targeting control; UTR, untranslated region; WT, wild-type; siRNA, small-interfering RNA.
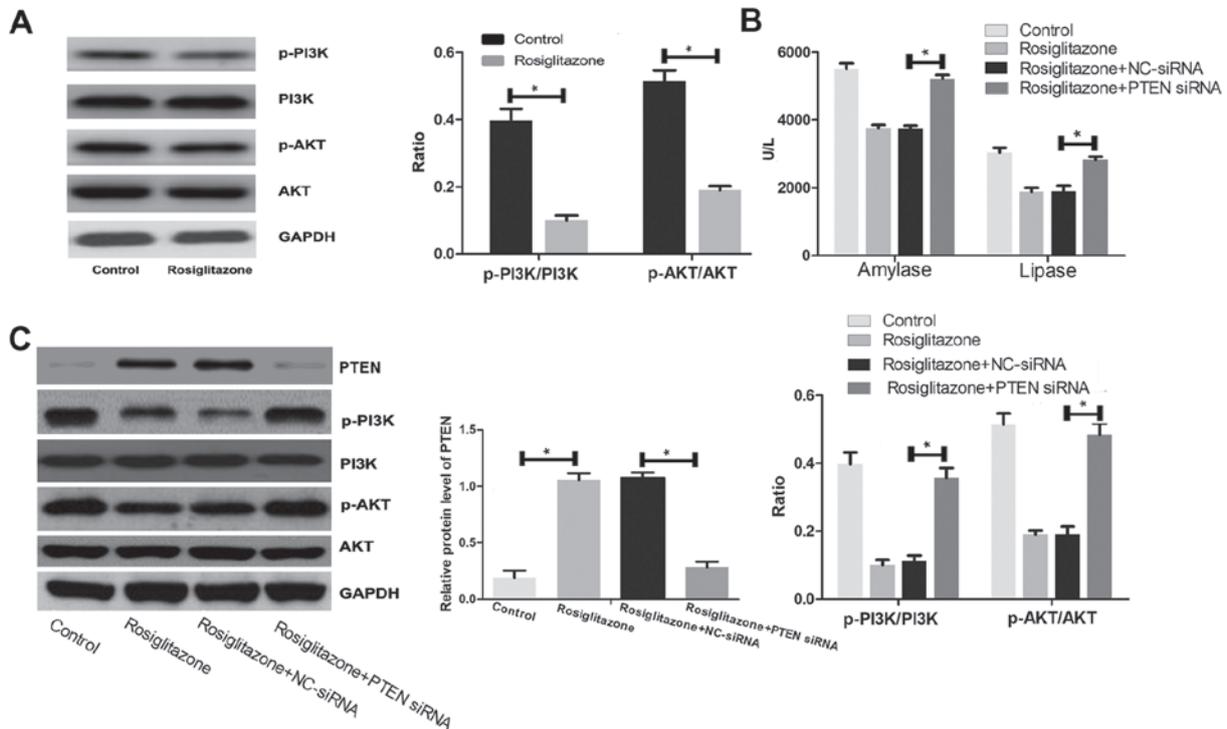
Figure 4. Rosiglitazone inhibits the PI3K/AKT pathway via PTEN. (A) Representative western blots and quantification demonstrating that the PI3K/AKT pathway was inhibited following pretreatment with rosiglitazone. (B) PTEN knockdown partially relieved the protective effect of rosiglitazone on AP. (C) PTEN knockdown partially relieved the inhibitory effect of rosiglitazone on the PI3K/AKT pathway. Each experiment was repeated three times. $^{*}P<0.05$. PTEN, phosphatase and tensin homolog; AP, acute pancreatitis; p, phosphorylated; NC, non-targeting control; siRNA, small-interfering RNA.

increased and miR-26a inhibitor significantly decreased expression compared with the miR-26a NC group (Fig. 3A). It was identified that miR-26a overexpression significantly suppressed PTEN expression and miR-26a inhibition significantly increased PTEN levels compared with the miR-26a NC group (Fig. 3B). Similar results were produced when detecting PTEN protein levels (Fig. 3C and D). The luciferase reporter gene assay demonstrated that miR-26a mimics significantly decreased the luciferase activity of cells co-transfected with PTEN-WT compared with those co-transected with miR-26a NC, which suggested that miR-26a could directly bind to PTEN and inhibit its expression (Fig. 3E). The expression of PTEN was significantly decreased in cells transfected with si-PTEN compared with those transfected with NC siRNA (Fig. 3F). By contrast, the expression of miR-26a was not changed significantly following PTEN knockdown (Fig. 3G).

Rosiglitazone suppresses the PI3K/AKT signaling pathway. A previous study demonstrated that the biological functions of PTEN were mediated by the PI3K/AKT signaling pathway (19). Therefore, the effect of rosiglitazone on the PI3K/AKT signaling pathway were investigated. Rosiglitazone pretreatment significantly suppressed the phosphorylation of key proteins involved in the PI3K/AKT signaling pathway compared with the control group (Fig. 4A). The serum levels of amylase and lipase were significantly increased in the PTEN knockdown group compared with the rosiglitazone + NC-siRNA group, indicating that PTEN knockdown reversed the beneficial effect of rosiglitazone on serum levels of amylase and lipase (Fig. 4B). In addition, the ratios of p-PI3K/PI3K and p-AKT/AKT were significantly increased in the PTEN knockdown group compared with the rosiglitazone + NC-siRNA group, indicating that PTEN knockdown reversed the inhibitory effect of rosiglitazone on the PI3K/AKT signaling pathway (Fig. 4C). These results suggest that rosiglitazone regulated the PI3K/AKT signaling pathway via PTEN.

Discussion

During the initial stage of AP, TNF-α is the main regulatory factor responsible for triggering the inflammatory cascade. Activation of the immune system elevates inflammatory signaling, and further leads to cell injury and necrosis. The TLR-4-mediated inflammatory reaction can active multiple cytokines (20,21). The present study demonstrated that expression levels of amylase, lipase, TNF-α, IL-6 and TGF-β were significantly increased following caerulein treatment, which suggested successful establishment of the AP model and pancreas injury. Rats pretreated with intraperitoneal injection of rosiglitazone significantly attenuated the inflammatory response and pancreas injury. Therefore, the present study next explored the underlying mechanism of rosiglitazone.

Previous studies have identified that miRNAs serve important roles in the progression of inflammatory diseases. Wu *et al* (22) reported that miRNA regulates macrophage polarity and thus controls the inflammatory reaction. In addition, miRNA is associated with various inflammatory diseases. For example, miR-365 directly suppresses the expression of histone deacetylase 4 and contributes to the development of rheumatoid arthritis (23). Sorbin and SH3 domain containing 2-mediated

cardiac dysfunction during sepsis is regulated by miR-21-3p (24). Since miRNA can regulate the expressions of several critical components and cytokines, it has become an important diagnostic and therapeutic target for rheumatoid arthritis (25). In the present study, rosiglitazone suppressed miR-26a expression, thus resulting in the elevated expression of the target gene PTEN.

PTEN and the PTEN-mediated pathway are involved in the occurrence and development of various diseases (26). Previous studies have demonstrated that the biological function of PTEN involved regulation of cell survival, cell proliferation and inflammation via the P13K/AKT signaling pathway (27,28). Inflammatory mediators can lead to the activation and chemotaxis of immune cells via the PI3K pathway (29). The present study demonstrated that decreased expression of PTEN reduced the inhibitory effect of miR-26a on the PI3K/AKT pathway, thereby regulating inflammation. However, the underlying mechanism of rosiglitazone suppression on the PI3K/AKT pathway remains poorly understood. Future work will use the PI3K/AKT inhibitor wortmannin to further investigate the underlying mechanism

In conclusion, rosiglitazone prevented AP progression through suppressing miR-26a expression, which elevated expression of PTEN. PTEN has been implicated in the development of various diseases therefore research into the gene can provide potential novel strategies for treatment.

Availability of data and materials

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

Authors' contribution

YC and CQ designed the study and performed the experiments. YC, WX and XL established the animal models. YC and DW collected the data. YC and WX analyzed the data. YC and CQ prepared the manuscript. All authors read and approved the final manuscript.

Ethics approval and consent to participate

This study was approved by the Soochow University Ethics Committee (Soochow, China).

Patient consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

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