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Fibroblast Growth Factor-5 Participates in the Progression of Hepatic Fibrosis

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Abstract: Non-alcoholic steatohepatitis (NASH) is characterized by the presence of steatosis, inflammation, and fibrosis and is believed to develop via a "two-hit process"; however, its pathophysiology remains unclear. Fibroblast growth factors (FGFs) are heparin-binding polypeptides with diverse biological activities in many developmental and metabolic processes. In particular, FGF5 is associated with high blood pressure. We investigated the function of FGF5 *in vivo* using spontaneously Fgf5 null mice and explored the role of diet in the development of NASH. Mice fed a high-fat diet gained little weight and had higher serum alanine transaminase, aspartate amino transferase, and non–high-density lipoprotein-cholesterol levels. Liver histology indicated marked inflammation, focal necrosis, fat deposition, and fibrosis, similar to the characteristics of NASH. FGF5 and a high-fat diet play significant roles in the pathophysiology of hepatic fibrosis and Fgf5 null mice may provide a suitable model for liver fibrosis or NASH.

Key words: FGF5, hepatic fibrosis, NAFLD, NASH, two-hit theory

Introduction

One of the most common liver pathologies, hepatic steatosis, has been traditionally believed to result from obesity and alcohol abuse; however, focus is now on non-alcoholic steatohepatitis (NASH), which does not carry the same associations [3]. NASH is characterized by steatosis, inflammation, and fibrosis [4, 6, 7, 22]. The development of NASH requires a "double hit," meaning that steatosis first develops in the liver, followed by the development of sufficient oxidative stress to initiate significant lipid peroxidation [9]. A recent study also reported that LDL-oxidizing antibodies play an important role in NASH pathogenesis [5], although the mechanisms of NASH development are unknown.

Fibroblast growth factors (FGFs) are heparin-binding polypeptides with diverse biological activities in many developmental and metabolic processes. The FGF gene family comprises 22 members [14]. Originally identified by Zhan *et al.* [24], FGF5 regulates hair growth [11, 19, 20, 21], and subsequent studies have found that Fgf5 knockout [11, 18] and null mice [15] have an angora phenotype. Although the role of FGF5 in pathology is not clear, recent studies have shown that FGF5 influ-

ences blood pressure [17, 23] and human glioblastomas [2]. We used Fgf5 null mice to investigate the *in vivo* function of FGF5 and the role of diet in the development of NASH.

Methods

Animals and diets

This study was approved by the National Defense Medical College Animal Care and Use Committee and conducted in accordance with the "Guide for the Care and Use of Laboratory Animals" of the National Academy of Sciences (USA). Male ICR (4 weeks old, wild-type, WT) mice were used as the control in all experiments (CLEA Japan, Tokyo, Japan). The mice were fed a high-fat (TD.88137, Harlan Laboratories, Madison, WI, USA) or control diet (CE-7, CLEA Japan) for 4 weeks. The control diet contained 3.8% fat, 17.7% protein, 59.4% carbohydrate, and 3.4 kcal/g. The high-fat diet contained 21.2% fat, 0.2% cholesterol, 17.3% protein, 48.5% carbohydrate, and 4.5 kcal/g. The mice had unlimited access to water and food during the study period.

Body weights were measured weekly. Serum high density lipoprotein (HDL), triacylglycerol (TG), total cholesterol (TCHO), serum alanine transaminase, and aspartate amino transferase were measured at 8 weeks of age using a DRI-CHEM 4000V analyzer (Fuji Film, Tokyo, Japan).

Polymerase chain reaction

Purified tail skin genomic DNA from Fgf5 null mice was amplified using primer sets for exon 1 (5′-TACCG-GCCGTGAGTACACA-3′, 5′-TCTAAGGAAACCCG-GTGTC-3′), exon 2 (5′-CTGAGAACAGTTGACG-TAGT-3′, 5′-CTATCTGGTAAACGGATTCC-3′), and exon 3 (5′-ATCTTGCCATTCTGAGATCAA-3′, 5′-TG-TATACAAACTAAACGGGCT-3′). The amplified fragments were cloned into pGEM-T-easy (Promega, Madison, WI, USA). Transformants were screened by PCR and sequenced using the T7 and S6 primers (Promega) on an ABI 3130XL Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

Morphological study

Mice were anesthetized with isoflurane (Mylan, Canonsburg, PA, USA) and sacrificed. Liver tissues were harvested and histology was performed. The livers were fixed with 10% formalin and slides were stained with hematoxylin and eosin to evaluate steatosis and steatohepatitis. Additional liver sections were stained with Masson trichrome.

Statistical analyses

All values are expressed as the mean ±SD. Statistical significance was determined using Excel Ver. 14.3.5 (Microsoft, WS, USA) by Student's *t*-test.

Results

Morphology and genome structure of the FGF5 null mouse

Figure 1 shows examples of the WT ICR (Fig. 1A) and Fgf5 null mice (Fig. 1B). ICR mice were obtained from CLEA Japan Co., Ltd. in the early 1980s, and have been maintained in our laboratory. The long hair (LH) mouse type found by our laboratory and has been maintained for over 20 years. PCR analyses were performed to determine the presence or absence of Fgf5 exons 1, 2, and 3. A 10-kb sequence, including exon 3, was deleted in the Fgf5 allele of the LH mice and a 0.5-kb sequence was inserted at the same site (Fig. 1C). This sequence shared more than 98% identity with an early transposon long terminal repeat (LTR). These LH mice may be genetically identical to mice recently reported by Mizuno *et al.* [15].

Body weight

At 8 weeks, the WT and LH mice fed the high-fat diet (WT-H, LH-H) weighed more than those fed the control diet (WT-C, LH-C). As shown in Fig. 2, the LH-H mice (28.6 ± 1.0 g) weighed slightly more than the LH-C mice (26.8 ± 0.7 g, *P*=0.023), but the WT-H mice (34.0 ± 1.7 g, *P*=0.002) demonstrated a marked increase in weight compared with the WT-C mice (27.2 ± 2.9 g).

Laboratory assessment

The LH-H mice had higher levels of alanine transaminase (150 ± 81 mg/dL) than the WT-C (30 ± 18 mg/dL, *P*=0.055), WT-H (42 ± 15 mg/dL, *P*=0.074), and LH-C (27 ± 4 mg/dL, *P*=0.055) mice. In addition, serum aspartate aminotransferase in the WT-H (226 ± 91 mg/dL, *P*=0.061) and LH-H (248 ± 94 mg/dL, *P*=0.046) mice was higher than in the control animals (WT-C, 107 ± 56 mg/dL, *P*=0.057; LH-C, 102 ± 38 mg/dL, *P*=0.046) (Fig. 3A). TG in the LH-H mice (80 ± 32 mg/dL) was
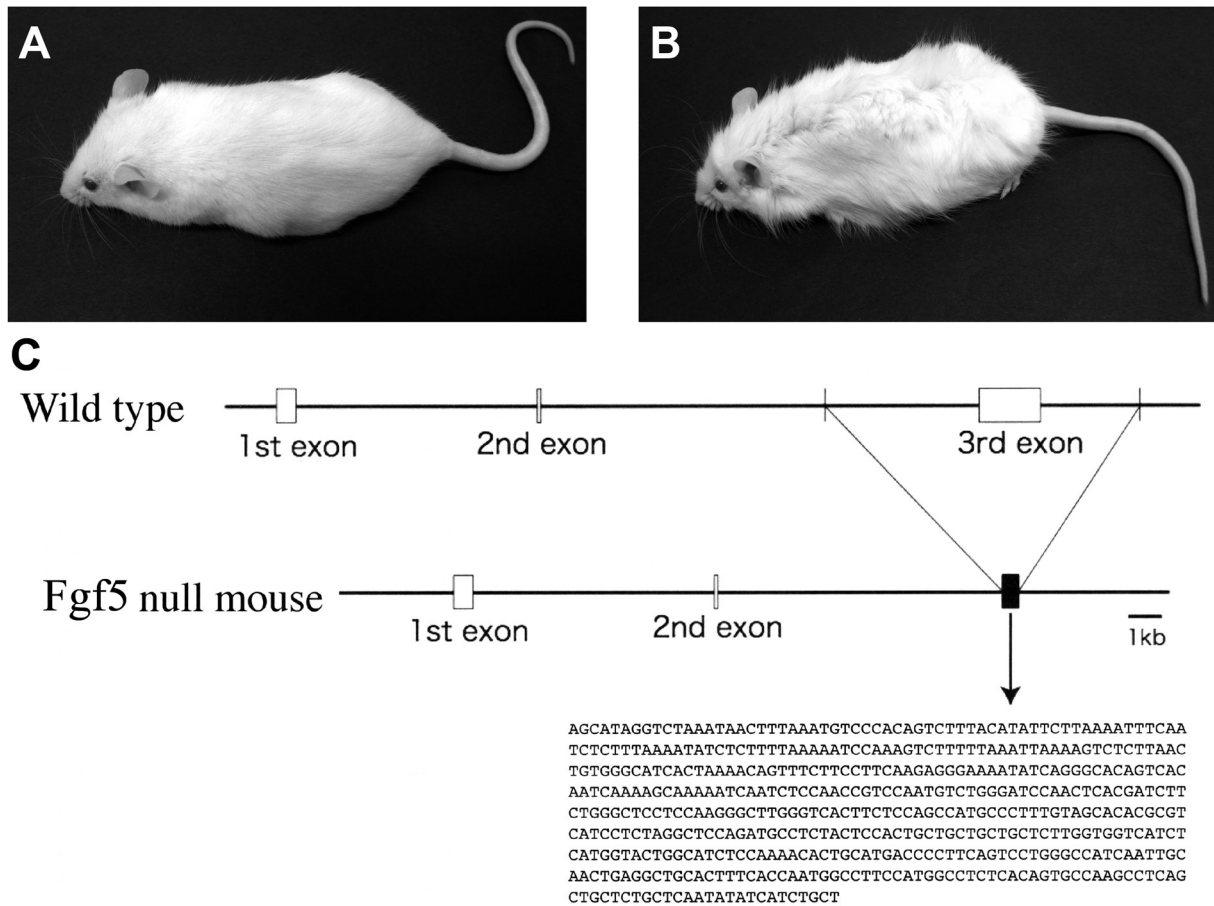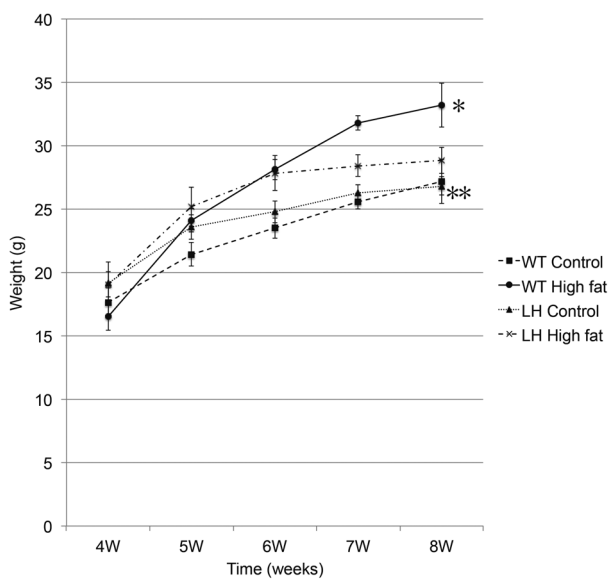
Fig. 1. Morphology of Fgf5 null mice and the Fgf5 deletion site. Fgf5 null mice (B) had long hair in comparison to wild-type animals (A). Genome structure of the wild-type and Fgf5 null alleles (C).

lower than in the WT-C (151 ± 18 mg/dL, P=0.015), WT-H (145 ± 50 mg/dL, P=0.013), and LH-C (179 ± 83 mg/dL, P=0.095) mice. Finally, TCHO was higher in LH-H mice (272 ± 76 mg/dL) than in the other groups (WT-C, 94 ± 3.0 mg/dL, P=0.013; WT-H, 203 ± 24.5 mg/dL, P=0.094; LH-C, 137 ± 12.5 mg/dL, P=0.09) (Fig. 3B). Non–high-density lipoprotein-cholesterol (non–HDL-C) was calculated as the difference between HDL and total cholesterol. Non–HDL-C in LH-H mice (54 ± 15.9 mg/dL) was also much higher than in the other groups (WT-C, 4 ± 4 mg/dL, P=0.03; WT-H, 2 ± 25 mg/dL, P=0.01; LH-C, −3 ± 10.6 mg/dL, P=0.01) (Fig. 3C).

Fig. 2. Body weight in Fgf5 null mice fed a high-fat diet. Body weights at ages 4, 5, 6, 7, and 8 weeks. Wild type (WT) control, WT mice fed a normal diet (n=5); WT High fat, WT mice fed a high fat diet (n=5); Long hair (LH) control, Fgf5 null mice fed a normal diet (n=4); LH High fat, FGF5 null mice fed a high fat diet (n=4). *P<0.01 and **P<0.05 compared to the LH High fat mice.
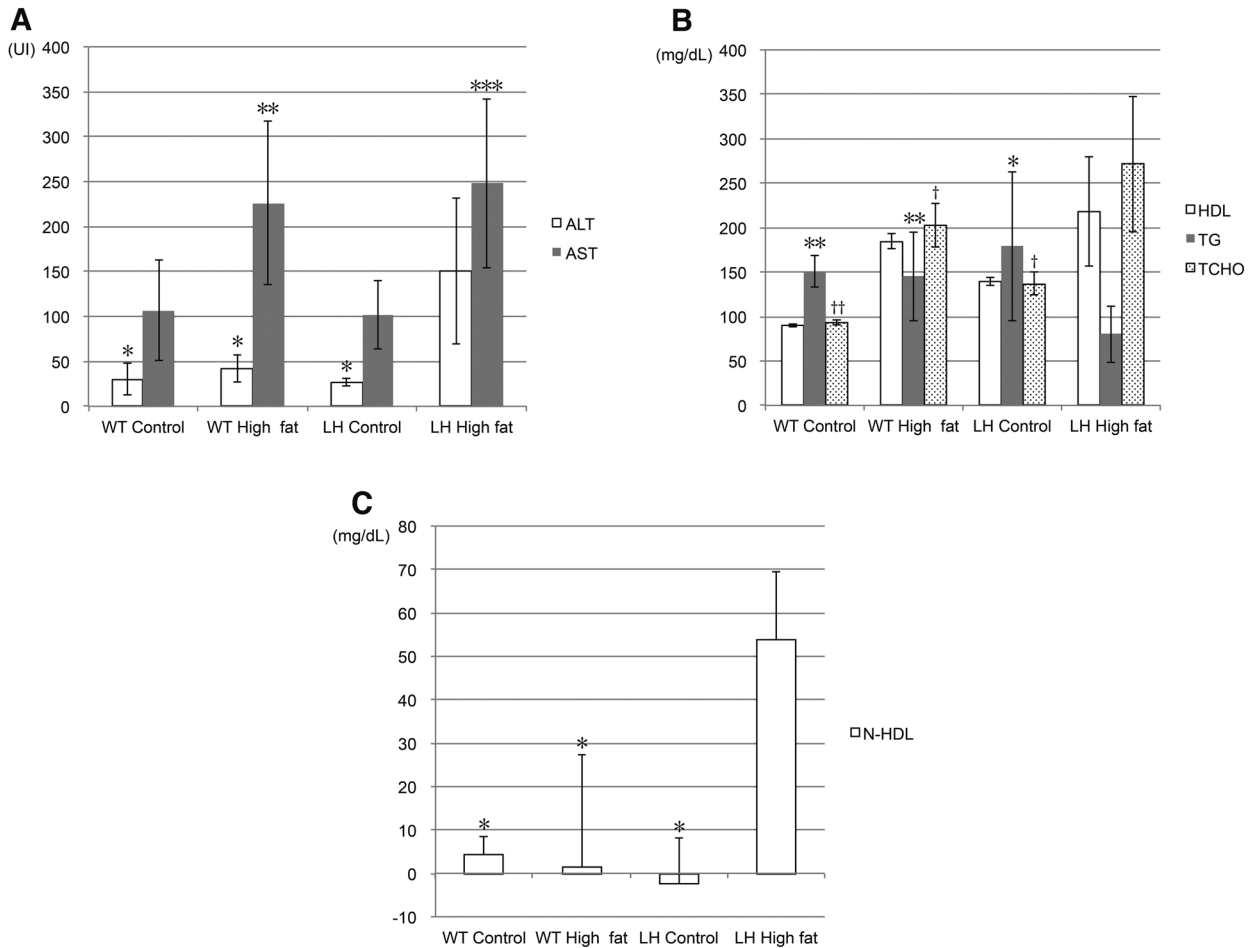
A

B

C

Fig. 3. Serum alanine transaminase (ALT), aspartate amino transferase (AST), high-density lipoprotein (HDL), triglyceride (TG), total cholesterol (TCHO) and Non–high-density lipoprotein-cholesterol (non–HDL-C). (A) Serum ALT and AST at age 8 weeks: Wild type (WT) control, WT mice fed a normal diet (n=3); WT High fat, WT mice fed a high fat diet (n=4–8); Long hair (LH) control, FGF5 null mice fed a normal diet (n=4); LH High fat, Fgf5 null mice fed a high fat diet (n=4). *$P<0.1$ compared to the LH-High fat group. **$P<0.1$ compared to the WT-Control group. ***$P<0.05$ compared to the LH-Control group. (B) Serum HDL, TG, and total TCHO levels at age 8 weeks: Wild type (WT) control, WT mice fed a normal diet (n=3); WT High fat, WT mice fed a high fat diet (n=4–8); Long hair (LH) control, FGF5 null mice fed a normal diet (n=4); LH High fat, Fgf5 null mice fed a high fat diet (n=4). *$P<0.1$ compared to the LH-High fat group. **$P<0.05$ compared to the LH-High fat group. †$P<0.1$ compared to the LH-High fat group. ††$P<0.05$ compared to the LH-High fat group. (C) Non–high-density lipoprotein-cholesterol (non–HDL-C) is calculated by subtracting HDL cholesterol level from the TC level. *$P<0.05$ compared to the LH-High fat group.

Liver histology

Histology of LH-H mice revealed marked infiltration of inflammatory cells in the periportal area (Zone 1) and mid-zonal parenchyma (zone 2) associated with massive piecemeal necrosis and focal necrosis (Figs. 4A and C). Swollen and vacuolated hepatocytes were observed around the central vein (Zone 3) (Fig. 4A, gray arrowhead). Higher magnification of this area revealed that these hepatocytes represented microvesicular steatosis and hepatocyte ballooning (Figs. 5A and B). Additionally, Masson-trichrome stain revealed severe fibrosis

specifically in the inflamed areas (Figs. 4B and D). LH-C mice exhibited normal liver histology, without steatosis, inflammation (Figs. 4E, 5C and 5D), fibrosis (Figs. 4F). Although enhanced hepatocyte steatosis and ballooning was observed in WT-H mice (Figs. 5E and F), they exhibited neither inflammation nor fibrosis (Figs. 4G and H). In human cases, NASH is diagnosed histopathologically using the NAFLD Activity Score (NAS) [1, 12]. In this experimental model, LH-H mice demonstrated moderate steatosis (score 2), prominent ballooning (score 2), and severe lobular inflammation (score 3),
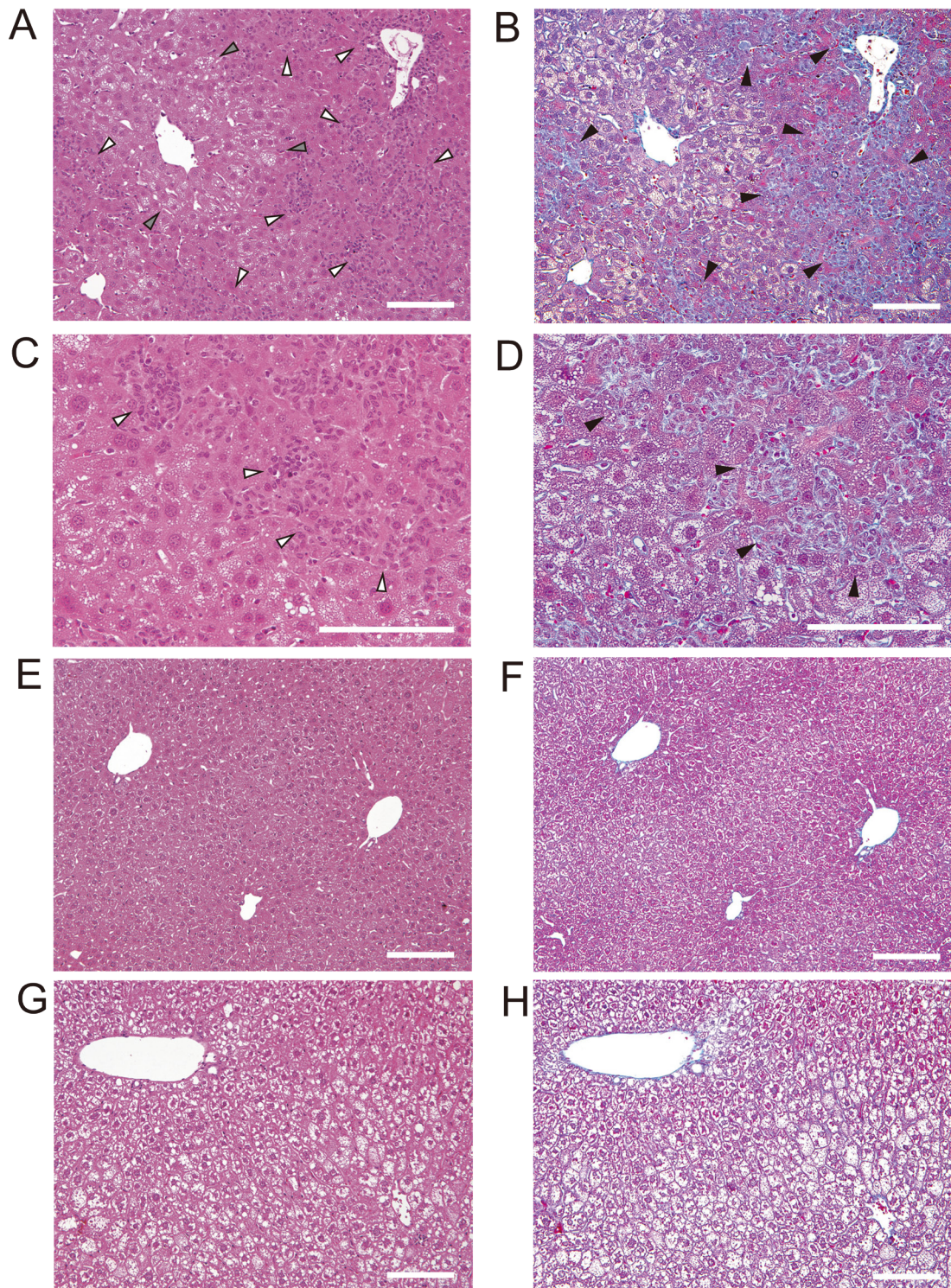
Fig. 4. Histology. Livers were collected from each mouse and fixed with 10% formalin for hematoxylin-eosin (HE) and Masson-Trichrome (Masson) staining. Livers from LH mice fed with high fat diet were examined at 200× magnification (A), 400× (C) after HE staining, and 200× (B), 400× (D) after Masson staining. White arrowheads indicate piecemeal and focal necrosis. Gray arrowheads indicate microvesicular steatosis and ballooning of hepatocytes. Black arrowheads indicate fibrosis. Livers from LH mice fed with control diet (E) (F) and WT mice fed with high fat diet (G) (H) were also compared. The white line in each picture indicates 100 μm.
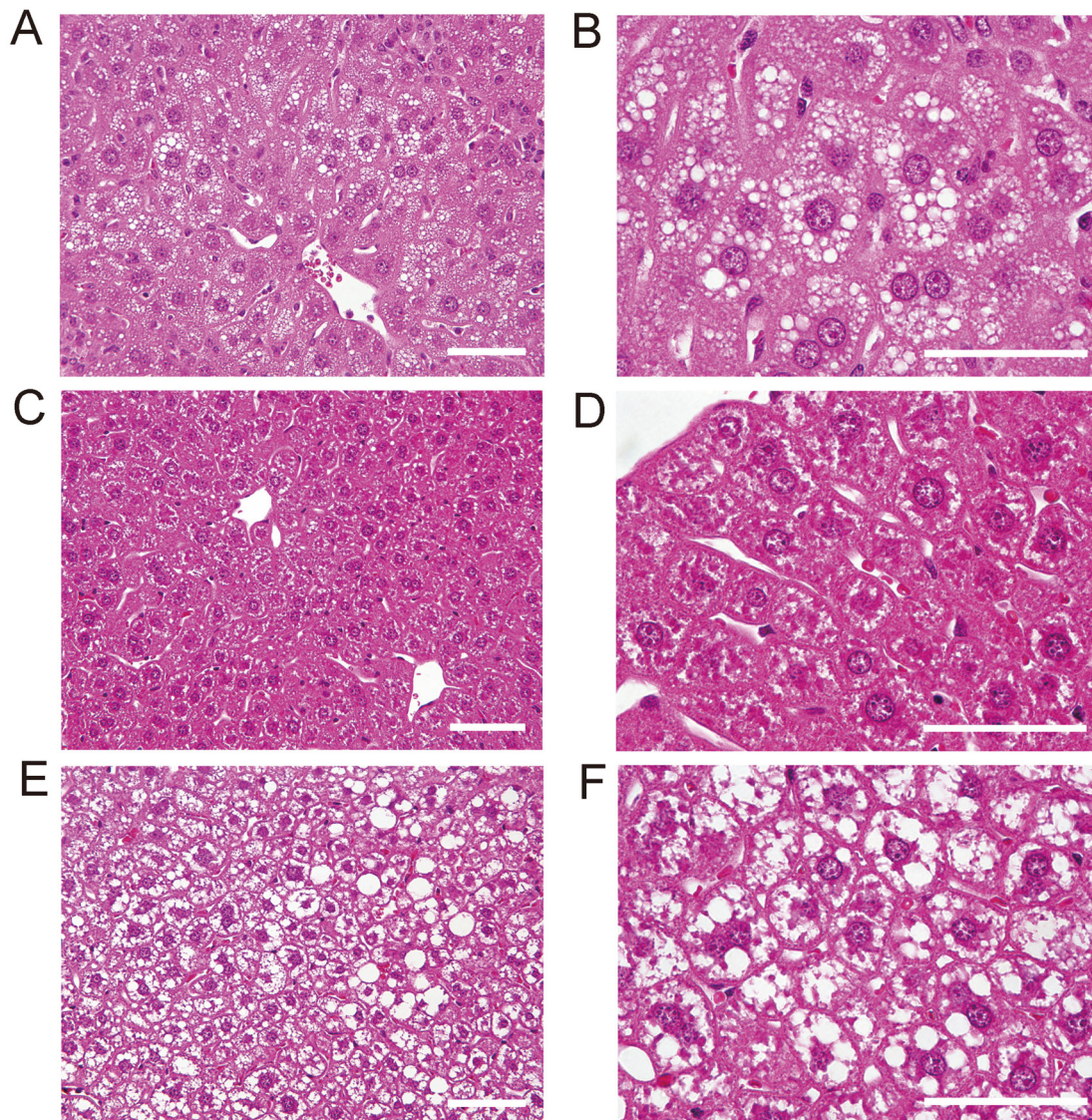
Fig. 5. Higher magnification of hepatic steatosis in LH mice and WT mice. Liver from high-fat diet-fed LH mouse at 400× (A) and 1,000× (B) after HE staining. Livers from Control Diet-fed LH mice at 400× (C) and 1,000× magnification (D) and high-fat diet-fed WT mice at 400× (E) and 1,000× (F) were compared. The white line in each image indicates 50 μm.

for a total NAS score of 7. Emergence of severe fibrosis is consistent with NASH. Although WT-H mice demonstrated severe steatosis (score 3) and prominent ballooning (score 2), the absence of lobular inflammation and fibrosis indicated fatty liver.

Discussion

FGF5 null mice fed a high-fat diet (LH-H) demonstrated clinical, laboratory, and hepatic histological characteristics similar to those observed in animals with NASH. Histology indicated marked inflammation, focal necrosis, fat deposition, and fibrosis in the livers of LH-H animals. NASH is also characterized by varying degrees of progressive steatosis, lobular inflammation, and fibrosis of the liver [16]. In this study, the LH-H mice demonstrated lower body weights, lower TG levels, and higher non–HDL-C levels, suggestive of a lipid metabolism disorder. In comparison, the WT-H mice had fatty livers and higher TG levels. The decreased fat deposition in LH vs. WT mice indicated possible impaired TG uptake in Fgf5 null mice. Thus, lipid balance

in the liver may be critical in the pathophysiology of NASH.

A recent study found that in patients who were not taking lipid-lowering agents, high levels of non–HDL-C were more closely associated with NASH than with steatosis. In that study, non-HDL-C was used to differentiate between NASH and steatosis [8]. The total cholesterol level was high in LH-H mice and non-HDL-C was significantly higher than in other groups. Hence, LH-H mice may have a metabolism disorder involving total cholesterol, and FGF5 may be involved in cholesterol metabolism, which may explain the association between FGF5 and the risk of hypertension observed in a recent genome-wide association study [13, 17].

NASH results from 2 distinct events, evolving from steatosis to fibrosis, known as a "two-hit process" [9, 10]. In our study, the WT-H livers exhibited steatosis but no fibrosis, and the LH-H livers exhibited both conditions. Thus, FGF5 is implicated in the progression from steatosis (first hit) to NASH (second hit). A high-fat diet also precipitated NASH in Fgf5 null mice. With 4.7 times more fat and 1.3 times more calories than the control diet, the high-fat diet likely has a role in the "two-hit process."

In conclusion, we demonstrated associations between hepatic fibrosis, steatosis, FGF5, and a high-fat diet; however, the mechanism underlying these associations is unclear, and a comprehensive analysis of the livers of WT and LH mice with microarrays and omics studies is needed. We believe that the Fgf5 null mouse with a high-fat diet can be a suitable animal model for liver fibrosis or NASH and suggest FGF5 may be a therapeutic target for hepatic fibrosis in NASH.

Acknowledgment

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