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ORIGINAL ARTICLE

Basic Study

Lack of hepcidin expression attenuates steatosis and causes fibrosis in the liver

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Abstract

AIM: To investigate the role of key iron-regulatory protein, hepcidin in non-alcoholic fatty liver disease (NAFLD).

METHODS: Hepcidin (*Hamp1*) knockout and floxed control mice were administered a high fat and high sucrose (HFS) or a regular control diet for 3 or 7 mo. Steatosis, triglycerides, fibrosis, protein and gene expression in mice livers were determined by histological and biochemical techniques, western blotting and real-time polymerase chain reaction.

RESULTS: Knockout mice exhibited hepatic iron accumulation. Despite similar weight gains, HFS feeding induced hepatomegaly in floxed, but not knockout, mice. The livers of floxed mice exhibited higher levels of steatosis, triglycerides and c-Jun N-terminal kinase (JNK) phosphorylation than knockout mice. In contrast, a significant increase in fibrosis was observed in knockout mice livers within 3 mo of HFS administration. The hepatic gene expression levels of sterol regulatory element-binding protein-1c and fat-specific protein-27, but not peroxisome proliferator-activated receptor-alpha or microsomal triglyceride transfer protein, were attenuated in HFS-fed knockout mice. Knockout mice fed with regular diet displayed increased carnitine palmitoyltransferase-1a and phosphoenolpyruvate carboxykinase-1 but decreased glucose-6-phosphatase expression in the liver. In summary, attenuated steatosis correlated with decreased expression of lipogenic and lipid storage genes, and JNK phosphorylation. Deletion of *Hamp1* alleles *per se* modulated hepatic expression of beta-oxidation and gluconeogenic genes.

CONCLUSION: Lack of hepcidin expression inhibits hepatic lipid accumulation and induces early development of fibrosis following high fat intake. Hepcidin and iron may play a role in the regulation of metabolic pathways in the liver, which has implications for NAFLD pathogenesis.

Key words: *Hamp*; Iron; Non-alcoholic steatohepatitis; Metabolic genes; Steatosis; Non-alcoholic fatty liver disease; Steatohepatitis

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Core tip: Due to obesity epidemic the incidence of non-alcoholic fatty liver disease (NAFLD) is on the rise. Iron contributes to disease severity and the expression of key iron regulatory hormone, hepcidin is modulated in NAFLD patients. The underlying mechanisms are unknown. We have generated hepcidin knockout mice with iron overload phenotype. This study investigates the role of hepcidin in NAFLD by using high fat and high sucrose-fed knockout mice as an experimental model of NAFLD. Our findings showed attenuated steatosis and early fibrosis development suggesting a role for hepcidin in the regulation of metabolic processes in the liver, and in NAFLD.

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INTRODUCTION

Non-alcoholic fatty liver disease (NAFLD) encompasses a spectrum of liver disease ranging from simple benign steatosis to non-alcoholic steatohepatitis (NASH). NASH, a more aggressive form of disease, is characterized by the presence of lobular inflammation, fibrosis, hepatocellular ballooning and Mallory-Denk bodies[1,2]. NASH with progressive fibrosis can progress to cirrhosis and end stage liver disease[1,3,4].

The precise mechanisms of NASH development are not well understood. Although a so-called "two-hit hypothesis"[5] has been widely adopted[6,7], NASH can also develop in the absence of insulin resistance and simple benign steatosis (*i.e.*, initial hit)[8]. The potential candidates regarded as the "second hit" include oxidative stress, inflammation and changes in mitochondrial function[7,9-12]. Iron is also considered as a "second hit" in liver injury[13] and a role for iron has been reported in NASH pathogenesis. Patients with NAFLD/NASH frequently display elevated serum iron indices and hepatic iron content[14,15]. A strong correlation between hepatic iron content and the level of liver fibrosis in NAFLD/NASH patients has been shown[16-18]. Phlebotomy has also been suggested to alleviate insulin resistance in NAFLD patients[19].

The mechanisms by which iron contributes to NAFLD/NASH pathogenesis have mainly been attributed to oxidative stress, which can induce lipid peroxidation[20] and ultimately the activation of fibrotic signaling[21]. Studies with genetic haemochromatosis (GH) patients have shown the association of primary iron overload with fibrogenesis[22]. By using dietary experimental models, some studies have also suggested a reverse connection between iron and steatosis in rat livers[23,24]. In contrast, another study with a mouse dietary model of iron and high fat failed to show any significant effect of iron on steatosis[25]. The consequences of altered iron homeostasis for lipid metabolism in the liver are therefore unclear.

In this study, we employed hepcidin knockout mice with iron overload phenotype as an experimental model to further study the role of iron metabolism in NAFLD/NASH. Hepcidin is the central regulator of iron homeostasis, which is primarily synthesized in hepatocytes as a circulatory protein[26]. Unlike humans, which express only one hepcidin gene, *HAMP*, mice express two hepcidin genes, hepcidin (*Hamp1*) and *Hamp2*[27]. *Hamp1*, the human equivalent of mouse hepcidin gene, is by itself sufficient to regulate iron metabolism[28,29]. Hepcidin controls iron homeostasis by decreasing iron absorption from the absorptive enterocytes in the duodenum and the release of iron from the macrophages[30]. The lack of hepcidin expression in knockout mice and in human iron disorders results in iron accumulation both in the liver and other organs[30-32]. GH patients also display impaired hepcidin expression[33]. Although changes in both serum and liver hepcidin expression levels have been reported in NAFLD/NASH patients[14,34-38], the significance of hepcidin in disease pathogenesis is unknown. Our findings in this study with *Hamp1* knockout mice administered a high fat diet for different time points suggest a role for hepcidin in NAFLD/NASH pathogenesis. This mouse model may also serve as a novel experimental model of NAFLD/NASH.

MATERIALS AND METHODS

Animal studies

Animal experiments were approved by the Institutional

Table 1 SYBR green real-time quantitative polymerase chain reaction primer sequences of mouse genes

Mouse genes	Forward primer (5'-3')	Reverse primer (5'-3')
Mttp	CTCTTGGCAGTGCTTTTTCTCT	GAGCTTGTATAGCCGCTCATT
Cpt1a	CTCCGCCTGAGCCATGAAG	CACCAGTGATGATGCCATTCT
Fsp27	ATGAAGTCTCTCAGCCTCCTG	AAGCTGTGAGCCATGATGC
G6pc	CGACTCGCTATCTCCAAGTGA	GTTGAACCAGTCTCCGACCA
Pck1	CTGCATAACGGTCTGGACTTC	CAGCAACTGCCCGTACTCC
Pparα	AGAGCCCCATCTGTCCTCTC	ACTGGTAGTCTGCAAAACCAAA
Srebp-1c	GCAGCCACCATCTAGCCTG	CAGCAGTGAGTCTGCCTTGAT
Gapdh	GTGGAGATTGTTGCCATCAACGA	CCCATTCTCGGCCTTGACTGT

Animal Care and Use Committee at the University of Nebraska Medical Center. *Hamp1* floxed mice and ubiquitous *Hamp1* knockout mice, lacking hepcidin expression in all the organs, were generated, as published previously[29]. All mice are on C57BL/6J genetic background. *Hamp* floxed mice have been donated to the Jackson Laboratory (Catalog No. 026872, 026873).

Male mice (4-6-wk-old) were randomly separated into groups to feed with custom-made regular control (17.2% kcal from fat, 100 g/kg sucrose) or high fat and high sucrose (HFS) [42% kcal from fat (54% saturated, 9.7% trans-fat), 0.4% cholesterol, 340 g/kg sucrose] diets for 3 or 7 mo (Harlan Laboratories; TD.97184; TD.120654). Water was given ad libitum, and contained sucrose (40 g/L) with HFS-fed groups to imitate the western diet with fat and soda consumption.

Liver histology

Formalin-fixed, paraffin-embedded liver tissues were sectioned and stained with hematoxylin and eosin at UNMC Histology Core Facility. To determine fibrosis, sections were stained with Picrosirius Red, as published previously[39] and histomorphometric analyses were performed using ImageJ ROI manager software.

Quantification of liver triglycerides

Triglycerides were isolated, as described[40] and quantified using a commercial kit (Thermo Scientific DMA kit 2750) according to manufacturer's instructions.

Real-time polymerase chain reaction

cDNA was synthesized from liver tissue RNA with Superscript Ⅱ reverse transcriptase (Invitrogen), as described[41]. Real-time polymerase chain reaction (PCR) reactions were performed using iTaq Universal SYBR Green Supermix (Bio-Rad) with a StepOnePlus instrument (Life Technologies). Glyceraldehyde 3-phosphate dehydrogenase (*Gapdh*) gene was used as the endogenous control and gene amplification was calculated using comparative Ct method, as described[41]. Primer sequences are shown in Table 1.

Western blotting

Western blots using whole liver tissue lysate proteins were performed, as published previously[41]. Antibodies were obtained commercially (Cell Signaling, Sigma) and immune-reactive bands were detected by the ImmunStar™ kits (Bio-Rad).

Statistical analysis

The significance of differences between groups was determined by Student's *t*-test or one-way ANOVA by using SPSS software. A value of $P < 0.05$ was accepted as statistically significant.

RESULTS

To study the interaction of hepcidin-induced iron overload and lipid metabolism, ubiquitous *Hamp1* knockout and floxed control mice were administered either high fat and HFS or regular (control) diets, as described in Material and Methods. Since NAFLD/NASH progression can occur over a long period of time, mice were fed up to 7 mo. We have previously shown that the deletion of both *Hamp1* alleles induces significant iron overload in the livers of *Hamp1* knockout mice by using inductively coupled mass spectrometry (ICP-MS)[29]. ICP-MS analysis did not detect any significant level of iron in the livers of homozygous *Hamp1* floxed control mice. Gradual iron deposition was also indicated macroscopically by the darker color of knockout mice livers compared to those of floxed control mice (Figure 1).

Macroscopic analyses have confirmed that HFS intake induced hepatomegaly and more pronounced visceral fat accumulation in floxed control mice compared to knockout mice (Figure 1). In agreement, the liver weights of floxed mice were significantly higher (3.5 ± 0.46 g) than those of knockout mice (2.42 ± 0.54 g) particularly following 7 mo of HFS administration (Figure 2A and B). However, HFS intake induced similar increases in body weights in both floxed (Figure 2C) and knockout (Figure 2D) mice after either 3 or 7 mo-long feeding, as compared to respective controls fed with regular diet.

To further understand these discrepancies between floxed and knockout mice, histological analysis were performed. Hematoxylin and eosin staining of livers showed significantly higher levels of steatosis in floxed than in knockout mice both after 3 and 7 mo-long HFS feeding (Figure 3). The quantification of hepatic triglycerides further confirmed that HFS intake signifi-

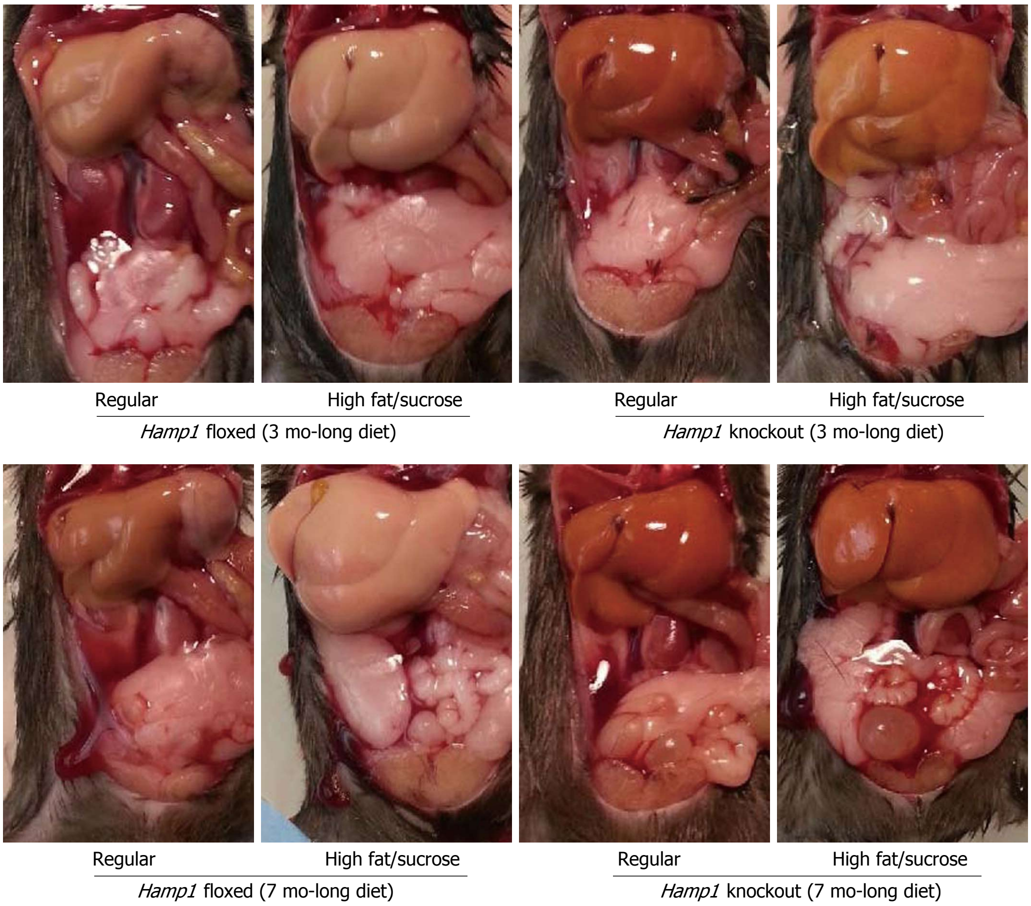

Figure 1 Macroscopic changes in *Hamp1* floxed and knockout mice fed with either a high fat and high sucrose or a regular control diet for 3 or 7 mo. Representative images showing the abdominal cavity of mice were obtained with a digital camera (Nikon).

cantly increased hepatic triglyceride content to different extents in floxed and knockout mice (Figure 4). At the end of 3 mo-long high fat intake, the level of hepatic triglyceride accumulation was 2.85-fold higher in floxed mice compared to knockout mice (1876.64 ± 370.84 and 657.98 ± 186.89 μmol/L per 100 g BW) (Figure 4A). Seven mo-long feeding yielded 2.07-fold higher hepatic triglyceride content in floxed than in knockout mice (1837.71 ± 118.12 and 886.91 ± 89.51 μmol/L per 100 g BW) (Figure 4B).

Sirius Red staining of liver sections showed that knockout, but not floxed, mice developed fibrosis within 3 mo of high fat intake (Figure 5A). The deletion of both *Hamp1* alleles per se has also caused weaker but significant level of fibrosis in the livers of knockout mice (Figure 5A). Quantification by ImageJ analysis has shown a 2.56-fold higher level of fibrosis in the livers of high fat-fed knockout than regular diet-fed knockout mice at 3 mo (Figure 5B) In contrast to 3 mo, 7 mo of high fat intake induced fibrosis in the livers of floxed mice (Figure 6A). Compared to 3 mo, regular diet feeding for 7 mo slightly increased the level of fibrosis in knockout mice livers (Figure 6A). Knockout mice with 7 mo-long high fat intake developed the highest level of fibrosis, as shown by Image J quantification (Figure 6B). The hepatic expression patterns of alpha smooth muscle actin (αSMA) protein, a marker for hepatic stellate cell activation, were in agreement with our histological analysis. Three months-long HFS feeding elevated liver αSMA expression in knockout, but not floxed, mice, as shown by Western blotting (Figure 7A). The deletion of *Hamp1* alleles by itself increased hepatic αSMA expression (Figure 7A). In contrast to 3 mo, 7 mo-long high fat intake increased αSMA expression in the livers of both floxed and knockout mice (Figure 7A).

Studies with JNK knockout mice fed with methionine-choline-deficient diet (MCD) diets have indicated a role for c-Jun N-terminal kinase (JNK) in steatosis[42]. JNK is activated by phosphorylation on serine residues[43]. The expression levels of phosphorylated JNK protein in the livers of *Hamp1* transgenic mice were therefore determined by western blotting using specific anti-phospho JNK antibodies (Figure 7B). Three-month-long high fat intake significantly stimulated JNK phosphorylation in the livers of floxed, but not knockout, mice (Figure 7B). In contrast, the effect of high fat intake on JNK phosphorylation in the liver was weakened by 7 mo-long feeding (Figure 7B).

To further investigate the underlying mechanisms of attenuated fat accumulation in the livers of knockout mice with high fat intake, mRNA expression levels of genes, which are known to be involved in lipid meta-

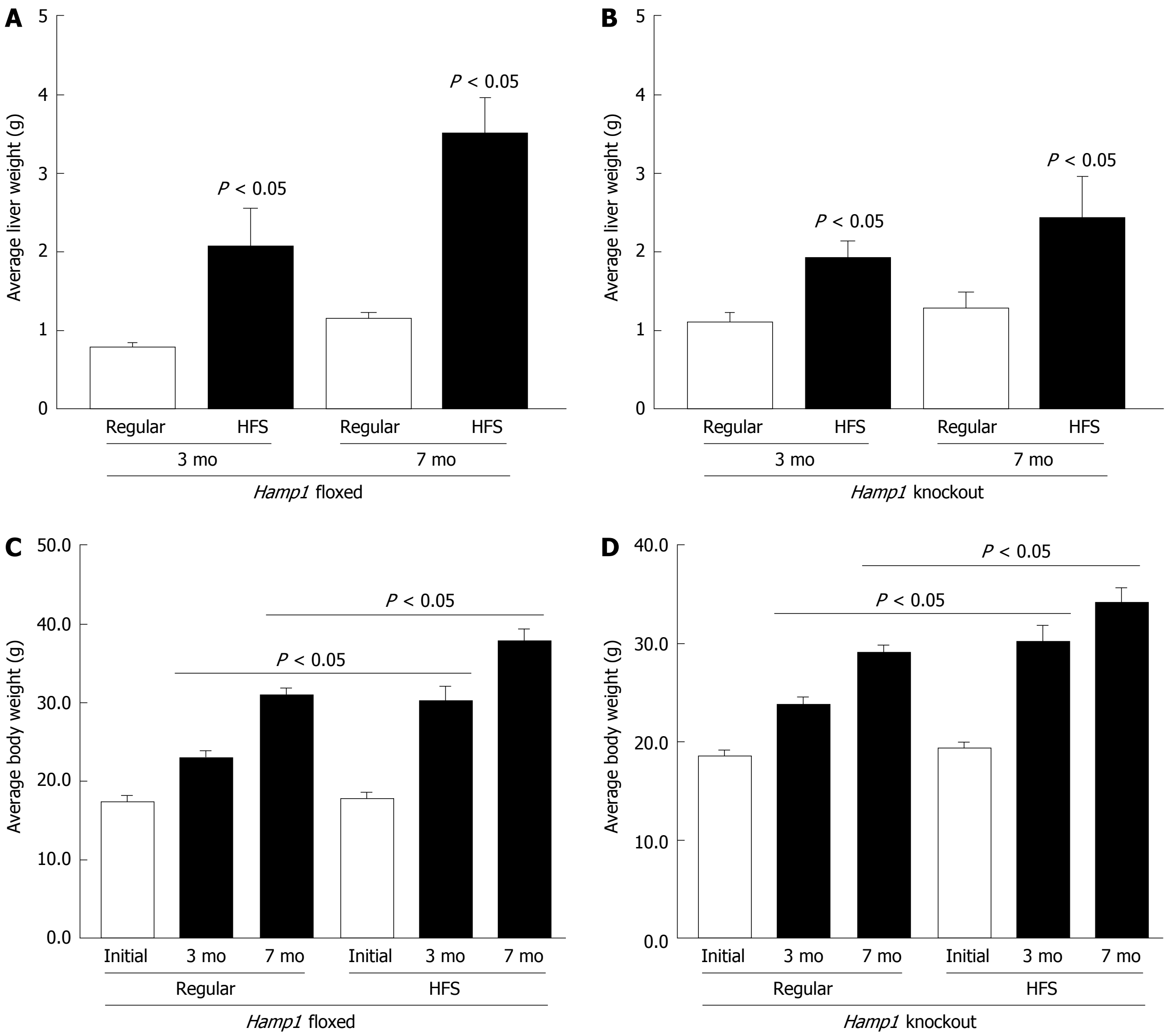

Figure 2 Liver and body weights of *Hamp1* floxed and knockout mice fed with high fat or regular diets. Average liver (A and B) and body (C and D) weights of floxed (A and C) and knockout (B and D) mice prior to (initial) and after feeding with high fat and sucrose (HFS) or regular control diets for 3 or 7 mo are shown as gram weight.

bolism, were examined by real-time PCR (Figure 8). The transcription factor, sterol regulatory element-binding protein-1c (Srebp-1c) is involved in *de novo* lipogenesis and its expression is also regulated at the transcriptional level[44,45]. The deletion of *Hamp1* alleles did not significantly alter basal hepatic expression levels of *Srebp-1c* (Figure 8A and B). Three months of high fat intake stimulated *Srebp-1c* expression by 13.39-fold in floxed and 7.40-fold knockout mice compared to controls (Figure 8A). In contrast, 7 mo of high fat intake elevated *Srebp-1c* expression only by 3.72-fold in floxed mice (Figure 8B). Furthermore, 7 mo-long high fat feeding did not significantly alter liver *Srebp-1c* expression in knockout mice (Figure 8B).

Fat-specific protein-27 (Fsp27) protein is involved in lipid droplet formation[46]. HFS feeding for 3 and 7 mo significantly induced *Fsp27* expression in the livers of floxed mice by 3.83- and 5.36-fold, respectively compared to regular diet-fed floxed mice (Figure 8C and D). The livers of knockout mice fed with HFS for 3 or 7 mo displayed significantly lower induction of *Fsp27* expression than floxed mice, which was more prominent at 7 mo (Figure 8C and D). Liver *Fsp27* expression was not significantly altered in knockout mice fed with regular diets for 3 or 7 mo compared to respective floxed controls (Figure 8C and D).

Microsomal triglyceride transfer protein (Mttp) protein is responsible for the production and secretion of VLDL particles[47]. The mRNA expression level of Mttp in the liver was not significantly altered in floxed and knockout mice after 3 mo of high fat intake (Figure 8E). However, high fat exposure for 7 mo significantly suppressed *Mttp* expression in the livers of both floxed and knockout mice (Figure 8F).

Changes in fatty acid oxidation in the liver play an important role in NAFLD pathogenesis. Peroxisome proliferator-activated receptor-alpha (Pparα) activates the transcription of genes involved in the regulation of

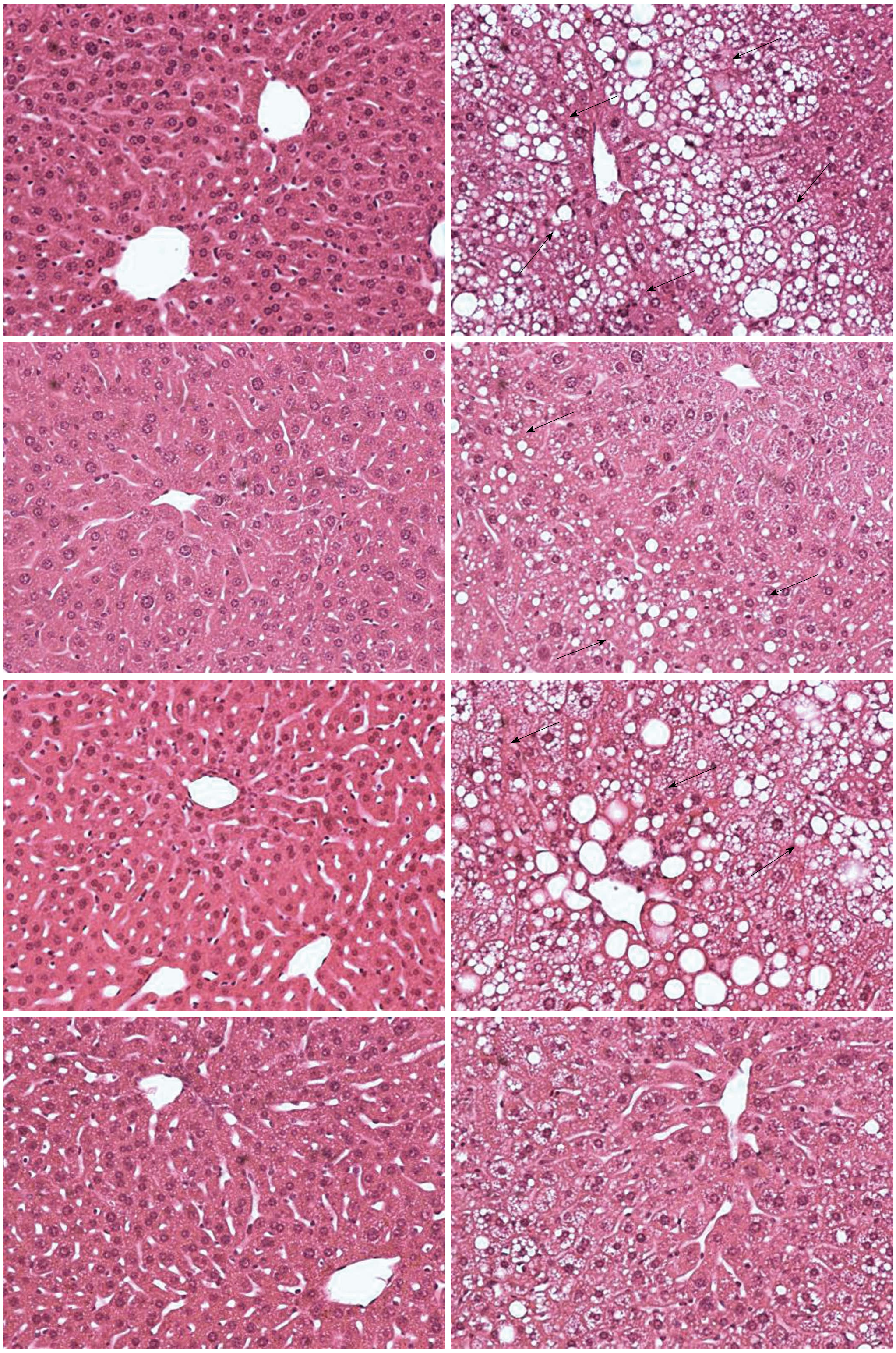

Figure 3 Liver histology in *Hamp1* floxed and knockout mice fed high fat or regular diets. Liver sections from floxed and knockout mice fed with high fat and sucrose (HFS) or regular diets for 3 and 7 mo were stained with hemotoxylin and eosin. Representative images obtained with a Nikon Eclipse E400 light microscope are shown (20 ×). Arrows indicate steatosis.

fatty acid β-oxidation[48]. The mRNA expression levels of Pparα were up-regulated at similar levels in the livers of both floxed and knockout mice within 3 mo of high fat feeding (Figure 9A). In contrast, the livers of floxed and knockout mice with 7 mo of high fat exposure displayed significantly inhibited Pparα expression (Figure 9B). Carnitine palmitoyltransferase-1 (Cpt1) is the rate-limiting enzyme in mitochondrial β-oxidation pathway[49].

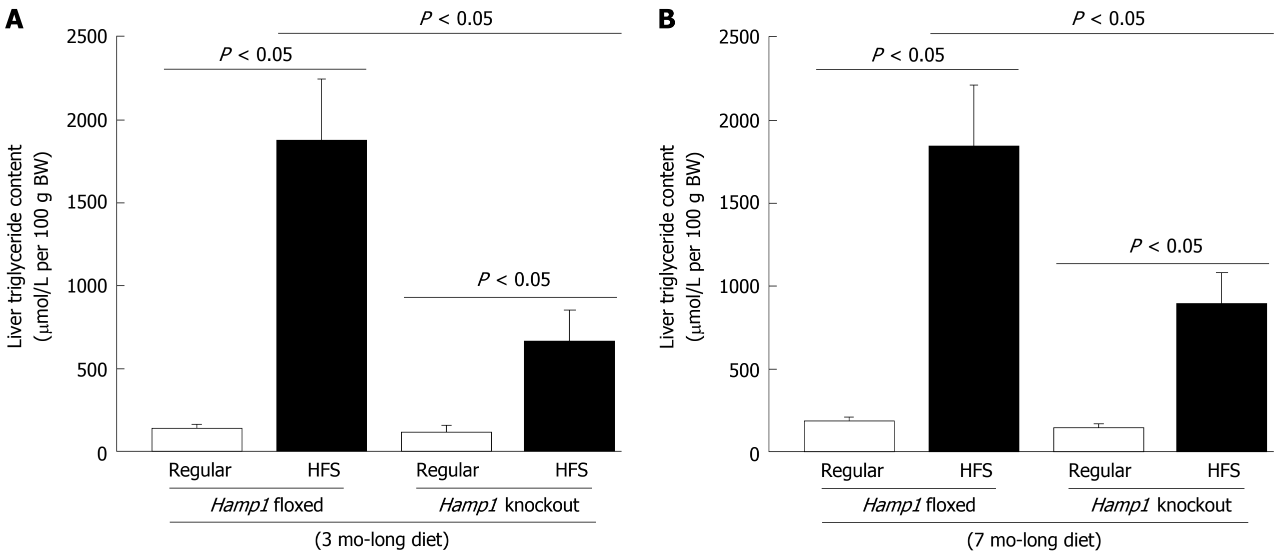

Figure 4 Liver triglyceride content in *Hamp1* floxed and knockout mice fed high fat or regular diets. Hepatic triglyceride content in floxed and knockout mice fed with regular or high fat sucrose (HFS) diets for 3 (A) or 7 (B) mo was quantified using 50 mg of wet liver tissue. Liver triglyceride amount was expressed as μmol per liver per 100 g body weight (μmol/L per 100 g BW).

Three month-long high fat administration did not exert a significant effect on hepatic *Cpt1a* expression in floxed and knockout mice (Figure 9C). On the other hand, the livers of knockout mice fed with regular diet for 7 mo expressed higher *Cpt1a* levels compared to floxed mice fed under similar conditions, suggesting a role for gradual iron deposition (Figure 9D). Seven month-long high fat intake did not alter hepatic *Cpt1a* expression in floxed mice (Figure 9D). In contrast, long-term high fat exposure significantly suppressed *Cpt1a* expression in the livers of knockout mice compared to knockout controls (Figure 9D).

Both phosphoenolpyruvate carboxykinase-1 (Pck1) and glucose-6-phosphatase (G6pc) are involved in gluconeogenesis. Similar to *Cpt1a*, the deletion of *Hamp1* alleles significantly up-regulated basal *Pck1* mRNA expression in the liver. In contrast, the absence of hepcidin expression suppressed basal hepatic *G6pc* mRNA expression (Figure 9E-H). Both 3 and 7 mo-long high fat exposure significantly inhibited *Pck1* and *G6pc* mRNA expression in the livers of both floxed and knockout mice (Figure 9E-H).

DISCUSSION

Changes in iron metabolism contribute to liver injury[22,50]. The deposition of iron in the liver correlates with disease severity in NAFLD patients[15]. The mechanisms by which excess iron contribute to NAFLD pathogenesis is unclear. Although inconclusive, some studies suggested a role for iron in the regulation of lipid metabolism[23-25]. Since hepcidin is the central regulator of iron metabolism, we investigated its role in fatty liver disease. We and others showed iron accumulation in *Hamp1* knockout mice[29,31,51]. *Hamp1* knockout mice were administered high fat diets for different time periods to generate pathological features in the liver, which are representative of NAFLD/NASH[2]. Collectively, our findings showed a strong correlation between hepcidin and lipid metabolism, and fibrosis in the liver.

The absence of hepcidin expression in *Hamp1* knockout mice exerted an inhibitory effect on hepatic lipid accumulation. This effect was not due to altered rates of diet consumption or weight gain and suggests the involvement of regulatory mechanisms. Previous studies showed a converse relationship between iron and lipid metabolism[22,23]. Since lack of hepcidin expression causes iron overload, elevated hepatic iron content may have interfered with fat accumulation in HFS-fed knockout mice. Furthermore, our findings suggest a role for JNK in this process. Namely, we showed a direct correlation between JNK phosphorylation and steatosis levels in floxed mice livers. In contrast, the livers of *Hamp1* knockout mice did not display significant JNK phosphorylation. Of note, the deletion of JNK1 reverses steatosis[52,53] and JNK is activated by phosphorylation[43]. Hepcidin-mediated changes in JNK activation may therefore be associated with attenuated steatosis in *Hamp1* knockout mice, particularly in early stages of high fat exposure.

Besides iron and JNK, altered metabolic gene expression in high fat-fed knockout mice may play a role in the inhibition of lipid accumulation. This is supported by our findings, which showed that the hepatic expression level of genes involved in lipogenesis and lipid storage do not adequately respond to high fat intake in *Hamp1* knockout mice. Namely, *Srebp-1c* and *Fsp27* expression were blunted in the livers of HFS-fed knockout, but not floxed, mice. These findings are significant because *Srebp-1c* and *Fsp27* expression are regulated at mRNA level[54]. Furthermore, the deletion of *Hamp1* alleles did not alter their basic expression levels.

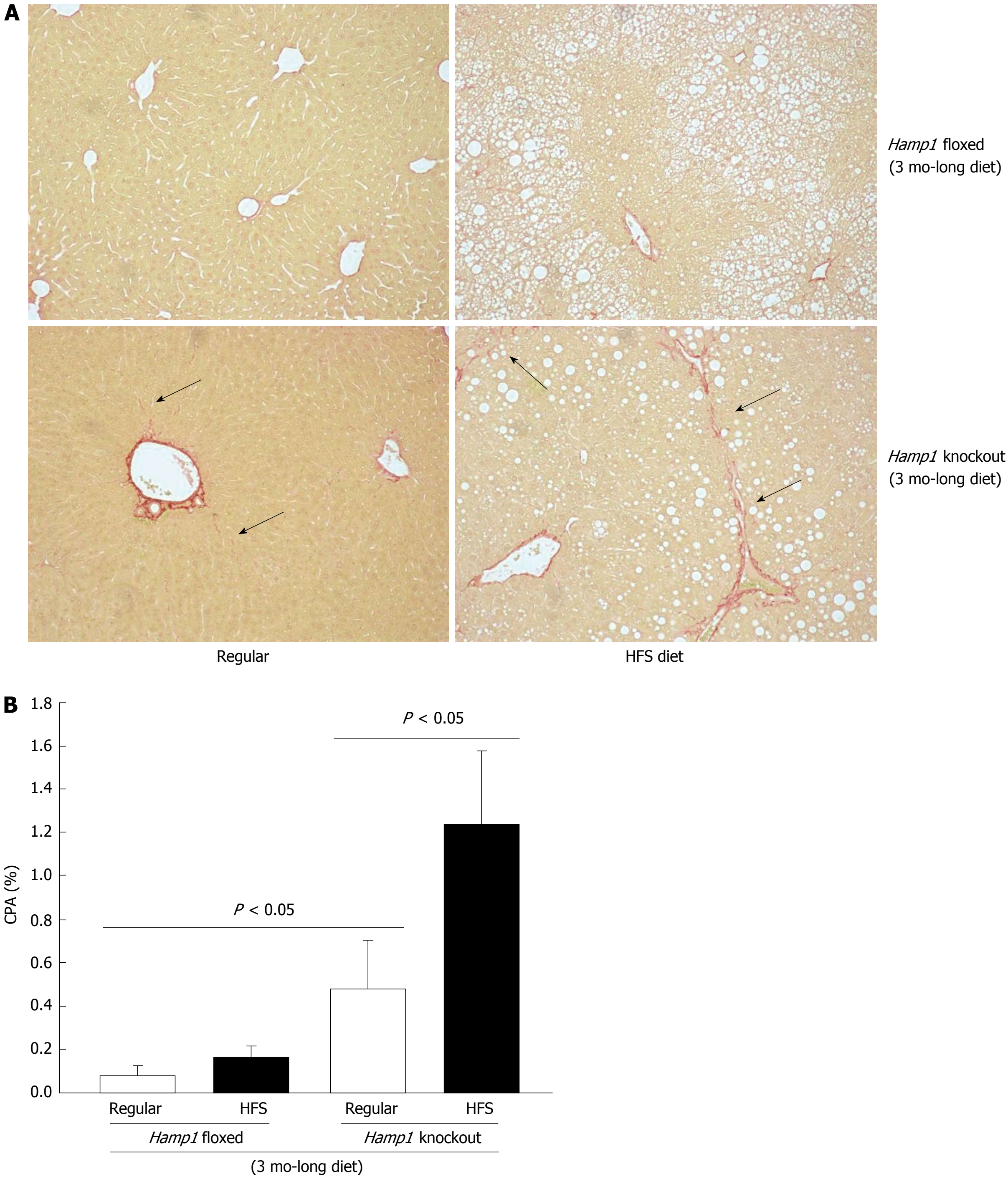

Figure 5 Fibrosis in *Hamp1* floxed or knockout mice fed high fat or regular diets for 3 mo. A: Fibrosis in the livers of floxed and knockout mice fed on regular or high fat sucrose (HFS) diets for 3 mo was detected by Sirius Red staining of tissue sections. Representative images obtained with Nikon Eclipse E400 light microscope are shown; B: 10 independent images (10 x) taken from each group were quantified using ImageJ ROI manager software. The collagen proportional area (CPA) was determined by calculating the percentage of collagen-occupied pixels against the total pixel values.

Iron-deficient rodents have been reported to display elevated lipogenic gene expression, which indirectly supports our findings[55-57]. Hepatic lipid homeostasis is also regulated by lipid export *via* VLDL secretion. The hepatic expression levels of *Mttp*, which is important in this process, were comparable between control and knockout mice. Our findings therefore suggest that decreased lipogenesis and lipid storage, but not increased lipid secretion, might lead to attenuated steatosis in high fat-fed *Hamp1* knockout mice.

Increased mitochondrial β-oxidation alleviates extra-hepatic fat burden in NAFLD by disposing of excess lipids[58]. Pparα, which induces the transcription of genes involved in β-oxidation, is itself regulated at the transcriptional level[59,60]. However, Pparα is not expected to contribute to liver pathology in *Hamp1*

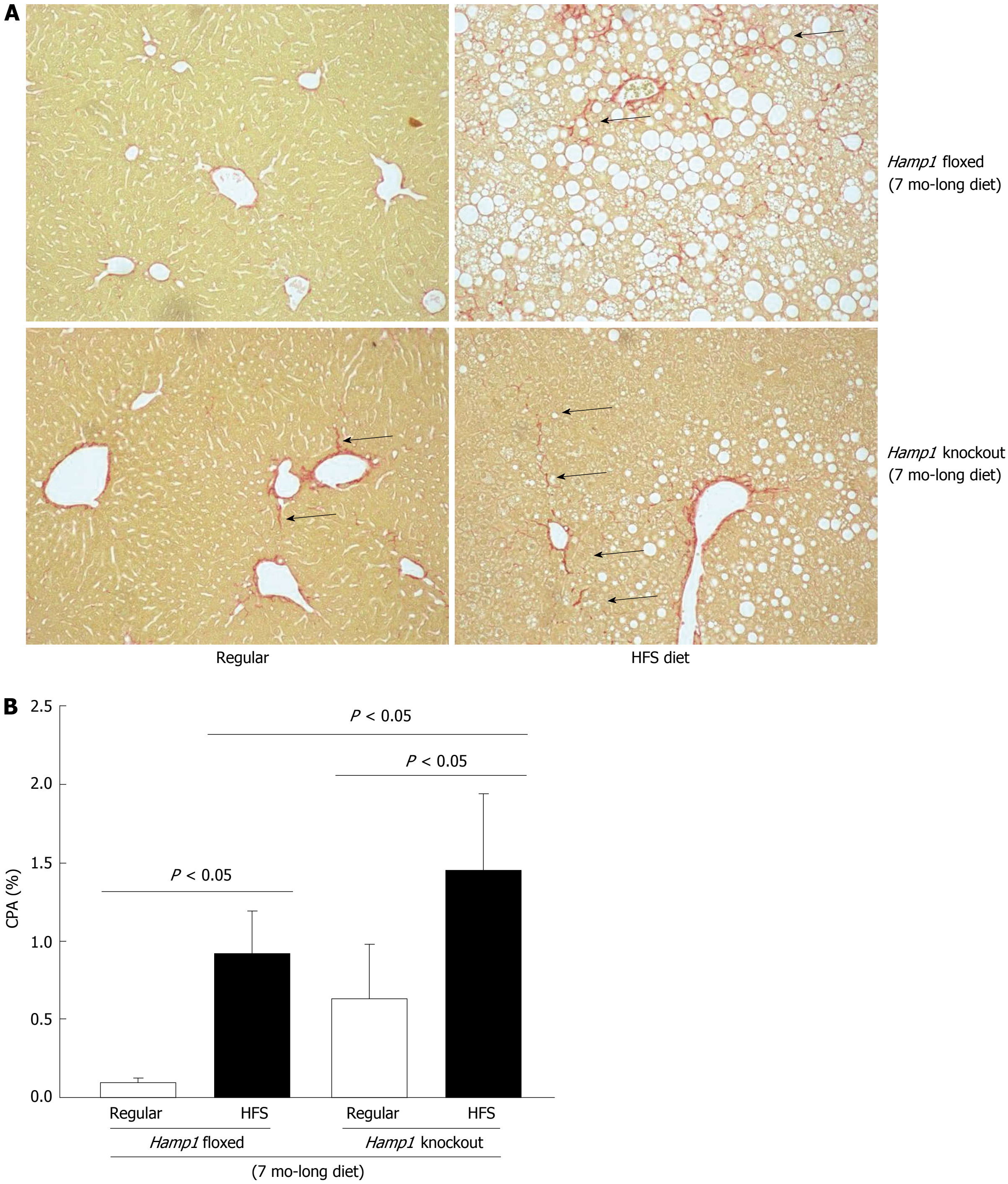

Figure 6 Fibrosis in *Hamp1* floxed or knockout mice fed high fat or regular diets for 7 mo. Liver fibrosis in floxed and knockout mice fed on regular or high fat sucrose (HFS) diets for 7 mo was detected (A) and quantified (B), as described above. CPA: Collagen proportional area.

knockout mice because HFS-fed floxed and knockout mice livers displayed similar levels of Pparα expression. Cpt1 is the rate-limiting enzyme in β-oxidation. Long-term high fat intake significantly suppressed *Cpt1a* expression only in knockout mice livers suggesting a role for it in attenuated steatosis in *Hamp1* knockout mice. Interestingly, *Hamp1* deletion by itself elevated hepatic *Cpt1a* expression. Besides β-oxidation, mitochondria is also important for iron metabolism[61]. It is feasible that iron accumulation caused by *Hamp1* deletion modulates metabolic gene expression in mitochondria. Of note, mitochondrial changes contribute to NAFLD/NASH pathology[11]. *Hamp1* deletion also altered the expression of gluconeogenic genes, *Pck1* and *G6pc*. Hepcidin serves as a gluconeogenic sensor in mice during starvation[62]. The reasons for the differential regulation of *Pck1* and *G6pc* expression in knockout mice livers are unclear. *Pck1* and *G6pc* are however regulated by various transcription factors including Foxo1[54] and iron regulates Foxo1 in adipocytes[63]. The net effect of hepcidin and

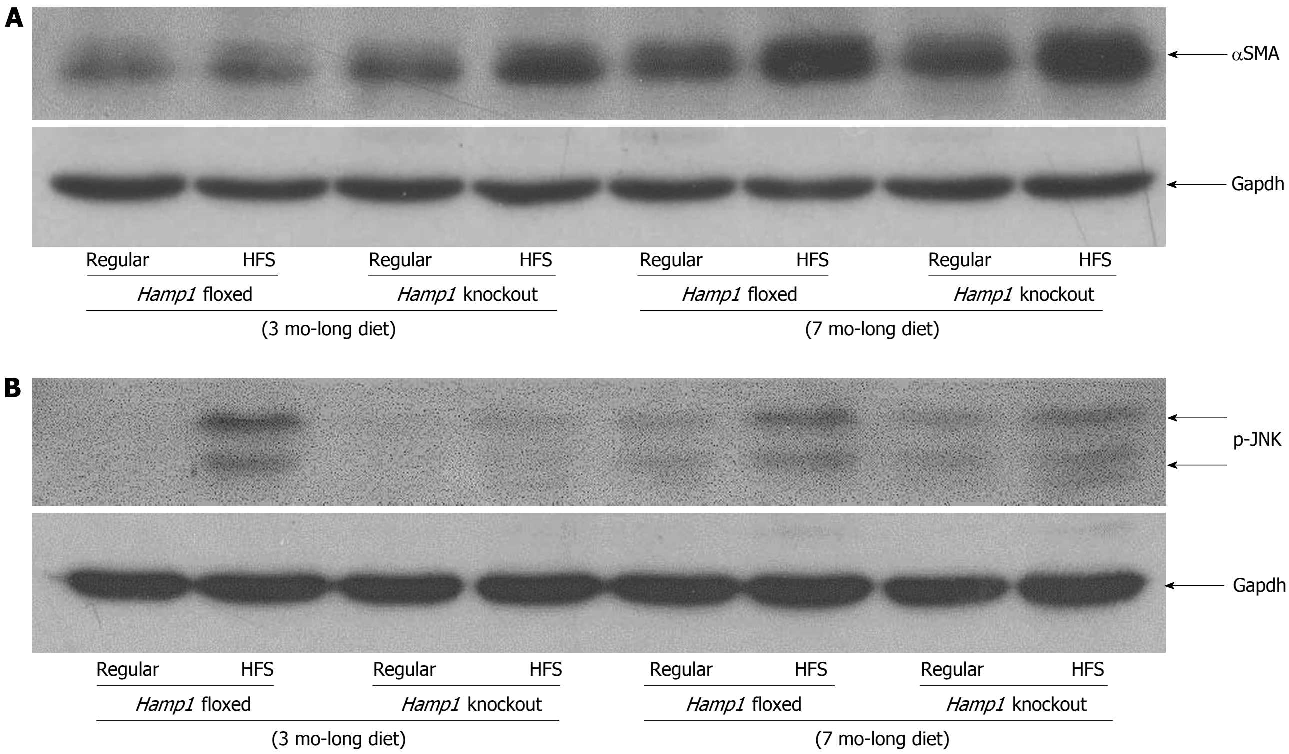

Figure 7 Protein expression levels of phosphorylated Jun N-terminal kinase and alpha smooth muscle actin in *Hamp1* floxed and knockout mice fed with high fat or regular diets for 3 or 7 mo. The expression levels of alpha smooth muscle actin (αSMA) (A) and phosphorylated Jun N-terminal kinase (p-JNK) (B) proteins in the livers of floxed and knockout mice fed with regular or high fat sucrose (HFS) diets for 3 or 7 mo was determined by western blotting, as described in Material and Methods. An anti-gapdh antibody was used as control to determine equal protein loading; Gapdh: Glyceraldehyde 3-phosphate dehydrogenase.

iron on metabolic processes in the liver requires further investigation.

Despite amelioration of steatosis, high fat administration caused injury in the livers of *Hamp1* knockout mice. In fact, knockout mice displayed an earlier and more pronounced development of fibrosis compared to control mice. Previous studies using MCD experimental models have shown that iron supplementation attenuates steatosis and triggers fibrosis[24,64]. Of note, MCD diet does not reproduce the metabolic changes observed in NAFLD/NASH patients and induces weight loss[65,66]. On the other hand, most high fat diet models induce metabolic changes but not fibrosis[66,67]. Furthermore, introduction of iron in the diet can create secondary effects by up-regulating liver hepcidin synthesis and thereby inhibiting the expression of iron exporter, ferroportin[68-70]. This will then lead to sequestration of iron in Kupffer cells and trigger inflammation. These artefacts are avoided in in our experimental system because iron accumulation is directly caused by the lack of hepcidin expression. Our high fat-fed *Hamp1* knockout mice, which develop early fibrosis, may therefore be an advantageous NAFLD/NASH model.

Simple steatosis is considered to be a benign condition in NAFLD patients. *In vivo* and *in vitro* studies have also shown this to be a beneficial process because triglycerides synthesis protects the liver from lipotoxicity induced by free fatty acid accumulation[64,71]. The decreased level of steatosis in synergy with iron might be responsible for early fibrosis development in the livers of HFS-fed *Hamp1* knockout mice.

In summary, our findings strongly suggest a role for hepcidin in the regulation of hepatic lipid and carbohydrate metabolism. There are currently a limited number of NASH experimental models[66]. *Hamp1* knockout mice will therefore be useful to investigate the molecular mechanisms of metabolic processes and fibrosis in NASH pathogenesis.

Lack of hepcidin expression due to the deletion of *Hamp1* alleles inhibited lipid accumulation in the liver following a high fat and high sucrose diet administration. Lack of c-jun kinase phosphorylation and the changes in the expression of metabolic genes, which are involved in lipogenesis and lipid storage, played a role in attenuated steatosis observed in hepcidin knockout mice. Knockout mice developed fibrosis within 3 mo of high fat exposure, which was more prominent at 7 mo. Deletion of *Hamp1* alleles by itself modulated hepatic expression of genes involved in mitochondrial fatty acid oxidation and gluconeogenesis. In summary, hepcidin is associated with the regulation of metabolic processes in the liver and the lack of hepcidin expression triggers early fibrosis development. High fat-fed hepcidin knockout mice may therefore serve as a useful animal model to study different aspects of fatty liver disease pathogenesis.

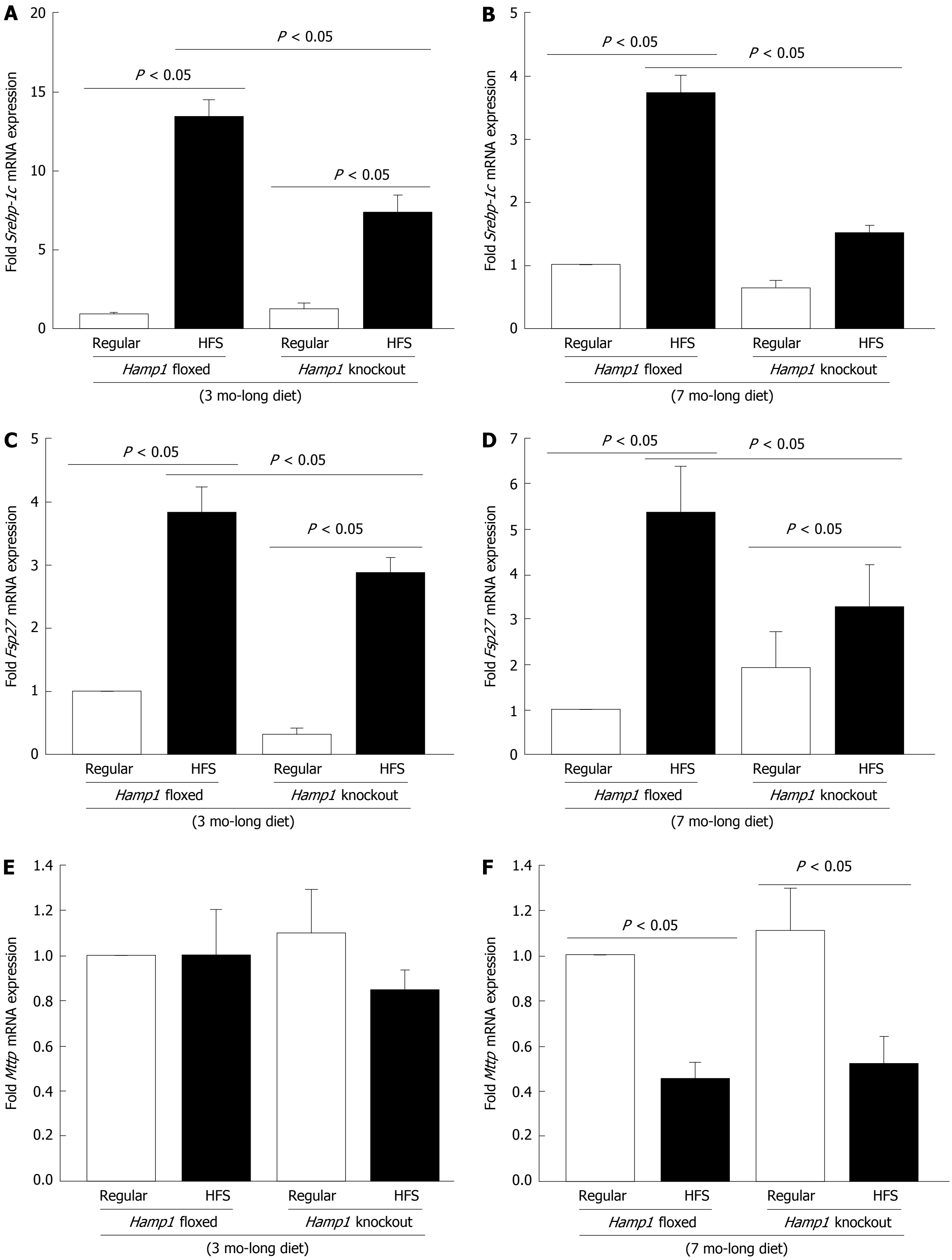

Figure 8 Expression of genes involved in lipogenesis, lipid storage and secretion. The mRNA expression levels of *Srebp-1c* (A and B), *Fsp27* (C and D), and *Mttp* (E and F) in the livers of floxed and knockout mice fed with regular and high fat sucrose (HFS) diets, was determined by real-time polymerase chain reaction. Gene expression in high fat-fed floxed or knockout and regular diet-fed knockout mice for 3 (A, C and E) or 7 mo (B, D and F) was expressed as fold change of that in floxed mice fed with a regular diet for the same time period.

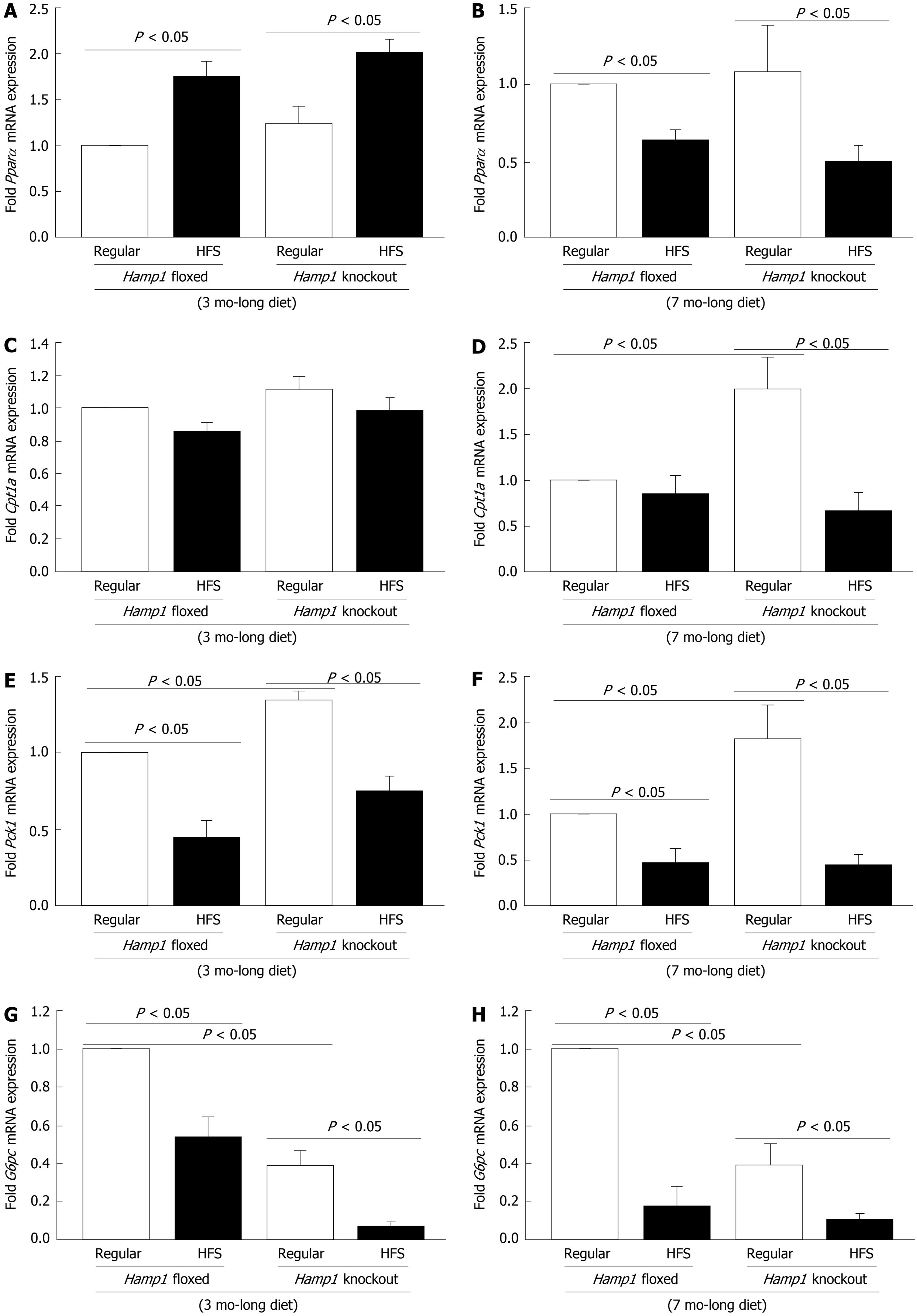

Figure 9 Expression of genes involved in β-oxidation and gluconeogenesis. The mRNA expression levels of *Pparα* (A and B), *Cpt1a* (C and D), *Pck1* (E and F) and *G6pc* (G and H), in the livers of *Hamp1* floxed and knockout mice fed with regular and high fat sucrose (HFS) diets, was determined by real-time polymerase chain reaction. Gene expression in high fat-fed floxed or knockout and regular diet-fed knockout mice for 3 (A, C, E and G) or 7 mo (B, D, F and H) was expressed as fold change of that in floxed mice fed with a regular diet for the same time period.

COMMENTS

Background

Obesity-related metabolic syndrome and its hepatic manifestation, non-alcoholic fatty liver disease (NAFLD) are important public health problems. Hepcidin, synthesized primarily by the liver, is the key iron-regulatory hormone. The authors have previously shown a role for hepcidin in alcoholic liver disease. Hepcidin expression is modulated in NAFLD patients but its significance is unknown. Furthermore, there are only a few animal models of NAFLD, which resemble human disease pathology. The authors are one of the few laboratories with hepcidin transgenic mice models, which were employed in this study to investigate NAFLD pathogenesis.

Research frontiers

NAFLD is a wide spectrum of disease ranging from simple benign fat accumulation (steatosis) to non-alcoholic steatohepatitis (NASH), which is characterized by inflammation (steatohepatitis) and fibrosis in the liver. A correlation between hepatic iron levels and disease severity in NAFLD/NASH patients has been clearly demonstrated. Since hepcidin is the central iron regulator, it is essential to understand its role in NAFLD/NASH.

Innovations and breakthroughs

The previously published studies with hepcidin knockout mice generated in the laboratory have demonstrated significant iron accumulation in the liver. To establish a novel NAFLD/NASH experimental model, hepcidin knockout mice were fed with a high fat diet for different time periods. By showing that hepcidin is directly involved in lipid storage and fibrogenesis in the liver following high fat intake, the authors underlined the importance of hepcidin and iron homeostasis in NAFLD/NASH pathogenesis.

Applications

This study indicated a role for hepcidin in the regulation of metabolic processes and early fibrosis development in the liver. These findings will further understanding of the mechanisms involved in NAFLD/NASH progression and liver fibrosis. Furthermore, the high fat-fed hepcidin knockout mice, as a novel experimental NAFLD/NASH model, can be useful in the search for functional biomarkers and therapeutics for NAFLD/NASH.

Terminology

Hepcidin is essential for systemic iron homeostasis. Chronic high fat intake and obesity ultimately lead to metabolic syndrome, which is characterized by dyslipidemia and insulin resistance. Obesity also impairs metabolic functions and histology of the liver causing fat accumulation (steatosis), inflammation (steatohepatitis) and scar tissue formation (fibrogenesis), as observed in patients with NAFLD/NASH.

Peer-review

This manuscript investigated the role of key iron-regulatory protein, hepcidin in non-alcoholic fatty liver disease in hepcidin (*Hamp1*) knockout and floxed control mice administered a high fat and high sucrose or a regular control diet for 3 or 7 mo. The authors suggest that *Hamp1* and iron may play a role in the regulation of metabolic pathways in the liver, which has implications for NAFLD pathogenesis. This manuscript was well designed *in vivo* experiments and well written with all the results obtained.

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