Overexpression of Hepcidin Alleviates Steatohepatitis and Fibrosis in a Diet-induced Nonalcoholic Steatohepatitis

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Abstract

Background and Aims: Iron overload can contribute to the progression of nonalcoholic fatty liver disease (NAFLD) to nonalcoholic steatohepatitis (NASH). Hepcidin (Hamp), which is primarily synthesized in hepatocytes, is a key regulator of iron metabolism. However, the role of Hamp in NASH remains unclear. Therefore, we aimed to elucidate the role of Hamp in the pathophysiology of NASH. Methods: Male mice were fed a choline-deficient L-amino acid-defined (CDAA) diet for 16 weeks to establish the mouse NASH model. A choline-supplemented amino acid-defined (CSAA) diet was used as the control diet. Recombinant adeno-associated virus genome 2 serotype 8 vector expressing Hamp (rAAV2/8-Hamp) or its negative control (rAAV2/8-NC) was administered intravenously at week 8 of either the CDAA or CSAA diet. Results: rAAV2/8-Hamp treatment markedly decreased liver weight and improved hepatic steatosis in the CDAA-fed mice, accompanied by changes in lipogenesis-related genes and adiponectin expression. Compared with the control group, rAAV2/8-Hamp therapy attenuated liver damage, with mice exhibiting reduced histological NAFLD inflammation and fibrosis, as well as lower levels of liver enzymes. Moreover, a smooth muscle actin-positive activated hepatic stellate cells (HSCs) and CD68-positive macrophages increased in number in the CDAA-fed mice, which was reversed by rAAV2/8-Hamp treatment. Consistent with the in vivo findings, overexpression of Hamp increased adiponectin expression in hepatocytes and Hamp treatment inhibited HSC activation. Conclusions: Overexpression of Hamp using rAAV2/8-Hamp robustly attenuated liver steatohepatitis, inflammation, and fibrosis in an animal model of NASH, suggesting a potential therapeutic role for Hamp.

Keywords: Liver fibrosis; Hepatic stellate cell; Hepcidin; NASH; CDAA.

Original Article

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correlated with obesity but not liver disease.\textsuperscript{15–17} Despite the fact that the mechanism through which Hamp induces NAFLD remains uncertain, excessive cytokines, such as interleukin (IL) 6, have a core function in Hamp production. More interestingly, Tsutsumi et al.\textsuperscript{18} recently found that there was a significant inverse correlation between Hamp immunoreactivity and fibrosis in pediatric NAFLD patients, suggesting that these patients experienced a reduction in the Hamp-producing ability of the liver in response to iron levels, leading to subsequent fibrosis.

Our preliminary data also demonstrated that low levels of Hamp are associated with murine carbon tetrachloride (CCL\textsubscript{4})-induced liver fibrosis, leading to the hypothesis that Hamp might be effective in treating NASH-related fibrosis. Therefore, we sought to determine if Hamp contributes to the severity of steatosis, inflammation, and fibrosis in a mouse model of NASH induced by a choline-deficient L-amino acid-defined (CDAA) diet. We used a recombinant adeno-associated virus genome 2 serotype 8 (rAAV2/8) vector to efficiently transfect a Hamp overexpressing plasmid into liver cells to investigate the effects of Hamp on NASH-related inflammation and fibrosis in a mouse model.

\textbf{Methods}

\textbf{Experimental animals}

This study was approved by the Institutional Animal Care and Usage Committee of the Beijing Friendship Hospital, Capital Medical University. C57BL/63 mice were fed a CDAA diet (M10530i; Moldiets). Recombinant adeno-associated virus subtype 2/8 vector expressing Hamp (rAAV2/8-Hamp) or its negative control vector (rAAV2/8-NC) were amplified by Obio (Shanghai, China). Mice were treated with either the rAAV2/8-NC or rAAV2/8-Hamp via tail vein injection at a dose of 3×10\textsuperscript{11} genome copies/mouse after 8 weeks of being fed either the CDAA or CSAA diet. The mice were housed on a 12 h light/dark cycle, with controlled temperature (23±2°C) at 40–60% humidity. Food intake and body weight were monitored weekly. After a total of 16 weeks on the CDAA or CSAA diet, animals were euthanized and sacrificed. Serum was collected and frozen, and the livers and spleens were dissected, weighed, and snap-frozen for further analysis.

\textbf{Hamp treatment}

The rat hepatic stellate cell (HSC) line-T6 was a gift from Dr. Scott Friedman (Mount Sinai Medical Center, New York, NY, USA). T6 cells were plated in Dulbecco’s modified Eagle medium (DMEM) supplemented with 10\% (v/v) fetal bovine serum (FBS). When cells reached 80–90\% confluence, a core function in Hamp production. More interestingly, Tsutsumi et al.\textsuperscript{18} recently found that there was a significant inverse correlation between Hamp immunoreactivity and fibrosis in pediatric NAFLD patients, suggesting that these patients experienced a reduction in the Hamp-producing ability of the liver in response to iron levels, leading to subsequent fibrosis.

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\textbf{Generation of Hamp-overexpressing hepatocyte cell line}

AML12 mouse hepatocytes (American Type Culture Collection, Manassas, VA, USA) were cultured in DMEM/F12 with 10\% FBS supplemented with 1\% Insulin-Transferrin-Selenium (ITS) (5150056; Invitrogen, Waltham, MA, USA), 40 ng/mL dexamethasone, and 1\% penicillin streptomycin mixture. The pIRE52-EGFP-NC and pIRE52-EGFP-Hamp plasmids were transfected into AML12 cells using the EndoFectin\textsuperscript{TM} Max transfection reagent (GeneCopoeia, Rockville, MD, USA) following the manufacturer’s instructions. Briefly, AML12 cells (5×10\textsuperscript{4} per well) were seeded into a 12-well plate. After 24 h, the cells were transfected with either 2 or 4 µg of the plasmids using the EndoFectin\textsuperscript{TM} Max transfection reagent.

\textbf{Quantitative real-time (q)PCR analysis}

Total mRNA was extracted using the TRIzol reagent (Sigma-Aldrich, St. Louis, MO, USA) according to the manufacturer’s protocol.\textsuperscript{19} Reverse transcription was performed with 1 µg total RNA using the SuperScript\textsuperscript{TM} VILO\textsuperscript{TM} Master Mix (Invitrogen). The SYBR Green Real-time PCR Master Mix (Invitrogen) was used for qPCR. Primers used for qPCR were listed in Table 1. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used to normalize the PCR results, and the ΔΔCt method was used for quantification.

\textbf{Western blot analysis}

Total cell lysates were homogenized in tissue lysis buffer (FNN0071; Invitrogen) supplemented with protease and phosphatase inhibitors (Roche, Basel, Switzerland).\textsuperscript{20} Proteins were resolved by SDS-PAGE and transferred to nitrocellulose membranes (Amersham Biosciences, Buckinghamshire, UK). Membranes were then incubated with primary antibodies against alpha-smooth muscle actin (αSMA) (diluted 1:500; Abcam, Cambridge, UK), tissue inhibitor of metalloproteinase (TIMP) 1 (diluted 1:500; Invitrogen), and β-actin overnight (diluted 1:2,000; Peprotech, Rocky Hill, NJ, USA) overnight at 4°C. On the following day, the membranes were incubated with the appropriate secondary antibodies (Cell Signaling Technology, Danvers, MA, USA) and proteins were visualized using a chemiluminescent substrate (Invitrogen).

\textbf{Histopathological analysis}

Liver samples were fixed with neutral-buffered formalin, embedded in paraffin, and cut into 4-µm thick sections that were stained with either hematoxylin and eosin (HE) or Sirius Red (SR), or prepared for immunohistochemistry (IHC) analysis of αSMA and CD68 expression. Histological assessment and scoring were performed by a pathologist blinded to the study. Steatosis and lobular inflammation scoring on liver histology were performed using the clinical criteria outlined by Kleiner et al.\textsuperscript{21} Activated HSCs and total macrophages were detected using an anti-αSMA antibody (diluted 1:200; Abcam) and an anti-CD68 antibody (diluted 1:500; Invitrogen), respectively. Morphometric quantification of SR staining (percent of area) was performed at 200× in 10 random fields per mouse from five individual animals using ImageJ software. αSMA- and CD68-positive cells were quantified as the number of positively stained cells per high-power field (HPF).

For Oil-red O staining, optimal cutting temperature-embedded frozen tissue was sectioned at 7 µm and fixed in 10% neutral buffered formalin. After washing with distilled water, dried slides were subsequently incubated with 60% isopropanol and Oil-red O solution, then rinsed with 60% isopropanol and distilled water and mounted with cover slips. Morphometric quantification of Oil-red O staining (percentage of area) was performed at 200× in 10 random fields per
Chen H. et al: Role of hepcidin in NASH

Table 1. Primers used for qPCR

| Gene      | Primer sequences                      | Product size | Accession No |
|-----------|---------------------------------------|--------------|--------------|
| Hamp      | F: 5’-CAATGTCGCTGCTCTCTTTTCT-3’       | 113 bp       | NM_032541.2  |
|           | R: 5’-TCTCTCCTGCTTCTCTCTCTTG-3’       |              |              |
| αSMA      | F: 5’-GATGAGGCCAGAGCAAGGAG-3’         | 87 bp        | XM_021152572.1|
|           | R: 5’-CCCTTTATGCTGTCCCGATG-3’         |              |              |
| COL1A1    | F: 5’-GACCGGAGAGATCTGGATCG-3’         | 158 bp       | NM_007742.4  |
|           | R: 5’-GCTTCTTTTTTCTGGGTTTC-3’         |              |              |
| CCR2      | F: 5’-GGCTGAGGATGACAGTTAA-3’          | 76 bp        | NM_011333.3  |
|           | R: 5’-CTTACTCTCTGATACCTTGCT-3’        |              |              |
| TGFβ-1    | F: 5’-GGGCTACCCGCTCTTG-3’             | 70 bp        | NM_011577.2  |
|           | R: 5’-GTGTGAGATGTCTTTGTTTTC-3’        |              |              |
| Il10      | F: 5’-GCTCTTACTGACTGGGATGAG-3’        | 105 bp       | NM_010548.2  |
|           | R: 5’-CGGAGCTCTAGGAGATGGT-3’          |              |              |
| TNFa      | F: 5’-TCCAGAGTTTCTCTCTAGGGA-3’        | 51 bp        | NM_001278601.1|
|           | R: 5’-GGTGAGGACACGATGCTGG-3’          |              |              |
| TIMP-1    | F: 5’-CCAGAGCCTCAGCTTCT-3’            | 126 bp       | NM_001294280.2|
|           | R: 5’-AGGAAGAGTAGCAGTGGTCTAGGCT-3’    |              |              |
| SREBP1c   | F: 5’-TGGGACACATCGCAAAACAG-3’         | 274 bp       | XM_030245748.1|
|           | R: 5’-GTTAGACAAACAGCCGACATC-3’        |              |              |
| ChREBP    | F: 5’-AGATGAGAACCCAGACGTATCA-3’       | 104 bp       | NM_001359237.1|
|           | R: 5’-ACTGACGGTCTGACAAGCTC-3’         |              |              |
| Acc       | F: 5’-GATGAAACATCTCCTCGTGGC-3’        | 65 bp        | XM_03024563.1|
|           | R: 5’-GACCCAAATTATGGAATGGGGAGTG-3’    |              |              |
| Scd1      | F: 5’-TGACTGAGGAGGAGCAGAA-3’          | 342 bp       | NM_009127.4  |
|           | R: 5’-ATGTGCGCCGCTACTCCA-3’           |              |              |
| Adiponectin| F: 5’-TGTTCCATTATCTCTTGCCA-3’         | 104 bp       | NM_009605.5  |
|           | R: 5’-CACAACCTGCCAGTATCTCCCT-3’       |              |              |
| GAPDH     | F: 5’-TCCACTCAGGCCAAATTCACA-3’        | 89 bp        | XM_017321385.1|
|           | R: 5’-CGCTTCTGGAAGATGGTAGT-3’         |              |              |

αSMA, smooth muscle alpha-actin; COL1A1, collagen type I alpha 1 chain; CCR2, C-C motif chemokine receptor 2; TGFβ-1, transforming growth factor-beta 1; Il10, interleukin 10; TNF, tumor necrosis factor; TIMP-1, tissue inhibitor of metalloproteinase 1; SREBP1c, sterol-regulatory element binding protein-1c; ChREBP, carbohydrate response element binding protein; Acc, acetyl coenzyme A carboxylase; Scd1, stearyl-coenzyme A desaturase 1; GAPDH, glyceraldehyde 3-phosphate dehydrogenase.

mouse from five individual animals using ImageJ software.

**Serum biochemistry**

Serum samples were stored at −80°C until analyses could be performed. Serum aspartate aminotransferase (AST), alanine aminotransferase (ALT), and iron were measured using Olympus Beckman Coulter AU480 automatic biochemistry analysis system reagents (InTec Products, Shenzhen, China) provided by the manufacturer. Hemoglobin (Hb) levels were measured using the Mindray Bc 3000 Automatic Blood Cell Analyzer according to the manufacturer’s recommendations.

**Statistical analysis**

Data were expressed as mean±standard error of the mean (SEM) and were analyzed using GraphPad Prism software (v.5; GraphPad Software, La Jolla, CA, USA). A Student’s t-test was used to compare values obtained from two groups. Data from multiple groups were compared using a one-way ANOVA followed by the Tukey’s post-hoc test. Finally, p-values <0.05 were considered significant.

**Results**

rAAV2/8-Hamp treatment increased Hamp expression in CDAO-induced NASH

To investigate the anti-steatotic effects of Hamp, we fed mice a CDAO diet for 16 weeks, which is commonly used to induce steatosis in mouse models of NASH. The mice were administered a total dose of 3×10^{11} genome copies per mouse of either rAAV2/8-Hamp or rAAV2/8-NC after 8 weeks of either...
Chen H. et al: Role of hepcidin in NASH

the CDAA or control CSAA diet (Fig. 1A). We found that Hamp mRNA levels were significantly lower in the CDAA-fed mice compared to the CSAA-fed mice, as expected. We also found that Hamp was increased in all liver tissues from mice injected with rAAV2/8-Hamp compared to rAAV2/8-NC (Fig. 1B).

**rAAV2/8-Hamp treatment reduced body weight gain without affecting iron status and Hb levels**

Both the CSAA and CDAA diets have been shown to cause a progressive and time-dependent increase in body weight. In our model, the CDAA-fed mice gained less body weight (~6% less) compared to the CSAA-fed mice after 16 weeks. It is worth noting that rAAV2/8-Hamp treatment decreased body weight gain in the control group (~5% lower) (Fig. 1C). Food intake (data not shown) following rAAV2/8-Hamp and rAAV2/8-NC treatment in both the CDAA- and CSAA-fed mice increased at similar rates and there were no differences in relative liver weight and spleen weight between the two groups (Fig. 1D–E).

Hamp is a master regulator of systemic iron homeostasis, and therefore tightly controls erythrocyte production. We measured serum iron and Hb levels among the four...
groups of mice. Serum iron levels were significantly higher in the CDAA-fed mice compared to the CSAA-fed mice, but rAAV2/8-Hamp treatment did not significantly decrease serum iron levels compared to rAAV2/8-NC treatment in either the CDAA- or CSAA-fed mice (Fig. 1F). Hb levels were similar among the four groups (Fig. 1G). Our data suggest that overexpression of Hamp in liver tissue does not induce systemic iron overload or affect Hb levels.

**rAAV2/8-Hamp treatment reversed steatosis in CDAA-fed mice**

We next determined whether increased Hamp expression following rAAV2/8-Hamp transduction reversed CDAA-induced hepatic steatosis. HE staining showed evidence of hepatic steatosis (primarily as micro- and macro-steatosis) in the CDAA-fed mice, and rAAV2/8-Hamp treatment remarkably decreased the hepatic lipid deposition in the CDAA-fed mice compared to the rAAV2/8-NC treatment (Fig. 2A). The semi-quantitative steatosis data were confirmed using lipid morphometry on Oil-red O stained liver sections; we observed an approximate three-fold decrease in the rAAV2/8-Hamp treatment group compared to the rAAV2/8-NC treatment group (2.80±0.42 vs. 1.70±0.67, respectively; p<0.05) (Fig. 2B). In addition, the steatosis score was lower in the rAAV2/8-Hamp treatment group compared to the rAAV2/8-NC treatment group (18.91±2.42% vs. 18.91±2.42%, respectively; p<0.05) (Fig. 2C).

The CSAA-fed mice group showed significant glucose intolerance compared to that of the CDAA-fed mice group after a 16-week feeding. rAAV2/8-Hamp treatment significantly improved oral glucose tolerance at 60, 90, and 120 m after gavage with glucose in the CSAA group; however, there was no difference between rAAV2/8-Hamp and rAAV2/8-NC administration in the CDAA group. These results indicate that Hamp effectively attenuated CSAA-induced changes in metabolic parameters (Fig. 2D).

**rAAV2/8-Hamp treatment modulates the expression of lipogenesis-related genes**

To further explore the underlying mechanisms of steatosis-hepatitis, we determined the difference in lipid metabolism following rAAV2/8-Hamp treatment in both the CDAA- and CSAA-fed mice. Compared to the CSAA diet, the CDAA diet significantly down-regulated the expression of genes involved in de novo lipogenesis (Fig. 3A). Consistent with the in vivo study, we found that expression of adiponectin was dramatically elevated in the AML12 cells transfected with plRES2-Hamp compared to plRES2-NC (Fig. 3B–C).

**rAAV2/8-Hamp treatment suppressed liver inflammation in CDAA-fed mice**

We next analyzed liver inflammation in the CDAA- and CSAA-fed mice treated with rAAV2/8-Hamp after 16 weeks. Immunostaining analysis of CD68, a well-established marker of activated macrophages, showed that CD68 expression was significantly increased in the CDAA-fed mice compared to the CSAA-fed mice. Treatment with rAAV2/8-Hamp significantly attenuated CDAA-induced macrophage infiltration and decreased the inflammation score (Fig. 4A–C).

Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) activity significantly increased in the CDAA-fed mice compared to the CSAA-fed mice. Treatment with rAAV2/8-Hamp in the CDAA-fed mice significantly decreased the serum ALT and AST levels compared to the rAAV2/8-NC treatment (Fig. 4D–E).

In line with these findings, livers from the CDAA-fed mice had increased mRNA levels of chemokine (C-C motif) receptor 2 (CCR2) and tumor necrosis factor alpha (TNFα) compared to the CSAA-fed mice, whereas expression of these genes was suppressed in CDAA-fed mice treated with rAAV2/8-Hamp (Fig. 4F–G). The mRNA levels of Il10, a potent anti-inflammatory cytokine, were significantly higher in the livers of the CDAA-fed mice compared to the CSAA-fed mice, and expression of Il10 was slightly increased in the CDAA-fed mice treated with rAAV2/8-Hamp (Fig. 4H).

**rAAV2/8-Hamp treatment ameliorated liver fibrosis in CDAA-fed mice**

Finally, we evaluated liver fibrosis in the CDAA- and CSAA-fed mice treated with rAAV2/8-Hamp after 16 weeks. Collagen deposition and HSC activation were significantly increased in the CDAA-fed mice compared to the CSAA-fed mice, and rAAV2/8-Hamp treatment reduced these fibrosis markers in the CDAA-fed mice, as assessed by SR staining and IHC detection of αSMA (Fig. 5A–C). Similarly, livers from CDAA-fed mice had increased mRNA levels of CO-L1A1, αSMA, and TIMP-1 compared to CSAA-fed mice, and rAAV2/8-Hamp treatment significantly suppressed the expression of these genes (Fig. 5D–F).

**Hamp supplementation showed anti-fibrotic effects in HSCs in vitro**

Considering the major pathophysiological role that HSCs have in fibrogenesis, we investigated the effect of Hamp in cultured HSC T6 cells. Specifically, we measured the expression of fibrogenetic genes (αSMA, TIMP-1, COL1A1, and transforming growth factor beta 1 [TGFβ-1]). Hamp (10 and 100 ng/mL) dose-dependently and significantly reduced the mRNA levels of fibrogenetic genes after 48 h of incubation compared to unstimulated HSCs (Fig. 6A–D). Reduction of αSMA and TIMP1 were also confirmed at the protein level, as determined by quantitative western blot analysis (Fig. 6E).

**Discussion**

Iron-load is prevalent in a third of NAFLD patients and can...
Fig. 2. rAAV2/8-Hamp treatment showed anti-steatotic properties in CDAA-fed mice. (A) Representative HE (original magnification 200×) and Oil-red O staining images (original magnification 100×) in liver sections of mice treated with rAAV2/8-Hamp or rAAV2/8-NC for up to 16 weeks. (B) Quantification of Oil-red O staining images of mice fed either the CDAA or CSAA diet. (C) Liver steatosis scores. (D) Oral glucose tolerance test. (E-I) Hepatic transcript levels of ChREBP, SREBP1c, Scd1, Acc, and adiponectin in mice fed either the CDAA or CSAA diet for 16 weeks. Data represent the mean±SEM of at least 10 animals per group. HE, hematoxylin-eosin; CDAA, choline-deficient L-amino acid-defined; CSAA, choline-supplemented amino acid-defined; rAAV, recombinant adeno-associated virus; ChREBP, carbohydrate response element binding protein; SREBP1c, sterol-regulatory element binding protein-1c; Scd1, steatoyl-coenzyme A desaturase 1; Acc, acetyl coenzyme A carboxylase.
accelerate the progression of steatosis, fibrosis, cirrhosis, and hepatocellular carcinoma. As such, iron removal therapy has become a potential treatment strategy for NASH.\textsuperscript{6,7} Given its role in regulating iron homeostasis, Hamp has gained attention as a promising therapeutic agent that can remove liver iron stores.\textsuperscript{13,14} In the present study, our data showed that rAAV2/8-Hamp treatment significantly suppressed CDAA diet-induced steatosis, hepatic inflammation, and subsequent liver fibrosis in mice.

A choline-deficient diet can increase the onset of steatohepatitis features and fibrosis in mice, similar to patients with rapid NASH progression. Since our main focus is understanding how to best treat NASH-related fibrosis, we established the CDAA mouse model to best mirror pathology and pathogenesis of human NASH. In agreement with previous studies, the CDAA diet resulted in moderate hepatic lipoapoptosis, liver inflammation, and fibrosis, while the CSAA diet led to severe insulin resistance and absence of inflammation and fibrosis.\textsuperscript{22–24} Mice on the CDAA developed macrovesicular steatosis in the liver, but the CDAA-fed mice developed a metabolic profile opposite to what is observed in human disease. We found that the CDAA diet significantly down-regulated the expression of genes involved in fatty acid synthesis, which might result from compensatory hepatic uptake of serum lipids or by impairment in very low-density lipoprotein secretion from the liver. NAFLD in patients has a complex and heterogeneous pathogenesis, thus it should be pointed out that animal models of NAFLD may not recapitulate all characteristics of human disease.

In our study, we first found that Hamp expression was significantly down-regulated and that iron stores were increased in the CDAA-fed mice, suggesting an important role for Hamp in the pathogenesis of NASH. Hamp is mostly produced by hepatocytes in response to iron loading in cells. As iron loads increase, Hamp expression also increases in hepatocytes, resulting in elevated serum Hamp level.\textsuperscript{25} Patients with chronic liver diseases have evidence of liver dysfunction and anemia associated with inflammation but surprisingly also have lower serum Hamp levels compared to control subjects.\textsuperscript{26} Similar findings have been reported in alcoholic chronic liver diseases,\textsuperscript{27} chronic hepatitis C,\textsuperscript{28,29} hepatitis B virus-related cirrhosis,\textsuperscript{30–32} and in autoimmune liver diseases.\textsuperscript{30} Hamp levels in NAFLD are difficult to interpret, since Hamp expression is likely to be regulated by complex mechanisms in response to diverse pathological stimuli. In the later stages of NAFLD/NASH, serum Hamp levels are not suppressed, and the levels eventually decrease in NAFLD with advanced fibrosis, similar to other liver diseases.\textsuperscript{18}

Although accumulating evidence shows that serum Hamp levels and iron metabolism are related to serum markers of steatosis, inflammation, fibrosis, and insulin resistance, no study has investigated if Hamp supplementation could be used as a therapeutic strategy for NASH. Here, we first investigated the possible effect of rAAV2/8-Hamp on CDAA diet-induced hepatic steatosis in mice. Our data demonstrated that treatment with rAAV2/8 significantly attenuated CDAA diet-induced hepatic steatosis without affecting iron and Hb levels. While the CDAA diet is a well-established nutritional NAFLD model, the metabolic profile of this diet does not completely reflect all properties of NAFLD. Specifically, other aspects of metabolic dysregulation are not necessarily accounted for because hepatic lipid accumulation in the model is mainly due to impaired secretion of very low-density lipoprotein. Our study showed that overexpression of Hamp corrected the abnormal expression of SREBP1c and ChREBP. This could partially explain the mechanism by which rAAV2/8-Hamp treatment can suppress CDAA diet-induced hepatic steatosis and regulate lipid metabolism. Furthermore, we noted that rAAV2/8-Hamp treatment normalized the loss in body weight in the CDAA diet-induced NASH model.

Increased inflammation is a hallmark of NASH. Thus, controlling liver inflammation may be a potential strategy to treat NASH. Macrophages are key cells that induce the release of inflammatory mediators, such as TNFα, CCR2, and
Fig. 4. rAAV2/8-Hamp treatment suppressed hepatic inflammation in CDAA-fed mice. (A) Representative CD68-positive macrophages (original magnification 200×) in liver sections of mice treated with rAAV2/8-Hamp or rAAV2/8-NC. (B) Quantification of images of mice fed either the CDAA or CSAA diet. rAAV2/8-Hamp treatment decreased (D) serum ALT and (E) AST levels in mice fed a CDAA diet compared with rAAV2/8-NC treatment. (F–H) Transcript levels of CCR2, TNFα and IL10. Data represent the mean±SEM of at least 10 animals per group. CDAA, choline-deficient L-amino acid-defined; CSAA, choline-supplemented amino acid-defined; rAAV, recombinant adeno-associated virus; ALT, alanine aminotransferase; AST, aspartate aminotransferase; NC, negative control; CCR2, C-C Motif chemokine receptor 2; TNF, tumor necrosis factor; IL10, interleukin 10.
IL1β in NASH. These inflammatory mediators further stimulate hepatocytes and HSCs to induce hepatocyte steatosis and fibrosis, respectively. As expected, overexpression of Hamp decreased the CDAA diet-induced levels of hepatic CD68-positive macrophages and altered the inflammatory response in our model, as indicated by a down-regulation in pro-inflammatory cytokines (TNFα and CCR2) and a slight up-regulation in the anti-inflammatory cytokine (IL10).

Hamp expression is controlled mainly at the transcriptional level by various stimuli, including inflammation, iron status, and hypoxia. The link between inflammation/infection and liver production of Hamp is attributed to IL6 produced at the sites of infection/inflammation. IL6 binds to the IL6-receptor and phosphorylates JAK-2/STAT3, which binds to and activates the Hamp promoter. Importantly, Hamp can also influence the function of macrophages.
Chen H. et al.: Role of hepcidin in NASH

tanova et al. reported that Hamp-induction by IL6 and/or iron could reduce the secretion of IL4 and IL13 in macrophages, thereby inhibiting cardiac repair. In contrast, Hamp-deficiency in macrophages promoted the release of IL4 and IL13 by recombinant IL6. It has been suggested that Hamp is an upstream repressor of reparatory cytokines (IL4 and IL13) secreted by cardiac macrophages. A similar study showed that Hamp induces M1 macrophage polarization. In contrast, another study showed that Hamp reduced M1 polarization of RAW264.7 macrophages. The authors explain that this discrepancy may be caused by factors such as differential simulation, iron concentration, and cell condition. We found that overexpression of Hamp in liver cells decreased the number of CD68-positive macrophages in the liver. Moreover, mRNA expression of pro-inflammatory cytokines, such as CCR2 and TNFα, decreased significantly, suggesting a role for Hamp in liver inflammation and macrophage infiltration.

Our study suggests an important role of Hamp in HSC activation, which results in hepatic fibrosis following a CDAA diet. The CDAA diet decreased Hamp expression, which was accompanied by HSC activation, as demonstrated by an increase in αSMA-positive cells. Furthermore, expression of fibrotic genes (αSMA, TIMP-1, and COL1A1) was significantly reduced in the livers of the CDAA-fed mice following rAVV2/8-Hamp treatment. Consistent with our data, pharmacological administration of Hamp has been shown to improve fibrosis by blocking activation of HSCs both in the CCl4 and bile duct ligation fibrosis models. Conversely, when challenged with an iron-overload diet, Hamp knockout mice displayed significant liver fibrosis associated with iron accumulation and stellate cell activation. In liver fibrosis, low Hamp levels cause high iron load and oxidative stress. Oxidative stress and lack of Hamp-induced suppression induces HSC activation, which results in scar tissue deposition and liver fibrosis. Cell-based assays provide a mechanism whereby exogenous Hamp hinders TGFβ1-induced SMAD3 phosphorylation in HSCs, inhibiting their activation. On the other hand, there have been contradictory reports as to the role of Hamp in NASH/NAFLD. Hamp knockout NAFLD mice develop liver damage. Although loss of Hamp is associated with a reduction in liver steatosis, liver fibrosis is present early and is more pronounced in the knockout mice compared to mice with normal Hamp expression. Different animal models of NASH involve several factors, including various degrees of hepatocyte damage, insulin resistance, inflammation, and lipid metabolism, all of which can affect the regulation of Hamp. Therefore, regulation of Hamp and consequently iron homeostasis need to be further investigated.

It is noteworthy that the beneficial effects of Hamp overexpression on lipid metabolism and inflammation may be due to HSC activation and induced adiponectin expression. Adiponectin is the predominant adipokine made by adipose tissue and is involved in the regulation of hepatic lipid metabolism. In addition to its metabolic effects, growing evidence suggests that adiponectin possesses potent anti-fibrotic and anti-inflammatory properties.

Fig. 6. Effect of hepcidin on HSCs activation. HSCs were stimulated with hepcidin (10 or 100 ng/ml) for 48 h. (A–D) Gene expression of (A) αSMA, (B) TIMP-1, (C) COL1A1, and (D) TGFβ-1 in HSCs. (E) Protein expression of αSMA and TIMP1 in HSCs. Data represent the mean±SEM. HSCs, hepatic stellate cells; αSMA, smooth muscle alpha-actin; TIMP-1, tissue inhibitor of metalloproteinase 1; COL1A1, collagen type I alpha 1 chain; TGFβ-1, transforming growth factor-beta 1.
Chen H. et al: Role of hepcidin in NASH

Altogether, we demonstrated for the first time that rAAV2/8-Hamp treatment ameliorated CDDA-induced inflammation and related liver fibrosis as well as improved lipid metabolic abnormalities, suggesting that rAAV2/8-mediated Hamp intervention may have beneficial effects on NASH. This increase in hepatic Hamp produced a marked induction of adiponectin both in vivo and in vitro. Furthermore, Hamp directly inhibited HSC fibrogenesis in vitro. To fully understand the protective and therapeutic function of Hamp, other dietary animal models, such as high fat- or western diet-induced NAFLD, should be explored.

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Conflict of interest

The authors have no conflict of interests related to this publication.

Author contributions

Study conception and drafting of the manuscript (HC, AY), and performance of experiments or analysis of the resultant data (HC, WZ, XY, TH, AY). All authors read or revised the manuscript.

Data sharing statement

All data are available upon request.

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Journal of Clinical and Translational Hepatology 2022 vol. 10(4)  |  577–588
Chen H. et al: Role of hepcidin in NASH

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