AKAP12 ameliorates liver injury via targeting PI3K/AKT/PCSK6 pathway

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Keywords: AKAP12, PCSK6, Inflammatory factor, Macrophage infiltration, Liver injury

ABSTRACT

A kinase anchor protein 12 (AKAP12) is a scaffold protein that is critical for cell structure maintenance and signal transduction. However, the role of AKAP12 in liver injury remains unclear. Here, we attempt to explore the potential contribution of AKAP12 in liver injury and elucidate its underlying molecular mechanism. We found that AKAP12 deletion in acute liver injury (ALI) activates the PI3K/AKT phosphorylation signaling pathway, induces the increased expression of PCSK6 and its downstream inflammation-related genes, and prompts macrophages to produce a large number of inflammatory factors. And knockdown of PCSK6 by in vitro siRNA assay reversed in liver injury AKAP12ΔΔmice, demonstrating that PCSK6 has an important role in ALI. Furthermore, we found that signal transducer and activator of transcription 3 (STAT3) and serine/threonine kinase Akt (AKT) were upregulated in AKAP12ΔΔmice of chronic liver injury. To sum up, our study here demonstrates that AKAP12 has a protective role in ALI and chronic liver fibrosis, at least in part through inhibition of the PI3K/AKT/PCSK6 pathway. Our findings provide a new potential treatment for liver injury with important clinical implications.

1. Introduction

A-kinase anchor protein 12 (AKAP12)/Src-suppressed C-kinase substrate (SSeCKS) is a member of the AKAP family and a component of the cytoskeleton structure [1]. AKAP12 maintains the stability of cells and tissues, and regulates cell proliferation and metastasis through activation of the phosphoinositide 3-kinase (PI3K)/protein kinase B (AKT) or protein kinase A (PKA)/protein kinase C (PKC) pathways [1–4].

AKAP12 is reportedly decreased, to varying degrees, and closely related to a poor prognosis and high recurrence rate in cancers of the prostate, breast, liver, and lung [5–8]. AKAP12 acts as a regulator of mitosis by anchoring key signaling proteins, such as PKA, PKC, and cyclins, thereby inhibiting the viability of tumor cells and inducing apoptosis via caspase-3 [4,9,10]. Notably, AKAP12 is reportedly downregulated in hepatocellular carcinoma (HCC) via promoter hypermethylation [11–14] and reduced in hepatic cirrhosis and precancerous lesions via upregulation of microRNA (miR)-183 and miR-186, which both target AKAP12. In addition, miR-1251-5p negatively regulates AKAP12 expression and plays a carcinogenic role in HCC [12]. Collectively, these findings indicate that AKAP12 acts a tumor suppressor in liver tumorigenesis.

Since AKAP12 protects against liver fibrosis and HCC progression [7, 15,16], it is reasonable to speculate that AKAP12 could influence acute liver failure and hepatocyte death [12,17]. Hence, the potential function and mechanism of AKAP12 in hepatocytes during acute liver injury (ALI) and chronic liver fibrosis were explored using hepatocyte-specific AKAP12 knockout (AKAP12Δhsp) mice. The results showed that disruption of AKAP12 in hepatocytes dramatically aggravated acute and chronic liver injury in a proprotein convertase subtilisin/kexin type 6 (PCSK6)-dependent manner.

PCSK6, also named PACE4, is a member of the encoding subtilisin-like proprotein convertase family [17,18]. The protease encoded by PCSK6 is expressed in many tissues, including the liver, intestine, and brain [18]. The PCSK6 gene is believed to affect the production of

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inflammatory factors and to play a role in tumor progression [19]. However, no study has yet analyzed the role of PCSK6 in ALI, and the relationship between AKAP12 and PCSK6 remains unclear.

ALI is the initial cause of most liver diseases. Elevated levels of alanine aminotransferase (ALT) and aspartate aminotransferase (AST) are established diagnostic markers of ALI [20,21]. ALI can cause inflammatory storms and even lead to death [22, 23]. Although the importance of preventing ALI and liver failure has been recognized for several decades, treatment to relieve symptoms is the most effective approach, as targeted therapies are lacking. At present, there is an urgent need for the identification of potential targets and development of ALI-targeted drugs. Hence, the aim of this study was to explore the potential function and mechanism of AKAP12 in hepatocytes during ALI and chronic liver fibrosis using AKAP12 hepΔ hepΔ mice to develop novel drug target for treatment.

2. Material and methods

2.1. Animals

The original AKAP12 knockout mouse was obtained from JiCuiYaoKang company in Nanjing, China. (C57BL/6 N genetic background). An alb-Cre tool mouse was provided by the Model Animal Research Center of Nanjing University in Nanjing, China. The breeding environment was standardized. (25 °C, 55% humidity, 12 h daylight and 12 h day/night light cycles). F1 generation mice, (AKAP12 systemic knockout mice) were crossed with alb-Cre tool mice to obtain F2 generation mice (AKAP12 hepΔ hepΔ). An equal volume of physiological saline via the abdominal cavity. After intraperitoneal administration of GalN/LPS, the survival rate of mice was measured (n = 8–12 per group). For the ALI model, carbon tetrachloride (CCl4) was administered by intraperitoneal injection at a dose of 4 ml/kg. The liver fibrosis model involved treatment with CCl4 mixed with olive oil (1:9 v/v), three times per week for 10 weeks. Lastly, siPCSK6 was injected intravenously (40 nmol per mouse).

2.3. Cell line construction and RNA interference

The mouse HCC cell line Hepa 1-6 was cultured in a 37 °C, 5% CO2 environment. The production of lentivirus for stable cell line construction was completed by GenePharma (Shanghai, China). For RNA interference, siRNA was purchased from GenePharma (Shanghai, China). Lipofectamine™ 3000 Transfection Reagent (L3000015 Thermo Fisher, Waltham, MA, USA) was used to transfect (si) RNA.

2.4. Immunohistochemical (IHC) analysis

Liver specimens were dewaxed, soaked in H2O2 for 20 min, washed three times with water, and incubated with an acid repair antigen for 5 min at 100 °C. Afterward, the specimens were washed three times, treated with blocking solution containing 1% bovine serum albumin for 10 min, then incubated with proteinase K solution for 10 min. Afterward, the specimens were washed three times, then incubated with TUNEL reaction solution for 1 h at 37 °C. The next day, the specimens were washed four times with phosphate-buffered saline (PBS) and probed with a secondary antibody for 30 min at 37 °C. After washing with PBS, the specimens were stained with 3,3′-diaminobenzidine (DAB) followed by hematoxylin for 10 min. Following dehydration, the specimens were coated with 50 μl of neutral resin.

2.5. Tissue protein extraction

Tissue samples (20 mg) were collected, frozen in liquid nitrogen, ground into powder, and placed in 1.5-ml centrifuge tubes. Following the addition of 1 ml of lysis buffer (P0013J Beyotime Institute of Biotechnology, Haimen, China), the samples were homogenized for 30 s at 1500×g, then centrifuged for 30 min. The supernatant was collected for further analysis.

2.6. Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) assay to assess apoptosis

After full deparaffinization and hydration, the samples were incubated with protease K solution for 10–30 min, washed with PBS, and incubated with TUNEL reaction solution for 1 h at 37 °C. Afterward, the samples were immersed in 0.3% H2O2 for 15 min, washed three times with PBS, reacted with 100 μl of horseradish peroxidase-conjugated streptavidin (diluted to 1:500 in PBS) for 30 min, then washed three times for 5 min with PBS. Finally, the samples were stained with DAB for 10 min, washed with PBS, and coated with neutral resin.

Abbreviations

| Abbreviation | Description |
|-------------|-------------|
| ALI | Acute liver injury |
| AKAP12 | A kinase anchor protein 12 |
| PCSK6/PACE4 | Proprotein convertase subtilisin/kexin type 6 |
| LPS | Lipopolysaccharide |
| GaIN | Galactosamine |
| CCL4 | Carbon tetrachloride |
| ROS | Reactive oxygen species |
| siRNA | small interference RNA |
| HCC | Hepatocellular carcinoma |
| AKAPS | A kinase anchoring protein |
| PI3K | phosphatidylinositol 3 kinase |
| AKT | serine/threonine kinase Akt/protein kinase B |
| ALT | Alanine aminotransferase |
| AST | Aspartate aminotransferase |
| WB | Western Blot |
| IL-6 | Interleukin-6 |
| IL-1β | Interleukin 1β |
| TNF-α | Tumor necrosis factor-α |
| TIMP | Tissue inhibitor of metalloproteinase |
| α-SMA | α-smooth muscle actin |
| Coll-1α | Collagen type 1 alpha 1 chain |
| NAC | N-acetyl cysteine |
| Bcl-2 | B-cell lymphoma-2 |
| Bax | Bcl-2 associated X protein |
| RASF | Rheumatoid arthritis synovial fibroblast |
| LSECs | Liver sinusoidal endothelial cells |
| STAT3 | Signal transducers and activators of transcription 3 |
| ERK | Extracellular regulated protein kinases |
| JNK | C-Jun N-terminal kinase |
2.7. Isolation of mouse primary hepatocytes

Mouse livers were perfused with eggtaic acid solution for 1–2 min at a flow rate of 5 mL/min, followed by pronase solution for 5 min and then infused with collagenase solution for 7 min. Afterward, the liver tissue was minced with 5 mL of pronase/collagenase solution and 1% DNase, then placed in a sterile petri dish. Following centrifugation at 180 rpm for 25 min, the tissue was filtered once through a 7-μm cell strainer and then centrifuged at 50 x g for 10 min at 4 °C. After aspiration of the supernatant, the cells were suspended in 120 μL of DNase I, then washed and centrifuged at 50 x g for 10 min at 4 °C.

2.8. mRNA level analysis

Mouse liver tissue was collected, added to Trizol, and stored at -80 °C until total RNA was extracted. The tissue was homogenized with ceramic beads, then RNA was extracted and reverse-transcribed into cDNA. The obtained cDNA was used for quantitative PCR (CFX96/384 TouchTM, Bio-Rad). Prior to use, each set of primers (Table 1) and RT-PCR conditions were extensively optimized to improve the efficiency of the reaction and avoid interference from primer dimers. The cycle parameters were as follows: 1 min at 65 °C, 45 cycles of 30 s at 94 °C, 1 min at 59 °C, and 1 min at 72 °C. Real-time quantitative PCR data were normalized to reference gene expression as the mean ± standard error. The two-tailed Student’s t-test was used to identify significant differences between two groups. Statistical analyses were conducted using GraphPad Prism 7.0 software (GraphPad Software, Inc., San Diego, CA, USA). Values are expressed as the mean ± standard error. The two-tailed Student’s t-test was used to identify significant differences between two groups. Comparisons were made using the two-tailed paired t-test. A probability (p) value of <0.05 was considered statistically significant.

| Table 1 | Primer sequence. |
|---------|------------------|
| Gene    | Sequences (5’-3’)                          |
| m-PCSK6 | Forward CAGGGCCGAGTGAAGCTCC                  |
|         | Reverse GAGGCAAGGAGCAGTTT                    |
| m-Fut8  | Forward GGTCCGCGGTTGGAATTG                   |
|         | Reverse TCAATGGGCGCCCCCTTGTTAT              |
| m-Kiif2a| Forward AAATGGAAGGTTGACGATCTG               |
|         | Reverse CAGTGCAGGGTGGGCATAGG                |
| m-Golgb1| Forward ATCGAGAGATGAGGAGTGA                 |
|         | Reverse GAGCTGATCTTGCCGTTG                  |
| m-Zfand6| Forward GTGCAAGAAACTAATACAGCAGCA            |
|         | Reverse GGTGCGTCTATTCATCAGTTG               |
| m-Sertad2| Forward AAAGAAGGAAAAGGAGTGTGAT              |
| m-Men1  | Forward TGAAATGGTCTGGCCTATGT                |
|         | Reverse AGGCTGATCTTGCCGTTG                  |
| m-Trim3 | Forward CTTGGGATTAATATCTGGCAC               |
| m-Dtx3  | Forward TGAGAAGACATTACATCAGG                 |
| m-Ap3m2 | Forward ACCAATGCTATCAGTCATATCAGGG           |
| m-β-actin| Forward CTTCTGACCCCTTGACG                   |
| m-Bcl2  | Forward CACACGAGGACCTGAGG                   |
| m-crc   | Forward GAGGTCAGGAGGATAGA                   |
| m-Prkcd | Forward CTCATCAGGAGAAGAGAGG               |
| m-AKAP12| Forward CTGACGAGGAGGAGGAGG                  |

2.9. Statistical analysis

Statistical analyses were conducted using GraphPad Prism 7.0 software (GraphPad Software, Inc., San Diego, CA, USA). Values are expressed as the mean ± standard error. The two-tailed Student’s t-test was used to identify significant differences between two groups. Comparisons were made using the two-tailed paired t-test. A probability (p) value of <0.05 was considered statistically significant.

3. Results

3.1. Knockdown of AKAP12 in hepatocytes exacerbated galactosamine (D-GalN)/lipopolysaccharide (LPS)-induced ALI

Hepatocyte-specific AKAP12 knockdown (AKAP12Δhep) mice were established by crossing alb-Cre mice with AKAP12 Δhep mice. AKAP12Δhep mice was validated by real-time quantitative polymerase chain reaction (qPCR) and IHC staining (Supplemental Figs. 1A–C). Subsequently, AKAP12 Δhep and AKAP12Δhep mice were subjected to a mouse model of ALI (low concentration group: 800 mg/kg GalN + 5 μg/kg LPS). As shown in Fig. 1A, mice were sacrificed at indicated time points and tissues and serum were collected. Another set of mice were used to assess survival after intraperitoneal injection of drugs to induce ALI (Fig. 1B). The results showed that liver congestion was more severe in the AKAP12Δhep group than the AKAP12 Δhep group at 4 h and 6 h (Fig. 1C). The liver of two groups at 4 h were stained with hematoxylin and eosin (H&E). As shown in Fig. 1D, AKAP12 deletion was associated with more severe liver damage. Glutathione (GSH) is an important regulator of intracellular metabolism. A decrease in serum GSH content is an established marker of oxygen free radical production and subsequent apoptosis [24,25]. Mouse serum was collected for detection of GSH and the liver function indicators ALT and AST. The results showed that serum GSH was significantly decreased, while serum ALT and AST were significantly increased in AKAP12Δhep group (Fig. 1E and F). The causes of liver congestion and necrosis were further investigated. The IHC results revealed that infiltration of F4/80-positive macrophages around the liver sinusoids was relatively increased in AKAP12Δhep mice (Fig. 1G). Furthermore, mRNA levels of the inflammatory cytokines interleukin (IL)-1β, IL-2, IL-6, and tumor necrosis factor (TNF)-α were significantly higher in the liver tissues of AKAP12Δhep mice as compared with AKAP12 Δhep mice (Fig. 1H). Finally, the expression levels of pro-apoptotic cleaved poly ADP-ribose polymerase (PARP) and cleaved caspase-3 were increased in AKAP12Δhep mice (Fig. 1I). Collectively, these results confirmed that liver damage was more severe in the AKAP12Δhep group and that knockout of AKAP12 promoted liver damage.

Another model of ALI (high concentration group: 800 mg/kg GalN + 20 μg/kg LPS) showed that liver damage was also more severe in the AKAP12Δhep group (Supplemental Figs. 2A–E). Together, these results demonstrate that AKAP12 plays an important role in maintaining the stability of cellular structures and resisting damage by ROS.

3.2. Hepatic AKAP12 deficiency aggravates carbon tetrachloride (CCL4)-induced ALI

To further confirm the role of hepatic AKAP12 in ALI, a mouse model of CCl4-induced ALI was constructed (Fig. 2A). Mice were sacrificed at 12 h after intraperitoneal administration of CCl4 (Fig. 2A). H&E staining of liver tissues demonstrated larger areas of hepatic necrosis in the AKAP12Δhep group as compared with the control group (Fig. 2B). TUNEL staining showed that the liver tissues of the AKAP12Δhep mice were enriched with apoptotic hepatocytes (Fig. 2C). In addition, serum GSH levels were significantly decreased in the AKAP12Δhep group mice as compared with the control group, indicating early activation of apoptosis (Fig. 2D). Meanwhile, serum levels of ALT and AST were increased in the AKAP12Δhep group mice (Fig. 2E). Finally, qPCR analysis showed that the mRNA expression of the inflammatory factors IL-1β, IL-6, and TNF-α were significantly increased in the liver tissues of AKAP12Δhep mice (Fig. 2F). Taken together, these results suggest that hepatocyte-specific knockout of AKAP12 triggers more severe liver injury during ALI.

3.3. Loss of AKAP12 results in more severe CCL4-induced liver fibrosis

The experimental results confirmed that AKAP12 knockout induces...
Fig. 1. AKAP12 knockout exacerbates LPS-GalN ALI. (A) Schematic diagram of the mouse experiments. (B) Statistical analysis of survival time (n = 5–8/group). (C) Mouse livers were collected at 2, 4, and 6 h after intraperitoneal injection of 800 mg/kg GalN + 5 μg/kg LPS. (D) H&E staining. (E) Serum levels of GSH (n = 5/group). (F) AST and ALT levels as markers of liver function (n = 5/group). (G) IHC staining. (H) mRNA expression levels of inflammatory factors in liver tissue (n = 3/group) (*p < 0.05, **p < 0.01, ****p < 0.0001). (I) Western blot (WB) analysis of the apoptotic proteins PARP, cleaved PARP, caspase-3, and cleaved caspase-3 (n = 3/group) (*p < 0.05, **p < 0.01, ****p < 0.0001).
more severe liver injury. To further explore the role of AKAP12 in liver fibrosis, AKAP12Δhep and AKAP12fl/fl mice were treated with CCl₄ for 10 weeks. The staining results (Masson, Sirius red, and IHC) demonstrated a dramatically increased level of liver fibrosis in the AKAP12Δhep group as compared with the AKAP12fl/fl group (Fig. 3A–C). Subsequently, markers of early fibrosis and inflammation were detected. The expression levels of α-smooth muscle actin (α-SMA) and tissue inhibitor of metalloproteinase 1 were significantly higher in the liver tissues of the AKAP12Δhep group than the control group at weeks 2, 4, 6, 8, and 10 (Fig. 3D). The expression levels of inflammatory factors were significantly increased in the liver tissues of AKAP12Δhep mice (Fig. 3E). WB analysis of α-SMA protein levels confirmed that liver fibrosis was more severe in the AKAP12Δhep group than the AKAP12Δhep group at 2, 6, and 10 weeks (Fig. 3F). To further investigate the mechanism by which AKAP12 deficiency exacerbates liver fibrosis, four pathways closely associated with liver fibrosis were examined. The results showed that signal transducer and activator of transcription 3 (STAT3) and serine/threonine kinase Akt (AKT) were phosphorylated (activated) in the liver tissues of the AKAP12Δhep group (Fig. 3G). However, there was no significant difference in the expression levels of phosphorylated extracellular regulated protein kinase (ERK) and c-Jun N-terminal kinase (JNK) between the two groups (Fig. 3G). Loss of AKAP12 activates the p-STAT3 and p-AKT pathways and aggravates liver fibrosis.

(A–C) Liver samples were collected from mice treated with CCl₄ at 0,
2, 4, 6, 8, and 10 weeks and stained (Masson trichrome, Sirius red, and IHC). (D) Detection of fibrosis-related genes (n = 3/group). (E) Detection of inflammatory factors (n = 3/group). (F) WB analysis of α-SMA protein expression at 2, 6, and 10 weeks (n = 3/group). (G) Phosphorylation pathway detection (n = 3/group) (*p < 0.05, **p < 0.01, ***p < 0.001, n.s. p > 0.05).

3.4. Limited role of antioxidants in GalN/LPS-induced ALI of AKAP12Δhep mice

The use of antioxidants has potential to alleviate acute liver damage. Therefore, we tested if antioxidant N-acetylcyesteine (NAC) could reverse exacerbated ALI in AKAP12Δhep mice. NAC were added to the drinking water of mice 24 h before the experiment, followed by intraperitoneal injection of NAC (150 mg/kg) at 30 min post GalN/LPS dosing. After NAC treatment, H&E staining, liver function and GSH results demonstrated that liver damage was alleviated in both AKAP12Δhep and AKAP12fl/fl mice.

Fig. 3. Knockout of AKAP12 results in more severe CCL4-induced liver fibrosis.
AKAP12\textsubscript{\textDelta hep} mice (Fig. 4A–C). However, the expression levels of liver inflammatory factors remained significantly elevated in the AKAP12\textsubscript{\textDelta hep} group as compared with the AKAP12 \textsubscript{\textbeta/\beta} group (Fig. 4D). The IHC staining results demonstrated that the liver tissues of AKAP12\textsubscript{\textDelta hep} mice had greater infiltration of macrophages (Fig. 4E). Moreover, the expression levels of apoptotic proteins were significantly elevated in AKAP12\textsubscript{\textDelta hep} mice (Fig. 4F).

NAC treatment had no significant effect on high concentration-GalN/ LPS and CCL\textsubscript{4} induced ALI. Although liver congestion and necrosis could be relieved, the levels of inflammatory factors in serum were still significantly increased (Supplemental Figs. 3A–D and Supplemental Figs. 4A–E). The results suggested that in addition to oxidative stress, there are other factors that exacerbate liver injury. Taken together, these results revealed that NAC has limited protection against AKAP12 knockdown-induced ALI and did not effectively reduce the production and infiltration of inflammatory factors, indicating that factors other than ROS trigger liver injury in AKAP12\textsubscript{\textDelta hep} mice.

3.5. PCSK6 is a potential target of AKAP12

RNA sequencing was performed to identify targets of AKAP12. Whole transcriptome sequencing of liver tissues from untreated AKAP12\textsubscript{\textDelta hep} and AKAP12 \textsubscript{\textbeta/\beta} mice (n = 3/group) identified 1275 genes that were upregulated and 987 that were downregulated. The results are presented in a heat map (Fig. 5A) and volcano plot (Fig. 5B). qPCR was performed to verify the changes of the top 10 upregulated genes (Table 2 and Fig. 5C). PCSK6 was the most significantly elevated gene in the liver of AKAP12\textsubscript{\textDelta hep} mice and is strongly associated with inflammation.

Fig. 4. NAC failed to relieve ALI. (A) Liver tissues were collected from mice with ALI within 4 h of NAC administration (150 mg/kg) for H&E and TUNEL staining (n = 5–12/group). (B) Serum GSH content (n = 5/group). (C) Serum ALT and AST levels (n = 5/group). (D) mRNA expression levels of inflammatory factors in liver tissues (n = 3/group). (E) IHC staining of F4/80. (F) Expression levels of apoptotic proteins in liver tissues (n = 3/group) (*p < 0.05, **p < 0.01, n.s. p > 0.05).
Fig. 5. PCSK6 expression was significantly increased in the liver tissues of AKAP12Δhep mice. (A) Whole transcriptome sequencing of liver tissues of untreated AKAP12Δhep and AKAP12fl/fl mice (n = 3/group). (B) A volcano map. (C) Verification of mRNA levels (n = 3/group). (D) IHC detection of PCSK6 expression. (E) WB detection of PCSK6 protein expression (F) Protein expression of AKAP12 and PCSK6 in primary hepatocytes of AKAP12Δhep mice (n = 2-3/group). (G) mRNA expression of PCSK6 downstream genes (n = 3/group) (*p < 0.05, **p < 0.01, n.s. p > 0.05).
autoimmune disease [26,27] (Fig. 5C). PCSK6, which is expressed in various tissues, including the liver, intestine, and brain [28,29], is reported to affect the production of inflammatory factors and to play a role in tumor progression. Specific knockout of PCSK6 in hepatocytes triggered increased PCSK6 expression (Fig. 5D and E). Notably, NAC had no effect on the expression levels of AKT1, PI3K, and AKT (Fig. 6A). To further determine the interaction between PCSK6 and AKAP12, a stable knockdown AKAP12 cell line was constructed using lentiviral transfection. Knockdown of AKAP12 was verified by qPCR and WB analyses (Fig. 6B and C). Consistent with the in vivo data, knockdown of AKAP12 increased PCSK6 protein expression, paralleled by increased phosphorylation of PI3K and AKT (Fig. 6C). Subsequently, simultaneous silencing of PCSK6 in AKAP12-silenced cell lines had no effect on the expression of phosphorylated PI3K/AKT (Fig. 6D). These results confirm that expression of PCSK6 has no effect on the PI3K/AKT pathway in AKAP12-silenced cell lines. Meanwhile, PCSK6 silencing reduced the expression levels of inflammatory genes in AKAP12-silenced cell lines (Fig. 6G), which strongly imply that activation of the PI3K/AKT pathway promotes the expression of PCSK6 (Fig. 6G), which revealed that AKAP12 overexpression inhibited the PI3K/AKT pathway (Fig. 6F). To determine whether AKAP12 regulates PCSK6 expression through the PI3K/AKT pathway, changes in PCSK6 expression in response to an AKT inhibitor were investigated. Pilot experiments have shown that the inhibitor works best at 5 min after Epidermal Growth Factor Receptor stimulation. The results demonstrated that the AKT inhibitor simultaneously inhibited the expression of p-AKT and PCSK6, which strongly imply that activation of the PI3K/AKT pathway promotes the expression of PCSK6 (Fig. 6G). To explore the function of PCSK6 in vivo, small interfering RNA targeting PCSK6 were synthesized to verify the knockdown effects at the mRNA and protein levels (Fig. 6H and I). Consistently, the mRNA levels of pro-inflammatory genes in the liver were significantly reduced (Fig. 6G). These results further confirmed that AKAP12 influences PCSK6 expression via the PI3K/AKT pathway.

### Table 2

**Differential gene list.**

| Gene   | F1          | F2          | F3          | C1          | C2          | C3          | FoldChange | C_vs_F_Pvalue |
|--------|-------------|-------------|-------------|-------------|-------------|-------------|------------|---------------|
| PCSK6  | 0           | 0           | 0           | 12.2        | 11.5        | 11.6        | 11.75      | 4.54E-132     |
| Eif4g3 | 0           | 0           | 0           | 10.1        | 10.6        | 8.3         | 9.67       | 2.87E-34      |
| Fut8   | 0           | 0           | 0           | 8.9         | 8.71        | 9.45        | 8.72       | 5.73E-36      |
| Kif21a | 0           | 0           | 0           | 5.49        | 7.75        | 9.33        | 7.52       | 2.32E-09      |
| Prkcd  | 0           | 0           | 0           | 6.38        | 7.14        | 7.28        | 7          | 4.03E-29      |
| Rspo1a | 0           | 0           | 0           | 7.1         | 6.03        | 7.22        | 6.78       | 3.92E-23      |
| Golgb1 | 0           | 0           | 0           | 7.24        | 6.34        | 6.54        | 6.71       | 6.38E-24      |
| Zfand6 | 0.33        | 5.1         | 9.54        | 9.34        | 9.24        | 6.55        | 6.35E-08    |

3.6. AKAP12 knockout activated the PI3K/AKT pathway and promoted PCSK6 expression, thereby exacerbating ALI

Various cell lines (PCSK6 silenced, AKAP12 silenced, AKAP12 overexpressed, and PCKS6/AKAP12 silenced) were constructed to explore the mechanism underlying regulation between AKAP12 and PCSK6. Silencing of PCSK6 reduced PCSK6 expression, but had no effect on the expression levels of AKAP12, PI3K, and AKT (Fig. 6A). To further explore the interaction between PCSK6 and AKAP12, a stable knockdown AKAP12 cell line was constructed using lentiviral transfection. Knockdown of AKAP12 was verified by qPCR and WB analyses (Fig. 6B and C). Consistent with the in vivo data, knockdown of AKAP12 reported that increased PCSK6 protein expression (Fig. 5F). In addition, several studies have confirmed that knockout of hepatocyte β-catenin increased inflammation-related genes [30,31]. In the present study, the liver tissues of 

| Gene   | F1          | F2          | F3          | C1          | C2          | C3          | FoldChange | C_vs_F_Pvalue |
|--------|-------------|-------------|-------------|-------------|-------------|-------------|------------|---------------|
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| Rspo1a | 0           | 0           | 0           | 7.1         | 6.03        | 7.22        | 6.78       | 3.92E-23      |
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| Gene   | F1          | F2          | F3          | C1          | C2          | C3          | FoldChange | C_vs_F_Pvalue |
|--------|-------------|-------------|-------------|-------------|-------------|-------------|------------|---------------|
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| Rspo1a | 0           | 0           | 0           | 7.1         | 6.03        | 7.22        | 6.78       | 3.92E-23      |
| Golgb1 | 0           | 0           | 0           | 7.24        | 6.34        | 6.54        | 6.71       | 6.38E-24      |
| Zfand6 | 0.33        | 5.1         | 9.54        | 9.34        | 9.24        | 6.55        | 6.35E-08    |

**Table 2**

### Differential gene list.

| Gene   | F1          | F2          | F3          | C1          | C2          | C3          | FoldChange | C_vs_F_Pvalue |
|--------|-------------|-------------|-------------|-------------|-------------|-------------|------------|---------------|
| PCSK6  | 0           | 0           | 0           | 12.2        | 11.5        | 11.6        | 11.75      | 4.54E-132     |
| Eif4g3 | 0           | 0           | 0           | 10.1        | 10.6        | 8.3         | 9.67       | 2.87E-34      |
| Fut8   | 0           | 0           | 0           | 8.9         | 8.71        | 9.45        | 8.72       | 5.73E-36      |
| Kif21a | 0           | 0           | 0           | 5.49        | 7.75        | 9.33        | 7.52       | 2.32E-09      |
| Prkcd  | 0           | 0           | 0           | 6.38        | 7.14        | 7.28        | 7          | 4.03E-29      |
| Rspo1a | 0           | 0           | 0           | 7.1         | 6.03        | 7.22        | 6.78       | 3.92E-23      |
| Golgb1 | 0           | 0           | 0           | 7.24        | 6.34        | 6.54        | 6.71       | 6.38E-24      |
| Zfand6 | 0.33        | 5.1         | 9.54        | 9.34        | 9.24        | 6.55        | 6.35E-08    |
were intraperitoneally injected with GalN/LPS (5 μg/kg) to induce ALI and treated with siPCSK6. Silencing of PCSK6 improved liver congestion and swelling in AKAP12Δhep mice (Supplementary Fig. 5A). The liver-to-body weight ratio and serum ALT and AST levels after in vivo siPCSK6 treatment were significantly decreased (Supplementary Figs. 5B and C). PCSK6 mRNA levels were significantly elevated in the livers of AKAP12Δhep mice with ALI (Fig. 7A). siPCSK6 was used to inhibit PCSK6 expression, which was validated by measuring mRNA levels (Fig. 7B). H&E staining showed that liver congestion and necrosis were significantly relieved in the siPCSK6 group (Fig. 7C). The expression of inflammatory factors and macrophage infiltration in the liver were significantly reduced following PCSK6 knockdown, as demonstrated by

Fig. 6. AKAP12 influences PCSK6 expression via the PI3K/AKT pathway. (A) Validation of the effect of PCS6 on the PI3K/AKT pathway and AKAP12. (B) AKAP12 mRNA expression. (C) Expression of PCSK6, PI3K, and AKT after AKAP12 silencing. (D) Simultaneous silencing of PCSK6 in AKAP12-silenced cell lines to detect PI3K/AKT expression. (E) Expression of inflammatory factors (n = 3/group). (F) Overexpression of AKAP12 verifies the expression of PI3K/AKT and PCSK6. (G) Detection of protein level expression following the use of AKT phosphorylation inhibitors. (H and I) In vivo siPCSK6 treatment and detection of PCSK6 mRNA and protein expression in liver tissue. (J) Detection of inflammatory factors (*p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001, n.s. p > 0.05).
IHC staining (Fig. 7D). In mice treated with siPCSK6, there was a significant decrease in the expression of MMP9, a downstream target of PCSK6, and as well as several pro-inflammatory genes (Fig. 7E and F). Further validation at the protein level found significant reductions in the expression levels of apoptotic proteins (cleaved PARP, cleaved caspase-3, cleaved caspase-9) in addition to TNFα (Fig. 7G). Collectively, these results confirmed that high PCSK6 expression promoted inflammatory factor expression and macrophage infiltration, leading to more severe liver injury in the ALI model.

4. Discussion

The present study confirm that activation of the PI3K/AKT pathway in the livers of AKAP12Δhep mice triggers increased expression of PCSK6, which promotes macrophage infiltration and inflammatory factor expression, thereby exacerbating liver injury.

Recent studies of endothelial cells, although these cells only account for 5%–18% of liver cells, whereas hepatocytes account for about 80% [7,15,34]. The role of AKAP12 in hepatocytes remains unclear, thus further studies are warranted. The results of the present study showed that AKAP12 deficiency promotes acute and chronic liver injury, although the specific mechanism underlying the development of liver fibrosis has yet to be elucidated. Several pathways closely related to liver fibrosis were identified and the phosphorylation levels of STAT3 and AKT were significantly increased in the liver tissues of AKAP12Δhep mice. Numerous studies have reported that STAT3 acts as an extracellular signaling molecule essential to chronic inflammation and promotes tumorigenesis and tumor-associated inflammation [35–37]. The PI3K/AKT signaling pathway plays an important regulatory role in the progression of liver fibrosis [37]. In addition, activation of the ERK and JNK pathways is significantly positively correlated with activation of hepatic stellate cells, which exacerbates liver fibrosis [37–40]. AKAP12 knockout in hepatocytes activated PI3K-AKT pathway while had little or no effect on the JNK and ERK pathways. In addition, AKAP12 knockout promoted the expression of MMP2/9 and inflammatory factors via activation of the PI3K/AKT/PCSK6 signaling pathway, which

Fig. 7. In vivo PCSK6 effectively relieved ALI. (A) PCSK6 mRNA expression after AKAP12 knockout (n = 3/group). (B) Verification of the efficiency of siPCSK6 (n = 3/group). (C) H&E staining. (D) IHC staining. (E) Expression of inflammatory factors in liver tissues (n = 3/group). (F) Detection of genes related to PCSK6 (n = 3/group). (G) WB analysis (n = 3/group) (**p < 0.01, ***p < 0.001, n.s. p > 0.05).
aggravates the progression of liver fibrosis [41,42]. Therefore, enhanced expression of PCSK6 might promote infiltration of inflammatory factors, thereby further aggravating liver fibrosis. The results of this study showed that AKAP12 has an inhibitory effect on the expression of PCSK6. In AKAP12-KO mice, the lack of AKAP12 during the development of ALI led to activation of the PI3K/AKT pathway and increased expression of PCSK6. Knockout of AKAP12 compromised the structural stability of hepatocytes and increased sensitivity to ROS [3,43]. Knockout of AKAP12 accelerated PCSK6-induced inflammation, oxidative stress, and apoptosis, leading to liver damage. Abnormal expression of PCSK6 is reported to promote activation of the vascular endothelium and subsequent vascular regeneration [44,45]. Previous studies have demonstrated that PCSK6 is abnormally expressed in several solid tumors (gastric cancer, breast cancer, melanoma, etc.) [17,46,47]. PCSK6 has also been shown to enhance proliferation, migration, invasion, and inflammation of rheumatoid arthritis fibroblast-like synovial (RASF) cells via activation of the NF-κB, STAT3, and ERK1/2 signaling pathways [17,47-50]. In rheumatoid arthritis, PCSK6 is reportedly significantly increased in RASF cells [51,52]. PCSK6 stimulates secretion of the inflammatory cytokines IL-1a, IL-1b, and IL-6 in RASF cells, and increases the expression of genes related to angiogenesis, hypoxia, proliferation, and inflammation [51-54]. In addition, PCSK6 is a secreted protein, which is transported from intracellular to extracellular [17,18]. The deletion of AKAP12 may directly affect the secretion of PCSK6, but no research has been reported and more experiments are still needed. Also, multiple pathways may be involved in the aggravation of ALI caused by AKAP12 deletion. This paper only explores the PI3K/AKT signaling pathway, which is limited. Increased expression of PCSK6 stimulates macrophages to increase production of inflammatory factors [28,29]. In PCSK6-aggravated liver damage, activated macrophages promote the secretion of various inflammatory cytokines, thereby enhancing the inflammatory response [52]. In the present study, NAC only partially alleviated liver damage, whereas inhibition of PCSK6 expression effectively alleviated ALI. Moreover, hepatocyte-specific knockout of AKAP12 increased apoptosis both in vivo and in vitro, which was reversed by PCSK6 knockdown. Taken together, these results demonstrate that aggravation of ALI is due to activation of PI3K/AKT and that AKAP12 knockout enhanced PCSK6 expression, highlighting AKAP12 and PCSK6 as potential drug targets for treatment of ALI.

**Ethics statements**

Our animal experiments are supported and supervised by the Animal Ethics Committee of Shanghai Tenth People’s Hospital (ID Number: SHDSYY-2021-3031).

**Declaration of competing interest**

The authors have declared that no competing interest exists.

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**Appendix A. Supplementary data**

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2022.102328.
