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Development of Non-alcoholic Steatohepatitis and Nodular Regenerative Hyperplasia in C57BL/6J Mice Fed a Fructose-containing Western Diet

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Abstract. *Background/Aim: We generated a novel disease mouse model in which a fructose-containing western diet (FD) induces development of non-alcoholic steatohepatitis (NASH). Materials and Methods: C57BL/6J mice were fed FD for 60 weeks and body weight and blood pressure were monitored. Plasma cholesterol level was measured at the end of the experiments. Histopathology of NASH was examined by hematoxylin and eosin staining, Masson-Trichrome staining, periodic acid-Schiff staining, and immunohistochemistry against a proliferation marker. Circadian gene expression levels were compared by sampling the livers in 4-h intervals, followed by quantitative RT-PCR analysis. Results: FD-fed mice developed obesity, transient hypertension, hypercholesterolemia, and liver adiposity. The mice spontaneously developed hepatic nodules, which were diagnosed as non-neoplastic nodular regenerative hyperplasia. FD-fed mice had increased expression of growth factor genes and cirrhosis markers compared to control mice. Circadian expression of lipid metabolism genes was deregulated by FD intake. Conclusion: C57BL/6J mice fed FD developed non-alcoholic steatohepatitis and nodular regenerative hyperplasia over time.*

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Fructose consumption has increased 100-fold over the past four decades and now accounts for about 10% of caloric intake in the United States (1). Accumulating evidence indicates a strong correlation between high fructose intake and hypertension (2, 3) as well as with non-alcoholic fatty liver disease (NAFLD) (4). Most lifestyle-related diseases gradually develop through exposure to various risk factors, but established rodent models can recapitulate immediate (acute) consequences of fructose intake (5-7), such as hypertension or steatosis. Although the timescale of disease progression differs between humans and rodents, prolonging the observation period (*e.g.*, up to one year) could clarify phenomena that have been overlooked. Here, we generated a novel disease mouse model in which a fructose-containing western diet induces NAFLD development. Prolonged nutritional challenge with FD for up to 60 weeks aggravated non-alcoholic steatohepatitis (NASH), culminating in lethal nodular regenerative hyperplasia.

Materials and Methods

Animals. The care and use of all mice were in accordance with the guidelines for proper conduct of experiments involving animals (Science Council of Japan). All experiments involving animals were approved by the Animal Care and Use Committee at Dokkyo Medical University. C57BL/6JJmsSlc (*Mus musculus musculus*) mice were purchased from Japan SLC, Inc. (Hamamatsu, Japan). Six-week-old male mice were housed at a constant temperature of 23±2˚C and a 12:12-h light-dark cycle and were given a normal chow diet *ad libitum* until the age of 8 weeks when experiments were initiated.

Nutritional challenge by fructose-containing western diet. We made a fructose-containing western diet (FD) by replacing sucrose in a standard western diet (D12079B, Research Diets Inc., New

Brunswick, NJ, USA) with an equal amount of fructose (40.5 kcal% fat, 29.1 kcal% fructose and 0.21 g% cholesterol: D08041801, Research Diets Inc.). The energy composition of each diet is shown in Table I. Eight-week-old C57BL/6JJmsSlc mice were divided into four groups (Figure 1A): (i) fed a normal diet (ND) for the 60-week observation as a control group (ND, n=24), (ii) fed a FD for the 60-week observation period (FD, n=33), (iii) fed FD and undertook physical exercise by forced swimming during the last 8 weeks of the 60-week observation period (FD + Ex8w, n=32) and (iv) fed FD and undertook exercise during the last 40 weeks of the 60-week observation period (FD + Ex40w, n=29). All mice in all experimental groups were given water *ad libitum*. Body weight gain was monitored once a week during the observation period (Figure 1B).

Forced swimming. Mice assigned to the FD + Ex8w or the FD + Ex40w groups undertook 20-min swimming sessions as described previously (8). Briefly, groups of 10 mice swam simultaneously in a 10 cm-deep tank containing tepid water (30˚C), which is a thermo-neutral temperature condition for mice. Water was circulated with a mild current to maintain swimming activity during the session.

Blood pressure measurement. Systolic blood pressure (SBP) and diastolic blood pressure (DBP) were measured using a non-invasive tail-cuff device (BP-98A, Softron Co., Ltd., Tokyo, Japan) at 20, 40, and 60 weeks during the nutritional challenge period (Figure 1A). During the measurement, mice were wrapped with a black pouch to minimize visual stimuli. The mean blood pressure was calculated as DBP+(SBP-DBP)/3 and is presented as individual data for each mouse.

Blood collection and analysis. Oral glucose tolerance tests were carried out at the end of the 60-week observation period. Mice that had been fasted for 5 h were given 2 g/kg glucose solution (15% wt/wt) administered by oral gavage, and then blood samples were taken from the tail vein before, and 30, 60, and 120 min after glucose intake. Plasma glucose levels were determined using a LabAssay Glucose kit (Wako Pure Chemical Industries, Osaka, Japan). Serum total cholesterol levels were measured with a LabAssay Cholesterol kit (Wako Pure Chemical Industries) in accordance with the manufacturer's instructions. Colorimetric signals were detected and quantified using a plate reader (Infinite F200 Pro, Tecan, Zurich, Switzerland).

Histological analysis. At the end of the 60-week observation period, the mice were euthanized and the livers were collected. Liver tissue was immediately fixed in 10% formalin for histological analysis. Paraffin-embedded tissues were cut into 5 μm-thick sections, which were deparaffinized and stained with hematoxylin and eosin, Masson-Trichrome (for collagen staining) and periodic acid-Schiff (PAS) (for glycogen staining). For immunohistochemistry, deparaffinized sections were immersed in target retrieval solution, pH 9 (Agilent Technologies, Santa Clara, CA, USA) and autoclaved at 105˚C for 1 min. Primary antibodies against a DNA replication licensing factor, MCM2 (#3619, Cell Signaling Technology, Danvers, MA, USA), horse-radish peroxidase-conjugated anti-rabbit IgG (#7074, Cell Signaling Technology) and Envision™+ (Agilent Technologies) were used to visualize nuclear MCM2 proteins in proliferating cells. Microscopic images of the stained sections were captured using an All-in-One Fluorescence Microscope BZ-X700 (Keyence, Osaka, Japan).

Measuring circadian gene expression by quantitative reverse-transcription polymerase chain reaction. Four mice in each experimental group were euthanized every four h over a 24-h period, *i.e.*, 3:00, 7:00, 11:00, 15:00, 19:00 and 23:00 Zeitgeber time [ZT], with ZT 0:00 defined as the beginning of the light period. Livers were collected from the mice and frozen. The copy number for each mRNA was quantified with real time RT-PCR as described elsewhere (9). Gene-specific Taqman® probes were purchased from Thermo Fisher Scientific (Waltham, MA, USA). The probes used were: *Acaca* (Mm01304289_m1), *Acly* (Mm01302282_m1), *Fasn* (Mm00662 319_m1), *Cyp7a1* (Mm00484152_m1), *Cyp7b1* (Mm00484157_m1), *Pparg* (Mm01184322_m1), *Ccl2* (Mm00441242_m1), *Il6* (Mm00446 190_m1), *Tnf* (Mm00443258_m1), *Trem2* (Mm04209424_g1), *Fgf2* (Mm00433287_m1), *Hgf* (Mm01135193_m1), *Pdgfb* (Mm0044 0677_m1), *Vegfa* (Mm01281449_m1) and *18S rRNA* (4352930E). The cycle of threshold (Ct) value was determined based on a threshold line 0.040. Relative $\log_2$ expression was calculated as 20 (arbitrary constant) + Ct (*18s sRNA*) - Ct (gene of interest).

Statistical analyses. For multiple comparison of mean values, Fisher's least significant difference procedure was applied. After an analysis of variance (ANOVA) by Kruskal–Wallis test, a Mann–Whitney *U*-test was carried out to compare results for every group. SPSS version 26 (IBM Corp., Armonk, NY, USA) was used for Kruskal–Wallis and Mann–Whitney *U*-tests. Gene expression data were analyzed by two-way ANOVA (Prism version 6, GraphPad software, San Diego, CA, USA) as follows. If there was an interaction between two factors, experimental groups and circadian time points, we interpreted the influence by the nutritional challenge (with or without exercise) depending on particular time windows. In other words, interaction of the two factors indicates that circadian rhythm could affect the experimental effects or *vice versa* (deregulated rhythm). Otherwise, the overall effect seen in the experiments was determined by Tukey's multiple comparisons test (Prism version 6, GraphPad software).

Results

FD induced weight gain and mild hypertension in C57BL/6J mice. Ad libitum FD feeding rapidly increased body weight in C57BL/6J mice, compared to ND feeding (Figure 1B). The mean blood pressure was similar between the ND and FD groups at 0 week (89.4±6.1 *vs*. 87.5±7.8 mmHg), but was higher at 20 weeks (91.7±8.4 *vs*. 97.9±9.5 mmHg: p=0.001), 40 weeks (90.0±6.9 *vs*. 98.3±7.2 mmHg: p<0.001), and 60 weeks (85.8±8.1 *vs*. 93.4±8.0 mmHg: p<0.001) for the FD group relative to the ND group (Figure 1C). Although blood pressure was transiently elevated at 20 weeks with FD feeding, this elevation gradually decreased over time (Figure 1C). Thus, FD feeding induced weight gain and mild hypertension in C57BL/6J mice.

Forced swimming did not ameliorate FD-induced weight gain in C57BL/6J mice. To examine whether physical exercise by forced swimming ameliorated FD-induced weight gain, we compared mean body weight among the groups (Figure 2A). The body weight of all FD-fed groups was significantly higher

Table I. *The composition of fructose-sweetened western diet.*

Diet type	Normal diet		Modified Western diet	
Company	Clea		Research diet	
ID	CE-2		D08041801*	
	g%	Kcal%	g%	Kcal%
Protein	25	25,3	20	17
Carbohydrate	50	62,8	50	42,5
Fructose	-	-	34	29,1
Fat	4,8	11,9	21	40,5
Cholesterol	-	-	0,21	-
Total	-	100	-	100
Kcal/g	3,499	-	4,686	-

Modified Western diet was purchased from Research Diets Inc. (D08041801*, New Brunswick, NJ, USA), normal diet were from CLEA Japan, Inc. (Tokyo). *Sucrose in western diet (D12079B) was replaced with fructose.

at 60 weeks relative to the ND group (34.9±2.8, 50.2±8.7, 53.5±6.7 and 44.1±6.4 g for ND, FD, FD + Ex8w and FD + Ex40w, respectively, (ND *vs*. others: $p<0.001$, FD *vs*. FD + Ex40w: $p=0.051$)). Next, we performed an oral glucose tolerance test at the end of the observation period. The plasma glucose level at each time point is shown in Table II and Figure 2B. FD significantly increased the fasting glucose level (ND *vs*. FD: $p=0.005$), but this increase was diminished in the Ex40 group (ND *vs*. FD + Ex40w: $p=0.182$). At 120 min after glucose ingestion, there was no significant difference among the groups ($p=0.112$). Overall, the glucose level promptly decreased in all experimental groups 60 and 120 min after glucose ingestion (Figure 2B) Thus, forced swimming did not ameliorate FD-induced weight gain in C57BL/6J mice and FD did not affect glucose tolerance.

Forced swimming ameliorated FD-induced hypercholesterolemia in C57BL/6J mice. The FD, which contains substantial amounts of cholesterol (Table I), induced hypercholesterolemia (Figure 2C). At the end of the 60-week observation period, the FD group had significantly higher serum cholesterol levels than the ND group (83.6±16.6 *vs*. 344.2±81.5 mg/dl, $p<0.001$). We next examined the effects of physical exercise on FD-induced hypercholesterolemia. In the FD + Ex8w group that undertook forced swimming for the final 8 weeks of the 60-week period, the serum cholesterol levels at 60 weeks (344.4±77.5 mg/dl) were similar to that of the FD group, but the FD + Ex40w group, which undertook exercise for 40 of the 60 weeks, had lower levels (149.68±40.91 mg/dl, $p<0.001$) compared to FD mice (Figure 2C). Thus, forced swimming for 40 weeks ameliorated hypercholesterolemia induced by FD intake in C57BL/6J mice.

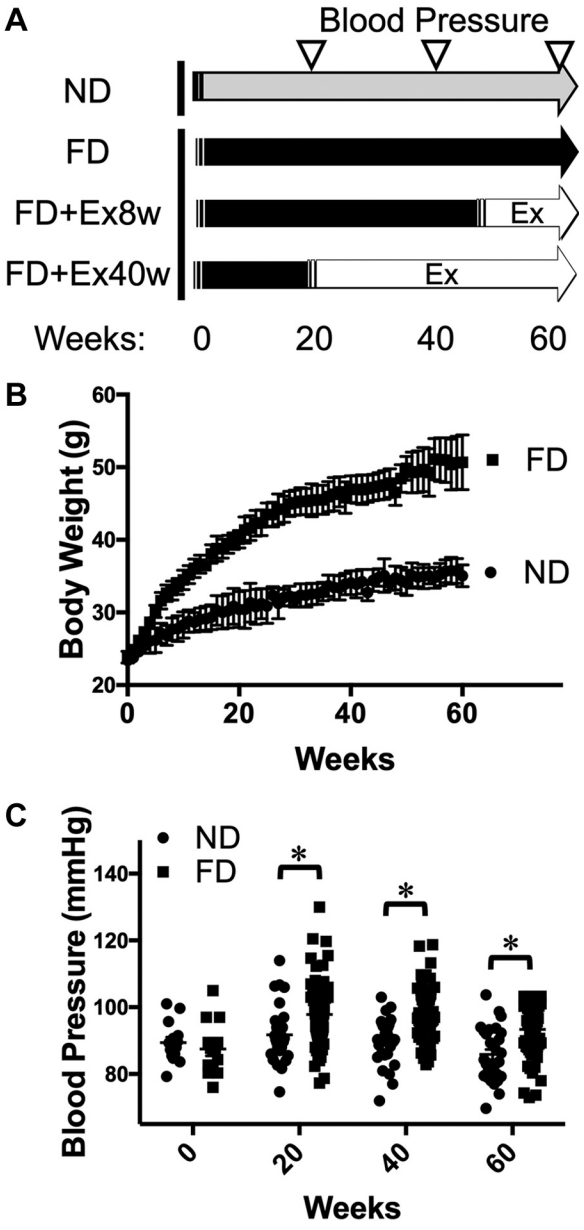

Figure 1. *Fructose-containing western diet induced weight gain and hypertension in C57BL/6J mice. (A) Nutritional challenge and intervention by physical exercise. ND (n=24): mice that were fed a normal diet (ND) as a control group; FD (n=33): mice fed a fructose-containing western diet (FD, see Table I for diet composition) for 60 weeks; FD + Ex8w (n=32): mice that were fed FD for 60 weeks and undertook physical exercise (20 min of forced swimming at the end of a fasting period) during the last 8 weeks of the observation period; FD + Ex40w (n=29): mice fed FD for 60 weeks that undertook physical exercise during the last 40 weeks of the observation period. Blood pressure was measured at 20, 40 and 60 weeks of FD ingestion. (B) Weekly changes in body weight of mice fed ND or FD. FD ingestion was associated with rapid increases in body weight compared with ND ingestion. Data plots and error bars represent mean and SE, respectively. (C) Scatter plot of mean blood pressure readings for individual mice. Bars indicate mean±SD. FD ingestion increased mean blood pressure compared with ND ingestion. Asterisks: p<0.001 by Mann–Whitney U-test. ND: Normal diet ingestion; FD: fructose-containing diet for 60 weeks.*

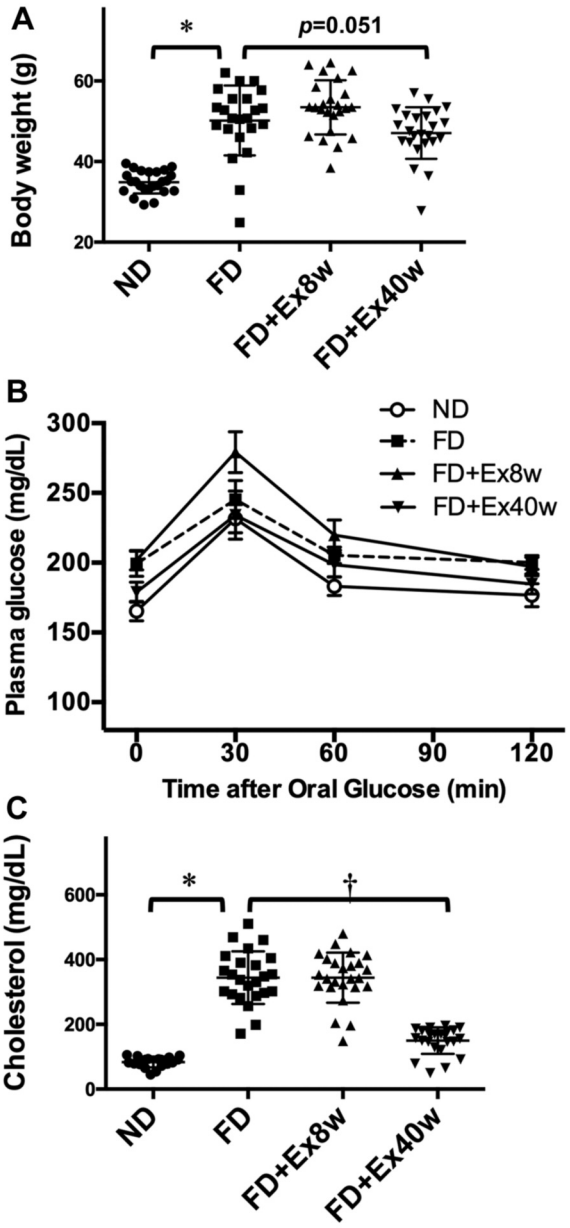

Figure 2. *Forced swimming had limited effects on weight gain and changes in glucose tolerance, but did ameliorate hypercholesterolemia in C57BL/6J mice fed FD. (A) Scatter plot represents body weight of individual mice at 60 weeks of FD ingestion. Bars indicate mean±SD. Asterisk: ND vs. FD: p<0.001. FD vs. FD + Ex40w: p=0.051 (Mann–Whitney U-test). (B) Change in plasma glucose levels after administration of oral glucose at 60 weeks of FD ingestion. Plots indicate a mean value for each group. Error bars represent SE. Glucose levels decreased 60 min after glucose ingestion, indicating normal glucose tolerance. (C) Plasma total cholesterol level for each mouse after 60 weeks of FD ingestion. Bars indicate mean±SD. Asterisk: ND vs. FD: p<0.001. Dagger: FD vs. FD + Ex40w: p<0.001 (Mann–Whitney U test). ND: mice fed a normal diet; FD: mice fed a fructose-containing western diet for 60 weeks; FD + Ex8w: mice fed FD for 60 weeks that undertook physical exercise during the last 8 weeks of the observation period; FD + Ex40w: mice fed FD for 60 weeks that undertook physical exercise during the last 40 weeks of the observation period.*

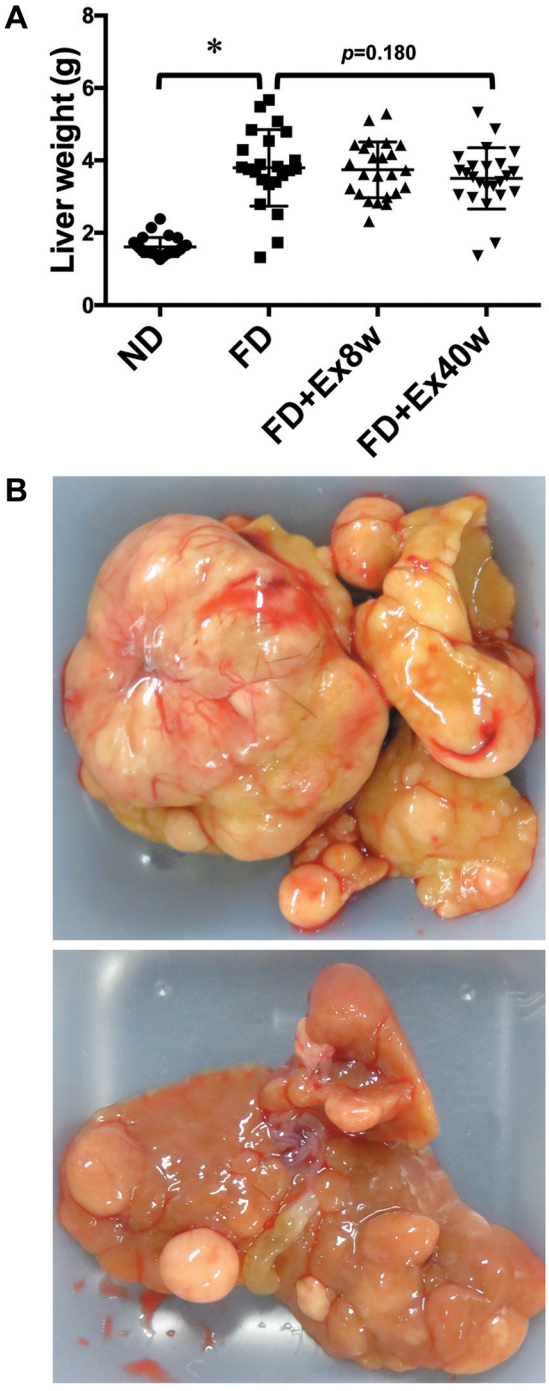

Figure 3. *Intake of FD caused liver weight gain and spontaneous hepatic nodules in C57BL/6J mice. (A) Scatter plot represents liver weight for each mouse at 60 weeks of FD ingestion. Bars indicate mean±SD. Asterisk: ND vs. FD: p<0.001. FD vs. FD + Ex40w: p=0.180 (Mann–Whitney U test). (B) Macroscopic images of dissected livers from FD group mice. The multiple hepatic nodules appeared spontaneously. ND: Mice fed a normal diet; FD: mice fed a fructose-containing western diet for 60 weeks; FD + Ex8w: mice fed FD for 60 weeks that undertook physical exercise during the last 8 weeks of the observation period; FD + Ex40w: mice fed FD for 60 weeks that undertook physical exercise during the last 40 weeks of the observation period.*

Table II. *Oral glucose tolerance test at the end of the observation period.*

	Fasting	30 min after glucose ingestion	60 min after glucose ingestion	120 min after glucose ingestion
ND	165.4±30.1	231.6±47.9	183.1±31.0	176.7±33.7
FD	199.3±42.4	245.3±62.2	205.4±26.2	200.2±21.5
FD + Ex8w	201.9±34.8	279.1±72.0	219.8±51.9	197.2±31.0
FD + Ex40w	178.9±35.2	234.1±80.5	198.5±40.6	184.8±34.7

The plasma glucose level at each time point (mg/dl). Data represent the mean±standard deviation. ND: mice fed a normal diet; FD: mice fed a fructose-containing western diet for 60 weeks; FD + Ex8w: mice fed FD for 60 weeks that undertook physical exercise during the last 8 weeks of the observation period; FD + Ex40w: mice fed FD for 60 weeks that undertook physical exercise during the last 40 weeks of the observation period.

Intake of FD caused liver weight gain and formation of spontaneous hepatic nodules in C57BL/6J mice. At the end of the 60-week observation period, all groups fed FD had significantly higher ($p<0.001$) liver weights compared to the ND group (1.61±0.26 for ND *vs*. 3.50±0.84, 3.74±0.77 and 3.50±0.84 g for in FD, FD + Ex8w and FD + Ex40w, respectively) (Figure 3A). Surface nodules were observed on the livers extracted from the mice in the FD groups, and mice that were fed FD but did not undertake exercise had a higher incidence of nodule formation (0/24 (0%), 7/33 (21.2%), 4/24 (12.5%) and 2/29 (6.9%) for ND, FD, FD + Ex8w and FD + Ex40w, respectively). Thus, FD intake caused liver weight gain and formation of hepatic nodules in C57BL/6J mice.

FD intake induced NASH and nodular regenerative hyperplasia in C57BL/6J mice. To characterize the liver tissue at a pathological level, histological analyses were performed. In mice fed FD, the liver parenchyma showed severe steatosis with swollen lipid droplets in the hepatocytes that was occasionally accompanied by neutrophil infiltration around the portal vein relative to mice fed ND (Figure 4A and B). Masson-Trichrome staining showed collagen fibers in the FD-fed mice (Figure 4D and E), suggesting fibrotic changes in the liver. In mice fed ND, PAS staining showed synthesized glycogen that was absent in some mice fed FD (Figure 4G and H). Immunohistochemistry for Mcm2 showed proliferating hepatocytes in livers from mice fed FD (Figure 4K). The findings in the liver parenchyma of mice fed FD were compatible with NASH. Focusing on the hepatic nodules in the mice fed FD, lipid droplets were mostly absent, but hepatocytes and their nuclei were enlarged; the hepatocytes were less organized but not fully dysplastic (Figure 4C). Some degree of glycogen synthesis was observed in the regenerative nodules by PAS staining (Figure 4I). The number of MCM2-positive proliferating hepatocytes was markedly increased in the regenerative nodules (Figure 4L). From these findings, the hepatic nodules were diagnosed as non-neoplastic nodular regenerative hyperplasia. Taken together, FD induced NASH and nodular regenerative hyperplasia in C57BL/6J mice.

FD intake deregulated circadian expression of genes related to lipid metabolism and increased expression of genes involved in inflammation or regeneration in C57BL/6J mice. To investigate the underlying mechanism of FD-induced NASH and nodular regenerative hyperplasia, we measured circadian expression of genes related to lipid metabolism (Figure 5A, *Acaca, Acly, Fasn, Cyp7a1, Cyp7b1* and *Pparg*) and inflammation (Figure 5B, *Ccl2, Tnf* and *Trem2*), as well as genes encoding growth factors (Figure 5C, *Fgf2, Hgf* and *Pdgfb*) in liver tissue. Two-way analysis of variance (ANOVA) rejected the null hypothesis concerning interaction effects between time difference and experimental groups for *Acaca, Acly, Fasn* and *Cyp7b1* (Figure 5A). This result suggests that the nutritional challenge alters the circadian rhythm of lipid metabolism. Two-way ANOVA retained the null hypothesis about interaction effects for other genes. Thus, we then examined the overall influence of the nutritional challenges with or without exercise. FD remarkably increased the expression of *Pparg*, *Ccl2*, *Tnf*, *Trem2*, *Fgf2*, *Hgf*, and *Pdgfb* (respective mean difference expressed as fold-increase for FD relative to ND: 2.39±1.07, 9.85±1.13, 5.98±1.12, 29.90±1.16, 3.00±1.08, 2.21±1.06, and 4.28±1.10; Figure 5A-C). Although Ex8w and Ex40w had completely normalized *Tnf* levels (Figure 5B), neither Ex8w nor Ex40w exhibited any beneficial influence on expression levels of *Pparg* (Figure 5A), *Ccl2* (Figure 5B), *Trem2* (Figure 5B), *Fgf2* (Figure 5C), *Hgf* (Figure 5C) and *Pdgfb* (Figure 5C). Ex40w partially normalized the deregulated expression of *Cyp7b1* seen with FD (Figure 5A), which is consistent with the cholesterol decrease seen for the FD + Ex40w group (Figure 2C). Taken together, FD intake deregulated circadian expression of genes related to lipid metabolism while increasing expression of genes related to inflammation and growth factors. Physical exercise ameliorated the deregulation of *Tnf* and *Cyp7b1*.

Discussion

A leading hypothesis for NAFLD progression is the two-hit model (10). The first hit involves ectopic lipid accumulation

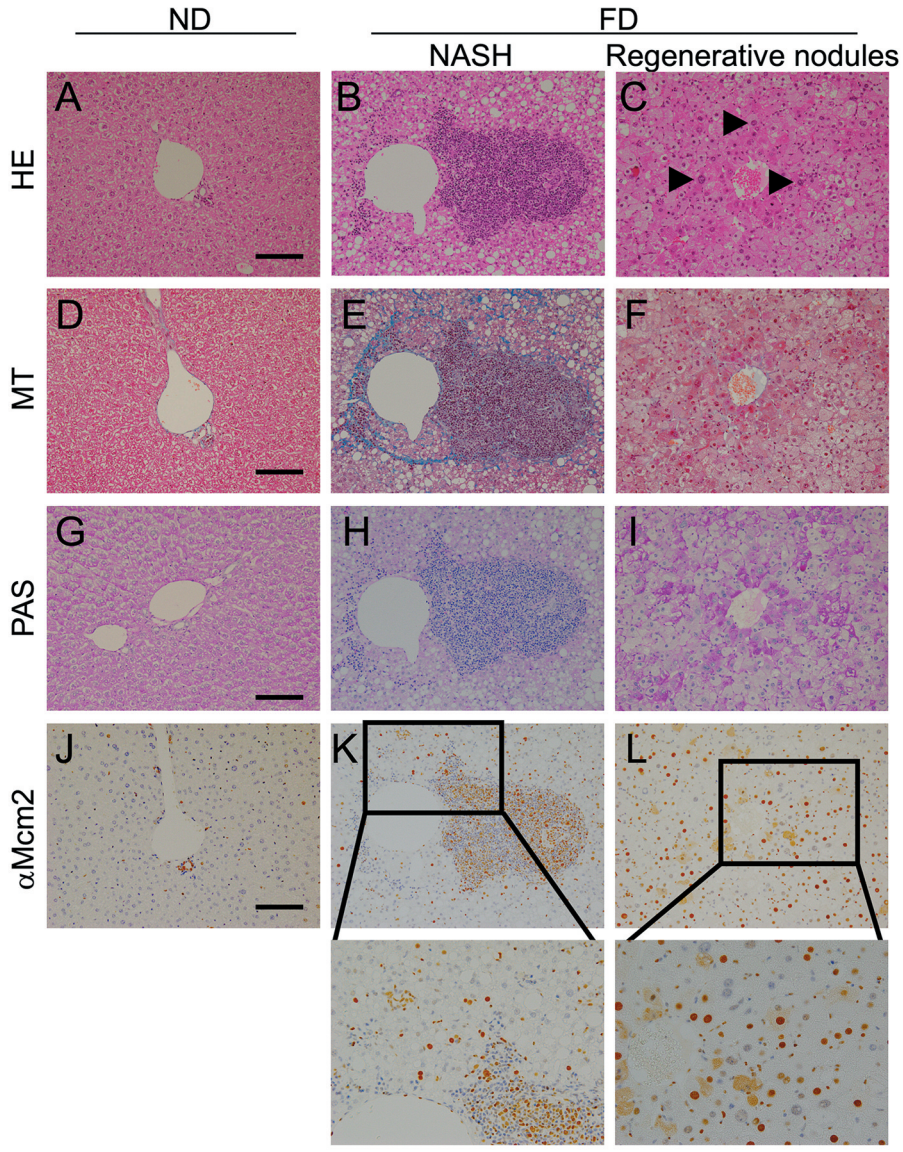

Figure 4. *FD intake induced NASH and nodular regenerative hyperplasia in C57BL/6J mice. Representative micrographs of liver tissue from mice fed ND (left column) or FD (middle column), and hepatic nodules from mice fed FD (right column). (A-C) HE staining. The liver parenchyma of FD mice included hepatocytes with large lipid droplets, indicating severe steatosis. (B) Neutrophils infiltrated around a portal vein. (C) In the hepatic nodules, hepatocyte nuclei were enlarged (arrowheads). (D-F) MT staining. E. Collagen was synthesized around the portal vein in the liver parenchyma of mice fed FD. G-I. PAS staining. (H) Glycogen synthesis was attenuated in the liver parenchyma of mice fed FD. (I) Glycogen synthesis was retained in the hepatic nodules. (J-K) Anti-Mcm2 staining. The number of proliferating hepatocytes appearing in the liver parenchyma of mice fed FD (K) and in the hepatic nodules was remarkably increased (L). A-L: ×200, K and L: ×200; HE: Hematoxylin eosin; MT: Masson trichrome; PAS: periodic acid-Schiff; Mcm2: Minichromosome Maintenance Complex Component; ND: normal diet ingestion; FD: fructose-contained diet ingestion for 60 weeks.*

in hepatocytes due to metabolic disorders including insulin resistance characterized by obesity, hypertension, and abnormal glucose and lipid metabolism. The second hit involves less well-characterized factors or combinations of factors, including genetic predisposition, inflammation, and oxidative stress (10). Multiple rodent models mimicking obesity reproducibly induce steatosis (11-13). However, whether the steatosis was followed by development of features typically associated with NAFLD progression, such as NASH, fibrosis and hepatocellular carcinoma, is unclear.

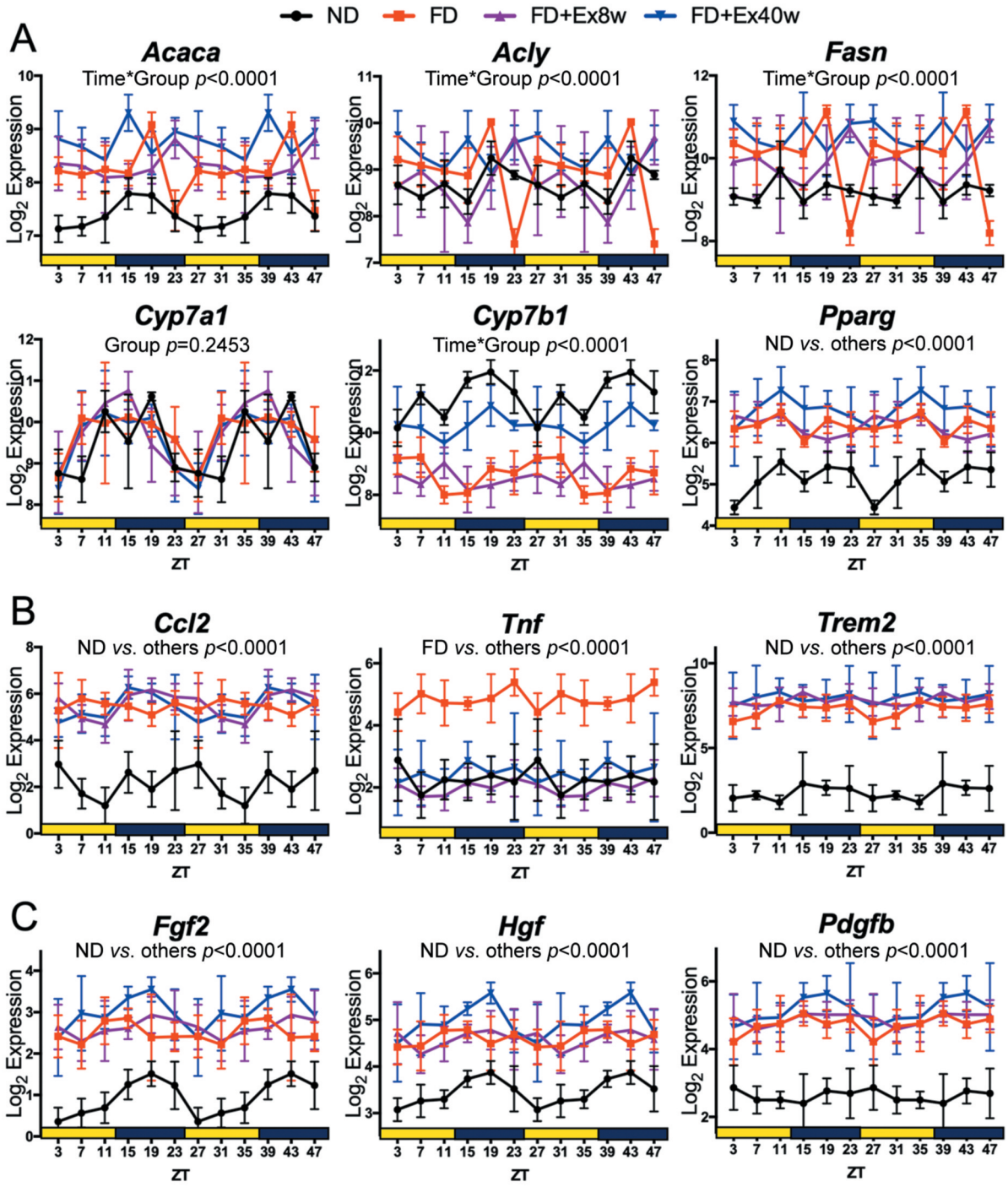

Figure 5. *FD intake deregulated circadian expression of lipid metabolism-related genes and increased expression of marker genes for inflammation or genes encoding growth factors in C57BL/6J mice fed FD. Expression levels of each indicated gene in the liver were measured by qRT-PCR at the indicated Zeitgeber time (ZT). ZT 0:00 was set as the beginning of the light period. Relative log2 expression values are shown. (A) Genes related to lipid metabolism. Note that FD deregulated the circadian pattern for Acaca, Acly, Fasn and Cyp7b1. Physical exercise for 40 weeks partially normalized Cyp7b1 deregulation. (B) Expression of genes related to inflammation. FD increased overall inflammatory gene expression, exercise for 8 or 40 weeks normalized Tnf expression. (C) Genes related to growth factors. FD increased overall expression of the examined growth factor genes. Although exercise failed to decrease overall expression levels, it normalized the circadian pattern (see blue and black lines for Fgf2 and Hgf). ND: mice fed a normal diet; FD: mice fed a fructose-containing western diet for 60 weeks; FD + Ex8w: mice fed FD for 60 weeks that undertook physical exercise during the last 8 weeks of the observation period; FD + Ex40w: mice fed FD for 60 weeks that undertook physical exercise during the last 40 weeks of the observation period.*

Recently, a Japanese biotechnology company established the STAM® model, in which streptozotocin (STZ)-treated mice fed a high-fat diet reproducibly exhibit NAFLD progression (14-16). STZ-treated model mice are a well-established model of type-1 diabetes, supporting evidence that hyperglycemia is a contributing factor to NAFLD. In this study, we found that C57BL/6J mice fed FD over a 60-week period developed obesity (Figure 1B), transient hypertension (Figure 1C), hypercholesterolemia (Figure 2C), and liver adiposity (Figure 4B), but maintained glucose tolerance (Figure 2B). In addition, mice fed FD developed spontaneous hepatic nodules (Figure 3B) that were histopathologically diagnosed as nodular regenerative hyperplasia (Figure 4). Mice fed FD also showed increased expression of genes encoding growth factors such as *Fgf2, Hgf,* and *Pdgfb* (Figure 5B and C) (17). Although hepatocytes in liver parenchyma from mice fed FD synthesized very little glycogen, regenerating hepatocytes in the nodules did retain glycogen synthesis activity (Figure 4I and L).

Physical exercise involving forced swimming undertaken for 20 min on each weekday had relatively mild effects on ameliorating FD-induced body weight gain (Figure 2A) and effects on liver adiposity (Figure 3A) were limited. Nevertheless, the physical exercise did ameliorate hypercholesterolemia (Figure 2C), and partially normalized expression of *Cyp7b1*, a gene related to cholesterol metabolism (Figure 5A) (18, 19). Although the expression of most inflammatory genes was not affected, *Tnf* expression was normalized by either shorter (8 weeks) or longer (40 weeks) duration of exercise (Figure 5B). This evidence suggests that even mild exercise has beneficial effects, especially on cholesterol metabolism.

We also found that circadian expression of genes related to lipid metabolism was deregulated by FD intake (Figure 5A). Genes involved in lipid and cholesterol metabolism are under the control of circadian rhythm, which indicates that clock genes have a crucial role in the development of NAFLD (20, 21). Circadian rhythms are continuously adjusted by two factors, light-dark cycles and nutritional input. The latter is especially important in the context of metabolism. Oda *et al*. (22) recently reported that a high-sucrose diet diminished oscillations in clock gene expression and augmented the expression of gluconeogenic enzymes in the small intestines of Wistar rats. Accumulating evidence indicates that correcting eating-fasting cycles, rather than total caloric restriction, is beneficial for pre-diabetic conditions (23-25). Therefore, time-restricted feeding of FD could normalize deregulated expression of genes such as *Acaca, Acly, Fasn,* and *Cyp7b1* (Figure 5A). This possibility could be addressed in future studies.

The FD model resembles the STAM model in that NAFLD progresses over steatosis and hepatic nodules spontaneously appear without genetic manipulation (14, 15). However, unlike the STAM model, we did not observe hyperglycemia and glucose intolerance in the FD model (Figure 2B). Development of carcinoma in the FD-ingestion model would likely require genetic manipulation such as oncogene transgene expression or knockout of a tumor suppressor gene (26, 27).

Conflicts of Interest

The Authors declare that they have no conflicts of interest in relation to this study.

Authors' Contributions

KI conceptualized the study. KI and HY performed the experiments and validated the data. TI and ST funded the project. KI wrote the manuscript. All the Authors critically read the manuscript.

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