



miR-148a suppresses autophagy by down-regulation of IL-6/STAT3 signaling in cerulein-induced acute pancreatitis

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ABSTRACT

Acute pancreatitis (AP) is a type of sterile inflammation of the pancreas, potentially leading to systemic inflammatory response syndrome or multiple organ failure. An emerging evidence that dysfunction of miRNA expression may alter pivotal physiological functions and lead to inflammation infiltration and complication of multiple diseases, including AP. Here, the AP model was successfully replicated using cerulein *in vitro* and *in vivo*. RT-qPCR was used to detect low expression of miR-148a in AP. This study verified that IL-6 was a direct target of miR-148a. Over-expression of miR-148a decreased the mRNA and protein levels of IL-6 by RT-qPCR and Elisa. Moreover, over-expression of miR-148a improved the pathological state of AP through H&E and MPO staining and transmission electron microscopy. After over-expressing miR-148a, Western blot and immunohistochemical method were used to confirm the reduction of autophagosomes and autolysosomes, blockade of the levels of p-STAT3, LC3-II, ATG7, ATG4c, Beclin1 and the increased p62 expression in AP. The expression of LAMP-2 was not significantly different. In addition, IL-6 and AG490, the IL-6/STAT3 signaling inhibitor, were used to verify the role of IL-6/STAT3 signaling in the regulation of miR-148a on autophagy in cerulein-induced AP *in vitro* and *in vivo*. Taken together, our findings indicate that miR-148a suppresses autophagy via regulating IL-6/STAT3 signaling in cerulein-induced AP *in vitro* and *in vivo*. The miR-148a appears to be a promising candidate for the gene therapy of AP.

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Introduction

Acute pancreatitis (AP) is the inflammatory disease with a rapidly evolving condition that occurs significant morbidity and mortality of human worldwide [1]. The current and common therapies of AP exhibit limited efficacy due to the lack of specific target [2]. Fortunately, recent advances in the understanding of the altered gene expression and signaling pathways in AP have offered the opportunities for new novel diagnostic and therapeutic approaches to improve the long-term survival for this disease [3].

The discovery and study of microRNAs (miRNAs), regulating post-transcription of target gene by binding to the 3'-UTR of target gene mRNA, has revealed a new thought regarding AP diagnosis and treatment. A report has identified that miR-148a-3p is the most abundant miRNAs at basal state in mouse pancreatic acinar cells [4].

Overexpression of miR-148 down-regulates LPS-induced inflammatory cytokines production, such as IL-6, and then avoids excessive inflammation response [5]. These study results suggest that miR-148 may be an effective tool for gene therapy against inflammation in AP.

Autophagy is a regulated process and a form of programmed cell death. At least 3 distinct autophagy pathways have been described: microautophagy, macroautophagy, and chaperone mediated autophagy. Autophagosomes, the sequestering of proteins in membrane bound vesicles, fuse with acidic lysosomes to degrade the autophagosome contents, which are formed in response to a number of environmental stimuli, such as inflammatory or stress conditions [6]. Autophagy also plays a role in many diseases. Numerous evidences have shown that impaired autophagy is involved in the pathogenesis of AP. During early AP, trypsinogen activation occurred in pancreatic acinar vacuoles. Across abnormal autophagy in AP, an increased number of zymogens accumulated in autolysosomes, resulting in trypsinogen activation [1]. Therefore, autophagy participates in the formation of acinar cell vacuoles in the

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process of AP and may be an important regulator of AP.

IL-6 is a pleiotropic cytokine with complex roles in inflammation and metabolic disease [7]. In patients and animals with AP, the serum IL-6 levels are significantly increased and positively correlate with the severity of the disease, predicting prognosis in cases of human AP [8,9]. Cytokines and mediators of inflammation are core mechanisms and straight factors in the course of lung injury caused by AP [10]. Some findings have discovered that inflammatory reaction could be activated by several signaling pathways [11], such as the most significant Janus kinase/signal transducers and activators of transcription 3 (JAK/STAT3) pathway. IL-6 can activate STAT3 and the autophagy enzyme GABARAPL1 in human islets [12]. AGER/RAGE-mediated autophagy promotes pancreatic tumorigenesis and bioenergetics through the IL6-pSTAT3 pathway [13]. These data imply that IL-6 may play a role in the process of autophagy through STAT3 signaling pathway.

However, the regulatory mechanism of miR-148a-mediated autophagy by regulation of IL-6/STAT3 signaling in AP remains unknown.

Here, we used cerulein to induce AP model by treatment with AR42J cells and intraperitoneal injection into mice. Then, we determined miR-148a expression in AR42J cells and mice with AP and analyzed the effect and mechanism of the overexpression of miR-148a on autophagy and IL-6/STAT3 signaling *in vitro* and *in vivo*.

Methods and materials

Cell lines and cell culture

The rat pancreatic acinar cell line AR42J (CRL 1492) was purchased from American Type Culture Collection (Manassas, VA, USA) and cultured in DMEM (Sigma, St. Louis, MO, USA) containing 10% FBS (GIBCO-BRL, Grand Island, NY, USA), 100 U/mL penicillin, and 100 µg/mL streptomycin, in a 37 °C incubator with 5% CO₂. The cells were divided into two groups: the control group, which was not specially treated; and the AP group, which was pre-treated with cerulein (10⁻⁸ M). Cerulein-induced pancreatitis is a well-characterized and widely used experimental model of AP [7].

Animal model

The experimental protocols were approved by the Institutional Animal Care and Use Committee of Capital Medical University. Healthy male 8-week-old BALB/c mice were kept in the room, which was air-conditioned at a steady temperature and humidity. Mice were acclimated to the facility for 1 week before the experiment and had access ad libitum to a commercial rodent pellet and sterilized drinking water.

The mice were divided into four groups: control group, which received neither cerulein nor miR-148a; AP model group, which received cerulein but not miR-148a; and treatment group, which received cerulein and empty adenoviral vector (Ad-miR-ctrl group) or miR-148a adenoviral vector (Ad-miR-148a group). In the model and treatment groups, for assessing the pathophysiology of AP and investigating treatment methods, AP was induced by intraperitoneal administration of 50 µg/kg cerulein, every hour for 7 h, as previously described [3].

The mice were euthanized by administration of pentobarbital followed by cervical dislocation. Blood samples were withdrawn from the heart to determine serum amylase, lipase, and cytokine levels. The pancreas was used for RT-qPCR analysis and hematoxylin and eosin (H&E) staining.

Construction of miR-148a expression adenoviral vector

Recombinant adenoviral vectors over-expressing miR-148a were constructed as described in previously published work [14,15]. The AdMax™ system (Genechem, Shanghai, China) was used in the process. The adenoviral vectors were then packaged and generated by transfection in HEK293 cells. The recombinant adenovirus titer was routinely concentrated to nearly 1 × 10¹¹ PFU/mL. To overexpressing miR-148a in the pancreas of mice, intraductal injection of adenovirus was performed prior to the start of intraperitoneal administration of cerulein, via the common bile duct in a final volume of 50 µL.

miR-148a mimics

miR-148a mimics and mimics control were commercially synthesized by GenePharma (Shanghai, China). Their sequences are as follows: miR-148a mimics, sense: 5'-UCAGUGCAUUCAGAA-CUUUG-3', antisense: 5'-AAGUUCUGUAAUGCACUGAUU-3'; mimics control, sense: 5'-UUCUCCGACGUGUCACGUTT-3', antisense: 5'-ACGUGACACGUUCGGAGAATT-3'. Each oligonucleotides was transfected into AR42J cells with a concentration of 30–100 nM for 24 h using Lipofectamine 2000™ (Invitrogen, Carlsbad, CA, USA). The relative RT-PCR analysis, Western blot analysis, and Elisa were carried on after transfection with miR-148a mimics and mimics control for 48 h.

Luciferase activity assay

As indicated in this study, the natural and mutated 3'-UTR segments of IL-6 were amplified and cloned into pMIR-GLO plasmids (Promega, CA, USA) to generate the IL-6-wt and IL-6-mut constructs, respectively. Then, luciferase reporter plasmids (100 ng/well) and 50 nmol/well miR-148a mimics or mimics control were co-transfected into HEK-293T cells using Lipofectamine 2000 (Invitrogen, USA). The relative luciferase activity after 48 h, was detected by a Dual-Luciferase Reporter Assay system (Promega, CA, USA) according to the manufacturer's instructions.

RNA extraction and quantitative RT-PCR analysis

Total RNA and sRNA (<200 nt) were extracted from cells and tissue samples using TRIzol (Invitrogen, Carlsbad, CA, USA) and miRcute miRNA Isolation Kit (Tiangen, Beijing, China), respectively, according to the manufacturer's instructions. The extraction concentration of total RNA was measured with a NanoDrop spectrophotometer. RT-qPCR analyses were conducted by an ABI 7300 machine (Applied Biosystems, Foster City, CA, USA) with SYBR® Green PCR kit (Takara, Dalian, China). β-actin and U6 served as an internal control. The sequences of RT-qPCR primers were listed in Table 1. The results were normalized and calculated by using the relative quantification (2^{-ΔΔCt}) method.

Table 1
Primer sequences for RT-qPCR.

Gene	Sequences
miR-148a	Forward: 5'- ATGCTCAGTGCCTACAGAA -3' Reverse: 5'- GTGCAGGGTCCGAGGT -3'
IL-6	Forward: 5'- GCGGTAAGGCATGGATAT -3' Reverse: 5'- GTTGTAGTTGGAAGGGCAG -3'
β-actin	Forward: 5'- GAGCCGCACGCTTCTTT -3' Reverse: 5'- CTGCTGTAGCCGAGGAC -3'
U6	Forward: ATTGGAACGATACAGAGAAGATT Reverse: GGAACGCTTCACGAATTTG

Biochemical analysis

In brief, medium of AR42J cells and serum pancreas and blood samples of mice after treatment were collected and centrifuged for supernatant. The levels of amylase and lipase in serum were measured using commercial assay kits (Nanjing Jiancheng Corporation, Nanjing, China). Trypsin activity, and the levels of trypsinogen activation peptide (TAP) in pancreas tissues were detected according to the method shown in the study [16].

The level of IL-6 in the media and serum were assessed using a commercially available ELISA kit (R & D Systems, Minneapolis, MN, USA) per the manufacturer's protocol. The absorbance value was measured at $\lambda = 450$ nm. The protein secretion was calculated according to the standard curve.

Histological analysis

Fresh specimens of mice were processed for H&E staining as shown previously [17]. The morphological changes were observed under a microscope by two pathologists in a blinded manner. An assessment of vacuolization, edema, acinar cell necrosis and inflammatory cell infiltration was carried out. Pancreatic injury was scored on a scale of 0–3 according to four items: edema (0 absent, 1 focally increased between lobules and 2 diffusely increased); inflammatory cell infiltrate (0 absent, 1 in ducts (around ductal margins), 2 in the parenchyma (<50% of the lobules) and 3 in the parenchyma (>50% of the lobules)); hemorrhage and fat necrosis (0 absent, 1 (1–2 foci), 2 (3–4 foci), 3 (>5 foci)); and acinar necrosis (0 absent, 1 periductal necrosis (<5%), 2 focal necrosis (5–20%) and 3 diffuse parenchymal necrosis (20–50%)), as previously described [8,9].

Immunohistochemistry

The protein expression of LC3 and Beclin-1 were examined by the streptavidin-peroxidase immunohistochemical method [12]. Formalin-fixed and paraffin-embedded surgical specimens were sequentially cut into 4 μ m sections. The sections were then dewaxed. Subsequently, antigen retrieval and hydrogen peroxide incubation were performed in sequence. Primary antibodies were used at a dilution of 1:200 and incubated at 4 °C overnight, with phosphate buffered saline (PBS) as a negative control. Further experimental steps were followed according to the instructions of a secondary biotinylated antibody kit purchased from ZSGB Biotech (Beijing, China). The slides were observed under a light microscope at a magnification of 200 \times . The degree of staining was scored as follows: 0 (0%), 1 (1–25%), 2 (26–50%), 3 (51–75%) and 4 (76–100%) for positive staining; and 0 (normal), 1 (weak), 2 (medium) and 3 (strong) for the staining intensity as described early [18].

Myeloperoxidase (MPO) staining [19].

Pancreas sections were incubated with a polyclonal rabbit anti-MPO antibody (Abcam, Cambridge, USA) in a dilution of 1:200 and visualized by DAB. The number of neutrophils was determined by counting MPO⁺ cells in ten high power fields (200 \times) per mouse.

Transmission electron microscopy (TEM)

TEM, an important technique for the observation autophagy, revealed autophagic vacuoles in the activated autophagy samples [17]. Fresh samples were fixed with 2.5% glutaral at 4 °C overnight. After post-fixation, the samples were dehydrated and embedded in an epoxy resin. The embedded tissue was then processed for TEM following the standard procedures. Finally, ultrathin sections were

examined with an electron microscope. For quantifications, the percentage of autophagic vacuoles per cytoplasm area was calculated on each print [20].

Western blot analysis

Using RIPA lysis buffer, protein can be extracted from cell lines. Protein concentrations depend on Bradford assay. Then equal number of proteins was separated for each sample by SDS-PAGE and blotted onto nitrocellulose filter membranes (EMD Millipore, Bedford, MA, USA). The membranes were incubated in 5% skim milk for 1–2 h, at room temperature, and subsequently with primary antibodies at 4 °C overnight. IL-6 (1:500 dilution), STAT3 (1:1000 dilution), p-STAT3 (1:500 dilution), LC3-I/II (1:500 dilution), ATG7 (1:500 dilution), ATG4c (1:500 dilution), Beclin-1 (1:500 dilution), p62 (1:500 dilution), Lysosomal-associated membrane protein 2 (LAMP2, 1:500 dilution) and β -actin (1:1000 dilution) were all purchased from Abcam (Cambridge, MA, USA). Following washing four times, the membranes were incubated with corresponding secondary antibody conjugated with horseradish peroxidase anti-rabbit immunoglobulin G (1:5000; ZSGB Biotech, Beijing, China) for 2 h at room temperature. Finally, the protein levels were quantified using an enhanced chemiluminescence (ECL) detection system (Thermo Scientific, CA, USA).

Statistical analysis

Statistical analysis were carried out by GraphPad Prism 6 (GraphPad Software, San Diego, CA, USA). All values are presented as the mean \pm standard deviation (SD). One-way ANOVA followed by Bonferroni's post hoc test was used to test the statistical differences. A value of $P < 0.05$ was considered to indicate a statistically significant difference.

Results

miR-148a is down-regulated in cerulein-induced AP

First, we analyzed the miR-148a expression in AR42J cells and the pancreatic tissues of mice stimulated with cerulein using RT-qPCR analysis. As shown in Fig. 1, the expression of miR-148a significantly decreased in AR42J cells and the pancreatic tissues of mice with AP compared to the control group ($P < 0.01$). The data demonstrate that miR-148a is down-regulated in cerulein-induced AP.

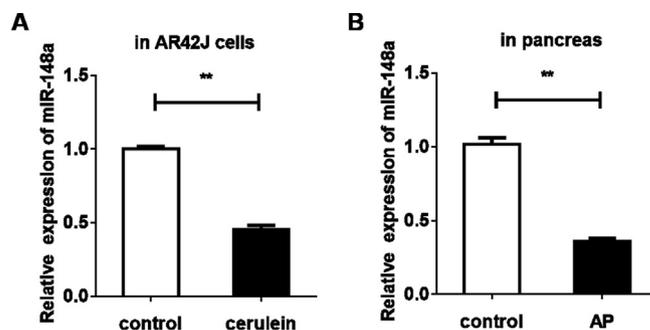


Fig. 1. The expression of miR-148a is low in cerulein-induced AP. RT-qPCR analysis was used to analyze the RNA level of miR-148a in AR42J cells (A) and the pancreatic tissues of mice (B) stimulated with cerulein. Data are presented as the means \pm SD from three independent triplicated experiments. ** $P < 0.01$ versus the control group.

miR-148a protects against cerulein-induced AP

To explore the effect of miR-148a on cerulein-induced AP, we transfected miR-148a mimics and Ad-miR-148a respectively into AR42J cells and the pancreatic tissues of mice, and then used RT-qPCR to confirm overexpression of miR-148a. As shown in Fig. 2A and C, RT-qPCR results revealed the higher expression of miR-148a after AR42J cells and the pancreatic tissues of mice respectively transfected with miR-148a mimics and Ad-miR-148a than the control group ($P < 0.01$). MTT assay (Fig. 2B) showed that cerulein inhibited cell proliferation in AR42J cells compared to the control group, which was counteracted by overexpression of miR-148a ($P < 0.01$).

As shown in Fig. 2D, the pancreatic tissues were H&E stained and examined for fibrosis, parenchymal edema, inflammatory cell

infiltration, acinar necrosis, and vacuolization in 20 randomly selected fields (5 mice per group). Focal acinar cell degeneration, inflammatory cell infiltration, and necrosis were decreased and the area of vacuoles within the acute pancreatitis tissue became smaller by using Ad-miR-148a treatment compared with the AP group ($P < 0.01$).

The pancreas pathological scores of the AP group was markedly higher than the control group ($P < 0.01$), and that of Ad-miR-148a treatment group was significantly lower than the AP group ($P < 0.01$) (Table 2). The results of MPO staining in Fig. 2D showed a significant increase of MPO positive cells in the AP group compared to the control group, and a significant reduction in Ad-miR-148a group compared to the AP group ($P < 0.01$). Previous study has described that cerulein pancreatitis was followed by the recovery of TAP, which is generated during activation of trypsinogen to trypsin,

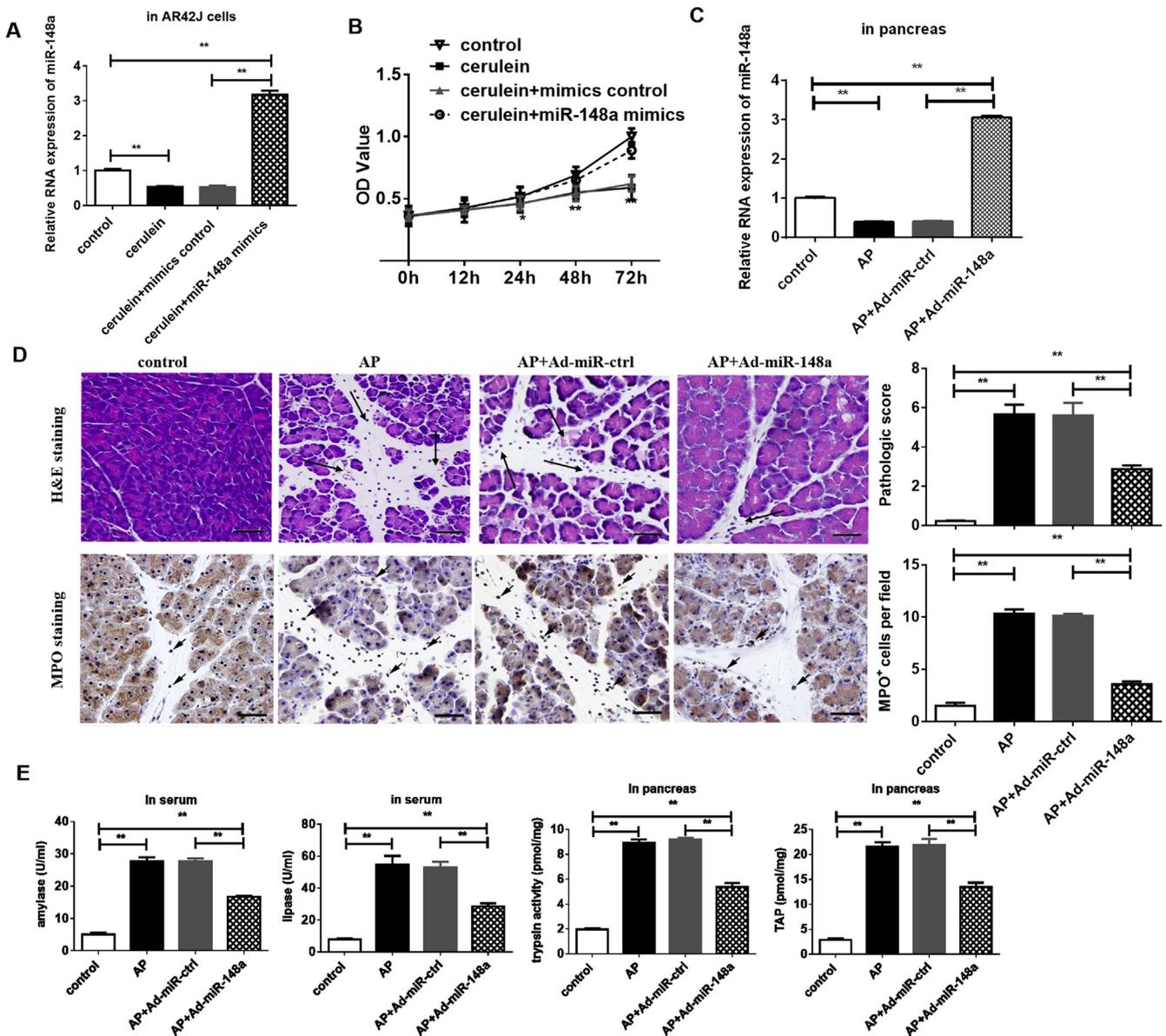


Fig. 2. Positive effects of overexpression of miR-148a on cell proliferation in AR42J cells with AP and on pathology in mice with AP. RT-qPCR analysis was used to detect overexpression of miR-148a after AR42J cells (A) and the pancreatic tissues of mice (C) respectively transfected with miR-148a mimics and Ad-miR-148a. (B) MTT analysis was used to detect effects of overexpression of miR-148a on cell proliferation in AR42J cells with AP. (D) Representative H&E and MPO stained sections of the pancreas. The representative acinar edema, vacuolization, inflammatory cells infiltration and acinar cell necrosis were indicated with arrows. Original magnification: $200 \times$, Scale bar = 50 μm . (E) The levels of amylase and lipase in serum, trypsin activity and TAP level in the pancreas homogenates were measured as described in text. Data are presented as the means \pm SD from three independent triplicated experiments. $**P < 0.01$ indicates significant difference.

Table 2
Effect of miR-148a on pancreas pathological scores.

Pathologic Changes	Acinar Edema	Vacuolization	Inflammation	Acinar Cell Necrosis
control	0.13 ± 0.16	0 ± 0	0.06 ± 0.03	0 ± 0
AP	2.88 ± 0.29**	0.57 ± 0.22**	1.01 ± 0.17**	1.24 ± 0.31**
AP + Ad-miR-ctrl	2.63 ± 0.40	0.74 ± 0.33	1.28 ± 0.21	0.99 ± 0.25
AP + Ad-miR-148a	1.19 ± 0.18##	0.35 ± 0.10##	0.55 ± 0.12##	0.43 ± 0.17##

Quantitation of the overall average tissue injury score. AP, acute pancreatitis. Data are presented as the means ± SD from three independent triplicated experiments. ***P* < 0.01 versus the control group; ##*P* < 0.01 versus the AP group.

and an increase in serum amylase and lipase levels [16]. To compare the changes of the severity of AP before and after overexpression of Ad-miR-148a, the levels of amylase and lipase in serum were measured. As shown in Fig. 2E, the levels of amylase and lipase in serum were significantly increased in cerulein-induced AP group compared with those in the control group (*P* < 0.01), indicating that AP model was successful. However, after administering Ad-miR-148a to the pancreatic tissues of mice, levels of both biomarkers were remarkably reduced as compared to those in the AP group (*P* < 0.01). Moreover, Trypsin activity and TAP levels in the pancreas homogenates showed a similar change to the levels of amylase and lipase in serum.

In sum, these results indicate that Ad-miR-148a effectively attenuates cerulein-induced AP in AR42J cells and mice.

miR-148a negatively regulates its target protein level of IL-6 in AR42J cells and mice with AP

Previous studies have indicated that IL-6 acts as a critical mediator in the pathogenesis of pancreatitis and reflects the severity of AP [7,21], and thus targeting IL-6 expression may suppress the process of AP. By searching online databases, including miRbase and TargetScan Human 7.1, the results of bioinformatics analysis predicted possible binding sites (Fig. 3F) and potential co-regulation of miR-148a with IL-6. The results of RT-qPCR and Elisa in Fig. 3A–E showed that cerulein significantly increased the mRNA and protein levels of IL-6 in AR42J cells and the pancreatic tissues of mice, which was inhibited by overexpression of miR-148a (*P* < 0.01).

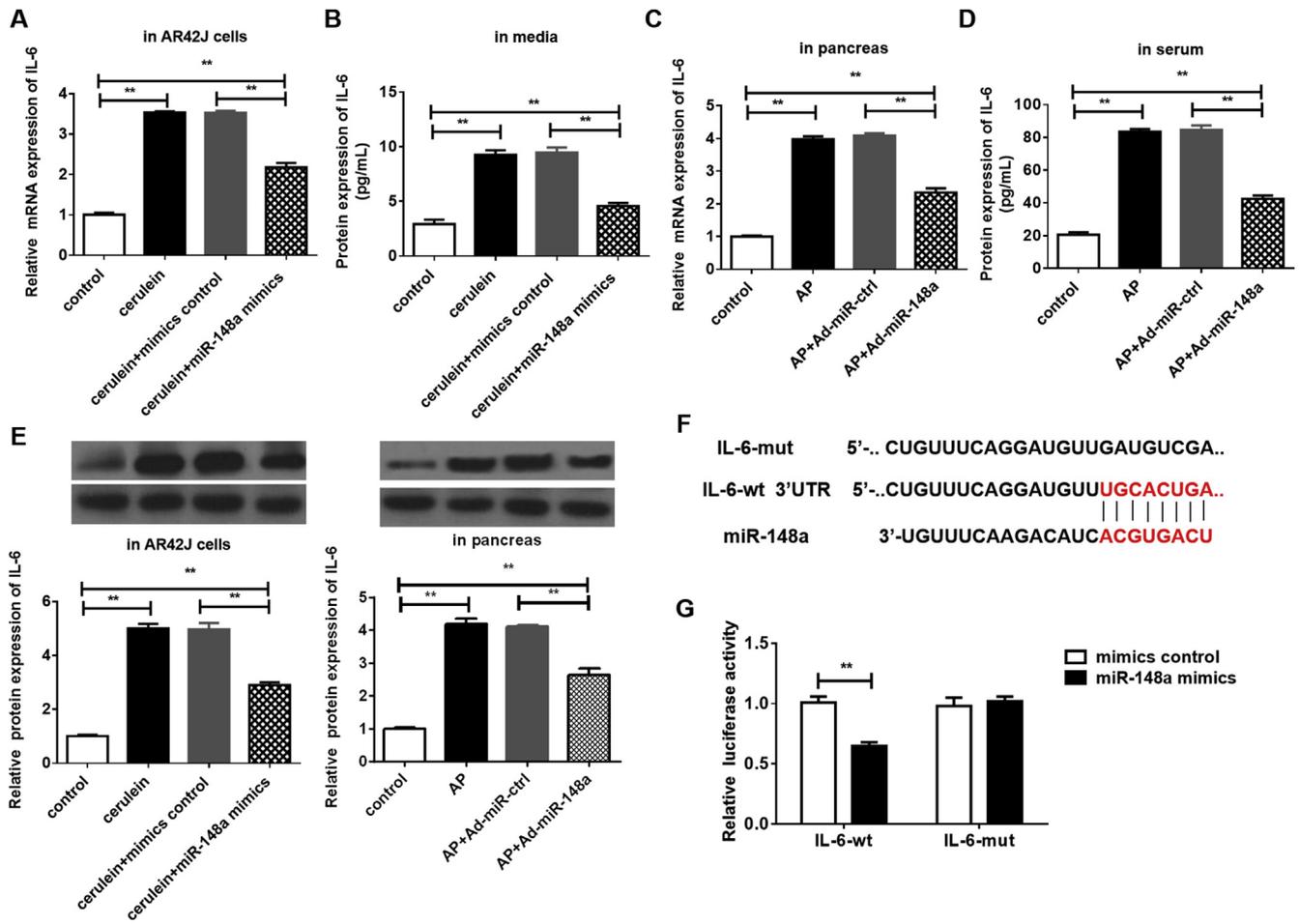


Fig. 3. miR-148a negatively regulate AP-induced the production of its target IL-6 protein. RT-qPCR was used to determinate the mRNA expression of IL-6 in AR42J cells (A) and the pancreatic tissues of mice (C) with cerulein-stimulated AP. Elisa and Western Blot were used to detect the protein level of IL-6 in AR42J cells (B, E) and the pancreatic tissues of mice (D, E) with cerulein-stimulated AP. (F) The binding sites of miR-148a and IL-6 was predicted by searching online databases. (G) Luciferase activity in HEK-293T cells co-transfected with miR-148a mimics and luciferase reporters containing IL-6-wt and IL-6-mut transcript. Data are presented as the means ± SD from three independent triplicated experiments. ***P* < 0.01 indicates significant difference.

Moreover, the binding of miR-148a with IL-6 in AP was verified by luciferase activity assay after transfection for 48 h. The results showed the luciferase activity (Fig. 3G) of the IL-6-wt vector was decreased in HEK-293T cells co-transfected with miR-148a mimics compared with the cells co-transfected with mimics control ($P < 0.01$). While the luciferase activity (Fig. 3G) of the IL-6-mut vector had no change in cells transfected with miR-148a mimics and mimics control group.

All the above data suggest that IL-6 is a direct target of miR-148a and that overexpression of miR-148a inhibits the production of IL-6 in AR42J cells and mice with cerulein-stimulated AP.

Inhibitory effects of miR-148a on regulation of autophagy in AR42J cells and mice with AP

The regulation of autophagy may alter the progression of AP induced by L-arginine or cerulein plus LPS in mice [17]. The formation of autophagosomes is a pivotal process in the development of autophagy [12]. To further investigate the effects of miR-148a on regulation of autophagy across the progression of AP *in vitro* and *in vivo*, AR42J cells and the pancreatic tissues of mice were treated for TEM to assess the autophagosomal and autolysosomal formation (Fig. 4A and B). Predominant population of vacuoles showed the presence of double membranes on empty autophagosomes consistent with their autophagic origin [22]. The intra-organellar content of the autophagosomes in AP group was filled with dense and irregular structures compared with the control group, while the autophagosomes in miR-148a group were less dense and

sometimes empty ($P < 0.01$). This is in accordance to the results of many studies that autophagy plays a critical role in the pathogenesis of AP [23].

Meanwhile, immunohistochemical changes confirmed that the levels of LC3-II and Beclin1 in pancreatic tissues of mice were increased significantly in the cerulein-induced AP group compared to the control group ($P < 0.01$), and that Ad-miR-148a decreased the expression of autophagy marker protein LC3-II and Beclin1 in pancreatic tissues of mice, compared to the AP group induced by cerulein ($P < 0.01$) (Fig. 4C). The Ad-vector control group was treated with cerulein and control adenoviral vector, and there was no significant change in the levels of LC3-II and Beclin1 in AP group and Ad-miR-ctrl with AP group.

Furthermore, autophagy markers play important roles in the process of autophagy. Such as, p62 is degraded by autolysosomal, and LAMP2 regulates the fusion of autophagosome with lysosome [12,17,20]. Western blot analysis was used to investigate the protein level of autophagy markers *in vitro* and *in vivo*. As shown in Fig. 5, the levels of p62, LC3-II, Beclin1, ATG4c, and ATG7 were assessed to have a statistically significant increase in the cerulein-treated group ($P < 0.01$). Compared to the cerulein-treated group, overexpression of miR-148a attenuated the levels of LC3-II, Beclin1, ATG4c, and ATG7, and relatively up-regulated p62 expression ($P < 0.01$). In addition, the expression of LAMP2, had no significant differences between the cerulein-induced AP group and overexpression of miR-148a group *in vitro* and *in vivo*.

Taken together, overexpression of miR-148a impairs autophagy *in vitro* and *in vivo*.

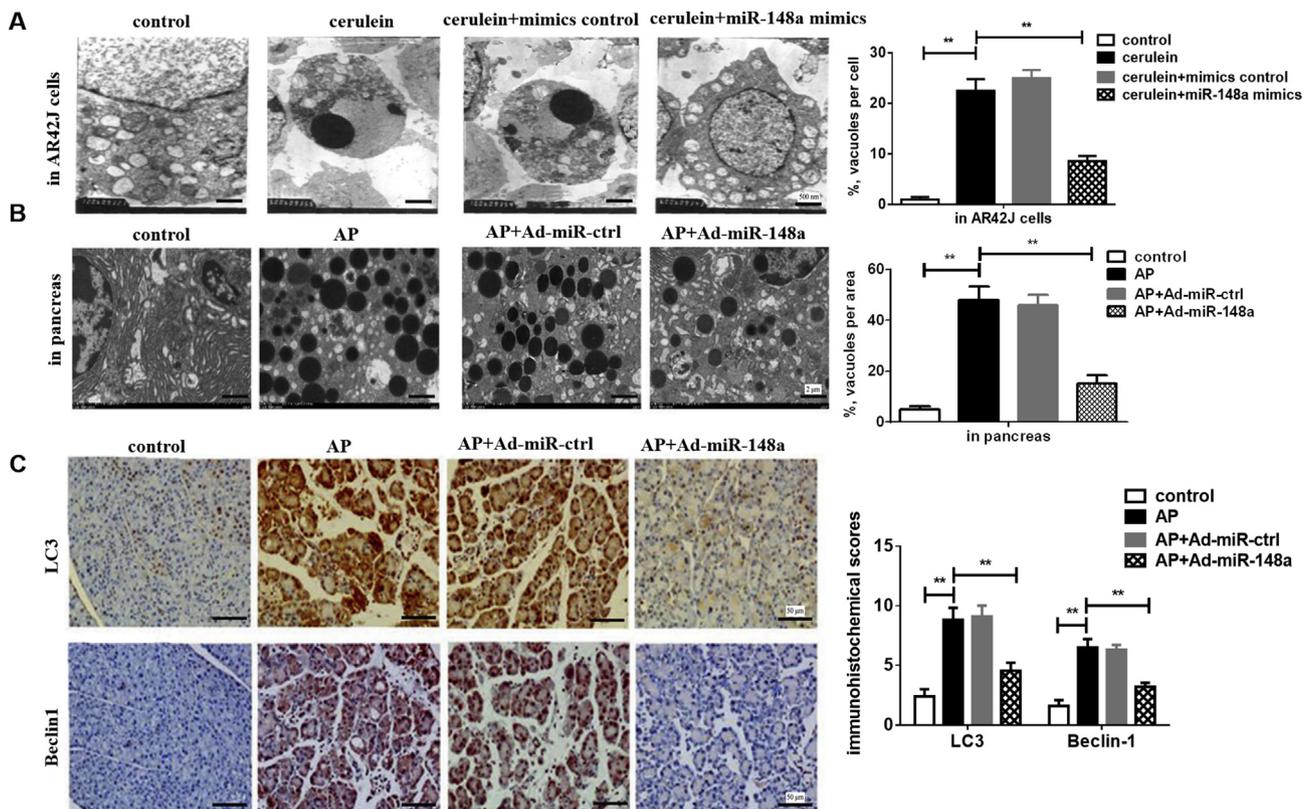


Fig. 4. Inhibitory effects of miR-148a on regulation of autophagy in AR42J cells and mice with AP. (A) Representative TEM image of AR42J cells (A) and pancreatic acinar cells of mice (B). The percentage of autophagic vacuoles per cytoplasm area was figured out. Original magnification: 12000 × . (C) Protein expression of LC3-II and Beclin1 in pancreatic tissues of mice was determined by immunohistochemical method. Original magnification: 200 × , Scale bar = 50 μm. The immunohistochemical scores of LC3 and Beclin-1 staining were analyzed. Data are presented as the means ± SD from three independent triplicated experiments. ** $P < 0.01$ indicates significant difference.

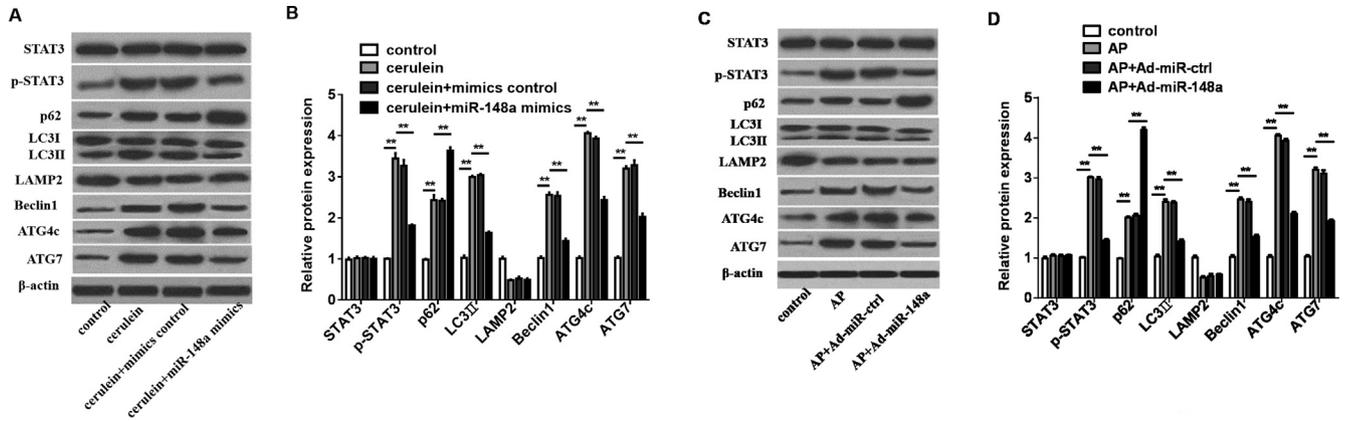


Fig. 5. Effects of miR-148a on the protein expression of autophagy markers in AR42J cells and mice with AP. The protein levels of autophagy markers LC3-I/II, Beclin1, ATG4c, ATG7, p62, and LAMP2 were determined by Western blot analysis in AR42J cells (A, B) and the pancreatic tissues of mice (C, D) with AP after treatment with miR-148a. Data are presented as the means ± SD from three independent triplicated experiments. ***P* < 0.01 indicates significant difference.

miR-148a may regulate autophagy through IL-6/STAT3 pathway in AR42J cells and mice with AP

In the present study, DHA induces PPAR γ activation and catalase expression, which inhibits ROS-mediated activation of JAK2/STAT3 and IL-6 expression in cerulein stimulated pancreatic acinar cells [7]. IL-6 can activate STAT3 and the autophagy enzyme GABARAP1 in human islets [12]. To elucidate the underlying mechanism, STAT3 activation in AR42J cells and mice treated with cerulein and miR-148a or IL-6/STAT3 inhibitor AG490 was determined. The levels of IL-6/STAT3 pathway and autophagy markers in Fig. 6 displayed that the protein expression of p-STAT3 was increased after treated with cerulein (*P* < 0.01), while total STAT3 expression showed no significant difference. IL-6 induced the levels of LC3-II, Beclin1, ATG4c, and ATG7 cut down by overexpression of miR-148a post AP, in contrast to the expression of p-STAT3 and p62 (*P* < 0.01). However, AG490 enhanced the effect of miR-148a and inhibited the function of IL-6 post AP (*P* < 0.01). In addition, the expression of LAMP2 and STAT3, had no significant difference *in vitro* and *in vivo* treated with overexpression of miR-148a plus IL-6 or AG490.

Taken together, overexpression of miR-148a may regulate autophagy through IL-6/STAT3 pathway *in vitro* and *in vivo*.

Discussion

AP is a severe disease with an incidence of 45/100,000, and 30% of patients suffered AP may develop severe acute pancreatitis (SAP),

leading to pancreatic necrosis, intestinal barrier dysfunction and bacterial translocation [21]. Recent studies have shown that the vacuoles in AP acinar cells originate from autophagy, which suggests that autophagy may play an important role in the process of intracellular trypsinogen activation in early AP [24]. However, the specific mechanism of autophagy in AP is still unclear.

A number of studies have implied that IL-6 and TNF- α are two pro-inflammatory cytokines expressed in response to local damage to the pancreas [21]. Pretreatment with IL-6 renders β cells resistant to apoptosis can activate STAT3 and the autophagy enzyme GABARAP1 in human islets [12]. In addition, some studies have demonstrated that miRNA can regulate the autophagic pathway by regulating the related genes in the AP process. For example, miR-141 binding to the miRNA 3'UTR of high mobility group box 1 (HMGB1) protein could inhibit autophagosome formation through the HMGB1/Beclin-1 pathway [14]. Down-regulated miR-148b-3p in the AR42J autophagy-induced cells up-regulated autophagy-associated target genes, thus promoting the autophagy process through the insulin signaling pathway [1]. Therefore, we used cerulein to stimulate AP model and then explored the expression of miR-148a and the interaction among miR-148a, IL-6 and autophagy.

In this study, we used cerulein to construct AP model successfully by confirming the activation of trypsinogen to trypsin in the pancreas, and an increase in the serum levels of amylase and lipase. Moreover, we revealed the low-expression of miR-148a in AP for the first time *in vitro* and *in vivo*, similar to the expression of miR-

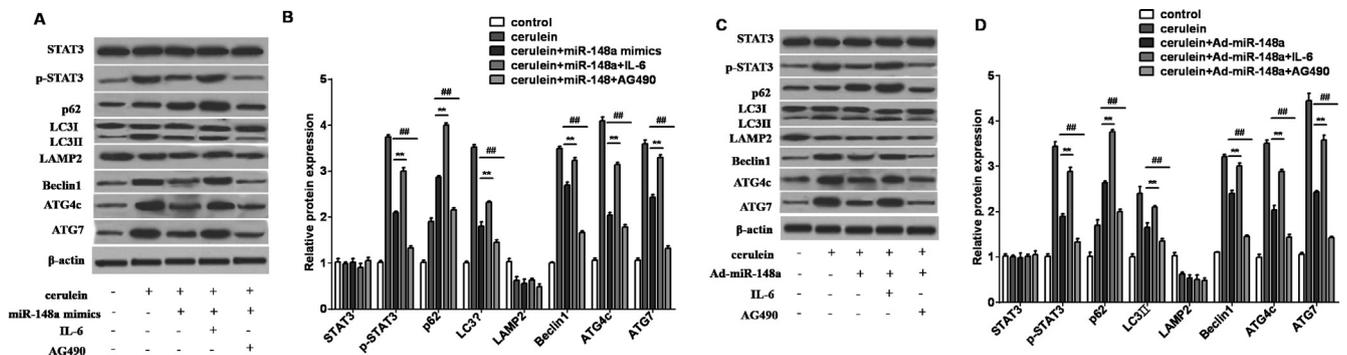


Fig. 6. miR-148a may regulate autophagy through IL-6/STAT3 pathway in AR42J cells and mice with AP. Western blot analysis were used to determine the protein levels of STAT3/p-STAT3 and autophagy markers LC3-I/II, Beclin1, ATG4c, ATG7, p62, and LAMP2 in AR42J cells (A, B) and the pancreatic tissues of mice (C, D) with AP after treatment with miR-148a plus IL-6 or AG490. Data are presented as the means ± SD from three independent triplicated experiments. ***P* < 0.01 and ###*P* < 0.01 indicate significant difference.

148b-3p. In order to confirm the effect of miR-148a on AP, we used miR-148a mimics and miR-148a adenoviral vector to overexpress miR-148a. Overexpressing miR-148a promoted cell proliferation and decreased the count of MPO positive cells, focal acinar cell degeneration, inflammatory cell infiltration, and necrosis, as well as the area of vacuoles within the AP tissue and the pancreas pathological scores. Additionally, cerulein increased the levels of amylase and lipase in serum and administering Ad-miR-148a post AP reduced the effect of cerulein. Moreover, Trypsin activity and TAP levels in the pancreas homogenates showed a similar change to the levels of amylase and lipase in serum, consistent with the previous study [16]. Hence, overexpression of miR-148a may attenuate cerulein-induced AP in AR42J cells and mice.

By searching online databases, we predicted possible binding sites and verified that IL-6 was a direct target of miR-148a by luciferase activity assay. Moreover, miR-148/152 inhibited the production of IL-6 expression and DC-initiated Ag-specific T cell proliferation by targeting CaMKII α [2]. Overexpression of miR-148 inhibited LPS-induced inflammatory cytokines production of IL-6, which then avoid excessive inflammation response [5].

The RT-qPCR and Western blot assay in our study showed that overexpression of miR-148a inhibited the production of IL-6 in AR42J cells and the pancreatic tissues of mice with cerulein-stimulated AP.

More and more studies imply that autophagy in acinar cells is a critical process in the pathogenesis of AP, and that impaired autophagic influx induced by defective function of lysosomes can contribute to intra-acinar trypsin activation and pancreatitis [19,23,25,26]. As shown in some findings, the regulation of autophagy may alter the progression of AP induced by L-arginine or cerulein plus LPS in mice [17,27], with miRNAs as a moderator in the autophagy process via regulating the expression of autophagy-associated genes [1,28]. Analogously, to further investigate the effects of miR-148a on regulation of autophagy in AP, AR42J cells and the pancreatic tissues of mice were treated for TEM. In our study, the intra-organellar content of the autophagosomes was filled with more dense and irregular structures after treatment with cerulein, which was offset by overexpression of miR-148a. Thus, miR-148a exhibits inhibitory effects on regulation of autophagy *in vitro* and *in vivo*, similar to the role of miR-148b-3p. To explore the regulatory mechanism of miR-148a, we detected the levels of autophagy-associated genes after the overexpression of miR-148a.

As all known, autophagy in experimental pancreatitis, is activated but its completion is inhibited [26], and a variety of proteins are involved in the process of autophagy. LC3 is divided into type I and type II proteins, and the content of LC3-II located on the membrane of autophagosomes within cells, is proportional to the number of autophagic vacuoles. LC3-II is ultimately degraded in the autolysosome and an increase in LC3-II indicates an increased number of autophagosomes [23]. While Beclin-1 plays a role in the formation of the pro-autophagic complex and mediates the localization of other autophagy proteins to the autophagosome membrane [29]. p62, a chaperone protein that brings ubiquitinated cargo to the autophagosome for destruction with degrading itself [30]. And the increase of p62 indicates that autophagy is impaired in autolysosome degradation [11]. ATG4c and ATG7 are both essential for autophagy, and lack of them suppresses the autophagic processes [31]. LAMP2 is a ubiquitous lysosomal membrane protein highly expressed in normal human pancreatic tissue and is required for the proper fusion of lysosomes with autophagosomes in the late stage of the autophagic process [6,32].

In our study, autophagy was induced by cerulein in AR42J cells and mice. The immunohistochemical results demonstrated that the increase in the relative protein expression of LC3-II and Beclin-1. Furthermore, in cerulein-stimulated AP, Western blot analysis

were used to detect the significant increase in the levels of LC3-II, Beclin1, ATG4c, and ATG7 while overexpression of miR-148a attenuated the effect of cerulein. MiR-148a promoted the expression of p62 induced by cerulein. An increase in both p62 and LC3-II indicates inefficient, and retarded autophagic flux [23]. Additionally, the expression of LAMP2, regulating the fusion of lysosome with autophagosome [14], had no significant differences between the cerulein-induced AP group and overexpression of miR-148a group *in vitro* and *in vivo*. Taken together, overexpression of miR-148a can block the process of autophagosomal formation and impair autophagy *in vitro* and *in vivo*.

Autophagy is a programmed cell death pathway, and the process includes the participation of numerous signaling pathways, such as Akt [33], MAPK, AMPK [1,34], TBK1 [35], RAS-cAMP-PKA [29], AGER/RAGE [13], STAT3 [12,36] and HMGB1/TLR4 [14,37], which regulate downstream autophagy-associated genes. Thus, to reveal the mechanism of miR-148a affecting the autophagy of AP, we confirmed the effect of miR-148a on IL-6/STAT3 signaling pathway. The mRNA and protein levels of IL-6 after over-expression of miR-148a were down-regulated via RT-qPCR and Elisa, in accordance with the previous study [2,5,38]. In addition, IL-6 and IL-6/STAT3 signaling pathway inhibitor AG490 were used to verify the role of IL-6/STAT3 signaling in the regulation of miR-148a on autophagy. We found that IL-6 stimulated the protein levels of p-STAT3, LC3-II, Beclin1, ATG4c, and ATG7 down-regulated by overexpression of miR-148a, in contrast to p62 expression. However, AG490 enhanced the effect of miR-148a and inhibited the function of IL-6. The expression of LAMP2 and STAT3, showed no significant differences *in vitro* and *in vivo* treated with overexpression of miR-148a plus whether IL-6 or AG490. These data imply that overexpression of miR-148a may regulate autophagy through IL-6/STAT3 pathway *in vitro* and *in vivo*.

Conclusion

The findings in this study provides new insight into the protective role of miR-148a in the process of AP. All the results indicate that miR-148a suppresses autophagy via down-regulating IL-6/STAT3 signaling in cerulein-induced AP *in vitro* and *in vivo*. The miR-148a appears to be a promising candidate for the gene therapy of AP.

Conflicts of interest

The authors declare that they have no conflicts of interest with the contents of this article.

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