1 **Deficiency of gluconeogenic enzyme PCK1 promotes murine NASH** 2 **progression and fibrosis by activation PI3K/AKT/PDGF axis**

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22

23 **Abstract:**

24 **Background and Aims:**

25 Nonalcoholic steatohepatitis (NASH) is a chronic liver disease characterized
26 by hepatic lipid accumulation, inflammation, and progressive fibrosis. However,
27 the pathomechanisms underlying NASH are incompletely explored.
28 Phosphoenolpyruvate carboxykinase 1 (PCK1) catalyzes the first rate-limiting
29 step of gluconeogenesis. This study was designed to determine the role of
30 PCK1 in regulating NASH progression.

31 **Methods:**

32 Liver metabolism, hepatic steatosis, and fibrosis were evaluated at 24 weeks
33 in liver-specific *Pck1*-knockout mice fed with NASH diet or chow diet. Gain- or
34 loss-of-function approaches were used to explore the underlying mechanism *in*
35 *vitro*. AKT and RhoA inhibitors were evaluated for NASH treatment *in vivo*.

36 **Results:**

37 Hepatic PCK1 was downregulated in patients with NASH and mouse models
38 of NASH. Mice with liver *Pck1* deficiency displayed hepatic lipid disorder and
39 liver injury fed with normal diet, while showed aggravated fibrosis and
40 inflammation when fed NASH diet. Mechanistically, PCK1 deficiency
41 upregulated genes involved in fatty acid transport and lipid droplet formation.
42 Moreover, metabolomics analysis showed the accumulation of glycerol
43 3-phosphate, the substrate of triglyceride synthesis. Furthermore, the loss of
44 hepatic PCK1 could activate the RhoA/PI3K/AKT pathway, which leads to

45 increased secretion of PDGF-AA and promotes the activation of hepatic
46 stellate cells. Accordingly, treatment with RhoA and AKT inhibitors alleviated
47 NASH progression in the presence of *Pck1* deletion *in vivo*.

48 **Conclusions:**

49 PCK1 deficiency plays a key role in the development of hepatic steatosis and
50 fibrosis by facilitating the RhoA/PI3K/AKT/PDGF-AA axis. These findings
51 provide a novel insight into therapeutic approaches for the treatment of NASH.

52

53 **Keywords:** phosphoenolpyruvate carboxykinase 1; gluconeogenesis;
54 non-alcoholic steatohepatitis; PI3K/AKT pathway; platelet-derived growth
55 factor AA

56

57 **Introduction**

58 Non-alcoholic fatty liver disease (NAFLD) is the most common chronic liver
59 disease worldwide affecting nearly 25% of U.S. and European adults.¹ NAFLD
60 is characterized by aberrant lipid accumulation in hepatocytes in the absence
61 of excessive alcohol consumption. NAFLD may progress to non-alcoholic
62 steatohepatitis (NASH), a more serious form of liver damage hallmarked by
63 irreversible pathological changes such as inflammation, varying degrees of
64 fibrosis, and hepatocellular damage, which is more likely to develop into
65 cirrhosis and hepatocellular carcinoma (HCC).² Although multiple parallel
66 insults, including oxidative damage, endoplasmic reticulum stress, and hepatic
67 stellate cell (HSC) activation have been proposed to explain the pathogenesis
68 of NASH, the underlying mechanisms remain incompletely elucidated.³

69

70 Gluconeogenesis is the process of generating glucose from non-carbohydrate
71 substrates such as glycerol, lactate, pyruvate, and glucogenic amino acids,
72 which occurs mainly in the liver to maintain glucose levels and energy
73 homeostasis. Phosphoenolpyruvate carboxykinase 1 (PCK1) is the first
74 rate-limiting enzyme in the gluconeogenesis which converts oxaloacetate
75 (OAA) to phosphoenolpyruvate (PEP) in the cytoplasm.⁴ Our previous studies
76 have shown that PCK1 deficiency promotes HCC progression by enhancing
77 the hexosamine-biosynthesis pathway.⁵ However, PCK1 has been found to
78 regulate not only glucose homeostasis but also lipogenesis by activating sterol

79 regulatory element-binding proteins (SREBPs).⁶ Patients lacking PCK1
80 function present diffuse hepatic macrosteatosis concomitant with
81 hypoglycemia and hyperlactacidemia.⁷ Likewise, mice with reduced *Pck1*
82 expression develop insulin resistance and exhibit hypoglycemia as well as
83 hepatic steatosis, indicating an important role of PCK1 in regulating both
84 glucose homeostasis and lipid metabolism.^{8,9} However, the exact role of
85 PCK1 during NASH progression is incompletely understood.
86
87 The phosphoinositide 3-kinase/protein kinase B (PI3K/ATK) pathway plays a
88 critical role in regulating cell growth and metabolism. This pathway can be
89 activated in response to insulin, growth factors, energy, and cytokines, and in
90 turn, regulates key metabolic processes, including glucose and lipid
91 metabolism, as well as protein synthesis.¹⁰ AKT promotes *de novo* lipogenesis
92 (DNL) primarily through SREBP activation.¹¹ PI3K/AKT dysregulation leads to
93 many pathological metabolic conditions, including obesity and type 2
94 diabetes.¹² NAFLD is characterized by disordered glucose and lipid
95 metabolism in liver. Although PI3K/AKT pathway is a key regulator for sensing
96 metabolic stress, its exact role in NAFLD/NASH progression is unclear.^{13, 14}
97
98 In this study, we explored the role of *Pck1* in a mouse NASH model. We
99 unraveled the molecular mechanisms underlying disordered lipid metabolism,
100 inflammation, and fibrosis induced by *Pck1* depletion. We also delineated the

- 101 functional importance of the PI3K/AKT pathway and its effectors in
- 102 steatohepatitis, providing a potential therapeutic strategy for treating NASH.

103 **Materials and Methods**

104 ***Animal models.***

105 *Pck1*^{loxp/loxp} mice with a 129S6/SvEv background were purchased from the
 106 Mutant Mouse Resource & Research Centers (MMRRC: 011950-UNC) and
 107 *Alb-Cre* mice with a C57BL/6 background were purchased from Model Animal
 108 Research Center of Nanjing University. To generate liver-specific
 109 *Pck1*-knockout mice (L-KO), *Alb-Cre* mice were crossed with *Pck1*^{loxp/loxp} mice.
 110 *Pck1*^{loxp/loxp} mice from the same breeding were used as control (wild-type, WT).
 111 Male WT and L-KO mice, 7-9-week-old, were fed NASH diet (Research Diets,
 112 D12492: 60% Kcal fat, with drinking water containing 23.1 g/L fructose and
 113 18.9 g/L glucose) (n=11 per group) or a control chow diet (Research Diets,
 114 D12450J: 10% Kcal fat, with tap water) (n=10 per group) for 24 weeks. Food
 115 and drinking water were provided *ad libitum*. All mice were housed in
 116 temperature-controlled (23°C) pathogen-free facilities with a 12-hour light-dark
 117 cycle. For the male L-KO mice in the treatment group, after fed with NASH diet
 118 for 16 weeks, mice were divided into 3 groups and intraperitoneally (*i.p.*)
 119 injected with vehicle solution (n=6), MK2206 (AKT inhibitor, 50 mg/kg, every 3
 120 days) (n=5) or Rhosin (RhoA inhibitor, 20 mg/kg, every 3 days) (n=6) for 8
 121 weeks, respectively. All mice were executed for further study after fed with
 122 NASH diet for 24 weeks. Animal experiments were approved by Animal
 123 Experimentation Ethics Committees of Chongqing Medical University and
 124 were carried out in accordance with the Guide for the Care and Use of

125 Laboratory Animals.

126

127 Liver tissues collection, ELISA, immunoblotting, transcriptomic analyses,

128 untargeted metabolomics, primer sequences, reagents, antibodies,

129 ChIP-qPCR, and other *in vitro* studies are provided in the **Supplementary**

130 **Materials and Methods.**

131

132 **Results**

133 **PCK1 is downregulated in patients with NASH and mouse models of** 134 **NASH**

135 To determine whether PCK1 is involved in NAFLD, we first examined hepatic
136 gene expression in a published transcriptome dataset (GEO: GSE126848)
137 containing samples from 14 healthy, 12 obese, 15 NAFLD, and 16 NASH
138 patients.¹⁵ Bioinformatics analysis showed that 32 genes were markedly
139 changed in obesity, NAFLD, and NASH; 12 genes were considerably
140 downregulated and 20 genes were upregulated ([Supplementary Figure 1A-C](#)).
141 Notably, *PCK1* was gradually reduced in obesity, NAFLD, and NASH patients
142 ([Figure 1A and B](#)). Downregulation of *PCK1* mRNA was also found in a similar
143 dataset (GSE89632) ([Figure 1B](#)). Moreover, q-PCR, immunoblotting, and
144 immunohistochemistry (IHC) assays showed that PCK1 expression were
145 dramatically downregulated in liver samples derived from NASH patients and
146 NASH model mice ([Figure 1C-E](#)).

147
148 Next, palmitic acid (PA) was used to mimic the liver steatosis of NAFLD
149 patients *in vitro*.¹⁶ Cell growth was assessed by CCK8 assay after treatment
150 with different concentration of PA ([Supplementary Figure 1D](#)). Interestingly,
151 PCK1 mRNA and protein levels were downregulated in a dose-dependent
152 manner during 24-hour PA stimulation ([Figure 1F and G](#)), suggesting that
153 transcription of PCK1 may be inhibited in response to lipid overload. We

154 screened several known regulators of *PCK1* ([Supplementary Figure 1E and F](#))
 155 and determined *ATF3*, a transcriptional repressor of *PCK1*,¹⁷ was upregulated
 156 upon PA stimulation ([Figure 1H](#)). Similarly, *ATF3* expression was remarkably
 157 upregulated in liver samples derived from NASH patients and NASH model
 158 mice ([Supplementary Figure 1G-I](#)). Chromatin immunoprecipitation assays
 159 revealed that the binding of *ATF3* to *PCK1* promoter was increased by PA
 160 administration ([Figure 1I](#)). *ATF3* knockdown restored the expression of *PCK1*
 161 in human hepatocytes under PA treatment ([Figure 1J](#)). These results indicate
 162 that increased lipids caused upregulation of the repressor *ATF3*, impairing
 163 *PCK1* transcription in NASH patients and mouse models.

164

L-KO mice exhibit a distinct hepatic steatosis phenotype

166 To explore the role of *Pck1* in fatty liver disease, WT and L-KO mice were fed
 167 with chow diet for 24 weeks ([Supplementary Figure 2A](#)). From 16 weeks, L-KO
 168 mice showed increased body weight compared with WT mice, however, there
 169 was no significant difference in the glucose tolerance test (GTT) and insulin
 170 tolerance test (ITT) ([Supplementary Figure 2B](#)). Moreover, significant
 171 hepatomegaly and increased liver weight were observed in L-KO mice
 172 ([Supplementary Figure 2C](#)). Alanine transaminase (ALT) and aspartate
 173 transaminase (AST) levels were higher in L-KO mice, indicating liver injury
 174 ([Supplementary Figure 2D](#)). In addition, total triglyceride (TG), total cholesterol
 175 (TC), and free fatty acids (FFAs) in liver tissues and serum were elevated in

176 L-KO mice ([Supplementary Figure 2E and F](#)). Histochemistry and ELISA
177 showed that L-KO mice exhibited prominent hepatic steatosis and higher
178 levels of TNF- α ([Supplementary Figure 2G-I](#)). These data suggest that L-KO
179 mice exhibited a distinct hepatic steatosis phenotype and liver injury even
180 when fed normal chow.

181

182 **Hepatic loss of *Pck1* promotes inflammation and fibrogenesis in NASH** 183 **mice**

184 To explore whether an unhealthy diet could exacerbate the pathologic
185 changes in L-KO mice, WT and L-KO mice were fed with high-fat diet with
186 drinking water containing fructose and glucose (NASH diet) for 24 weeks
187 ([Figure 2A](#)).^{18, 19} From 4 weeks, L-KO mice showed significant weight gain
188 ([Figure 2B](#)). GTT and ITT showed that L-KO mice developed a more severe
189 form of glucose intolerance and insulin resistance ([Figure 2C and D](#)). L-KO
190 mice had pale and heavier livers ([Figure 2E](#)), although there was no significant
191 difference in liver weight ratio ([Supplementary Figure 3A](#)). Insulin, AST, ALT,
192 TC, TG, and FFAs were increased in serum or liver homogenates of L-KO
193 mice, suggesting more serious liver injury and lipid metabolism disorder
194 ([Figure 2F; Supplementary Figure 3B and C](#)). Analyses of L-KO liver sections
195 revealed increased fat droplets, more severe fibrosis, and greater macrophage
196 infiltration ([Figure 2G and H](#)). Furthermore, L-KO mice had higher NAFLD
197 activity score (NAS score), and higher TNF- α and IL-6 levels ([Figure 2I and J](#)).

198 In addition, the expression of inflammatory factors, lipogenic enzymes, and
199 fibrogenesis associated genes were upregulated in L-KO mice
200 ([Supplementary Figure 3D-F](#)). In summary, mice lacking hepatic *Pck1* showed
201 substantial liver inflammation and fibrosis when fed NASH diet.

202

203 **Transcriptomic and metabolomics analyses confirm loss of *Pck1***
204 **promotes hepatic lipid accumulation**

205 To comprehensively investigate the role of *Pck1* in NASH, we performed
206 RNA-seq analysis of liver samples from L-KO and WT mice fed normal chow
207 or NASH diet for 24 weeks. Gene ontology analysis indicated that lipid
208 metabolic processes were remarkably upregulated in L-KO mice fed with
209 NASH diet ([Figure 3A](#)). Volcano plot showed that genes involved in fatty acid
210 uptake, such as *Slc27a1* and *Cd36*, and lipid droplet synthesis, such as *Cidec*
211 and *Cidea*, were upregulated in response to NASH diet ([Figure 3B](#)). Gene Set
212 Enrichment Analysis (GSEA) revealed that the PPAR signaling pathway was
213 prominently upregulated in L-KO mice fed either diet ([Figure 3C](#);
214 [Supplementary Figure 4A and B](#)). Several genes selected from the data set
215 were independently validated by q-PCR and immunoblotting and found to be
216 significantly overexpressed in L-KO mice ([Figure 3D and E](#)). Furthermore,
217 genes involved in the glycerol 3-phosphate (G3P) pathway were also
218 upregulated in L-KO mice ([Figure 3F](#)). Metabolomics analysis showed that
219 compared with WT mice fed NASH diets, L-KO mice had significantly higher

220 G3P and PA levels ([Figure 3G and H](#)). Since G3P is a substrate for TG
221 synthesis and PA is a key intermediate metabolite in DNL, these data
222 suggested that *Pck1* ablation could promote the substrates accumulation for
223 lipid synthesis.

224

225 To further examine the function of PCK1 on steatosis *in vitro*, we
226 overexpressed (*PCK1*-OE) using the AdEasy adenoviral vector system and
227 knocked out PCK1 (*PCK1*-KO) using the CRISPR-Cas9 system in human
228 hepatocytes ([Supplementary Figure 4C and D](#)), and found that *PCK1*-OE
229 attenuated the accumulation of lipid droplets, whereas *PCK1*-KO facilitated
230 lipid accumulation ([Supplementary Figure 4E and F](#)). Collectively, these results
231 suggested that hepatic *Pck1* deficiency leads to lipid accumulation by
232 promoting the expression of lipogenic genes and the accumulation of
233 substrates related to lipid synthesis ([Supplementary Figure 4G](#)).

234

235 **Hepatic *Pck1* deficiency leads to HSC activation via PI3K/AKT pathway**

236 RNA-seq analysis indicated that the PI3K/AKT pathway was also specifically
237 activated in L-KO mice fed NASH diet ([Figure 4A and B](#)). Immunoblotting
238 revealed p-AKT (S473) and p-AKT (T308), two activated forms of AKT, and
239 downstream c-MYC were significantly upregulated in L-KO livers ([Figure 4C](#)).
240 Consistently, q-PCR also confirmed the high expression of genes related to
241 PI3K/AKT pathway in L-KO mice ([Supplementary Figure 5A](#)). Similarly, p-AKT

(S473 and T308) significantly decreased in human *PCK1*-OE cells, but increased in *PCK1*-KO cells after 0.2 mM PA treatment ([Figure 4D and E](#)).

To clarify the role of PI3K/AKT pathway activation, transcriptome data were further analyzed. Interestingly, *Col1a1*, *Col3a1*, and *Lama2*, which are primary components of the extracellular matrix (ECM), were upregulated as shown in the heat map of the PI3K/AKT pathway ([Supplementary Figure 5B](#)). Moreover, GSEA analysis revealed that ECM-receptor interaction was upregulated in L-KO mice ([Supplementary Figure 5C](#)). Since ECM deposition is usually considered as the key event underlying liver fibrosis, we suspected that activation of the PI3K/AKT pathway may promote fibrosis in L-KO mice.

Considering HSCs are major ECM secretors, we performed human hepatocyte (MIHA) and HSC (LX-2) co-culture assays ([Figure 4F](#)). Interestingly, mRNA levels of *ACTA2* (α -SMA, an HSC activation marker), *COL1A1*, and *COL3A1* were increased in HSCs co-cultured with *PCK1*-KO cells, but were decreased in HSCs co-cultured with *PCK1*-OE cells ([Figure 4G and H](#)). Likewise, *COL1A1*, *COL3A1*, and α -SMA expression was increased in liver tissues and primary HSCs of L-KO mice, which was confirmed by IHC analysis of *COL3A1* ([Figure 4I-K](#)). However, these increases could be partially reversed by MK2206, an AKT inhibitor ([Figure 4L and M](#)). Collectively, these data suggested that loss of *PCK1* in hepatocytes induces HSCs activation and ECM formation via activating the PI3K/AKT pathway.

264

265 **Paracrine PDGF-AA from hepatocytes promotes HSC activation**

266 Hepatocytes elicit several fibrogenic actions in a paracrine fashion to promote
 267 the activation of HSCs.²⁰ Thus, PCK1-mediated hepatic fibrosis may be
 268 involved in paracrine disorders. To test this hypothesis, several pro-fibrotic
 269 factors were screened, and *Pdgfa* was significantly elevated in liver tissues of
 270 L-KO mice (Figure 5A). Bioinformatics analysis confirmed that *PDGFA* were
 271 significantly increased in NAFLD and NASH patients (Figure 5B). *Pdgfa*
 272 encodes a dimer disulfide-linked polypeptide (PDGF-AA), and the chronic
 273 elevation of PDGF-AA in mice liver induces fibrosis.²¹ Immunoblotting and
 274 ELISA demonstrated increased PDGF-AA expression in liver tissues and
 275 primary hepatocytes of L-KO mice (Figure 5C-E). Moreover, PDGF-AA
 276 concentration was markedly increased in the culture medium of *PCK1-KO*
 277 cells, but decreased in that of *PCK1-OE* cells treated with 0.2 mM PA (Figure
 278 5F). Correspondingly, platelet-derived growth factor receptor alpha (*PDGFRA*),
 279 which encodes the PDGF-AA receptor, was increased in HSCs co-cultured
 280 with *PCK1-KO* cells, while decreased in HSCs co-cultured with *PCK1-OE* cells
 281 (Figure 5G). To determine whether the pro-fibrogenic effect was mediated by
 282 PDGF-AA secretion, we used a neutralizing antibody against PDGF-AA. As
 283 expected, the increase of α -SMA, COL1A1, and COL3A1 in HSCs co-cultured
 284 with *PCK1-KO* cells can be reversed by anti-PDGF-AA treatment (Figure 5H).

285

286 Reviewing transcriptome data, we found that *Pdgfa* appeared in the heat map
287 of the PI3K/AKT pathway ([Supplementary Figure 5B](#)). IHC results showed that
288 p-AKT (S473) was positively correlated with PDGF-AA ([Figure 5I](#)). The AKT
289 inhibitor MK2206 significantly blocked the increase of *PDGFA* expression
290 levels in the supernatants and cells lysates of *PCK1*-KO cells ([Figure 5J and](#)
291 [K](#)). Taken together, these data confirmed that *PCK1* deficiency promoted the
292 expression of PDGF-AA through the PI3K/AKT pathway, and activated HSCs
293 through hepatocyte-HSC crosstalk.

294

295 **PCK1 deficiency promotes the activation of the PI3K/AKT/PDGF-AA axis**
296 **by activating RhoA signaling in hepatocytes**

297 Rho GTPases, which cycle between active GTP-bound and inactive
298 GDP-bound conformations, are known to activate the PI3K/AKT pathway.²²⁻²⁴
299 Considering *PCK1* catalyzes the conversion of OAA to PEP, consuming GTP
300 to generate GDP, we speculated that *PCK1* deficiency may alter intracellular
301 GTP homeostasis. To test this hypothesis, we examined several Rho GTPase
302 protein levels in mice liver tissues and found that GTP-bound RhoA
303 significantly increased but inactivated RhoA, p-RhoA (S188) reduced in L-KO
304 mice ([Figure 6A-6C](#)). Consistently, after PA treatment, levels of GTP-bound
305 RhoA decreased but p-RhoA (S188) expression increased in *PCK1*-OE cells,
306 while *PCK1*-KO cells displayed the opposite effects ([Figure 6D-6G](#)). Next,
307 Rhosin was used to determine whether PI3K/AKT activation is dependent on

308 RhoA. Immunoblotting, ELISA, and q-PCR assays showed that Rhosin
309 blocked the increase of activated forms of AKT and PDGF-AA in *PCK1*-KO cell
310 lysate and supernatant, as well as *ACTA2*, *COL1A1*, and *COL3A1* expression
311 in HSCs co-cultured with *PCK1*-KO hepatocytes (Figure 6H-J). Moreover,
312 PCK1 and p-RhoA (S188) were downregulated in NASH patient samples,
313 while p-AKT (S473) and PDGF-AA levels were upregulated (Figure 6K). Taken
314 together, these data indicate that PCK1 ablation stimulated the
315 PI3K/AKT/PDGF-AA axis by activating RhoA.

316

317 **Therapeutic treatment with RhoA and AKT inhibitors reduced** 318 **progressive liver fibrosis *in vivo***

319 To explore whether blocking RhoA/PI3K/AKT could rescue the NASH
320 phenotype in L-KO mice, Rhosin and MK2206 were used *in vivo* (Figure 7A).
321 Treatment of L-KO mice with Rhosin or MK2206 showed improved glucose
322 intolerance (Supplementary Figure 6A) and insulin resistance (Supplementary
323 Figure 6B). Furthermore, the increased liver weight was also prevented
324 (Figure 7B), whereas body weight was reduced only in the MK2206 treatment
325 group (Supplementary Figure 6C). Additionally, Rhosin or MK2206
326 administration attenuated AST and ALT levels, as well as TG and FFA levels in
327 serum and liver tissues (Figure 7C; Supplementary Figure 6D and E). Similarly,
328 histochemistry showed reduced liver steatosis, inflammation, and fibrosis in
329 Rhosin or MK2206 treated mice (Figure 7D; Supplementary Figure 6F), which

330 was confirmed by decline in liver TNF- α and IL-6 levels ([Figure 7E](#)).

331 Additionally, α -SMA, COL1A1, COL3A1, PDGF-AA, p-AKT (S473, T308)

332 expression and GTP-bound RhoA levels were also decreased, while the

333 expression of p-RhoA (S188) was increased in treatment group ([Figure 7F](#);

334 [Supplementary Figure 6G](#)). MK2206 or Rhosin treatment also reduced the

335 expression of genes related to inflammation and fibrosis ([Figure 7G](#)). These

336 data suggested that the RhoA/PI3K/AKT axis plays a key role in NASH

337 progression in L-KO mice.

338 Discussion

339 This study revealed that the hepatic gluconeogenic enzyme PCK1 plays an
340 important role in NASH progression. The expression of PCK1 was diminished
341 in livers from patients or mice with NASH. Moreover, deletion of PCK1
342 significantly exacerbated hepatic steatosis, fibrosis, and inflammation in
343 mouse models fed NASH diet. Mechanistically, loss of PCK1 not only
344 promotes steatosis by enhancing lipid deposition, but also induces fibrosis by
345 HSC activation via the PI3K/AKT/PDGF-AA axis, thus promoting the
346 progression of NASH.

347

348 Abnormal lipid metabolism is a characteristic of NAFLD and NASH. Previous
349 studies assumed that altered lipid homeostasis was usually caused by
350 abnormal expression of genes related to lipid metabolism.²⁵ However, recent
351 studies have demonstrated that disruption of gluconeogenesis also leads to
352 abnormal lipid metabolism. Deficiency of fructose-1,6-bisphosphatase 1 (FBP1)
353 and glucose-6-phosphatase catalytic subunit (G6PC), key enzymes of
354 gluconeogenesis, results in severe hepatic steatosis and hypoglycemia,
355 indicating that suppression of gluconeogenesis could also disrupt lipid
356 homeostasis.^{26, 27} As the first rate-limiting enzyme in gluconeogenesis, it is
357 currently not clear whether PCK1 plays a critical role in NAFLD/NASH
358 development. In this study, we identified a robust decrease in PCK1
359 expression in the livers of NASH mice and NAFLD/NASH patients, causing

360 severe hepatic steatosis and confirming that disordered hepatic
361 gluconeogenesis can affect lipid homeostasis.
362
363 Previous reports have shown that PCK1 expression is increased in several
364 obesity/diabetes mouse models, such as ZDF rats, *ob/ob* and *db/db* mice, and
365 the disease progression of NASH is positively correlated with obesity and type
366 2 diabetes mellitus (T2DM).²⁸⁻³⁰ Interestingly, we found that PCK1 expression
367 was down-regulated in diet-induced murine NASH model. Such a discrepancy
368 might be due to the differences in animal models. The widely used rodent
369 models of genetic forms of obesity and diabetes, such as *ob/ob* and *db/db*
370 mice, have increased plasma glucocorticoids, which may drive PCK1
371 expression.^{30, 31} Another explanation is that high-fat diet supplemented with
372 high fructose/glucose in drinking water is capable of suppressing PCK1
373 expression, which is consistent with the previous report.³² Furthermore, we
374 identified ATF3, a member of the basic leucine zipper (bZIP) family of
375 transcription factors,³³ transcriptionally repressed *PCK1* upon PA overload *in*
376 *vitro* or in NASH mouse model. This is in line with previous studies suggesting
377 that ATF3 was upregulated in NAFLD patients and murine NASH model, and
378 could inhibit the expression of PCK1 in alcoholic fatty liver disease.^{16, 34, 35}
379 Therefore, in the current study, we found that PCK1 markedly decreased in
380 NASH, and PA inhibited *PCK1* transcription via the upregulation of ATF3.
381

382 Accumulating studies using PCK1 agonists or whole-body *Pck1* knockdown
 383 mice have verified that PCK1 may affect lipid metabolism.^{36, 37} In the present
 384 study, we found that liver-specific *Pck1* knockout induced significant hepatic
 385 steatosis even under normal feeding conditions. This is a very important
 386 phenomenon, since it is uncommon for a single gene ablation to cause
 387 spontaneous steatosis unless a high-fat diet is used. Moreover, we observed
 388 that mice with liver *Pck1* deficiency showed aggravated inflammation when fed
 389 high-fat high-fructose diet, which was completely different from a previous
 390 study showing that whole-body *Pck1* knockdown prevented hepatic
 391 inflammation.³⁸ We speculate that this discrepancy is possibly due to
 392 differences between diet plan and animal models, since whole-body *Pck1*
 393 knockdown may have unforeseen effects on glucolipid metabolism.

394

395 Lipid accumulation is the essence of steatosis. Emerging evidence has
 396 indicated that increased fatty acid uptake is associated with lipid
 397 accumulation.^{39, 40} In this study, genes involved in fatty acid uptake such as
 398 *Cd36* and *Slc27a1* were highly expressed in L-KO mice. In addition, *Cidec*, a
 399 lipid droplet-associated protein that promotes their formation, was increased
 400 by both chow and NASH diets, and recently it was claimed to upregulated in
 401 NALFD patients and mice, suggesting that PCK1 ablation also promotes lipid
 402 droplet formation.^{41, 42} Abnormal levels of metabolites also contribute to TG
 403 accumulation in liver. The G3P pathway contributes to over 90% of TG

404 synthesis.⁴³ Since our metabolomics data showed that G3P and PA were
 405 significantly upregulated in L-KO mice, we propose that PCK1 deficiency
 406 promotes hepatic lipid accumulation by enhancing the expression of *Cd36*,
 407 *Slc27a1*, and *Cidec* and the levels of metabolic substrates such as G3P and
 408 PA. However, the exact mechanism by which PCK1 regulates G3P pathway
 409 and the expression levels of *Cd36*, *Slc27a1* remains to be further explored.
 410
 411 Fibrosis is another characteristic of NASH and drives the transition from simple
 412 steatosis to NASH. Activation of HSCs through the secretion of profibrotic
 413 cytokines, such as TGF- β and PDGF, is a key event in liver fibrosis.⁴⁴ A recent
 414 study identified high mobility group protein B1 (HMGB1), secreted by
 415 FBP1-deficient hepatocytes, as the main mediator to activate HSCs, showing
 416 the important crosstalk between hepatocytes and HSCs via paracrine
 417 signaling.²⁶ Herein, we found that PDGF-AA was secreted by PCK1-deficient
 418 hepatocytes and acted in a paracrine manner to activate HSCs. Increased
 419 deposition of extracellular matrix and activation of HSCs were shown in
 420 PDGFA-transgenic mice, however, the underlying mechanism mediating
 421 PDGF-AA upregulation in fibrosis remains unclear.²¹ Here, we demonstrated
 422 that PCK1 deficiency promoted PDGF-AA secretion via activation of the
 423 RhoA/PI3K/AKT pathway. Mechanistically, we hypothesize that PCK1 deletion
 424 may increase intracellular GTP levels, thus promoting the activation of RhoA
 425 and further activating the PI3K/AKT pathway. Based on our *in vitro* findings,

426 we used pharmacological AKT and RhoA inhibitors, MK2206 and Rhosin
427 respectively, in L-KO NASH mice. Hepatic steatosis, fibrosis, and inflammation
428 were significantly attenuated in treated mice. Although RhoA and AKT
429 inhibitors are currently only in phase 3 trials or preclinical studies for the
430 treatment of liver fibrosis or clinical tumors, these compounds may also have a
431 promising therapeutic potential for NASH.⁴⁵⁻⁴⁷

432

433 In conclusion, this study demonstrated that hepatic PCK1 deficiency could
434 promote lipid deposition and fibrosis in murine NASH model. Moreover,
435 hepatic PCK1 loss activates the RhoA/PI3K/AKT pathway, which increases
436 secretion of PDGF-AA and promotes HSC activation. AKT/RhoA inhibitors
437 could reduce progressive liver fibrosis, providing a therapeutic window for
438 NASH treatment.

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459

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462

463 **Author contributions:** NT, AH, and KW conceived and designed the study.

464 QY, YL and GZ performed most experiments and analyzed the data. HD, CC

465 and KW conducted bioinformatics analysis. XP assisted with mice experiments.

466 XW, JF, and QP provided human NAFLD/NASH samples. QY, KW, and NT

467 wrote the manuscript with all authors providing feedback. The order of the

468 co-first authors was assigned on the basis of their relative contributions to the

469 study.

470

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472

473 **Abbreviations:**

474 AKT, protein kinase B; ALT, Alanine transaminase; AST, aspartate
475 transaminase; α -SMA, alpha-smooth muscle actin (ACTA2); ATF3, Activating
476 Transcription Factor 3; bZIP, Basic Leucine Zipper; ChIP, chromatin
477 immunoprecipitation; CIDEc and CIDEA, cell death inducing DFFA like
478 effector C and A; COL1A1, COL1A3, recombinant collagen type I alpha 1 and
479 alpha 3; CD36, fatty acid translocase; DNL, *de novo* lipogenesis; FFA, free
480 fatty acid; G3P, glycerol 3-phosphate; GTT, glucose tolerance test; GSEA,
481 Gene Set Enrichment Analysis; HE, hematoxylin and eosin; IHC,
482 immunohistochemistry; ITT, insulin tolerance test; Lama2, laminin subunit
483 alpha 2; L-KO, liver-specific *Pck1*-knockout mice; NASH, non-alcoholic
484 steatohepatitis; NAFLD, non-alcoholic fatty liver disease; NAS, NAFLD activity
485 score; PCK1, Phosphoenolpyruvate carboxykinase 1; PCK1,
486 Phosphoenolpyruvate carboxykinase 1; PDGF-AA, platelet-derived growth
487 factor AA; PI3K, phosphatidylinositol 3-kinase; PPAR, Peroxisome
488 Proliferator-Activated Receptor; RhoA, Ras homolog family member A;
489 SLC27A1, solute carrier family 27 member 1; TC, total cholesterol; TG,
490 Triglyceride

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- 640 Author names in bold designate shared co-first authorship.

641 **Figure legends**

642 **Figure 1. PCK1 is downregulated in patients with NASH and mouse**

643 **models of NASH.** (A) Genes downregulated in patients with obesity (n=12),
644 NAFLD (n=15), and NASH (n=16) from GSE126848 dataset. (B) Relative
645 *PCK1* mRNA levels in GSE126848 and GSE89632 datasets. (C) *PCK1*
646 expression in normal individuals and patients with NASH. Scale bars: 50 μ m.
647 (D-E) mRNA and protein levels of *PCK1* in the livers of WT mice fed with chow
648 diet or NASH diet. (F-G) *PCK1* mRNA and protein levels in MIHA cells treated
649 with palmitic acid (PA) or BSA. (H) Relative levels of indicated genes in MIHA
650 cells treated with 0.2 mM PA. (I) ChIP assays were performed in MIHA cells
651 with or without PA treatment using an antibody against ATF3, IgG or H3. (J)
652 Protein levels of *PCK1* in MIHA cells infected with either shControl or shATF3
653 treated with 0.2 mM PA. Data expressed as mean $\pm$ SEM; **P* < 0.05, ** *P* <
654 0.01, ****P* < 0.001. *P* values obtained via 2-tailed unpaired Student's *t* tests or
655 one-way ANOVA with Tukey's post hoc test.

656 **Figure 2. PCK1 ablation accelerates inflammation and fibrogenesis in**
657 **NASH model.** (A) Schematic diagram of mouse model fed with NASH diet.
658 (B-D) Body weight, GTT, and ITT were measured in WT and L-KO mice (n=11).
659 (E) Representative gross liver morphology, whole body photo, and liver weight.
660 (F) Serum levels of insulin, ALT and AST were measured. (G)
661 Paraffin-embedded liver sections were stained with H&E, Sirius Red, α -SMA
662 and F4/80. Frozen sections stained with Oil Red O. Scale bars: 50 μ m. (H)
663 Quantifications of Oil red O staining, Sirius red staining, and IHC staining. (I)
664 NAS scores of each group. (J) Levels of TNF- α and IL-6 in liver tissues were
665 examined using ELISA (n=8). Data expressed as mean $\pm$ SEM; * P < 0.05, ** P
666 < 0.01, *** P < 0.001. P values obtained via 2-tailed unpaired Student's t tests.

667 **Figure 3. Loss of PCK1 promotes lipid accumulation confirmed by**
668 **transcriptome and metabolome.** RNA sequencing was performed on livers
669 of WT and L-KO mice fed NASH diet (n=4-5). (A) Gene ontology analysis of all
670 significantly changed genes in top 10 biological processes. (B) Volcano plot
671 representation of significantly up- and downregulated genes. (C) GSEA plot
672 (left) of enrichment in “PPAR signaling pathway” signature; Heatmap (right)
673 presentation of significantly upregulated PPAR target genes. (D-E) q-PCR and
674 immunoblot analysis of indicated genes or protein expression in mice liver
675 tissues. (F) Relative mRNA expression of key genes in G3P pathway (n=8). (G)
676 Upregulated metabolites detected by untargeted metabolomics (n=6). (H) The
677 relative level of G3P and PA in mice liver tissues (n=6). Data expressed as
678 mean $\pm$ SEM; * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$. P values obtained via
679 2-tailed unpaired Student's t tests.

680 **Figure 4. Hepatic PCK1 deficiency leads to HSC activation via PI3K/AKT**
681 **pathway.** (A) Pathway enrichment analysis of significantly upregulated genes
682 in L-KO mice. (B) GSEA plot of enrichment in PI3K/AKT pathway. (C-E)
683 Immunoblot analysis of AKT and p-AKT (S473 or T308) in mice liver tissues or
684 *PCK1*-OE and *PCK1*-KO MIHA cells with or without 0.2 mM PA treatment. (F)
685 Schematic flow chart of co-culture models. (G-H) q-PCR analysis of fibrosis
686 related gene in HSC (LX-2) cells co-cultured with *PCK1*-KO or *PCK1*-OE MIHA
687 cells. (I-J) Western blot of fibrosis related protein in liver tissues or primary
688 HSCs (n=3). (K) COL3A1 immunostaining in mice liver sections. Scale bars:
689 50 μ m. (L-M) Relative mRNA expression and immunofluorescence images of
690 *ACTA2*/ α -SMA, *COL1A1* and *COL3A1* in LX-2 cells co-cultured with *PCK1*-KO
691 MIHA cells treated with AKT inhibitor MK2206 (10 μ M). Scale bars: 25 μ m.
692 Data expressed as mean $\pm$ SEM; * P < 0.05, ** P < 0.01, *** P < 0.001. P values
693 obtained via 2-tailed unpaired Student's t tests or one-way ANOVA with
694 Tukey's post hoc test.
695

696 **Figure 5. Paracrine PDGF-AA from hepatocytes promotes HSC activation.**

697 (A) Expression levels of genes related to fibrogenesis. (B) Relative *PDGFA*
698 mRNA levels in GSE126848 and GSE89632 datasets. (C-D) PDGF-AA protein
699 levels in liver tissue detected by Western blot and ELISA. (E) PDGF-AA protein
700 levels in primary hepatocytes (n=3). (F) Secreted PDGF-AA levels in the
701 conditional medium of *PCK1*-KO or *PCK1*-OE MIHA cells with 0.2mM PA
702 treatment. (G) mRNA levels of *PDGFRA* in cell lysate of LX-2 co-cultured with
703 *PCK1*-KO or *PCK1*-OE MIHA cells treated with PA. (H) Indicated protein level
704 in LX-2 cells co-cultured with *PCK1*-KO MIHA cells containing nonspecific
705 rabbit IgG or a PDGF-AA blocking antibody. (I) IHC analysis of PCK1, p-AKT
706 (S473) and PDGF-AA in mice liver sections. Scale bars: 50 μ m. (J) Levels of
707 PDGF-AA or *PDGFA* in the conditional medium or cell lysate of *PCK1*-KO
708 MIHA cells treated with AKT inhibitor MK2206 (10 μ M). (K) Indicated protein
709 levels in *PCK1*-KO MIHA cells treated with AKT inhibitor MK2206 (10 μ M).
710 Data expressed as mean $\pm$ SEM; * P < 0.05, ** P < 0.01, *** P < 0.001. P values
711 obtained via 2-tailed unpaired Student's t tests or one-way ANOVA with
712 Tukey's post hoc test.

Figure 6. PCK1 deficiency promotes the activation of PI3K/AKT/PDGF-AA axis by activating RhoA in hepatocytes. (A) Immunoblotting analysis of indicated protein in mice liver tissues. (B) IHC analysis of p-RhoA (S188) in mice liver tissues. Scale bars: 50 μ m. (C-E) Relative levels of active RhoA were measured by G-LISA in mice liver tissues, *PCK1*-OE and *PCK1*-KO MIHA cells treated with 0.2 mM PA. (F-G) Immunoblots of p-RhoA (S188) and RhoA in *PCK1*-OE and *PCK1*-KO MIHA cells with or without 0.2 mM PA treatment. (H) Expression of indicated proteins in *PCK1*-KO MIHA cells after addition of Rhosin (30 μ M). (I) Levels of PDGF-AA in the supernatant of *PCK1*-KO MIHA cells treated with Rhosin (30 μ M). (J) Relative mRNA expression of *ACTA2*, *COL1A1*, and *COL3A1* in LX-2 cells co-cultured with *PCK1*-KO MIHA cells treated with Rhosin (30 μ M). (K) IHC analysis of PCK1, p-RhoA (S188), p-AKT (S473), and PDGF-AA in normal individuals and patients with NASH. Scale bars: 50 μ m. Data expressed as mean $\pm$ SEM; * P < 0.05, ** P < 0.01, *** P < 0.001. P values obtained via one-way ANOVA with Tukey's post hoc test.

729 **Figure 7. AKT and RhoA inhibitors prevent the development of NASH *in***
730 ***vivo*.** L-KO mice were fed NASH diet for 24 weeks, and therapeutic treatment
731 with AKT or RhoA inhibitor was initiated at 16 weeks. (A) Schematic diagram of
732 L-KO mice treated with DMSO (n=6), Rhosin (n=6) or MK2206 (n=5). (B-C)
733 Representative whole body, gross liver morphology, liver weight, and serum
734 ALT and AST. (D) Paraffin-embedded liver sections were stained with HE, or
735 immunostained for F4/80, COL3A1 and α -SMA. Frozen sections stained with
736 Oil Red O. Scale bars: 50 μ m. (E) Levels of TNF- α , IL-6 in liver tissues. (F)
737 Expression of indicated protein in mice liver tissues. (G) mRNA levels of genes
738 associated with lipid metabolism, fibrogenesis, and inflammatory infiltration.
739 Data expressed as mean $\pm$ SEM; * P < 0.05, ** P < 0.01, *** P < 0.001; n.s., not
740 statistically significant. P values obtained via one-way ANOVA with Tukey's
741 post hoc test.

Figure 1

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Figure 7