LONP1 ameliorates liver injury and improves gluconeogenesis dysfunction in acute-on-chronic liver failure

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Abstract

Background: Acute-on-chronic liver failure (ACLF) is a severe liver disease with complex pathogenesis. Clinical hypoglycemia is common in patients with ACLF and often predicts a worse prognosis. Accumulating evidence suggests that glucose metabolic disturbance, especially gluconeogenesis dysfunction, plays a critical role in the disease progression of ACLF. Lon protease-1 (LONP1) is a novel mediator of energy and glucose metabolism. However, whether gluconeogenesis is a potential mechanism through which LONP1 modulates ACLF remains unknown.

Methods: In this study, we collected liver tissues from ACLF patients, established an ACLF mouse model with carbon tetrachloride (CCl_4), lipopolysaccharide (LPS), and D-galactose (D-gal), and constructed an *in vitro* hypoxia and hyperammonemia-triggered hepatocyte injury model. LONP1 overexpression and knockdown adenovirus were used to assess the protective effect of LONP1 on liver injury and gluconeogenesis regulation. Liver histopathology, biochemical index, mitochondrial morphology, cell viability and apoptosis, and the expression and activity of key gluconeogenic enzymes were detected to explore the underlying protective mechanisms of LONP1 in ACLF.

Results: We found that LONP1 and the expressions of gluconeogenic enzymes were downregulated in clinical ACLF liver tissues. Furthermore, LONP1 overexpression remarkably attenuated liver injury, which was characterized by improved liver histo-pathological lesions and decreased serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) in ACLF mice. Moreover, mitochondrial morphology was improved upon overexpression of LONP1. Meanwhile, the expression and activity of the key gluconeogenic enzymes were restored by LONP1 overexpression. Similarly, the hepatoprotective effect was also observed in the hepatocyte injury model, as evidenced by improved cell viability, reduced cell apoptosis, and improved gluconeogenesis level and activity, while LONP1 knockdown worsened liver injury and gluconeogenesis disorders.

Conclusion: We demonstrated that gluconeogenesis dysfunction exists in ACLF, and LONP1 could ameliorate liver injury and improve gluconeogenic dysfunction, which would provide a promising therapeutic target for patients with ACLF.

Keywords: Acute-on-chronic liver failure; Lon protease-1; Gluconeogenesis disturbance; Mitochondrial dysfunction; Metabolism

Introduction

Acute-on-chronic liver failure (ACLF) is a life-threatening syndrome resulting from acute decompensation of chronic liver disease, manifesting with severe jaundice, encephalopathy, and coagulative disorders.[1] ACLF progresses rapidly and causes a high mortality rate with 28-day mortality ranging from 23.3% in ACLF grade 1 to 75.5% in ACLF grade 3 without effective treatment.[2] To date, the mechanisms of development and progression of ACLF have not been fully understood. As such, deciphering ACLF pathogenesis and preventing its progression are of great significance.

The liver acts as a pivotal organ for the dynamic regulation of liver glucose metabolism, maintaining normal glycemia by regulating the processes of glycogen synthesis, glycogenolysis, and gluconeogenesis.[3] Patients with cirrhosis are in accelerated starvation, where an overnight fast in cirrhosis has been equated with 72 h of fasting in healthy individuals.[4] As ACLF often develops in patients with liver cirrhosis suffering from acute insults,[5] an abnormal metabolic state might also exist in ACLF and is even more severe. In response to fasting, liver glycogen storage is depleted and gluconeogenesis becomes the primary source of glucose production to maintain normoglycemia.[6] ACLF is always accompanied by massive

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hepatocyte death,[7,8] contributing to impaired glycogen synthesis. In addition, gluconeogenesis, contributing to 90% of the circulating glucose during starvation, is also impaired in ACLF.[9] In these settings, patients with ACLF always experience episodes of clinical hypoglycemia with a surge in the morning. Of note, hypoglycemia is associated with increased mortality in patients with ACLF.[10] Therefore, there is a critical need to better understand the characteristics of glucose metabolism in ACLF and identify novel targets that could improve or even reverse this metabolic disturbance.

Lon protease-1 (LONP1), a protease of the mitochondrial matrix and a member of the highly conserved AAA+ superfamily, is responsible for protein quality control processes and maintaining mitochondrial morphology and function by degrading misfolded and damaged proteins.[11,12] Homozygous deficiency of LONP1 leads to early embryonic lethality.[13] Recent studies also show that LONP1 could protect against ischemia–reperfusion injury through preventing oxidative damage of proteins and lipids, and maintaining mitochondrial redox balance.[14] The mice in which LONP1 was ablated showed impaired mitochondrial ultrastructure and functions.[13] Apart from the important role in mitochondrial homeostasis,[15] LONP1 is also involved in energy and substance metabolism.[16] Specifically, LONP1 is associated with basal oxygen consumption and adenosine triphosphate (ATP) production and induces metabolic reprograming of the oxidative phosphorylation (OXPHOS) system by decreasing the flux from nicotinamide adenine dinucleotide (NADH) electrons while maintaining the flux through the flavin adenine dinucleotide (FAD)-dependent enzymes in melanoma cells.[17] Similarly, LONP1 deficiency retained the metabolic shift from anaerobic glycolysis to mitochondrial oxidative phosphorylation, which further impaired heart development.[18] In addition, LONP1 could also reverse insulin resistance and improved glucose tolerance in diabetic mice.[19] Despite increasing evidence highlighting the pivotal biological role of LONP1 and its regulatory effect in glucose metabolism, little is known regarding the regulatory effect of LONP1 in ACLF as well as the underlying mechanisms. Therefore, the present study aimed to investigate whether LONP1 plays a beneficial role in attenuating liver damage and gluconeogenesis disorder in ACLF.

Methods

Patients

Liver tissues in this study were obtained from ACLF patients who underwent liver transplantation for treatment in the Beijing You-An Hospital of Capital Medical University between January 2019 and June 2021. Normal liver tissues collected from trimmed scrap portions of donor livers were used as healthy control (HC). All the ACLF patients meet the Asia Pacific Association for the Study of the Liver (APASL) criteria.[20] Patients with diabetes or other disorders known to affect glucose metabolism were excluded. Importantly, human sample collection was in accordance with the *Declaration of Helsinki* and

approved by the Ethical Committee in Beijing You-An Hospital (No. [2019]012).

Mice and experimental design

C57BL/6 mice were purchased from Beijing Weitong Lihua Experimental Animal Ltd. Co. (Beijing, China) at 6–8 weeks of age. The mice were housed in a light (12 h light; 12 h dark cycle) and temperature (22°C) controlled environment with free access to food and water for a week before experimentation. The ACLF animal model was established as follows: mice were intraperitoneally injected with a mixture of carbon tetrachloride (Sigma-Aldrich, St. Louis, MO, USA; CCl_4/olive oil volume = 1:4) at a dose of 0.2 mL twice a week for 8 weeks and then three times a week for 4 weeks to generate a chronic liver injury model. Twenty-four hours after the last dose, lipopolysaccharide (LPS) (Sigma-Aldrich; 10 μg/kg) and D-galactose (Solaibao Biological Technology Co., Ltd., Beijing, China; D-gal; 800 mg/kg) were injected as acute insults.

Mice were randomly divided into four groups. Group 1, normal control ($n = 3$): mice received 0.2 mL of saline in water (twice a week for 8 weeks and thrice a week for 4 weeks); Group 2, ACLF ($n = 5$): ACLF models were constructed as described above; Group 3, ACLF + adenovirus-mediated overexpression of LONP1 (ad-LONP1) ($n = 3$): LONP1 overexpressing adenoviruses (5×10^8 pfu per mouse) were injected through the tail vein once a week for 2 weeks at the beginning of the 10th week; Group 4, ACLF + short interfering RNAs-mediated knockdown of LONP1 (siRNA-LONP1) ($n = 3$): LONP1 siRNA adenoviruses (5×10^8 pfu per mouse) were injected through the tail vein once a week for 2 weeks at the beginning of the 10th week. LONP1 adenoviruses were designed, validated, and synthesized by Shanghai Ji Kai Gene Technology Co. Ltd (Shanghai, China).

All animal experiments were approved by the Animal Experiment Committee at Capital Medical University and performed according to their guidelines (No. AEEI-2020-195).

Liver histopathology

The liver tissues were fixed with 4% paraformaldehyde, followed by dehydration, and then embedded in paraffin blocks and sectioned. Then, these sections were stained with hematoxylin and eosin (HE) and Masson's trichrome staining for assessment of histological injury and liver fibrosis.

Liver function assays

Serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) were tested with auto-biochemical analyzer (Olympus Company, Tokyo, Japan).

Western blot

Liver tissues and cells were lysed in radioimmunoprecipitation assay (RIPA) buffer (Solaibao Biological

Technology Co., Ltd.), with protease inhibitor cocktail (Biyuntian Biotechnology Co., Ltd., Shanghai, China) for 30 min on ice and centrifuged at 12,000 r/min for 30 min at 4°C. Supernatant was collected and the protein concentration was determined with a bicinchoninic acid (BCA) protein assay kit (Biyuntian Biotechnology Co., Ltd.). Proteins were denatured with 5× loading buffer (Pulilai Gene Technology Co., Ltd., Beijing, China) and boiled for 5 min in a 100°C metal bath. Next, proteins were separated on 8%–12% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) gels and transferred onto polyvinylidene fluoride (PVDF) membrane (Bio-Rad, CA, USA). This was followed by blocking for 1 h in 5% skim milk (Bo Maide Biotech Co., Ltd., Beijing, China), after which the membrane was incubated with different antibodies (Glyceraldehyde-3-phosphate dehydrogenase[GAPDH]: Cell Signaling Technology, Danvers, MA, USA; LONP1, phosphoenolpyruvate carboxykinase [PCK1], pyruvate carboxylase [PC]: Abcam, Cambridge, UK; glucose-6-phosphatase [G6Pase]: Abcam and Abcepta, Suzhou, China) overnight at 4°C. Blots were washed thrice in tris buffered saline with tween (TBST) and incubated with secondary antibodies (horseradish peroxidase [HRP]-conjugated secondary antibodies, Cell Signaling Technology) for 1 h at room temperature, and then washed thrice more with TBST. Finally, membranes were subjected to chemiluminescence and developed.

Quantitative real-time polymerase chain reaction (qRT-PCR)

The total RNA was extracted using TRIzol (Invitrogen, Waltham, MA, USA) and reverse transcribed into complementary DNA (cDNA) with PrimeScript RT reagent kit (TaKaRa Biotechnology, Beijing, China). Then PCR assays were performed using ABI ViiA 7 Real-Time PCR System (Life Technologies, Grand Island, NY, USA). Finally, the data were analyzed using $2^{-\Delta\Delta CT}$ method. The primers used in our study were synthesized by Sangon Biotechnology (Shanghai, China). The primer sequences are shown in Supplementary Table 1, http://links.lww.com/CM9/B847.

Transmission electron microscopy (TEM)

The mitochondrial morphology was examined by TEM (JEM-1200; Jeol Ltd., Tokyo, Japan). Liver tissues were fixed in 2.5% glutaraldehyde (Solaibao Biological Technology Co., Ltd.), and postfixed in 1% osmium tetroxide (Solaibao Biological Technology Co., Ltd.). Then, the tissues were dehydrated in a graded series of ethanol and embedded in paraffin. Finally, blocks were cut into 60–80 nm ultrathin sections, followed by staining with uranium acetate and lead citrate, and observed with TEM.

Cell culture

L02 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Gaithersburg, MD, USA) supplemented with 10% fetal bovine serum (FBS) (Gibco).

L02 cells were infected with LONP1 overexpression or knockdown lentiviruses for 48 h. Then, stably transfected cells were selected using 1 µg/mL puromycin for 2 weeks. Cells were divided into the following groups: Group 1, Control group; Group 2, Model group; Group 3, Model + LV-NC group; Group 4, Model + LV-LONP1 group; Group 5, Model + sh-NC group; and Group 6, Model + sh-LONP1 group. L02 cells from Control group were grown under standard cell culture conditions (37°C, 5% CO_2) for 48 h. L02 cells from Group 2 and stably transfected cells from Groups 3–6 were treated with 20×10^{-3} mol/L NH_4Cl (Sangon Biotechnology) in a 1% O_2 hypoxic environment for 48 h.

Cell viability assay

L02 cells in different groups were incubated in 96-well plates (Corning Inc., Corning, NY, USA) with 5×10^3 cells per well under the corresponding environment. After 48 h, cell counting Kit-8 (CCK8) reagent (AbMole BioScience, Inc., Houston, TX, USA) was added into each well for 2 h incubation at 37°C. The absorbance at 450 nm was measured using a microplate reader (Thermo Fisher Scientific Inc., Waltham, MA, USA).

Flow cytometry

Samples were stained with Annexin V-PE/7-AAD staining Kit (Biolegend, San Diego, CA, USA) and then analyzed by flow cytometry (FACSCalibur, BD, San Jose, CA, USA) to evaluate cell death and apoptosis.

Activities of gluconeogenic enzymes

The activities of G6Pase and phosphoenolpyruvate carboxykinase (PCK1) were determined with the G6Pase and PCK assay kits (Solaibao Biological Technology Co., Ltd.), following the manufacturers' instructions.

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.0 software (GraphPad, San Diego, CA, USA). Experimental data were expressed as means ± standard deviation. Data were conducted by unpaired Student's t-test and Mann–Whitney U test. $P < 0.05$ was regarded as an indication of significant statistical differences.

Results

LONP1 is significantly downregulated and gluconeogenesis disturbance exists in ACLF

Western blot was used to analyze the expression of LONP1 in liver tissues from eight patients with ACLF and four liver transplant donors (HCs). Supplementary Figure 1, http://links.lww.com/CM9/B847 shows the histopathological images of ACLF patients and Supplementary Table 2, http://links.lww.com/CM9/B847 lists the baseline characteristics of ACLF patients. Compared with HCs, LONP1 was obviously downregulated, with protein expression being lower in ACLF liver tissues ($P < 0.0001$). As gluconeogenesis is a primary source of glucose during

fasting, we analyzed the expression level and activity of enzymes modulating gluconeogenesis. We found that the protein expression and activities of rate-limited gluconeogenic enzymes were significantly decreased in ACLF [Figure 1A,B].

In vitro, we constructed a hypoxia and hyperammonemia-induced hepatocyte injury model. Consistent with the results from ACLF liver tissues, LONP1 protein ($P = 0.0229$) and mRNA ($P = 0.0013$) expression decreased in the injury setting [Figure 1C,D]. As expected, we also found a significant decrease in the expression of key gluconeogenic enzymes, including G6Pase, PCK1, and PC at both protein and RNA levels [Figure 1C,D]. In addition, the gluconeogenic enzymes with the lower activity were found in the hepatocyte injury model [Figure 1E]. Collectively, gluconeogenesis dysfunction existing in ACLF and LONP1 might play an important regulatory role in the disease progression in ACLF.
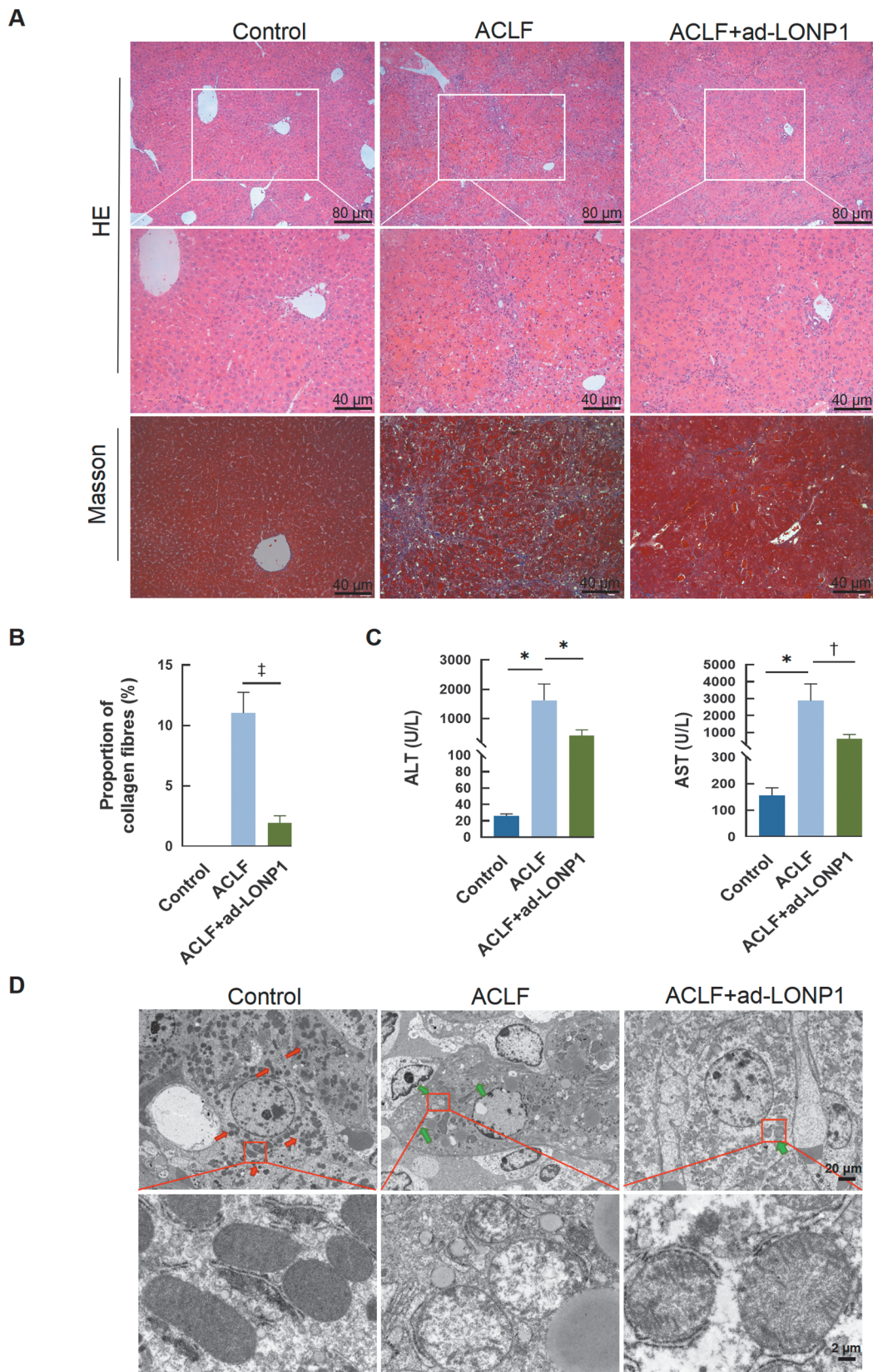
Overexpression of LONP1 ameliorated liver injury and improved gluconeogenesis dysfunction in vivo

To verify the above hypothesis, we established an ACLF mice model using CCl4, LPS, and D-gal [Supplementary Figure 2, http://links.lww.com/CM9/B847]. As shown in the Supplementary Figure 3, http://links.lww.com/CM9/B847, the liver of normal mouse was soft and smooth with sharp edges, while in the ACLF group, the liver was smaller and harder with a rough surface and gray nodules. HE and Masson staining showed that the structure of liver lobules was damaged, with obvious hepatocyte death, massive inflammatory cell infiltration, and substantial collagen fiber deposition in the ACLF models [Figure 2A]. All these histopathological lesions were typical trails of ACLF, which indicated that we successfully established ACLF mice models. Next, we upregulated the expression of LONP1 in the liver by tail vein injection of the LONP1 overexpression adenovirus. We observed improved ACLF-specific histopathological lesions upon LONP1 overexpression, as evidenced by a smaller area of hepatocyte death, decreased inflammatory cell infiltration, and reduced fibrotic levels [Figure 2A,B]. In line with the attenuated histopathological lesions, serum levels of ALT ($P = 0.0125$) and AST ($P = 0.0095$) were also reduced after LONP1 overexpression [Figure 2C]. LONP1 is the main mitochondrial matrix protease that plays a crucial role in maintaining mitochondrial morphology. We further examined the liver mitochondrial ultrastructural under TEM. The hepatocyte mitochondria
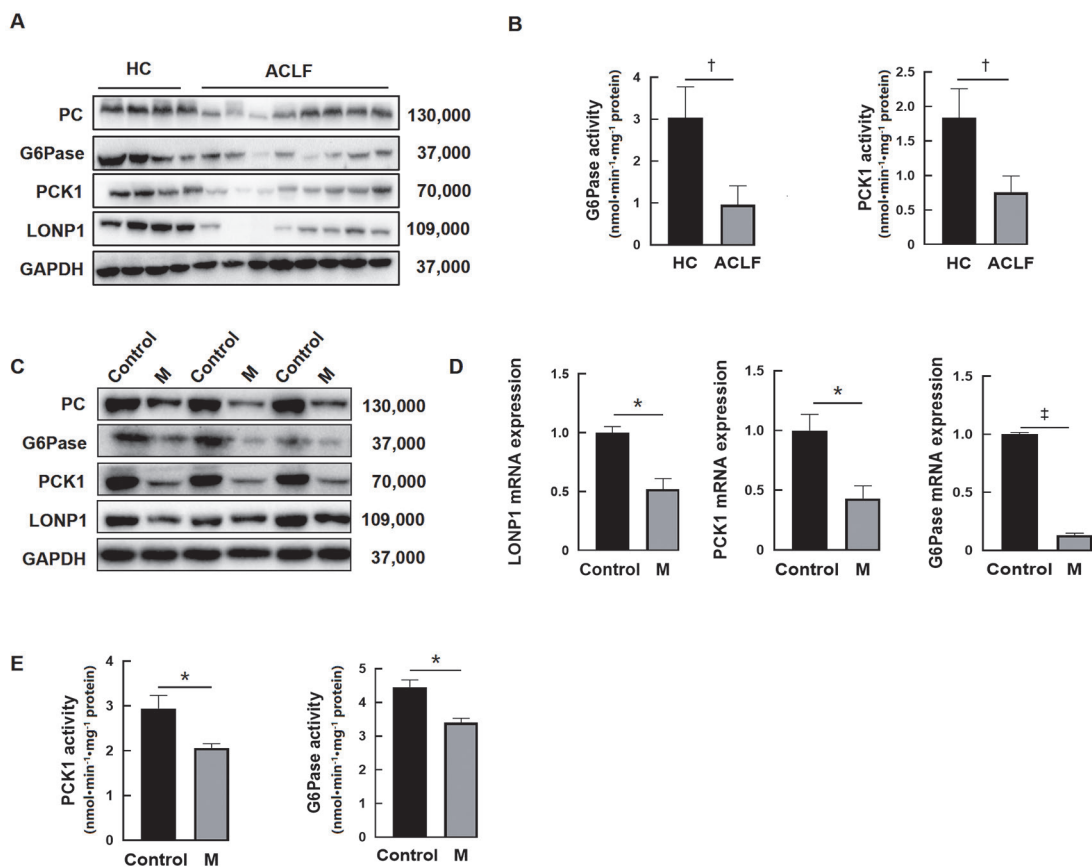
Figure 1: LONP1 is significantly downregulated and gluconeogenesis disturbance exists in ACLF. (A) Protein level of LONP1 and gluconeogenic enzymes (G6Pase, PCK1, PC) in liver tissue of HCs and ACLF patients (HC: $n = 4$, ACLF: $n = 8$). (B) Activity of G6Pase and PCK1 in liver tissue of HCs and ACLF patients. (C) Protein expression of LONP1 and gluconeogenic enzymes in hypoxia and hyperammonemia -induced hepatocyte injury model. (D) mRNA levels of LONP1, PCK1, and G6Pase were measured by qRT-PCR. (E) Activity of PCK1 and G6Pase in hepatocyte injury model. Data are expressed as mean ± standard deviation. *$P < 0.01$, †$P < 0.001$, ‡$P < 0.0001$. ACLF: Acute-on-chronic liver failure; G6Pase: Glucose-6-phosphatase; HC: Healthy control; LONP1: Lon protease-1; M: Hepatocyte injury model; PC: Pyruvate carboxykinase; PCK1: Phosphoenolpyruvate carboxykinase; qRT-PCR: Quantitative real-time polymerase chain reaction.

Figure 2: Overexpression of LONP1 ameliorated liver injury *in vivo*. (A) Representative images of histopathological features of control, ACLF, and ACLF + LONP1-overexpression mice. (B) Collagen fiber content was quantified in liver tissue using Masson trichrome staining. (C) Serum ALT and AST levels were decreased upon LONP1 overexpression. (D) Mitochondrial ultrastructure of liver tissues in different groups. Larger and swollen mitochondrial and disrupted cristae were seen in ACLF mice, while LONP1 overexpression improved these morphological changes. The red arrows indicate normal mitochondria; green arrows indicate larger and swollen mitochondria. $^*P <0.05$, $^†P <0.01$, $^‡P <0.001$. ACLF: Acute-on-chronic liver failure; ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; HE: Hematoxylin and eosin; LONP1: Lon protease-1.

from the control group displayed small spherical or rod-shaped structures with double membranes and densely packed lamellar cristae, while mitochondria of the ACLF mice displayed larger and swollen mitochondria, along with disrupted cristae. As expected, mitochondrial of the LONP1-overexpression group exhibited a more ordered crista and denser matrix [Figure 2D]. Moreover, the expression levels of G6Pase and PCK1 were significantly increased upon LONP1 overexpression [Figure 3A,B]. In addition, LONP1 overexpression enhanced the activity of these enzymes [Figure 3C]. Overall, these data implied that LONP1 may have a protective effect on liver injury and improve gluconeogenesis disorder.

Knockdown of LONP1 aggravated liver injury and gluconeogenesis dysfunction in vivo

Next, to further validate the above findings, we knocked down the expression of LONP by siRNA. Compared with the ACLF group, the LONP1 knockdown group exhibited more severe histopathologic lesions, manifesting with obvious hepatocyte death, massive inflammatory cell infiltration, and blue strip-like thick fibers that separated the liver lobules [Supplementary Figure 3, http://links.lww.com/CM9/B847, Figure 3D,E]. Convergently, liver damage evaluated by serum ALT ($P = 0.0010$) and AST ($P = 0.0003$) were also aggravated in the LONP1 knockdown group [Figure 3F]. Meanwhile, morphologic damage of mitochondrial was more evident in the LONP1 knockdown group, as evidenced by the decrease of the matrix density and the disintegration of cristae [Figure 3G]. In parallel with aggravated liver damage, the expression level and activity of G6Pase and PCK1 were much lower in the LONP1 knockdown group than that in the ACLF group [Figure 3A–C]. Together, these findings indicate that knockdown of LONP1 may drive liver damage and impair gluconeogenesis function.
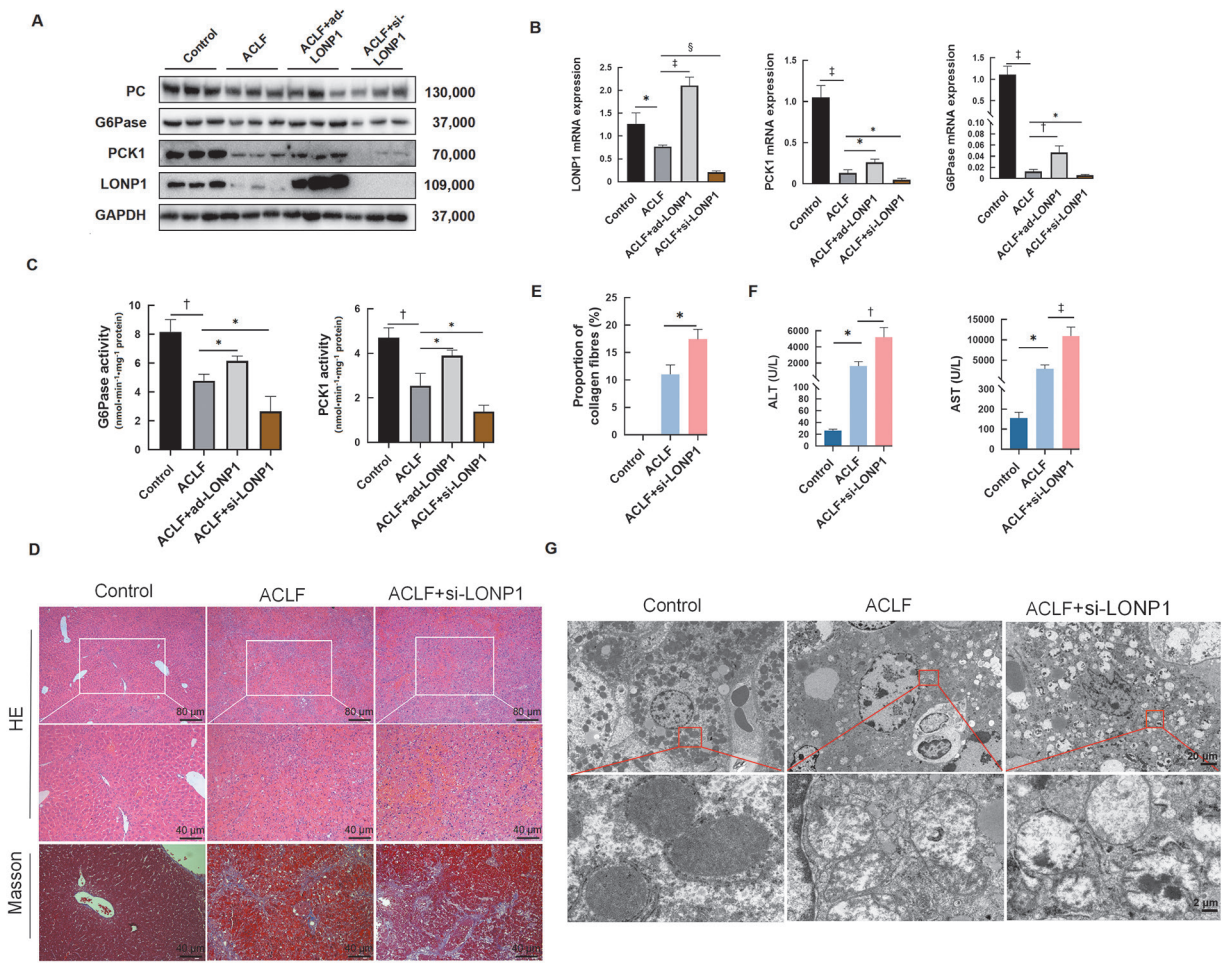
Figure 3: Knockdown of LONP1 aggravated liver injury and gluconeogenesis dysfunction *in vivo*. (A) Effect of LONP1 on the protein expression level of key enzymes of gluconeogenesis (G6Pase, PCK1, PC) in the liver of ACLF mice. (B) Effect of LONP1 on the RNA expression levels of G6Pase and PCK1 in the liver of ACLF mice. (C) Effect of LONP1 on the activity of G6Pase and PCK1 in the liver of ACLF mice. (D) The mice in LONP1 knockdown group demonstrated a more severe liver injury compared with ACLF mice, evidenced as obvious hepatocytes death, massive inflammatory cells infiltration, and blue strip-like thick fibers that separated the liver lobules. (E) Collagen fiber content was quantified in liver tissue using Masson trichrome staining. (F) Significantly increased serum ALT and AST suggested LONP1 knockdown aggravated liver damage. (G) Mitochondrial ultrastructure of liver tissues in ACLF mice with LONP1 knockdown. Red arrows indicate normal mitochondria; green arrows indicate larger and swollen mitochondria. $^*P <0.05$, $^†P <0.01$, $^‡P <0.001$, $^§P <0.0001$. ALT: Alanine aminotransferase; AST: Aspartate aminotransferase; ACLF: Acute-on-chronic liver failure; G6Pase: Glucose-6-phosphatase; LONP1: Lon protease-1; PC: Pyruvate carboxykinase; PCK1: Phosphoenolpyruvate carboxykinase; siRNA: Small interfering RNAs.

LONP1 protected against hypoxia and hyperammonemia-induced hepatotoxicity and improved gluconeogenic level in vitro

To explore the effects of LONP1 on cell injury, L02 cells were treated with LONP1-overexpressing and knockdown lentivirus, respectively. As shown in Figure 4A, LONP1 overexpression improved cell viability, while LONP1 knockdown had opposite effects. The morphological cell shape changes also follow this tendency [Figure 4B]. Under bright-field light, L02 cells became elongated and skinner in response to hypoxia and ammonia exposure. This morphological injury was improved by overexpression of LONP1, whereas obvious floating dead cells were observed in the LONP1 knockdown group [Figure 4B]. Furthermore, flow cytometry after Annexin PE/7-AAD staining was used to assay cell apoptosis and death. Overexpression of LONP1 significantly reduced apoptotic cells, whereas an increased ratio of apoptotic cells was observed upon LONP1 downregulation [Figure 4C]. To elucidate whether overexpression of LONP1 could improve gluconeogenesis levels that had been reduced by hypoxia and ammonia treatment, we tested the key enzymes of gluconeogenesis. As shown in Figure 4D, levels of G6Pase and PCK1 were increased by LONP1 overexpression. The RNA levels were also consistent with increased protein levels [Figure 4E]. In addition, the activity of gluconeogenic enzymes increased upon overexpression of LONP1 [Figure 4F]. Conversely, knockdown of LONP1 yielded a decrease in such enzymes in protein levels as well as RNA level and activity [Figure 4F,G,H]. Contrary to our expectation, the protein level of PC did not follow the tendency. Taken together, these results indicate that LONP1 is responsible for the increased hepatic gluconeogenesis.

Discussion

In the study, we verified the downregulation of LONP1 expression in patients with ACLF. In addition, our study confirmed that patients with ACLF suffer from gluconeogenesis disturbance, which is underpinned by downregulated protein expression and activity of key gluconeogenic enzymes. We provided *in vivo* and *in vitro* evidence that LONP1 overexpression attenuated liver damage, whereas LONP1 knockdown aggravated liver injury. Specially, our data reveal, for the first time, that LONP1 may be a potential target in ACLF, which confers a hepato-protective effect and improves gluconeogenesis dysfunction. Whether the beneficial effect of LONP1 on liver injury in ACLF through targeting gluconeogenesis function is unclear in this present study, which is replenishing in our follow-up experiments.

Gluconeogenesis is an important process for maintaining euglycemia during starvation. Whereas, massive hepatocyte death would lead to a dramatic reduction of glycogen storage and impairment of gluconeogenic capacity, which eventually leads to a significant reduction in blood glucose in ACLF.[21,22] Furthermore, severe hypoglycemia has been reported to be associated with adverse outcomes.[10] Clinically, our previous studies have confirmed that glucose metabolism dysfunction is present in ACLF, which is characterized by reduced carbohydrate oxidation.[23] Importantly, a carbohydrate-predominant late-evening snack treatment could not only decrease the risk of hypoglycemia but also improve liver function in patients with liver disease.[24] In this study, by detecting the protein expression and activities of key gluconeogenic enzymes, we confirmed that gluconeogenesis disturbance exists in ACLF patients.

LONP1 is widely expressed in human metabolic organs, including the liver and kidney.[25] It is known that LONP1 plays an important role in regulating the response of mitochondria to stress by removing oxidized proteins from the mitochondrial matrix.[26] During early stages of injury, LONP1 degraded damaged proteins to maintain mitochondrial homeostasis, while the expression of LONP1 is reduced under persistent and severe oxidative stress.[27] In the present study, we reported low expression of LONP1 in liver tissues of ACLF, because ACLF is a process of long-time oxidative injury. A decreased LONP1 expression was also observed in both ACLF mice models and hepatocyte injury models. Therefore, we hypothesized that LONP1 might participate in the development and progression of ACLF.

We further constructed LONP1 overexpression mice to explore the role of LONP1 in ACLF. Earlier studies showed that ATP dependent LONP1 involved in pathological and stressful conditions and reduced sensitivity to cardiac ischemia–reperfusion.[28] In the study, we found that LONP1 overexpression significantly alleviated liver damage characterized by improved histological lesions and decreased serum AST and ALT levels in ACLF mice, indicating the protective effects of LONP1 in liver injury. The *in vitro* data also demonstrated that the upregulation of LONP1 improved the morphology and viability of damaged hepatocytes. Accumulating evidence indicates that enhanced hepatocyte apoptosis is a typical molecular event of ACLF.[8,29] We observed that LONP1 could decrease hepatocytes' apoptosis and cell death. As a multifunctional protease, LONP1 participates in removing oxidatively damaged proteins from the mitochondria and thereby maintains mitochondrial homeostasis. Moreover, growing lines of studies have demonstrated that mitochondrial dysfunction is also a typical hallmark of ACLF.[30,31] Consistent with the above finds, in this study, we found the mitochondrial morphology was improved in response to LONP1 overexpression. Studies have shown that LONP1 deficiency could cause abnormal gluconeogenesis and hepatic insulin resistance,[19] indicating the important role of LONP1 in glucose metabolism. In addition, studies have shown that liver-specific loss of G6Pase would cause hyperlipidemia, lactic acidosis, and hepatomegaly with glycogen buildup and hepatic steatosis.[32] And mice with systemic deletion of PCK1 died within 3 days after birth.[33] In parallel with alleviated liver damage, we also found that LONP1 overexpression increased both G6Pase and PCK1 expression level and activity, indicating an improved gluconeogenesis dysfunction in ACLF. Our data indicated that in the presence of overexpressed LONP1 in ACLF, gluconeogenesis is upregulated and liver injury is ameliorated.
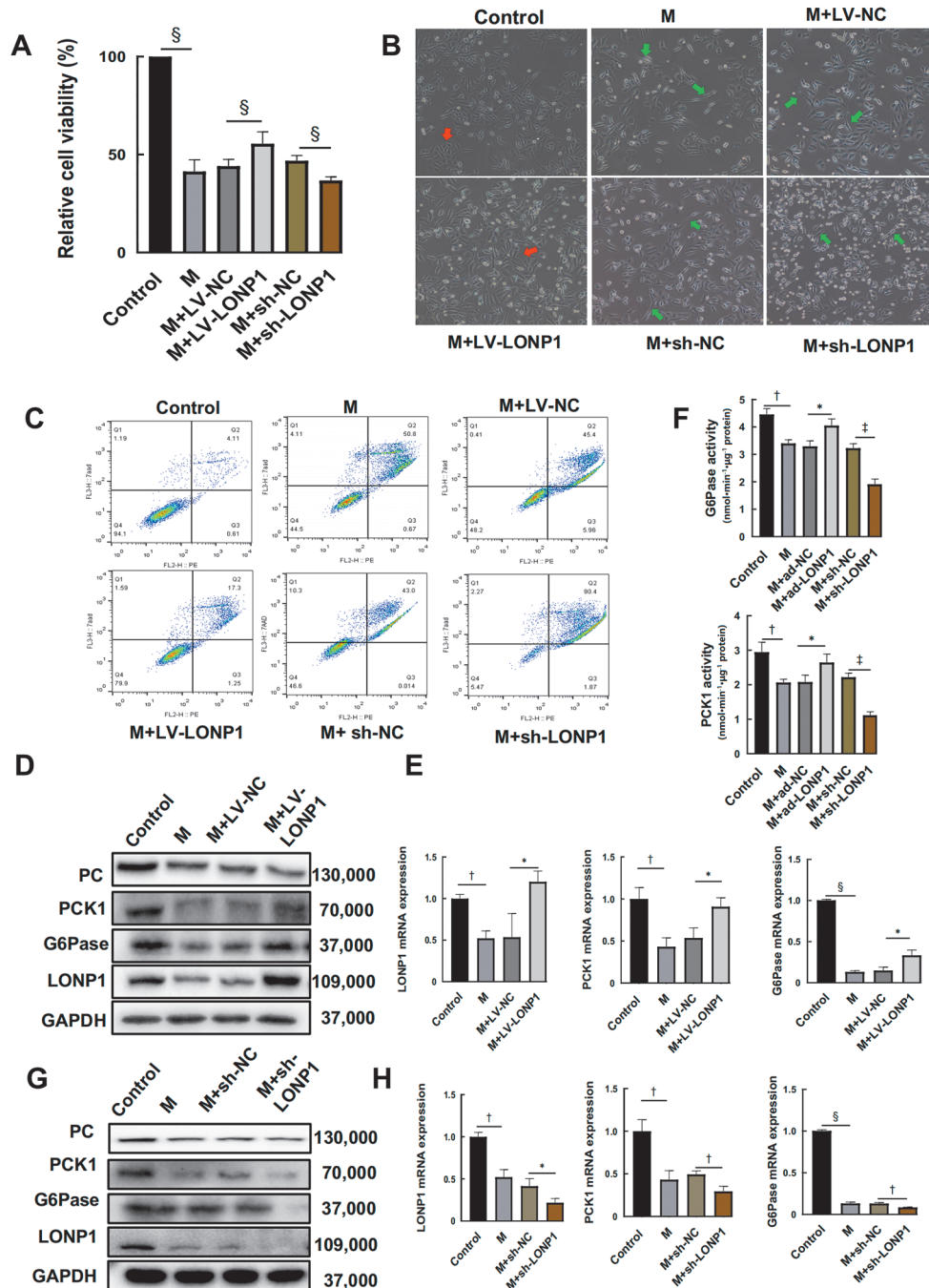
Figure 4: LONP1 protected against hepatotoxicity via improving gluconeogenesis *in vitro*. L02 cells were transfected with LONP1-overexpressing lentivirus and lentiviral shRNA, respectively. (A) CCK-8 assay was used to detect the viability of L02 cells in different groups. LONP1 overexpression improved cell viability, while LONP1 knockdown had opposite effects. (B) The cell morphology change was detected by microscope. Red arrows indicate normal mitochondria; green arrows indicate larger and swollen mitochondria. (C) Flow cytometry was performed to observe the cell apoptosis and cell death. (D) Changes in protein expression level of gluconeogenic enzymes (G6Pase, PCK1, PC) in LONP1-overexpressed cells. (E) The mRNA expression levels of LONP1, PCK1, and G6Pase in LONP1-overexpressed cells were measured by qRT-PCR. (F) Effect of LONP1 on the activity of G6Pase and PCK1 in hepatocyte injury model. (G) LONP1 knockdown decreased protein expression of gluconeogenic enzymes (G6Pase, PCK1). (H)The mRNA expression of LONP1, PCK1, and G6Pase was elevated in response to LONP1 knockdown. $^{*}P < 0.05$, $^{†}P < 0.01$, $^{‡}P < 0.001$, $^{§}P < 0.0001$. CCK-8: Cell Counting Kit-8; G6Pase: Glucose-6-phosphatase; LONP1: Lon protease-1; LV-LONP1: Lentivirus-mediated overexpression of LONP1; M: Hepatocyte injury model; PC: Pyruvate carboxykinase; PCK1: Phosphoenolpyruvate carboxykinase; qRT-PCR: Quantitative real-time polymerase chain reaction; shRNAs: Short hairpin RNAs.

To confirm the above findings, we next constructed LONP1 knockdown ACLF mice to further explore the regulatory effect of LONP1 in ACLF. As expected, more severe liver injury was observed in the LONP1 knockdown mice, as evidenced by the worsening of histological lesions and significantly increased levels of the biochemical index.

Furthermore, the level and activity of G6Pase and PCK1 were significantly downregulated. Unexpectedly, the level of PC failed to follow this tendency. Beyond serving as key enzymes of gluconeogenesis, PC also oxidizes pyruvate into oxaloacetate, providing one of the tricarboxylic acid (TCA) cycle intermediates.[34] The metabolomics data

from liver tissues from ACLF patients revealed enhanced glutamine anaplerosis.[35] LONP1 is known to degrade aconitase and glutaminase in the TCA cycle, contributing to changes in oxaloacetate (TCA cycle intermediates) levels and reduction in glutamine anaplerosis.[11] Thus, the expression of PC does not merely reflect the gluconeogenesis level but might be affected by both the gluconeogenesis process and TCA flux. This may partially explain why PC was not changed as expected in response to LONP1 overexpression or knockdown in ACLF.

Overall, in both *in vitro* and *in vivo* models, our data reveal that LONP1 may protect liver injury and improve gluconeogenesis dysfunction, whereas knockdown of LONP1 exacerbated the gluconeogenesis disturbance and aggravated liver damage in ACLF.

Despite several novel and important observations, our study has several limitations. Although LONP1 is protective against liver injury and gluconeogenesis dysfunction, the definite conclusions that LONP1 regulates gluconeogenesis function to mediate the hepatoprotective effect could not be drawn from the present results, because we only examined the association of LONP1 with gluconeogenesis function and liver injury, instead of establishing whether gluconeogenesis function is a potential mechanism through which LONP1 impacts liver injury. Whether knockdown of key gluconeogenic enzymes could aggravate liver function in LONP1 overexpression ACLF mice deserves further investigation. Additionally, our results demonstrated that LONP1 was downregulated in liver tissues of ACLF patients, but no significant association was identified between the expression of LONP1 and liver function, which may be due to the low sample number. We are considering collecting more clinical samples and exploring their linear correlation in later studies.

In summary, we find a protective effect of LONP1 on liver injury. Also, this study implies that targeting LONP1 may be a potential therapeutic target to improve gluconeogenesis disturbance and correct hypoglycemic symptoms in ACLF.

Funding

Conflicts of interest

None.

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