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Hepatocyte-specific depletion of *Nnmt* protects mice from non-alcoholic steatohepatitis

To the Editor:

NNMT is an enzyme that mediates the conversion of nicotinamide (NAM) to 1-methylnicotinamide (MNA), while consuming a methyl group from S-Adenosyl methionine (SAM). The important role of NNMT in metabolic homeostasis has been investigated in various context.[1–3] With great interest, we read the research article by Song *et al.* The authors demonstrated that the expression of *Nnmt* can be regulated by unfolded protein response (UPR)-related PERK-ATF4 signaling in the context of alcohol-induced liver damage.[4] Notably, similar phenotypes have also been identified in the context of palmitate-induced hepatocyte damage in cell culture.[5]

But the specific role of NNMT in the development of non-alcoholic steatohepatitis (NASH) has not been systemically investigated. Previously, many investigators including Song *et al.* and ourselves have tried to study the specific effects of NNMT in livers using adenovirus- or adeno-associated virus (AAV)-based transient overexpression or knockdown. The fact that the viruses will be eliminated in a short period of time has prevented us from performing long-term experiments. Additionally, virus-induced immune responses may also confound such experiments. A non-virus-based tool to study the long-term physiological function of NNMT in a tissue-specific manner is still missing.

To address these issues, a conditional knockout (KO) mouse model of *Nnmt* was established by knocking in the *LoxP* cassettes around the second exon ($Nnmt^{flox/flox}$). By crossing the $Nnmt^{flox/flox}$ mice to *Alb-cre* mice, we created the hepatocyte-specific *Nnmt* KO mouse model *Alb-cre::*$Nnmt^{flox/flox}$ (referred to as *Nnmt* KO mice hereafter) (Fig. 1A). The KO efficiency was validated by reverse-transcription quantitative PCR and western blot (Fig. 1B,C and Fig. S1A,B). The *Nnmt* KO mice have similar body weight as control mice ($Nnmt^{flox/flox}$) when fed a normal chow diet (Fig. S1C).

The mice were then challenged with carbon tetrachloride (intraperitoneally), fructose and glucose (via drinking water) and western diet to establish a NASH model. No bodyweight differences were observed between *Nnmt* KO mice and the control mice (Fig. S1D). Lower concentrations of total cholesterol, LDL-c, ALT and AST were observed in the serum of *Nnmt* KO mice (Fig. 1D-E). A lower proportion of liver was also identified in the *Nnmt* KO mice (Fig. 1F). Notably, the serum concentrations of TC, LDL-c, ALT and the proportion of liver in the *Nnmt* KO mice were comparable to the control mice on chow diet (Fig. S1E-G). The changes in serum lipid profile as well as the liver proportion were not described in the original articles from Song *et al.* but are consistent with their major conclusions.

The expression of genes related to fibrosis and inflammation in the liver was downregulated in *Nnmt* KO mice (Fig. 1G-H). We then applied RNA-seq analysis to understand the genome-wide transcriptional changes in the liver. Principle component analysis revealed dramatic differences in gene expression profiles (Fig. S1H). We identified 1,752 genes with at least 2-fold changes and $p < 0.05$ using *t* tests (Fig. S1I). The top differentially expressed genes were presented in a heatmap (Fig. S1J). Gene set enrichment analysis showed downregulation of genes enriched in gene sets related to fibrosis and inflammation (Fig. 1I-J). The results of Picrosirius red, α-SMA and CD68 staining assays indicated less fibrosis and inflammation in the livers of *Nnmt* KO mice (Fig. 1K). In contrast, no differences in fibrosis or inflammation were observed in the livers of *Nnmt* KO mice fed a chow diet (Fig. S1K). These phenotypic changes suggested *Nnmt* KO protected the mice from NASH. Similarly, the protective effects of *Nnmt* knockdown or pharmacological inhibition against alcohol-induced liver damage were also observed by Song *et al.*

As to the mechanism, Song *et al.* proposed that *Nnmt* knockdown protected against liver damage by suppressing the *de novo* lipogenesis induced by alcohol or endoplasmic reticulum stress. We thus investigated the effects of *Nnmt* depletion on lipid metabolism in mouse primary hepatocytes. Few lipid contents were detected in the *Nnmt* KO hepatocytes with the oil red staining assay, when the cells were treated with palmitic acid and oleic acid (Fig. 1L-M). In addition, genes related to lipogenesis were also downregulated by *Nnmt* KO (Fig. 1N). The inhibitory effects of *Nnmt* depletion on fatty acid-induced *de novo* lipogenesis are consistent with the phenotypes Song *et al.* observed in alcohol-induced *de novo* lipogenesis.

It has been reported that whole body depletion of *Nnmt* leads to profound metabolic changes in mice,[6] but the functions of

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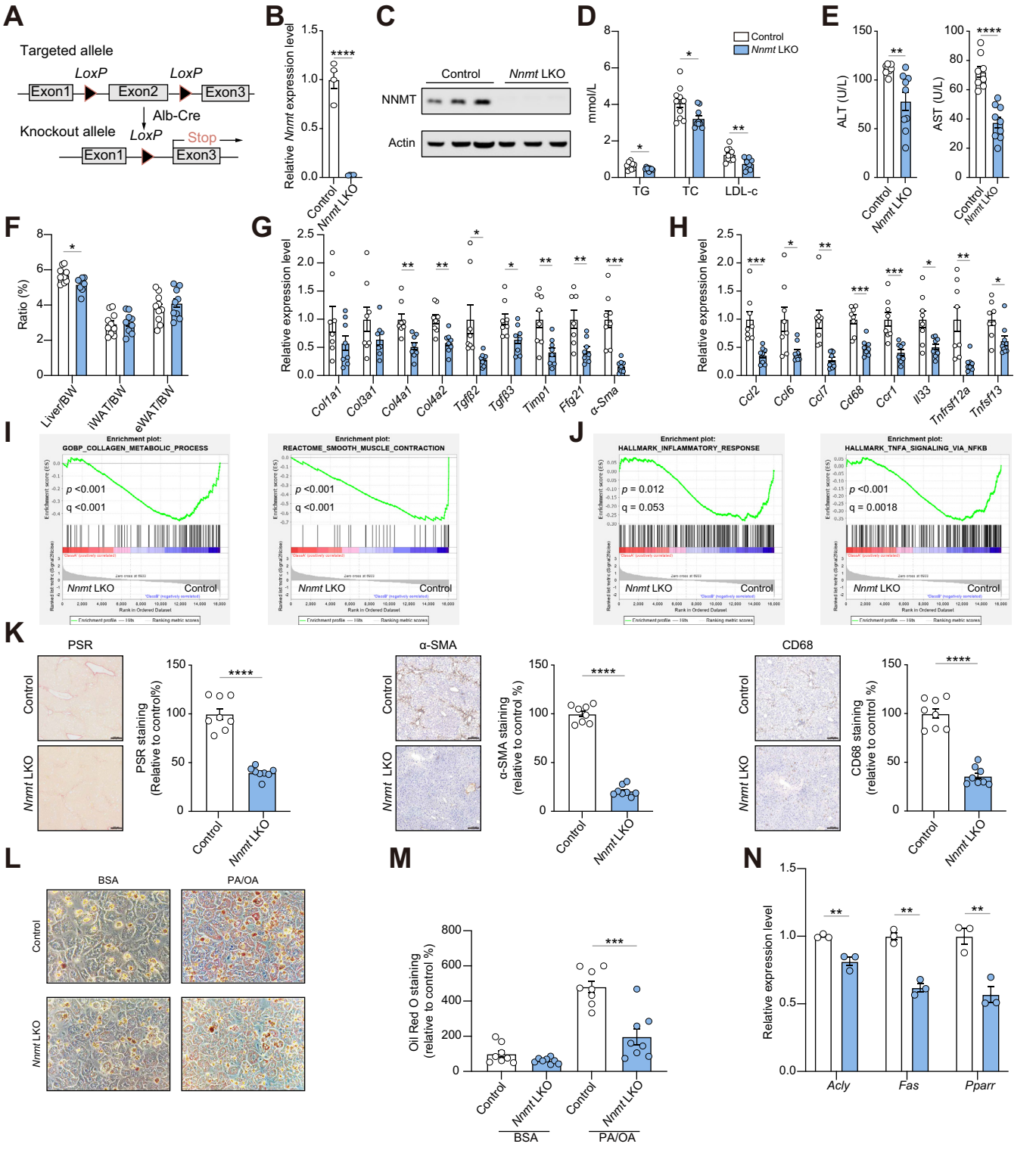

Fig. 1. *Nnmt* depletion protected mice from NASH. (A) Establishment of hepatocyte-specific *Nnmt* KO mouse model; (B,C) The expression of *Nnmt* mRNA (B) and NNMT protein (C) in liver; (D) Serum lipid profile; (E) Serum ALT and AST; (F) Body composition; (G-H) The expression of genes related to fibrosis (G) and inflammation (H); (I-J) GSEA showing the enrichment in (I) fibrosis and (J) inflammation; (K) PSR, α-SMA and CD68 staining on the liver. (L) Images and (M) Quantifications of oil red staining; (N) Relative expression of lipogenesis related genes. Scale bar, 200 μm. N = 5-10. *p <0.05; **p <0.01; ***p <0.001; ****p <0.0001. GSEA, gene set enrichment analysis; KO, knockout; NASH, non-alcoholic steatohepatitis. (This figure appears in color on the web.)

NNMT in different tissues are variable.[7] The *Nnmt* $^{flox/flox}$ mice we generated are a useful tool for the community to study the physiological function of NNMT in a tissue-specific manner. We identified the protective effects of *Nnmt* depletion on NASH, which is consistent with its protective effects on alcohol-induced liver damage. We therefore propose that NNMT is a general and

key mediator of liver damage. UPR-NNMT signaling has the potential to be developed as a therapeutic target in liver disease.

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Conflicts of interest

The authors claim no conflict of interests.

Please refer to the accompanying ICMJE disclosure forms for further details.

Authors' contributions

Conceptualization, D.L., C.Y., H.H., S.H. and J.L.; Investigation, D.L., C.Y., H.H., S.H. and J.L.; Analysis, D.L., C.Y., H.H., S.H. and J.L.; Writing, D.L., C.Y., H.H., S.H. and J.L.; Data Visualization, D.L., C.Y., H.H., S.H. and J.L.; Funding Acquisition, H.H., S.H. and J.L.; Supervision, S.H., J.L..

Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jhep.2022.03.021.

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Cirrhosis: What are all those factor VIII and protein C for?

To the Editor:

Over the last decades our understanding of cirrhotic coagulopathy has undergone considerable changes. Clinical, experimental, and pathophysiological observations helped us to view cirrhosis not as the epitome of acquired bleeding disorders, but as a disease characterized by rebalanced hemostasis and possibly even the increased risk of thrombosis.[1] Scheiner *et al.* recently published an interesting paper on this topic.[2] Based on retrospective evaluation of coagulation biomarkers and clinical observations, the authors conclude that the factor (F)VIII/protein C(PC) ratio in cirrhosis is independently associated with liver-related events/death. However, investigations of thrombomodulin-modified thrombin generation (TG) in another overlapping cohort of patients suggest that the FVIII/PC ratio is apparently unrelated with hypercoagulability.[2]

The conclusions of the above study give rise to the following considerations.

Pathophysiological considerations on FVIII and PC: One may wonder what are all those FVIII and PC for? If they are unrelated to hypercoagulability. High FVIII, being associated in non-cirrhotic patients with hypercoagulability and risk of first/recurrent thrombosis,[3] is a prototype procoagulant driver for TG. On the other hand, (thrombomodulin-activated) PC is one of the most potent naturally-occurring anticoagulants responsible for thrombin downregulation – non-cirrhotic patients with congenital PC deficiency are at increased hypercoagulability (high TG) and thrombotic risk.[4] The fact that (thrombomodulin) activated PC is the physiological inhibitor of FVIII and that FVIII and PC are respectively increased or decreased in cirrhosis, accords perfectly with the concept of the FVIII-PC axis as a crucial determinant of hypercoagulability. Consequently, the results reported by Scheiner *et al.*[2] make one wonder what underlies the contrast in the

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