

Original Article

LncRNA-PVT1 aggravates severe acute pancreatitis by promoting autophagy via the miR-30a-5p/Beclin-1 axis

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Abstract: Severe acute pancreatitis (SAP) is a serious abdominal disease associated with increased morbidity and high mortality rates. The initial pancreatic injury and inflammatory response, which begins within acinar cells, play vital roles in promoting SAP severity. Previous studies have indicated that overactivated autophagy in acinar cells increases the risk of SAP. Autophagy is affected by various signaling pathways, partially through long noncoding RNA (lncRNA)-PVT1. However, few studies have focused on the effect of lncRNA on autophagy in pancreatitis. Our results demonstrate that sodium taurocholate (STC) induces abnormal activation of the autophagic response in pancreatic acinar cells *in vitro* and *in vivo*. The lncRNA-PVT1 level was significantly upregulated in this process and was capable of targeting the miR-30a-5p/Beclin-1-mediated autophagy signaling pathway. Additionally, STC-induced pancreatic acinar cells injury and autophagy activation were all abrogated with the downregulation of lncRNA-PVT1 by shRNAs *in vitro*. Furthermore, we confirmed that the lncRNA-PVT1/miR-30a-5p/Beclin-1 axis induces abnormal autophagy in the pancreas of SAP rats. Collectively, these results demonstrate that the lncRNA-PVT1/miR-30a-5p/Beclin-1 axis is a potential target for improving SAP, thus providing a foundation for further development of therapeutics in the future.

Keywords: Severe acute pancreatitis, acinar cells, autophagy, lncRNA-PVT1, miR-30a-5p

Introduction

Severe acute pancreatitis (SAP), a common and serious inflammatory disorder, can lead to the development of systemic inflammatory response syndrome and multiple organ failure [1-3]. Because no specific therapy is available for SAP, its severe complications and invasive interventions result in a mortality rate as high as 30% [4, 5]. To date, SAP remains a significant and unresolved challenge for clinicians due to its complicated pathology and multifactorial features [6].

Mechanistic studies have reported that inappropriate activation of trypsinogen (a key initiator for zymogen activation) and a lack of efficient exclusion of active trypsin in SAP, both play vital roles in the occurrence and development of SAP [7, 8]. It is understood that each of

the aforementioned processes results in severe pathological changes in the pancreatic acinar cells. Autophagy is a cellular metabolic mechanism that transports intracellular substances to lysosomes for degradation, prompting the synthesis of new substances [9, 10]. Numerous autophagy studies have revealed a critical homeostatic role in the “quality control” of intracellular organelles, metabolic adaptation, and differentiation and development [9]. Deregulation of autophagy appears in the pathogenesis of several diseases, including inflammatory and neurodegenerative disorders and cancer [11-13]. The exocrine pancreas has been shown to have a higher basal level of autophagy than in other organs such as the heart, liver, kidney, and endocrine pancreas, especially when activated in response to starvation [14, 15]. However, overactivation of pancreas-specific autophagy causes excessive accumulation of

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zymogen granules in acinar cells, further increasing the risk of SAP [16-19]. Therefore, the elucidation of autophagy mechanisms in SAP, as well as how they affect the development of pancreatitis, may provide new therapeutic targets or approaches for SAP.

Long noncoding RNA (lncRNA) is a type of RNA with a transcript length of more than 200 nucleotide residues. Recent studies have found that lncRNA can activate or inhibit the expression of target genes by directly binding to target genes [20, 21]. lncRNA participates in gene expression regulation with multiple actions, including histone modification, transcription factor recruitment, and miRNA endogenous competition [22, 23]. However, there are few reports evaluating lncRNA actions in pancreatitis. Previous studies have shown that lncRNA plasmacytoma variant translocation 1 (lncRNA-PVT1) can promote the occurrence and development of multiple cancers by affecting cell proliferation, apoptosis, migration, and invasion [24]. In addition to this, lncRNA-PVT1 can affect autophagy by regulating various signaling pathways and biological processes [25, 26]. Thus, lncRNA-PVT1 may be involved in the autophagic process of acinar cells in SAP.

In the present study, models of SAP were constructed with sodium taurocholate (STC) *in vitro* and *in vivo* to explore the effect of lncRNA-PVT1 on acinar cell autophagy and to uncover the regulatory mechanism, providing an experimental basis for the clinical treatment of SAP.

Materials and methods

Cell viability and morphology assays

Rat pancreatic acinar cells AR42J (ATCC®CRL-1492™) were cultured in Ham's F-12K medium supplemented with 20% fetal bovine serum. According to a previously reported method, AR42J cells were cultured in 96-well plates at a density of 1×10^5 cells/mL for 48 hours, and subsequently treated with 500 μ M (IC50 value) STC (Solarbio; Beijing, China) for 2 hours [27]. Cells in the control group were cultured under normal conditions during the entire experiment. Cell morphology was imaged using a phase contrast microscope (Nikon; Tokyo, Japan).

Target identification of lncRNA-PVT1

To elucidate the potential mechanism of lncRNA-PVT1 in SAP, its target miR-30a-5p was

predicted using lncRNABase, Starbase2.0, and RNAhybrid databases, where possible binding sites were identified in the BiBiserv database. The dual luciferase reporter assay was performed and the miR-30a-5p binding site of lncRNA-PVT1 was cloned into the GP-miRGLO vector to construct the wild-type (WT) plasmid. Site-directed mutagenesis of the seed sequence of lncRNA-PVT1 was performed to generate a mutant (MUT) plasmid. Plasmids containing PVT1-MUT and PVT1-WT were purchased from GenePharma Co., Ltd. (Jiangsu, China). AR42J cells were cultured in medium and cotransfected with plasmid DNA (PVT1-MUT or PVT1-WT) and miR-30a-5p agomir (50 nM) or the negative control for 24 hours. Luciferase activities were then assessed using a double-luciferase reporter assay kit (Promega; Madison, WI, USA) with a microplate reader (Thermo; Waltham, MA, USA) and a normalized ratio of firefly and renilla luciferase signals.

To further verify this target, we performed the biotin-labeled pull-down assay in AR42J cells. Briefly, AR42J cells (1×10^6 cells/mL) were seeded in 10-cm plates in duplicate. The next day, biotin-labeled miR-30a-5p was transfected into the cells to final concentration of 50 nM. After 48 hours, whole cell lysates were harvested; and Dynabeads M-280 Streptavidin (50 μ L each sample) (Invitrogen; Carlsbad, CA, USA) were coated with yeast tRNA (10 μ L per sample, stock 10 mg/mL), and incubated with rotation at 4°C for 2 hours. Simultaneously, the pre-coated beads were washed with 500 μ L lysis buffer and resuspended in 50 μ L lysis buffer. Sample lysates were mixed with the beads (50 μ L per sample) and incubated at 4°C for 3 hours in a rotator. Beads were then washed 5 times with 500 μ L ice cold lysis buffer to remove unbound materials. To isolate the RNA, 750 μ L TRIzol and 250 μ L nuclease free water were added to both input and pull-down samples. Finally, RNA was precipitated and then subjected to real-time quantitative PCR to detect miR-30a-5p and lncRNA-PVT1 levels.

RNA fluorescence in situ hybridization assay

To further detect the expression of lncRNA-PVT1, the RNA fluorescence in situ hybridization (FISH) assay was conducted in STC-treated AR42J cells using a FISH kit (GenePharma; Jiangsu, China) in accordance with the manufacturer's directions. Streptavidin-biotin system labeled PVT1 RNA probe was used as the

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probe mix. Samples were observed using a confocal laser scanning microscope (LeicaSP8, Wetzlar, Germany) at 630 × magnification.

LncRNA-PVT1 knockdown experiments

AR42J cells were cultured in 6-well plates (1×10^5 cells/mL) containing a serum-free medium and transfected with LncRNA shRNAs (pGPU6/GFP/Neo-Pvt1-RAT-NC, -1, -2, -3, and -4) or mature miR-30a-5p agomir mixed with GP-transfect-Mate according to the manufacturer's instructions (GenePharma; Jiangsu, China). In addition, 48 hours after transfection, the cells were cultured in the presence or absence of STC for 2 hours.

Animals

Male Sprague-Dawley rats weighing 200 ± 20 g were obtained from the Experimental Animal Center at Dalian Medical University (Dalian, China). Rats were housed in a temperature-controlled ($25 \pm 2^\circ\text{C}$) room with a 12-hour light/dark schedule and free access to food and water. All animal experimental procedures were approved by the Animal Care and Use Committee at Dalian Medical University (No. AEE18072) and complied with the principle for replacement, refinement, or reduction.

Twenty rats were randomly divided into 2 groups ($n = 10$): a sham operation (SO) group and a SAP model group. Experimental models were established as previously described [27]. Briefly, rats were fasted for 12 hours with free access to water, then anesthetized via intraperitoneal injection of sodium pentobarbital (30 mg/kg). The SAP model was established using a retrograde infusion of 5.0% STC (0.1 mL per 100 g body weight) into the biliopancreatic duct. In the SO group, the abdomen was surgically opened and then closed. Blood samples were collected from the abdominal aorta for biochemical analyses 24 hours after establishment of the model. Pancreatic head samples were fixed in 10% buffered formalin for histopathological and immunofluorescence assays. The other pancreatic tissues were maintained at -80°C until real-time PCR and western blotting analyses.

Hematoxylin and eosin and immunofluorescence staining

Formalin-fixed pancreas tissues were embedded in paraffin and sectioned into 5- μm slices.

The slices were stained with hematoxylin and eosin and photographed using a light microscope (Olympus BX53, Japan) at 200 × magnification. For immunofluorescence staining, AR42J cells or pancreatic sections were incubated in a humidified chamber overnight at 4°C with rabbit microtubule-associated protein 1 light chain 3-II (LC3-II) antibody (dilution ratio 1:100) (Abcam; Cambridge, UK). After this step, the samples were incubated with TRITC-conjugated goat anti-rabbit IgG for 1 hour at 37°C . The samples were re-stained with 4',6-diamidino-2-phenylindole (5 $\mu\text{g}/\text{mL}$) for 5 min and observed under an Olympus BX63 fluorescence microscope (Olympus; Tokyo, Japan).

Enzyme-linked immunosorbent assay analysis

The levels of amylase, tumor necrosis factor- α (TNF- α), and interleukin 1β (IL- 1β) in plasma and cell supernatant were assessed using rat enzyme-linked immunosorbent assay (ELISA) kits (Langdun Biotech; Shanghai, China) according to the manufacturer's instructions. The absorbance was measured at 450 nm using a microplate reader (Thermo; Waltham, MA, USA).

Transmission electron microscope assay

Cells and pancreatic tissues were harvested and fixed overnight at 4°C in 2% glutaraldehyde. Next, the samples were fixed in 1% osmium tetroxide for 2 hours and washed 3 times (each for 15 min) in 0.1 M sodium cacodylate buffer, before being dehydrated in gradient ethanol solutions. Subsequently, pretreated samples were used for ultramicrotomy and collected on copper grids. Finally, the samples were stained and observed with a transmission electron microscope (TEM) (JEM-2000EX; JEDL, Japan) with 20,000 × magnification.

Real-time quantitative PCR

Total RNA samples were extracted from the cells and pancreatic tissues using a total RNA extraction kit according to the manufacturer's protocol. The purity of the extracted RNA was determined, then reverse-transcribed using a PrimeScript[®]RT reagent kit (Takara; Dalian, China) based on the manufacturer's instructions. Relative quantitation was performed using a SYBR[®] Premix Ex Taq[™] II (TliRNaseH Plus) kit (Takara; Dalian, China) following the manufacturer's instructions, with GAPDH or U6 as an endogenous control, and the fold chang-

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Table 1. Primer sequences used for real-time quantitative PCR in AR42J cells and pancreatic tissues of rats

Name	Sequences
lncRNA PVT1	Forward: 5'-ACCAGGATATAAACCTAGTGATAAA-3' Reverse: 5'-AAGCACCCAATGCAGAATAG-3'
miR-30a-5p	TGTAAACATCCTCGACTGGAAG
GAPDH	Forward: 5'-ATGTTCCAGTATGACTCTA-3' Reverse: 5'-CACCCATTGATGTTAG-3'
U6	Forward: 5'-GGAACGATACAGAGAAGATTAGC-3' Reverse: 5'-TGGAACGCTTCACGAATTTGCG-3'
IL-1 β	Forward: 5'-ACAGCAGCATCTCGACAAGAGC-3' Reverse: 5'-CCACGGGCAAGCATAGGTAGC-3'
TNF- α	Forward: 5'-CAAGAGCCCTTGCCCTAAGG-3' Reverse: 5'-CGGACTCCGTGATGTCTAAGTACTT-3'

es were calculated for each gene. The primer sequences are listed in **Table 1**.

Western blotting assay

Total protein samples of cells and pancreas tissues were extracted using cold lysis buffer containing 1 mM phenylmethylsulfonyl fluoride according to the manufacturer's protocol (Beyotime; Jiangsu, China), and the concentration of the extracted protein was quantified with the BCA Protein Assay Kit (Beyotime; Jiangsu, China). The samples were then separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to polyvinylidene difluoride membranes. Membranes were incubated overnight at 4°C with rabbit LC-3 and Beclin-1 antibodies (1:1000) (Abcam; Cambridge, UK) and incubated at 37°C for 2 hours with the secondary antibody (1:2000) (Abcam; Cambridge, UK). Finally, the expression levels of extracted proteins were normalized to β -actin using the enhanced chemiluminescence method.

Statistical analysis

All data were presented as the means \pm standard deviation (SD) and were analyzed using GraphPad Prism 5.0 software (San Diego, CA, USA). One-way ANOVA followed by post hoc Tukey's test was used for the analysis of the differences between multiple independent groups, and the unpaired Student's t-test was used for two groups' comparison according to the homogeneity of variances. The results were considered to be statistically significant at p values less than 0.05 or 0.01.

Results

STC induces AR42J cell injury by promoting autophagy

The morphology of AR42J cells was observed using a phase contrast microscope, and the results revealed that STC notably caused the reduced density and number of bright and transparent normal cells and increased morphological changes characteristic of cell death (**Figure 1A**). We performed an ELISA to measure amylase, a biochemical marker of pancreatic disease, and found that STC significantly induces the release of amylase (**Figure 1B**). Moreover, as shown in **Figure 1B**, the levels of inflammatory mediators, TNF- α and IL-1 β , in the cell were markedly increased in AR42J cells after treatment with STC. TEM technology was used to clearly observe the ultrastructure of acinar cells, and the accumulation of autophagosomes and autophagic vacuoles (as denoted by the red arrows) was observed in STC-stimulated AR42J cells (**Figure 1C**). Western blot results indicated that Beclin-1 and LC3-II protein expressions are significantly upregulated in AR42J cells treated with STC (**Figure 1D** and **Supplementary Figure 1**). Furthermore, immunofluorescence analysis demonstrated that STC notably increases LC3-II expression (red area) (**Figure 1E**).

Downregulation of lncRNA-PVT1 alleviates STC-induced AR42J cell injury

As shown in **Figure 2A**, the lncRNA-PVT1 level was significantly upregulated in STC-treated AR42J cells. The RNA FISH assay demonstrated that lncRNA-PVT1 transcripts are abundant in the cytoplasm of AR42J cells treated with STC (**Figure 2B**). Furthermore, the *in vitro* transfection approach of lncRNA-PVT1 shRNA was used to observe the regulatory role of lncRNA-PVT1 following pancreatic acinar cell injury. We chose the most suitable shRNA plasmid, using the real-time PCR assay, which revealed that shRNA-3 and shRNA-4 can effectively inhibit lncRNA-PVT1 expression in AR42J cells (**Figure 2C**). As shown in **Figure 2D**, the gene level of lncRNA-PVT1 was also notably decreased by shRNA-3 and shRNA-4 in STC-treated AR42J cells. Meanwhile, the upregulation of amylase, TNF- α , and IL-1 β release in STC-treated cells

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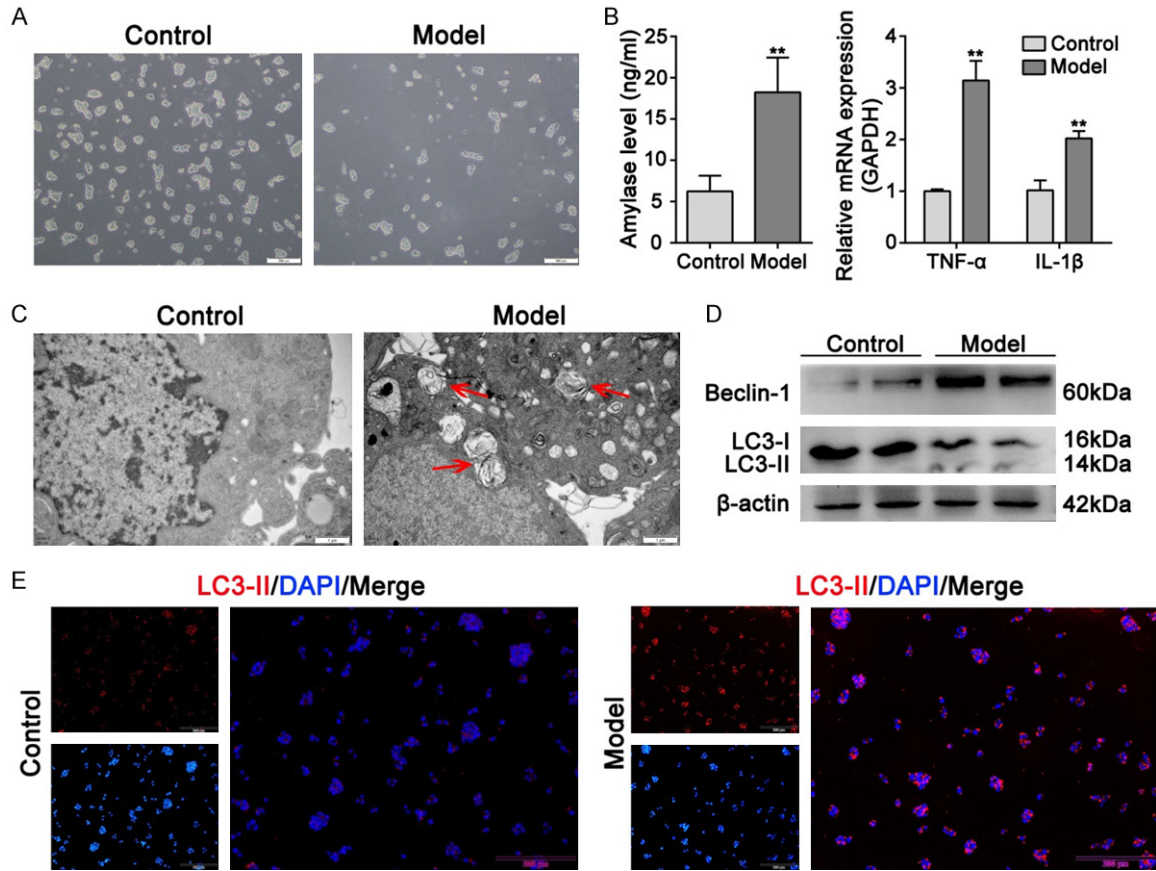


Figure 1. STC induced AR42J cell injury by promoting autophagy. AR42J cells were cultured in plates for 48 hours, then treated with 500 μ M (IC50 value) STC for 2 hours. A. Morphology images of AR42J cells from an optical microscope (100 \times magnification). B. Amylase (n = 6) detected by ELISA and TNF- α and IL-1 β (n = 3) detected by real-time PCR in AR42J cells. C. Typical cellular ultrastructure observed by TEM (20000 \times magnification). D. Expression levels of Beclin-1 and LC3 proteins in AR42J cells assayed by western blotting. E. LC3-II protein expression (red fluorescence) observed by immunofluorescence analysis (100 \times magnification). Significance was tested by independent Student's t-tests. Data are presented as the means \pm SD. **P < 0.01 vs. control group.

was significantly inhibited by lncRNA-PVT1 shRNA plasmid (**Figure 2E**). Furthermore, the morphology of AR42J cells was observed in different treatment groups, revealing that the inhibition of lncRNA-PVT1 expression by shRNAs alleviates STC-induced AR42J cell death and morphological changes characteristic for cell death (**Figure 2F**). Therefore, these results indicate that the downregulation of lncRNA-PVT1 alleviates STC-induced AR42J cell injury.

Downregulation of lncRNA-PVT1 inhibits autophagy in STC-injured AR42J cells

As shown in **Figure 3A**, TEM results demonstrated a downregulation of lncRNA-PVT1 by shRNAs and a reduction in the number of autophagic vacuoles (as indicated by the red arrows) in STC-stimulated AR42J cells. The expression lev-

els of Beclin-1 and LC3-II proteins were all significantly downregulated by lncRNA-PVT1 shRNAs compared to the negative control group (**Figure 3B** and [Supplementary Figure 2](#)). Furthermore, the immunofluorescence results revealed that lncRNA-PVT1 shRNAs decrease LC3-II expression (red area) (**Figure 3C**). Therefore, these results suggest that the inhibition of autophagy in STC-treated AR42J cells occurs with downregulation of lncRNA-PVT1.

lncRNA-PVT1 targets miR-30a-5p in AR42J cells

Based on bioinformatic approaches, we predicted that miR-30a-5p is a potential target of lncRNA-PVT1 (**Figure 4A**). To further verify this finding, the dual luciferase reporter assay was performed. In this assay, luciferase activity was

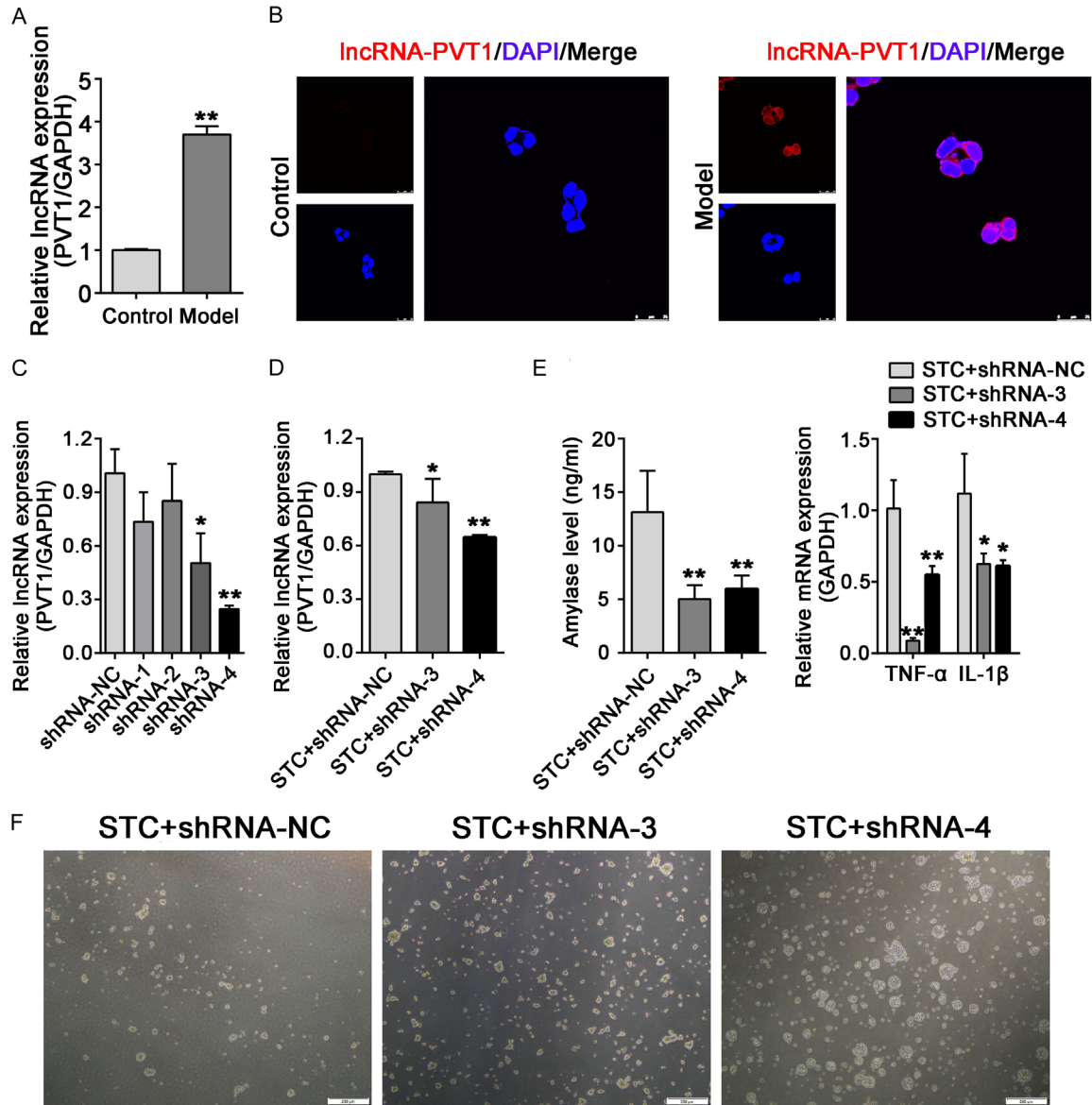


Figure 2. Downregulation of lncRNA-PVT1 alleviated STC-induced AR42J cell injury. AR42J cells were cultured in 6-well plates containing a serum-free medium and transfected with lncRNA shRNAs mixed with GP-transfect-Mate. After a 48-hour transfection, the cells were cultured in the presence or absence of STC for 2-hours. (A) Relative expression level of lncRNA-PVT1 in AR42J cells detected by real-time PCR (n = 3). (B) lncRNA-PVT1 expression in AR42J cells assayed by RNA FISH (630 × magnification). (C) Relative expression level of lncRNA-PVT1 in AR42J cells transfected with lncRNA-PVT1 shRNA plasmid (n = 4). (D) Relative expression level of lncRNA-PVT1 in STC-injured AR42J cells transfected with lncRNA-PVT1 shRNA plasmid (n = 4). (E) Amylase (n = 6), TNF- α , and IL-1 β (n = 3) levels in STC-injured AR42J cells transfected with lncRNA-PVT1 shRNA plasmid. (F) Morphology images of STC-injured AR42J cells transfected with lncRNA-PVT1 shRNA plasmid (100 × magnification). Significance between two groups (A) was tested by independent Student's t-tests; significance between multiple independent groups (C-E) was tested by one-way ANOVA followed by post hoc Tukey's test. Data are presented as the means \pm SD. *P < 0.05 or **P < 0.01 vs. control or NC group.

significantly repressed in the PVT1-WT plasmid by miR-30a-5p agomir; however, this effect was not observed in the PVT1-MUT plasmid group (Figure 4B). Additionally, the level of lncRNA-PVT1 was markedly elevated in the pull-down

material isolated from AR42J cells, following transfection with biotin-labeled miR-30a-5p, compared to the control (Figure 4C). Furthermore, the input samples showed that lncRNA-PVT1 significantly decreased, where miR-30a-

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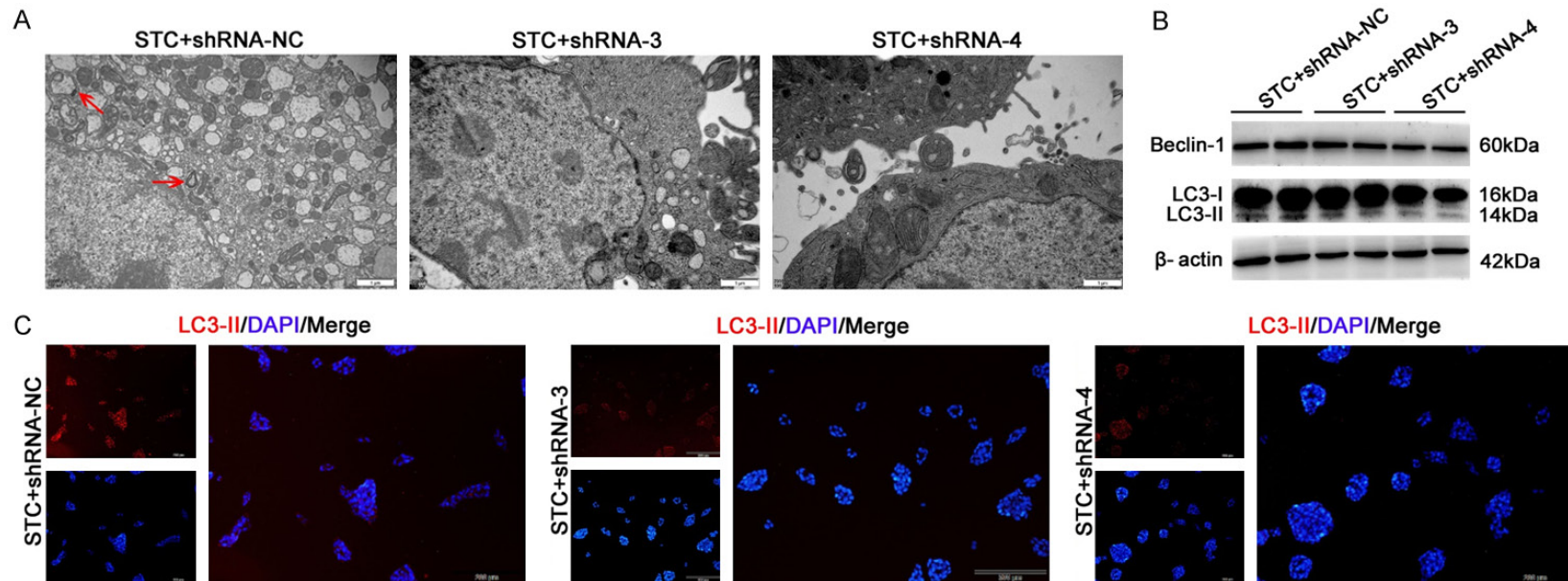


Figure 3. Downregulation of lncRNA-PVT1 inhibits autophagy in STC-injured AR42J cells. AR42J cells were cultured in 6-well plates containing a serum-free medium and transfected with lncRNA shRNAs mixed with GP-transfect-Mate. After a 48-hour transfection, the cells were cultured in the presence or absence of STC for 2 hours. A. Typical cellular ultrastructure in STC-injured AR42J cells transfected with lncRNA-PVT1 shRNA plasmid (20000 \times magnification); B. Expression levels of Beclin-1 and LC3 proteins in STC-injured AR42J cells transfected with lncRNA-PVT1 shRNA plasmid detected by western blotting; C. LC3-II protein expression (red fluorescence) in STC-injured AR42J cells transfected with lncRNA-PVT1 shRNA plasmid observed by immunofluorescence analysis (100 \times magnification).

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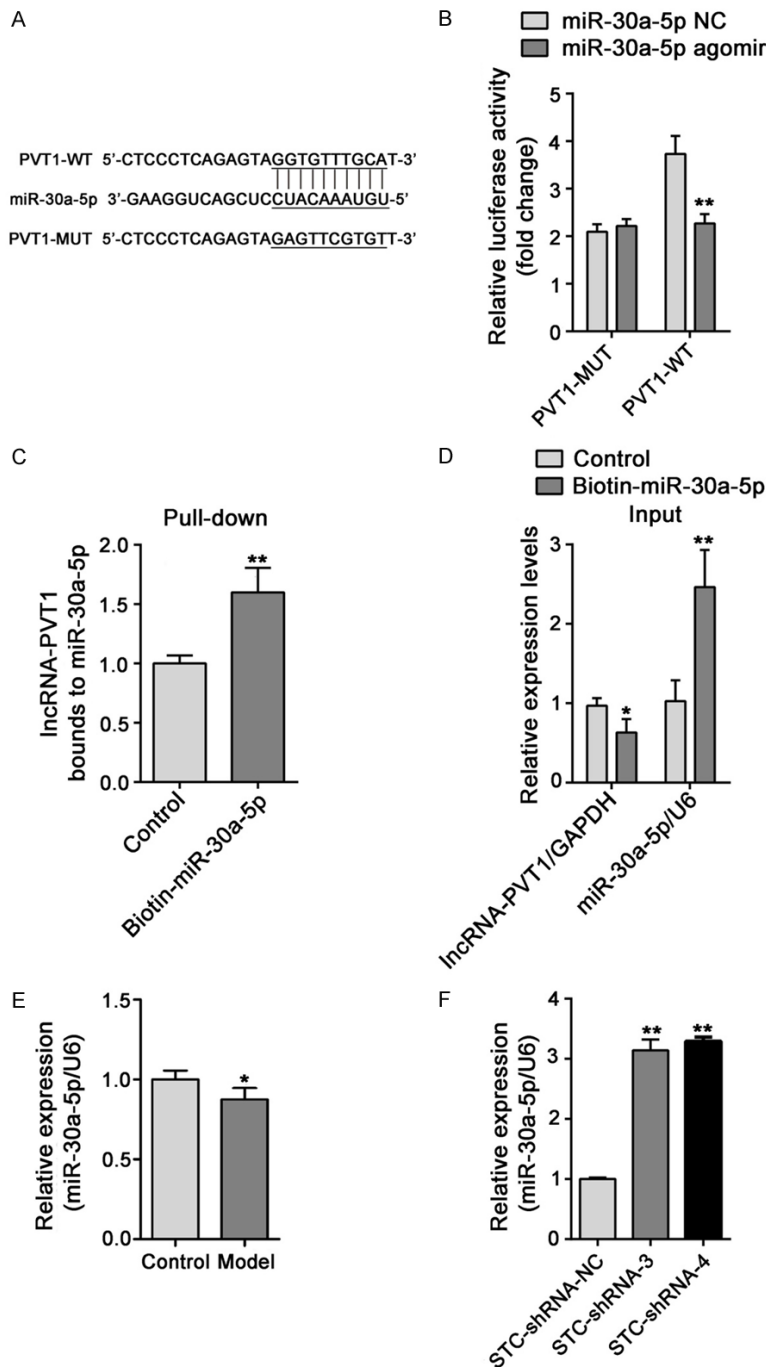


Figure 4. LncRNA-PVT1 targets miR-30a-5p in AR42J cells. (A) The binding site of LncRNA-PVT1 to miR-30a-5p. One target of LncRNA-PVT1, miR-30a-5p, was predicted by IncRNABase, Starbase2.0, and RNAhybrid databases, and then the possible binding sites were identified in the BiBiserv database. (B) Dual luciferase reporter assay of LncRNA-PVT1 and miR-30a-5p in AR42J cells (n = 5). AR42J cells were cultured in medium and co-transfected with plasmid DNA (PVT1-MUT or PVT1-WT) and miR-30a-5p agomir (50 nM) or the negative control for 24 hours. (C) Relative expression level of LncRNA-PVT1 in the materials pulled down by biotin-miR-30a-5p in AR42J cells. Biotin-labeled pull-down assay in AR42J cells was performed. (D) Relative expression level of the total input of LncRNA-PVT1 and miR-30a-5p in AR42J cells. Biotin-labeled pull-down assay in AR42J cells was performed. (E) Relative expression level of miR-30a-5p in AR42J cells (n = 4). AR42J cells were cultured in

plates for 48 hours, then treated with 500 μ M (IC50 value) STC for 2 hours. (F) Relative expression level of miR-30a-5p in STC-injured AR42J cells transfected with LncRNA-PVT1 shRNA plasmid. AR42J cells were cultured in 6-well plates containing a serum-free medium and transfected with LncRNA shRNAs mixed with GP-transfect-Mate. After 48-hour transfection, the cells were cultured in the presence or absence of STC for 2 hours. Significance between two groups (B-E) was tested by independent Student's t-test; significance between multiple independent groups (F) was tested by one-way ANOVA followed by post hoc Tukey's test. Data are presented as the means \pm SD. *P < 0.05 or **P < 0.01 vs. control or NC group.

5p was significantly increased, in AR42J cells treated with biotin-labeled miR-30a-5p compared with the control (Figure 4D). These results indicate that LncRNA-PVT1 can target miR-30a-5p and inhibit its expression.

We also detected a downregulation of miR-30a-5p expression level in STC-treated AR42J cells (Figure 4E). This is the opposite effect observed in STC-treated AR42J cells treated with LncRNA-PVT1 shRNAs, where a downregulation of LncRNA-PVT1 significantly upregulated miR-30a-5p (Figure 4F). These results show that LncRNA-PVT1 can target miR-30a-5p in AR42J cells.

LncRNA-PVT1/miR-30a-5p/Beclin-1 axis-mediated autophagy was activated in the acinar cells of SAP rats

As shown in Figure 5A, hematoxylin and eosin staining results clearly demonstrated SAP-induced pancreatic edema, necrosis, hemorrhage,

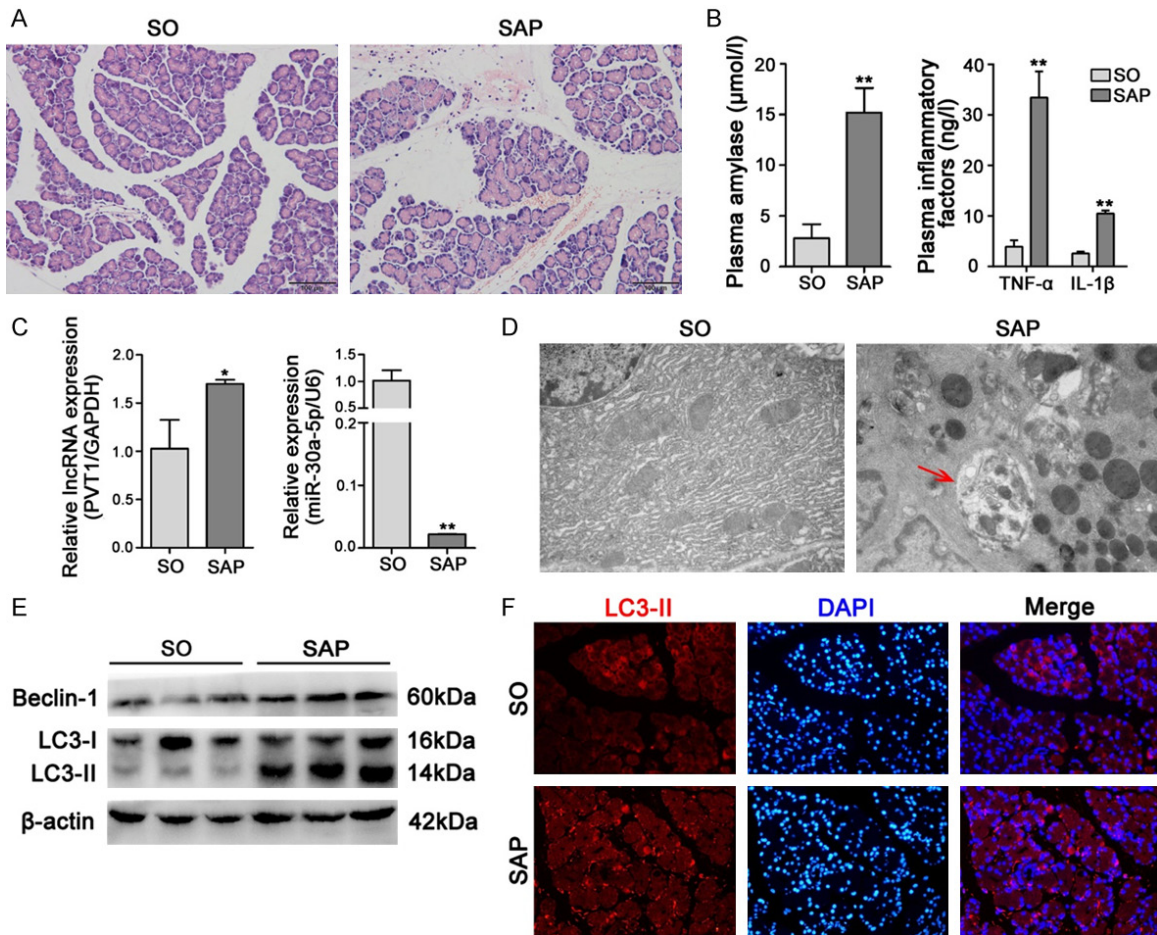


Figure 5. LncRNA-PVT1/miR-30a-5p/Beclin-1 axis-mediated autophagy was activated in the acinar cells of SAP rats. Twenty male Sprague-Dawley rats were randomly divided into 2 groups (n = 10): sham operation (SO) group or SAP model group. Briefly, the SAP model was established by retrograde infusion of 5.0% STC (0.1 mL per 100 g body weight) into the biliopancreatic duct. In the SO group, the abdomen was only surgically opened and closed to serve as a control. Samples were collected for biochemical analyses at the 24 hour timepoint following establishment of the model. A. Pancreatic tissue observed in SAP rats using an optical microscope (400 × magnification). B. Plasma levels of amylase, TNF-α, and IL-1β in SAP rats (n = 10). C. Relative expression levels of lncRNA-PVT1 and miR-30a-5p in the pancreas of SAP rats (n = 3). D. Typical cellular ultrastructure in the pancreas of SAP rats observed by TEM (20000 × magnification). E. Expression levels of Beclin-1 and LC3 proteins in the pancreas of SAP rats detected by western blotting. F. LC3-II protein expression (red fluorescence) in pancreatic tissues of SAP rats observed by immunofluorescence analysis (400 × magnification). Significance between two groups was tested by independent Student's t-tests. Data are presented as the means ± SD. *P < 0.05 or **P < 0.01 vs. SO group.

and inflammatory cell infiltration. ELISA results revealed that significantly higher levels of plasma amylase, TNF-α, and IL-1β in the SAP group than in the SO group (Figure 5B). The SAP group also had significantly higher levels of lncRNA-PVT1 expression, but notably lower levels of miR-30a-5p expression (Figure 5C). Although the SO group showed acinar cells with normal cellular structure, the SAP group exhibited massive autophagosome accumulation and autophagic vacuoles (as indicated by the red arrows) (Figure 5D). Compared with the SO group, the SAP group had notably upregulated protein expressions of Beclin-1 and LC3-II

(Figure 5E and Supplementary Figure 3). Meanwhile, immunofluorescent results indicated that the pancreas tissue of SAP rats exhibited more LC-3-II-positive area (red fluorescence) than that of SO rats (Figure 5F). Therefore, these results showed that lncRNA-PVT1/miR-30a-5p/Beclin-1 axis-mediated autophagy is activated in the acinar cells of SAP rats.

Discussion

SAP exhibits a complex disease progression associated with a high mortality rate. The onset of SAP is marked by acinar cell injury caused by

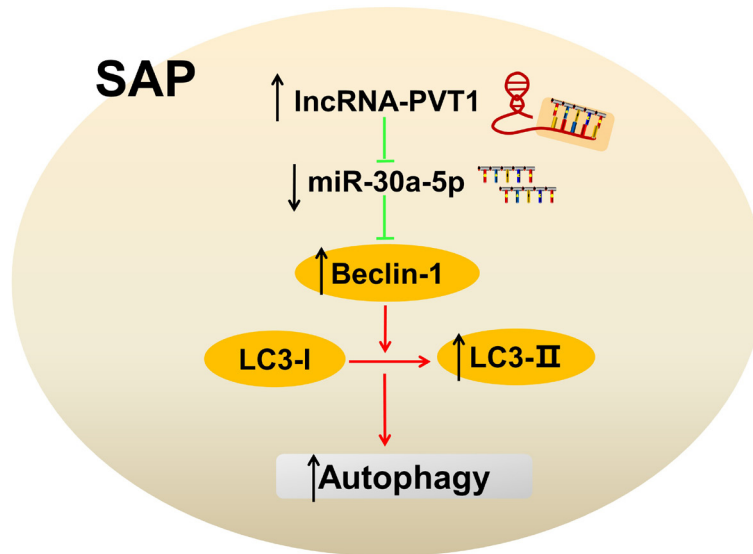


Figure 6. Proposed models for LncRNA-PVT1/miR-30a-5p/Beclin-1 axis-mediated autophagy in SAP. LncRNA-PVT1-mediated downregulation of miR-30a-5p expression in pancreatic acinar cells is essential for activating autophagy through the activation of the Beclin-1 signaling pathway. The LncRNA-PVT1/miR-30a-5p/Beclin-1 axis represents a novel drug target to improve pancreatic acinar cell injury and has potential implications in SAP pathology.

the pathological activation of trypsinogen [18, 28]. In the present paper, our *in vitro* and *in vivo* results cumulatively indicate that SAP notably increases the accumulation of autophagosomes and autophagic vacuoles in pancreatic acinar cells, as well as the expression of LC3-II protein. During autophagy, the cytoplasmic form (LC3-I) of LC3 is lipidated and converted into LC3-II, where it is then transported to the autophagosome membrane as a molecular marker for autophagy [29]. SAP induced the upregulation of autophagy in acinar cells *in vitro* and *in vivo*.

LncRNA-PVT1, located at 8q24.21, is upregulated in many cancers and associated with cell proliferation, apoptosis, and autophagy [25, 26, 30]. Our real-time PCR and RNA FISH results showed that LncRNA-PVT1 was also significantly upregulated in the cytoplasm of injured acinar cells, in both *in vitro* and *in vivo* assays. Moreover, the inflammatory response and acinar cell injury was alleviated *in vitro* following downregulation of LncRNA-PVT1 by shRNAs. These results indicate that LncRNA-PVT1 is a potential new therapeutic target of SAP.

Additional studies found that LncRNA-PVT1 may be an endogenous miRNA sponge that inhibits the expression of miR-30a-5p, a member of the

miRNA-30 family that regulates of several cell processes including cell growth, differentiation, and apoptosis [31]. Previous studies have also indicated that miR-30a-5p can inhibit autophagy by targeting the Beclin-1 gene [32-35]. Beclin-1, the first effector identified in the initial stage of autophagy, interacts with phosphatidylinositol 3-kinase to form a protein complex that can recruit the autophagy marker, LC3, and initiate autophagy flux [36, 37]. In this study, we found that in both SAP rats and STC-treated AR42J cells, there was a significant downregulation of miR-30a-5p expression and an upregulation of Beclin-1 and LC3-II, compared with their respective control groups. Furthermore, the down-

regulation of LncRNA-PVT1 by shRNAs notably inhibited STC-induced autophagy in STC-treated AR42J cells. These mechanistic studies support the notion that inhibition of LncRNA-PVT1 expression protects pancreatic acinar cells from injury during SAP by targeting the miR-30a-5p/Beclin-1 signaling pathway. Therefore, the LncRNA-PVT1/miR-30a-5p/Beclin-1 axis is a critical factor in autophagy during SAP and is a potential target for SAP therapy.

In summary, we demonstrated that the LncRNA-PVT1-mediated downregulation of miR-30a-5p expression in pancreatic acinar cells is essential for activating autophagy through activation of the Beclin-1 signaling pathway (**Figure 6**). Therefore, the LncRNA-PVT1/miR-30a-5p/Beclin-1 axis represents a novel drug target to improve pancreatic acinar cell injury and has potential implications for SAP pathology. The insights obtained from this study can further advance the development of future therapeutics to treat SAP.

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Disclosure of conflict of interest

None.

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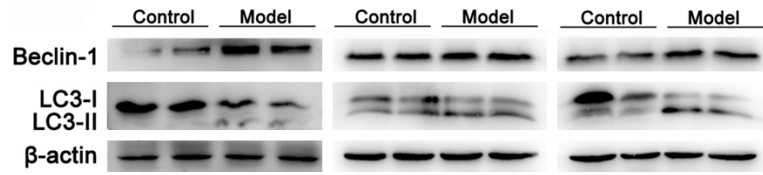
References

- [1] Lankisch PG, Apte M and Banks PA. Acute pancreatitis. *Lancet* 2015; 386: 85-96.
- [2] van Dijk SM, Hallensleben NDL, van Santvoort HC, Fockens P, van Goor H, Bruno MJ and Besselink MG; Dutch Pancreatitis Study Group. Acute pancreatitis: recent advances through randomised trials. *Gut* 2017; 66: 2024-2032.
- [3] Hines OJ and Pandol SJ. Management of severe acute pancreatitis. *BMJ* 2019; 367: i6227.
- [4] Trikudanathan G, Wolbrink DRJ, van Santvoort HC, Mallery S, Freeman M and Besselink MG. Current concepts in severe acute and necrotizing pancreatitis: an evidence-based approach. *Gastroenterology* 2019; 156: 1994-2007, e1993.
- [5] Leppäniemi A, Tolonen M, Tarasconi A, Segovia-Lohse H, Gamberini E, Kirkpatrick AW, Ball CG, Parry N, Sartelli M, Wolbrink D, van Goor H, Baiocchi G, Ansaloni L, Biffi W, Coccolini F, Di Saverio S, Kluger Y, Moore E and Catena F. 2019 WSES guidelines for the management of severe acute pancreatitis. *World J Emerg Surg* 2019; 14: 27.
- [6] Forsmark CE, Vege SS and Wilcox CM. Acute pancreatitis. *N Engl J Med* 2016; 375: 1972-1981.
- [7] Xiang H, Zhang Q, Qi B, Tao X, Xia S, Song H, Qu J and Shang D. Chinese herbal medicines attenuate acute pancreatitis: pharmacological activities and mechanisms. *Front Pharmacol* 2017; 8: 216.
- [8] Sandler M, Weiss FU, Golchert J, Homuth G, van den Brandt C, Mahajan UM, Partecke LI, Döring P, Gukovsky I, Gukovskaya AS, Wagh PR, Lerch MM and Mayerle J. Cathepsin B-mediated activation of trypsinogen in endocytosing macrophages increases severity of pancreatitis in mice. *Gastroenterology* 2018; 154: 704-718, e710.
- [9] Parzych KR and Klionsky DJ. An overview of autophagy: morphology, mechanism, and regulation. *Antioxid Redox Signal* 2014; 20: 460-473.
- [10] Kuma A, Komatsu M and Mizushima N. Autophagy-monitoring and autophagy-deficient mice. *Autophagy* 2017; 13: 1619-1628.
- [11] Choi AM, Ryter SW and Levine B. Autophagy in human health and disease. *N Engl J Med* 2013; 368: 651-662.
- [12] Kroemer G. Autophagy: a druggable process that is deregulated in aging and human disease. *J Clin Invest* 2015; 125: 1-4.
- [13] Tao S and Drexler I. Targeting autophagy in innate immune cells: angel or demon during infection and vaccination? *Front Immunol* 2020; 11: 460.
- [14] Gukovskaya AS, Gukovsky I, Algül H and Habtezion A. Autophagy, inflammation, and immune dysfunction in the pathogenesis of pancreatitis. *Gastroenterology* 2017; 153: 1212-1226.
- [15] Gukovskaya AS and Gukovsky I. Autophagy and pancreatitis. *Am J Physiol Gastrointest Liver Physiol* 2012; 303: G993-G1003.
- [16] Iwahashi K, Hikita H, Makino Y, Shigekawa M, Ikezawa K, Yoshioka T, Kodama T, Sakamori R, Tatsumi T and Takehara T. Autophagy impairment in pancreatic acinar cells causes zymogen granule accumulation and pancreatitis. *Biochem Biophys Res Commun* 2018; 503: 2576-2582.
- [17] Dolai S, Liang T, Orabi AI, Holmyard D, Xie L, Greitzer-Antes D, Kang Y, Xie H, Javed TA, Lam PP, Rubin DC, Thorn P and Gaisano HY. Pancreatitis-induced depletion of syntaxin 2 promotes autophagy and increases basolateral exocytosis. *Gastroenterology* 2018; 154: 1805-1821, e1805.
- [18] Saluja A, Dudeja V, Dawra R and Sah RP. Early intra-acinar events in pathogenesis of pancreatitis. *Gastroenterology* 2019; 156: 1979-1993.
- [19] Iovanna JL. Autophagy induced during pancreatitis promotes KRAS-dependent transformation in the pancreas. *Front Oncol* 2016; 6: 226.
- [20] Peng WX, Koirala P and Mo YY. LncRNA-mediated regulation of cell signaling in cancer. *Oncogene* 2017; 36: 5661-5667.
- [21] Ferrè F, Colantoni A and Helmer-Citterich M. Revealing protein-lncRNA interaction. *Brief Bioinform* 2016; 17: 106-116.
- [22] Quinn JJ and Chang HY. Unique features of long non-coding RNA biogenesis and function. *Nat Rev Genet* 2016; 17: 47-62.
- [23] Paraskevopoulou MD and Hatzigeorgiou AG. Analyzing miRNA-lncRNA interactions. *Methods Mol Biol* 2016; 1402: 271-286.
- [24] Pan X, Zheng G and Gao C. LncRNA PVT1: a novel therapeutic target for cancers. *Clin Lab* 2018; 64: 655-662.
- [25] Alessio E, Buson L, Chemello F, Peggion C, Grespi F, Martini P, Massimino ML, Pacchioni B, Millino C, Romualdi C, Bertoli A, Scorrano L, Lanfranchi G and Cagnin S. Single cell analysis reveals the involvement of the long non-coding RNA Pvt1 in the modulation of muscle atrophy and mitochondrial network. *Nucleic Acids Res* 2019; 47: 1653-1670.

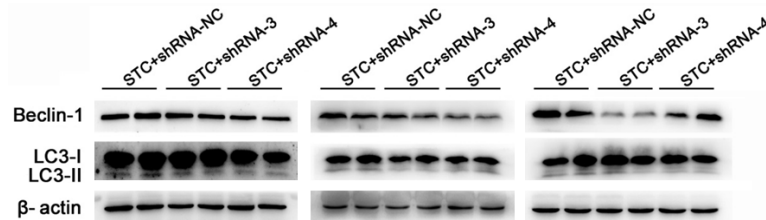
LncRNA-PVT1 aggravates severe acute pancreatitis

- [26] Huang F, Chen W, Peng J, Li Y, Zhuang Y, Zhu Z, Shao C, Yang W, Yao H and Zhang S. LncRNA PVT1 triggers Cyto-protective autophagy and promotes pancreatic ductal adenocarcinoma development via the miR-20a-5p/ULK1 Axis. *Mol Cancer* 2018; 17: 98.
- [27] Xiang H, Tao X, Xia S, Qu J, Song H, Liu J and Shang D. Emodin alleviates sodium taurocholate-induced pancreatic acinar cell injury via microRNA-30a-5p-mediated inhibition of high-temperature requirement A/transforming growth factor beta 1 inflammatory signaling. *Front Immunol* 2017; 8: 1488.
- [28] Lee PJ and Papachristou GI. New insights into acute pancreatitis. *Nat Rev Gastroenterol Hepatol* 2019; 16: 479-496.
- [29] Tanida I, Ueno T and Kominami E. LC3 and autophagy. *Methods Mol Biol* 2008; 445: 77-88.
- [30] Wu H, Wei M, Jiang X, Tan J, Xu W, Fan X, Zhang R, Ding C, Zhao F, Shao X, Zhang Z, Shi R, Zhang W and Wu G. LncRNA PVT1 promotes tumorigenesis of colorectal cancer by stabilizing miR-16-5p and interacting with the VEGFA/VEGFR1/AKT axis. *Mol Ther Nucleic Acids* 2020; 20: 438-450.
- [31] Quan X, Li X, Yin Z, Ren Y and Zhou B. p53/miR-30a-5p/SOX4 feedback loop mediates cellular proliferation, apoptosis, and migration of non-small-cell lung cancer. *J Cell Physiol* 2019; 234: 22884-22895.
- [32] Wu X, Jia R, Wang M, Chen S, Liu M, Zhu D, Zhao X, Yang Q, Wu Y, Yin Z, Zhang S, Huang J, Zhang L, Liu Y, Yu Y, Pan L, Tian B, Rehman MU, Chen X and Cheng A. Downregulation of microRNA-30a-5p contributes to the replication of duck enteritis virus by regulating Beclin-1-mediated autophagy. *Virology* 2019; 16: 144.
- [33] Yang X, Bai F, Xu Y, Chen Y and Chen L. Intensified beclin-1 mediated by low expression of mir-30a-5p promotes chemoresistance in human small cell lung cancer. *Cell Physiol Biochem* 2017; 43: 1126-1139.
- [34] Kumar S, Xie H, Shi H, Gao J, Juhlin CC, Björnhagen V, Höög A, Lee L, Larsson C and Lui WO. Merkel cell polyomavirus oncoproteins induce microRNAs that suppress multiple autophagy genes. *Int J Cancer* 2020; 146: 1652-1666.
- [35] Huang X, Han X, Huang Z, Yu M, Zhang Y, Fan Y, Xu B, Zhou K, Song L, Wang X, Lu C and Xia Y. Maternal pentachlorophenol exposure induces developmental toxicity mediated by autophagy on pregnancy mice. *Ecotoxicol Environ Saf* 2019; 169: 829-836.
- [36] Sun Y, Yao X, Zhang QJ, Zhu M, Liu ZP, Ci B, Xie Y, Carlson D, Rothermel BA, Sun Y, Levine B, Hill JA, Wolf SE, Minei JP and Zang QS. Beclin-1-dependent autophagy protects the heart during sepsis. *Circulation* 2018; 138: 2247-2262.
- [37] Kang R, Zeh HJ, Lotze MT and Tang D. The Beclin 1 network regulates autophagy and apoptosis. *Cell Death Differ* 2011; 18: 571-580.

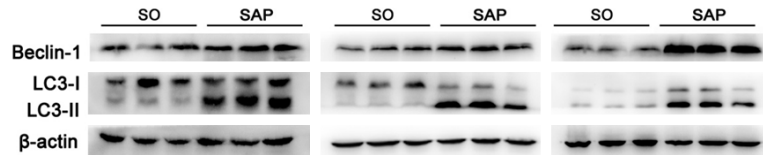
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Supplementary Figure 1. The additional Original western images of Figure 1D.



Supplementary Figure 2. The additional Original western images of Figure 3B.



Supplementary Figure 3. The additional Original western images of Figure 5E.