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Accumulation of endoplasmic reticulum stress and lipogenesis in the liver through generational effects of high fat diets

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Background & Aims: The dramatic rise of nonalcoholic fatty liver disease (NAFLD) among children in the past decade cannot be solely explained by the increased high fat diet (HFD) intake in kids. Recent studies suggest that the offspring of HFD-fed mothers develop a worse form of NAFLD when weaned on the HFD than when weaned on the normal chow (NC), indicating that a feed-forward circle may exacerbate the syndromes throughout multiple generations. In the present study, the aforementioned feed-forward circle was investigated in mice by employing continuous HFD feeding for three generations.

Methods: C57BL/6 mice were fed with either a HFD or NC for three consecutive generations (F0, F1, and F2). Body weight, food intake, hepatic histology; levels of insulin, leptin, and triglycerides; expression of factors involved in lipogenesis and endoplasmic reticulum (ER) stress pathways; and histone methylation status were investigated in male offspring.

Results: Obesity occurred earlier, became more severe through generations (F2 >F1 >F0), and was accompanied by a gradual increase of histological scoring of steatosis in male mice with transgenerational HFD feeding. The highest degree of steatosis occurred in HFD-treated F2 mice and was associated with the highest levels of insulin and leptin. The latter mice were characterized by enhanced lipogenesis and ER stress with a trend of transgenerational changes was detected for LXRα, ERO1-α, histone methylations, and H3K9 histone methyltransferase. Furthermore, chromatin immunoprecipitation (CHIP) assay demonstrated a significantly reduced accumulation of methylated histones in *LXRα* and *ERO1-α* gene promoters.

Conclusions: Under HFD feeding stress, the male offspring of the F2 generation (derived from both grand-maternal and maternal obesity) are extremely susceptible to developing obesity and hepatic steatosis. This is presumably a consequence of transgenerational accumulation of epigenetic modifications leading to up-regulation of lipogenesis and ER stress pathways in the liver.

Keywords: NAFLD; Maternal obesity; Lipogenesis; ER stress; Histone methylation.

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Abbreviations: NAFLD, nonalcoholic fatty liver disease; T2DM, type 2 diabetes mellitus; HFD, high fat diet; NC, normal chow; HF F0, high fat-fed offspring of normal chow fed mice; HF F1, high fat-fed offspring of HF F0 mice; HF F2, high fat-fed offspring of HF F1 mice; NC F0, normal chow-fed offspring of normal chow fed mice; NC F1, normal chow-fed offspring of NC F0 mice; NC F2, normal chow-fed offspring of NC F1 mice; ER, endoplasmic reticulum; BW, body weight; GTT, glucose tolerance test; ITT, insulin tolerance test; SREBP-1c, sterol regulatory element binding protein 1c; LXR, liver X receptor; PPAR, peroxisome proliferator-activated receptor; FASN, fatty acid synthase; NFBG, non-fasted blood glucose; FBG, fasted blood glucose; pSREBP-1, precursor SREBP-1; mSREBP-1, mature SREBP-1; eIF2α, eukaryotic initiation factor 2α; BIP, binding immunoglobulin protein; ERO1-α, oxireductase endoplasmic reticulum oxidoreductin1-α.

Introduction

Nonalcoholic fatty liver disease (NAFLD) is the most common cause of chronic liver diseases, with a prevalence of 10–31% worldwide [1,2]. NAFLD is usually associated with obesity and type 2 diabetes mellitus (T2DM) [2], and can gradually develop from simple steatosis to steatohepatitis, advanced fibrosis, and eventually cirrhosis [3].

In the past decades, the global prevalence of obesity and NAFLD has dramatically increased in both adults and children [4,5]. The disproportionately early-onset of obesity and NAFLD among children can be partially explained by Baker's "Fetal origins of adult disease" hypothesis [6], which is supported by the fact that high fat diet (HFD) intake during pregnancy increases the rates of obesity and metabolic disorders, including NAFLD, in offspring [7–10]. To date, most studies on rodent models have focused on the relationships between diet-induced obesity in female rodents (F0) and the development of metabolic diseases in adult offspring (F1) weaned on normal chow (NC). Under this condition, the HFD may impact the F1 generation mostly through developmental over-nutrition. However, a recent study suggests that alterations of neural circuitry in offspring from maternal obesity (F0) may lead to the offspring (F1) preferring HFD or hyperphagia [11]. Under this condition, the effects of a HFD on the F1 generation are through both developmental and adult over-nutrition. In fact, the F1 offspring under both developmental and adult over-nutrition insults (F1 offspring of maternal obesity weaned on a HFD) develop nonalcoholic steatohepatitis, a worse form of NAFLD, when they become adults. However, F1 offspring under only developmental over-nutrition insult (F1 offspring of maternal obesity weaned on NC) develop only NAFLD when they

become adults [12]. This suggests a possible feed-forward circle that may exacerbate the syndromes of fatty liver disease throughout multiple generations.

We hypothesized that the feed-forward circle may result in a transgenerational exacerbation of obesity and associated diseases, such as NAFLD, under continuous HFD feeding stress. In this work, HFD and NC were used to feed mice for three consecutive generations to generate six experimental groups. The onset of obesity and the degree of NAFLD were compared among these groups. Lipogenesis and endoplasmic reticulum (ER) stress signaling were also investigated, which was motivated by the accumulating evidence that increased lipid storage by *de novo* lipogenesis and ER stress in the liver is an important factor contributing to the development of NAFLD [13–16]. Epigenetic modifications and alterations of lipogenic and ER stress pathways were demonstrated as well, and the observation that environmental factors may cause the epigenetic transgenerational effects in disease etiology [17] has led us to speculate that epigenetic changes may be involved in the effects of feed-forward circle. Our results provide new insights into the underlying molecular mechanisms underpinning the world epidemic of NAFLD.

Materials and methods

Animals, diets, and experimental design

Breeding pairs of C57BL/6 mice were obtained from the Wuhan University Animal Laboratory and housed in ventilated microisolator cages with free access to water and NC to get the F0 generation. At 4–6 weeks of age, male and female F0 offspring were fed with the NC or HFD (60% kcal fat; Research Diets Inc., New Brunswick, NJ) and three generations of continuous HFD-fed or NC-fed were generated as shown in Fig. 1A. Physiological parameters of experimental animals such as body weight (BW) and food intake were measured weekly. Only male mice were used in this study. Organs from different groups were dissected under a microscope and weighed. Animals were handled according to the Guidelines of the China Animal Welfare Legislation, as provided by the Committee on Ethics in the Care and Use of Laboratory Animals of Wuhan University.

Glucose tolerance test (GTT) and insulin tolerance test (ITT)

GTT and ITT tests were performed in all experimental groups at 3 months of age. For GTT assays, mice were fasted overnight and 1.5 g/kg BW of glucose was injected intraperitoneally. Blood glucose was measured at different times after the injection. The data were plotted as blood glucose concentration over time. For ITT assays, mice were fasted for 6 h and 0.5 U/kg BW of insulin (Lily France S.A.S., France) was injected intraperitoneally. Blood glucose was measured at different times after the injection. The data were plotted as percentage of initial blood glucose concentration over time.

Serum levels of insulin, leptin, and triglyceride

Serum samples were collected for insulin, leptin, and triglyceride analyses. Measurements were performed using an insulin ELISA kit (Millipore Corporation, Billerica, MA), a leptin ELISA kit (Millipore Corporation), and a triglyceride assay kit (Cayman, Ann Arbor, MI), according to the manufacturers' instructions.

Histological analysis and pathological evaluation

Liver samples were routinely embedded in paraffin and cut. Sections were stained with hematoxylin–eosin (H&E). Pictures of 4–6 different fields per sample were taken under an Olympus BX60 microscope equipped with a digital CCD, and semi-quantitative analyses of liver histology were performed in a blinded fashion by experienced liver pathologists, as previously reported [18].

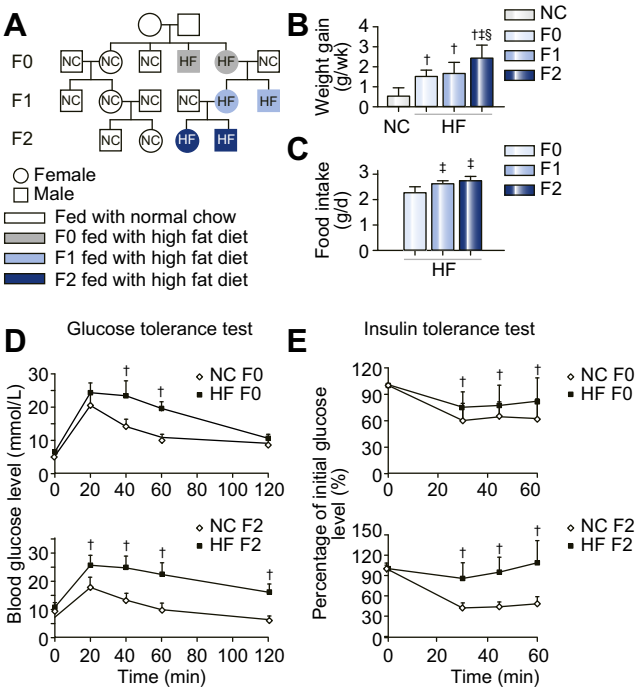

Fig. 1. Increased body weight gain, glucose intolerance, and insulin resistance in the HF F2 male mice. (A) Experimental design. (B) Weight gain and (C) food intake are shown. NC mice, normal chow-fed mice (males, n = 16); HF F0 mice, high fat diet fed mice F0 generation (males, n = 5); HF F1 mice, high fat diet fed mice F1 generation (males, n = 4); HF F2 mice, high fat diet fed mice F2 generation (males, n = 14). (D) GTT and (E) ITT were performed as described in Materials and methods, with the upper panels showing results for the F0 generation and the bottom panels showing results for the F2 generation (males, n = 4–7 per group). † p <0.05 compared to the NC mice; $^{\ddagger}p$ <0.05 compared to the HF F0 mice; $^{\S}p$ <0.05 compared to the HF F1 mice. (This figure appears in colour on the web.)

Histone extraction

Extraction of liver histones was performed as previously described [19]. Briefly, livers were homogenized and the nuclear fraction was collected. H_2SO_4 (0.2 M) was added to the nuclear fraction to extract histones. Histones were precipitated by TCA, and washed with ice-cold acetone three times. After quantification with the Bio-Rad Protein Assay (Bio-Rad, Hercules, CA), purified histones were aliquoted and stored at −80 °C until use.

Western blotting

Liver tissues were sonicated in RIPA buffer (Beyotime Biotechnology, Jiangsu, China) to obtain whole cell lysates. Western blot analysis was performed as previously reported [20]. Antibodies for sterol regulatory element binding protein 1c (SREBP-1c), liver X receptors (LXRs) and peroxisome proliferator-activated receptor γ (PPARγ) were obtained from Santa Cruz Biotechnology (Santa Cruz, CA), while the remaining antibodies were purchased from Cell Signaling Technologies (Danvers, MA). The expression levels of target proteins were quantified with the Quantity One 1-D Analysis Software (Bio-Rad) and normalized to the Coomassie staining in the same sample. The levels of modified histones were quantified relative to the levels of histone H3 in the same sample.

Real-time PCR

Total RNA was extracted using RNAiso Plus (Takara Biotechnology Co., Dalian, China). One microgram of RNA of each sample was reverse transcribed into cDNA using ReverTra Ace-α kit (Toyobo Co., Osaka, Japan). The PCR was performed with specific primers for the targeted genes. Transcriptional levels of fatty acid

synthase (*FASN*) and stearoyl-CoA desaturase-1 (*SCD-1*) were quantified using 18S rRNA in the same sample as internal control. The sequences of primers used are described in Supplementary Table 2.

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) was performed as previously reported [21] with some modifications. Briefly, liver tissues were cut and fixed with 1% formaldehyde, the reaction was then stopped by glycine. Pellets were resuspended in RIPA buffer (Beyotime Biotechnology). DNA was sheared into 200–700 bp in length, and an aliquot was saved as input. Twenty-five micrograms of DNA from each sample were resuspended in RIPA buffer containing Me-K antibody (Abcam ab7315) plus salmon sperm DNA–protein G agarose for overnight incubation. Immune complexes were collected, washed, and eluted with elution buffer (1% SDS, 100 mM $NaHCO_3$). The DNA–protein cross-links were reversed by incubating overnight at 65 °C. DNA was extracted and PCR analyses were performed on *LXRα* and *ERO1-α* promoter regions (primer sequences shown in Supplementary Table 2). The input samples were used as an internal control for comparison between samples.

Statistical analysis

All results were expressed as mean ± SD (standard deviation). Statistical significance was determined by analyzing the data of variance by the Kruskal–Wallis test, followed by the Mann–Whitney test. Differences were considered statistically significant when p <0.05. The changes were considered as trends when 0.05< p <0.08.

Results

Effects of HFD-feeding on the general physiological features of experimental groups

The general physiological characteristics of experimental groups are provided in Table 1. Since there were no significant differences among the control groups of NC F0, NC F1, and NC F2 (Supplementary Table 1), data on these three groups were combined and referred to as the NC group. As expected, HFD-feeding induced a significant increase in BW, body mass index (BMI), and epididymal fat weight in the HF F0 group compared to those of the NC group. In the second (HF F1) generation, the liver weights were significantly heavier than those of the NC group. Surprisingly, the third (HF F2) generation showed dramatic and significant increases in BW, BMI, and liver weight under the same HFD condition as the HF F0 and HF F1 generations. A clear trend of transgenerational accelerated BW gain was identified (Fig. 1B). However, a trend of increasing food intake in these three-generation HFD-fed mice was not observed (Fig. 1C). Furthermore, the levels of non-fasted blood glucose (NFBG) and fasted blood glucose (FBG) were significantly increased in the HF F2 and F1 groups compared to the NC group (Table 1).

Transgenerational effects on glucose tolerance, insulin sensitivity and serum levels of insulin, leptin and triglyceride in HFD-fed obese mice

As expected, the HF F0 mice showed impaired glucose tolerance compared with the NC F0 mice, as demonstrated by GTT. The HF F2 group showed an even more severe glucose intolerance compared to the HF F0 group, as the returning rate of blood glucose to the normal level was significant slower in the HF F2 mice compared to the NC F2 mice (Fig. 1D). The ITT results revealed that the HF F0 group also showed reduced insulin sensitivity compared to the NC F0 mice. The HF F2 mice showed more severely impaired insulin sensitivity compared with the HF F0 mice, since the amount of insulin administrated could not determine any significant decrease of blood glucose levels during the testing period (Fig. 1E). In the HF F1 mice, no further increase in glucose intolerance or further decrease in insulin sensitivity were observed when compared to HF F0 mice (data not shown).

Consistent with the GTT and ITT results, serum levels of insulin were also significantly increased in the HF F2 mice compared to the HF F0 and NC groups (Table 1). Moreover, serum leptin levels were significant increased in the HF F0 and HF F1 groups when compared with those in the NC group, and were further significantly increased in the HF F2 group compared to the HF F1 and HF F0 groups (Table 1). Similarly, levels of serum triglyceride were also significantly increased in the HF F1 and HF F2 groups compared to the NC mice (Table 1).

Transgenerational effects on hepatic steatosis score in HFD-fed obese male mice

The grade of hepatic steatosis was amplified in the HF F0 and HF F1 groups compared to the NC group, and a further increased severity of steatosis was observed in the HF F2 mice when com-

Table 1. Physiological characteristics of different experimental groups.

	NC	HF		
		F0	F1	F2
Body weight (g)	24.9 ± 1.6	35.4 ± 2.9†	39.0 ± 2.0†	52.4 ± 6.4†‡§
BMI (kg/m²)	3.3 ± 0.2	4.2 ± 0.4†	4.3 ± 0.4†	5.3 ± 0.3†‡§
Liver weight (g)	1.03 ± 0.11	1.08 ± 0.21	1.26 ± 0.11†	2.26 ± 0.94†‡§
Epididymal fat weight (g)	0.18 ± 0.04	0.96 ± 0.20†	1.27 ± 0.31†	1.33 ± 0.32†
NFBG (mmol/L)	10.2 ± 2.2	10.8 ± 2.3	14.1 ± 1.4†‡	12.8 ± 1.9†
FBG (mmol/L)	7.75 ± 2.7	9.33 ± 1.2	15.9 ± 5.5†	12.4 ± 2.7†
Insulin level (ng/ml)	0.11 ± 0.13	0.56 ± 0.85	0.78 ± 1.03	2.29 ± 1.37†‡
Leptin level (ng/ml)	1.19 ± 1.2	37.1 ± 13.2†	38.4 ± 5.3†	63.8 ± 3.8†‡§
Serum TG level (mg/dl)	21.3 ± 5.0	29.5 ± 7.7	50.6 ± 28.4†	32.7 ± 11.6†

NC, normal chow-fed male mice from F0, F1, and F2 generations; HF F0–F2, high fat diet fed male mice F0–F2 generation; BMI, body mass index; NFBG, non-fasted blood glucose; FBG, fasted blood glucose; TG, triglyceride. n = 6–12/group. Statistically significant differences are indicated as follows: †p <0.05 compared with NC; ‡p <0.05 compared with HF F0; §p <0.05 compared with HF F1.

pared to the HF F0 and HF F1 groups. As shown in H&E stained liver sections (Fig. 2A), an increase in the severity of hepatic steatosis over generations was observed. Steatosis was mild in the HF F0 and HF F1 mice, becoming more severe with frequent cytologic ballooning in the HF F2 group (Fig. 2B). In accord with the score of hepatic steatosis, the locations of steatosis invasion were zone 1 or azonal in the HF F0 and HF F1 mice, and panacinar in the HF F2 group. Furthermore, prominent ballooned hepatocytes were found in the HF F2 group, whereas no ballooning was observed in the NC and HF F0 mice. Together, these data suggest that under the same duration of HFD stress, the HF F2 group developed a much more severe form of steatosis compared to the HF F0 and HF F1 groups.

Transgenerational effects on lipogenesis-related factors in HFD-fed obese mice

To address the underlying mechanisms of the transgenerational increases in liver weight and aggravated hepatic steatosis in the continuous HFD-fed mice, mRNA and/or protein levels of multiple transcription factors and enzymes involved in lipogenesis were assessed. We found that mRNA levels of *FASN* (fatty acid synthase) were significantly increased in the HF F1 and HF F2 groups compared with the NC group (Fig. 3A). FASN protein levels were gradually and significantly increased in the HF F0 and HF F1 groups compared to the NC group; a slight further increase was observed in the HF F2 group compared to the HF F1 group, although the data did not achieve statistical significance (Fig. 3B). The mRNA levels of *SCD-1* (stearoyl-CoA desaturase-1) were significantly increased in the HF F2 mice compared to the other groups (Fig. 3A). The protein levels of both precursor SREBP-1 (pSREBP-1) and mature SREBP-1 (mSREBP-1) in the HF mice were increased compared to the NC group. The levels of mSREBP-1 were 1.5-fold, 2.5-fold, and 2.3-fold higher in the HF F0, HF F1, and HF F2 groups, respectively (Fig. 3C). Furthermore, there was also a significant increase of the mSREBP-1 protein in the HF F1 and HF F2 groups compared to the HF F0 group (Fig. 3C). Although the protein levels of pSREBP-1 were increased in the HF F0 (4.3-fold) and HF F1 (5.1-fold) groups compared to the NC mice, they were decreased in the HF F2 group compared with the HF F0 and HF F1 groups, but still significantly higher than the NC group (Fig. 3C). The levels of LXRα show a clear transgenerational accumulation in the liver with a gradual increase in the HF F0 (4.8-fold), HF F1 (7.4-fold), and HF F2 (13.2-fold) groups compared with the NC group (Fig. 3D). Similarly, markedly increased levels of LXRβ were observed in the HF F2 group compared with the other groups (Fig. 3D). No difference in the levels of PPARγ was observed in the HFD-fed mice through generations (Fig. 3B). Changes in multiple lipogenic transcription factors and enzymes in the HF F2 group indicate that increased lipogenesis may be one of the pathogenetic mechanisms responsible for the development of severe hepatic steatosis in the HF F2 group.

Transgenerational effects on hepatic ER stress markers in HFD-fed obese mice

The ER is the main site for lipid synthesis in the cell. The excess acyl-CoA produced by liver mitochondria after HFD stress is shuttled to the ER as key substrate for lipid synthesis [12]. The stimulation of lipid synthesis after HFD intake disrupts the ER homeostasis and leads to ER stress [22]. Previous studies have also suggested that activation of ER stress after over-nutrition

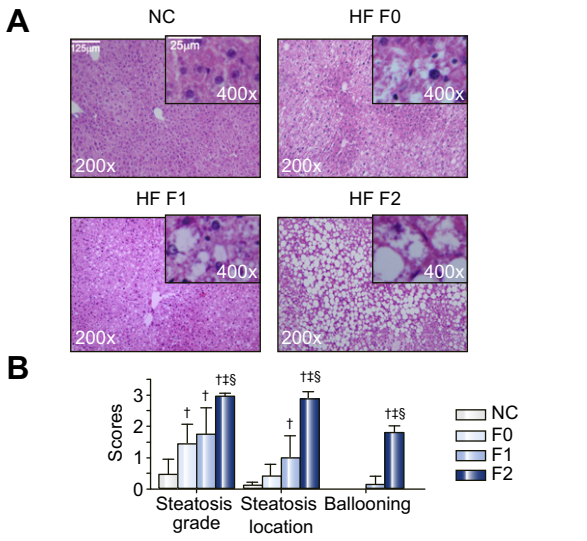

Fig. 2. Gradually severe hepatic steatosis in HFD-fed male mice through generations. (A) Representative pictures of H&E stained liver sections of the NC, HF F0, HF F1, and HF F2 groups. (B) Scores of hepatic steatosis grade, steatosis location and ballooning of experimental groups. Males, n = 6–9 per group. †p <0.05 compared to the NC mice; ‡p <0.05 compared to the HF F0 mice; §p <0.05 compared to the HF F1 mice.

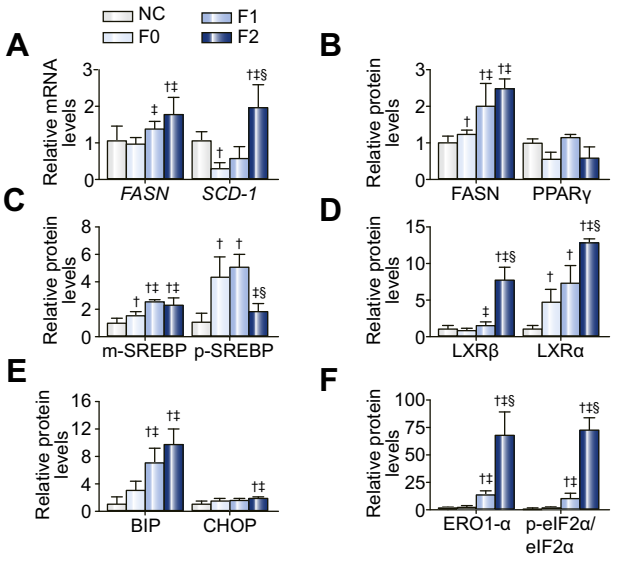

Fig. 3. Accumulation of hepatic lipogenesis-related factors and ER-stress markers in HFD-fed male mice through generations. (A) Quantitative results of relative mRNA levels of *FASN* and *SCD-1* in different experimental groups. (B–F) Quantitative results of relative protein levels of FASN, PPARγ, SREBP-1 (precursor, p and mature, m), LXRs, BIP, CHOP, ERO1-α, phosphorylation of eIF2α and total eIF2α in different experimental groups. †p <0.05 compared to the NC mice; ‡p <0.05 compared to the HF F0 mice; §p <0.05 compared to the HF F1 mice.

contributes to the development of hepatic steatosis by regulating the process of lipogenesis [23]. This results in a positive regulation between ER stress and lipid synthesis. The PKR-like eukaryotic initiation factor 2α kinase branch of ER stress, including the levels of phosphorylated eukaryotic initiation factor 2α (p-eIF2α) and C/EBP homologous protein (CHOP) were investigated. Levels

of two ER chaperones, binding immunoglobulin protein (BIP) and protein disulfide isomerase (PDI), and an ER oxireductase, ERO1-α (oxireductase endoplasmic reticulum oxidoreductin 1-α) were also evaluated. The expression levels of BIP were gradually and significantly increased in the HF F0 (2.9-fold), HF F1 (5.4-fold), and HF F2 (7.0-fold) groups compared to the NC group. Similarly, CHOP, the executor of ER stress dependent cell death, was significantly up-regulated in the HF F2 group compared to the HF F0 and NC groups (Fig. 3E). Furthermore, the levels of ERO1-α were found to be gradually increased in the HF F1 (14-fold) and HF F2 (66-fold) groups compared to the NC group, accompanied by a gradual increase of p-eIF2α/eIF2α in the HF F1 (10-fold) and HF F2 (71-fold) groups (Fig. 3F). However, there was no difference in the levels of PDI among all experimental groups (Supplementary Fig. 1). These results suggest that the accumulation of ER stress markers may synergize with up-regulation of lipogenesis inducers to induce severe hepatic steatosis in the HF F2 mice.

Transgenerational effects on histone methylation in HFD-fed obese mice

Since genomic mutation induced by HFD-feeding tends to be very low, it is plausible that epigenetic changes might contribute to this transgenerational amplification of the NAFLD phenotype in male offspring. Thus, histone methylation levels, the static epigenetic markers of gene repression [24], were investigated using enriched histone extracted from the livers of different experimental groups. Levels of methylated histone H3 showed a trend of gradual decrease in the HF F1 and HF F0 groups compared to the NC group, with a further dramatic decrease in the HF F2 group compared to the other three groups (Fig. 4A). Dimethylation of histone H3 lysine 9 (H3K9Me2), a critical epigenetic marker of gene repression [25], was also studied. The levels of H3K9Me2 also showed a trend to decrease (p <0.08 compared with HF F0) in the HF F1 and HF F2 groups compared to the HF F0 and NC groups. However, the levels of H3K27Me3, the epigenetic marker usually associated with tumorigenesis [26], were similar in all experimental groups (Fig. 4A). Levels of G9a, a histone methyltransferase responsible for H3K9 dimethylation [27], were dramatically down-regulated in the HF F2 group compared with the other groups (Fig. 4B). Significantly less methylated histones were bound to *LXRα* and *ERO1-α* gene promoters in the HF F2 mice when compared with the NC mice, as demonstrated by ChIP (Fig. 4C). These findings suggest that the dramatic up-regulation of lipogenesis and ER stress in the HF F2 group may be the results of epigenetic regulation through less methylated histones bound to their promoter regions.

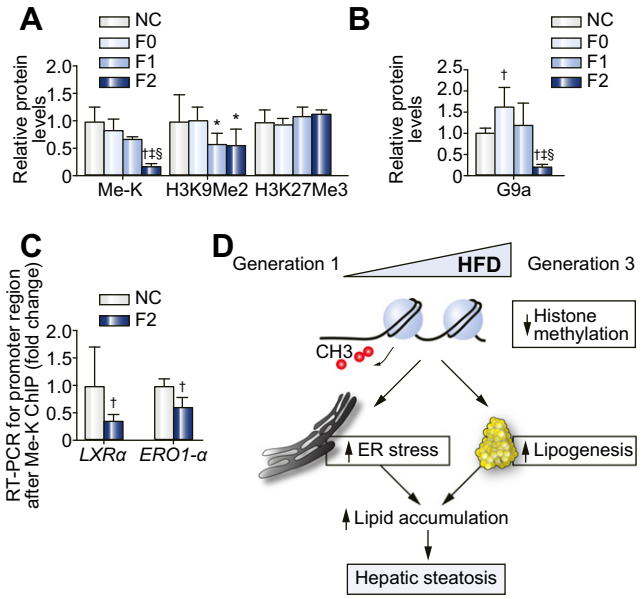

Fig. 4. Gradual reduction of histone methylation in HFD-fed male mice through generations. Quantitative results of relative protein levels of H3K9Me2, H3K27Me3, Me-K (A), and G9a (B) in different experimental groups. (C) Reduction of methylated histones binding to *LXRα* and *ERO1-α* promoters in the HF F2 mice. (D) Possible mechanism of development of a gradually severe hepatic steatosis over generations after HFD feeding. $^{\dagger}p$ <0.05 compared to the NC mice; $^{\ddagger}p$ <0.05, 0.05< $^{*}p$ <0.08 compared to the HF F0 mice; $^{\S}p$ <0.05 compared to the HF F1 mice.

Discussion

The obesity and T2DM epidemics are leading to an accelerated prevalence of NAFLD worldwide. The phenomenon of markedly increased childhood NAFLD calls attention to the effects of maternal obesity on offspring NAFLD development. Increasing evidence suggests that excessive maternal fat-intake during pregnancy and/or lactation may program offspring to develop NAFLD in adulthood. However, the mechanisms underlying the latter event remain poorly understood [9,10,12]. Most previous studies have been focused on comparing physiological changes between offspring from obese and lean mothers weaning on a normal diet, while few studies have investigated the offspring weaning on a HFD. In addition, research on the transgenerational effects of continuous HFD feeding through generations is limited. However, it has been shown that the F1 generation from obese mothers has a more severe fatty liver phenotype when weaned on the HFD than that weaned on the NC [12]. In the present study, we observed that an extremely severe NAFLD phenotype is developed in the HF F2 male mice compared to other generations under the same duration of HFD feeding stress. The surprisingly exacerbated hepatic steatosis in the HF F2 mice is accompanied by a dramatic increase in liver weight in the HF F2 mice compared to the other groups (Fig. 2 and Table 1). This suggests that a feed-forward circle due to a continuous HFD feeding results in transgenerational amplification effects on male offspring, with consequent NAFLD development. Consistent with our observations, a similar finding on lipid accumulation in the livers of the HF F2 female mice was reported [28] during the preparation of this manuscript. However, gradual increases in fat weight over generations were not observed in male offspring and grand-offspring in our study compared to a recent report on a Western-like diet feeding [29], which may be due to the use of different diets.

Although the biochemical mechanisms underlying NAFLD are not well understood, it is evident that NAFLD is associated with insulin resistance [30]. It has been shown that maternal obesity can lead to offspring (F1) insulin resistance [31]. However, the severity of insulin resistance has not been compared between offspring (F1/F2) of maternal/grand-maternal obesity and the HFD induced obesity (F0) in previous studies [10,32,33]. The HF F2 mice in our study show extremely severe insulin resistance, glucose intolerance and elevated serum insulin levels compared to the HF F0 mice (Fig. 1D and E and Table 1). To our knowledge, this

is the first evidence indicating that continuous HFD feeding over generations induces extremely severe insulin resistance in grand-offspring.

Like insulin resistance, leptin resistance is also proposed to play a role in NAFLD [34]. A recent pediatric NAFLD study demonstrated that higher levels of leptin may be associated with increased severity of liver damage, including steatosis and ballooning score, in obese children [35]. Consistent with this clinical study, we demonstrated dramatically increased serum leptin levels in the HF F2 mice associated with a severe liver damage (Table 1 and Fig. 2). Our results also suggest that serum leptin levels have a stronger correlation with the histopathological lesions in liver over generations after continuous HFD feeding than serum levels of insulin.

Lipid accumulation in the liver is a key marker for the development of NAFLD. Increased insulin resistance and leptin resistance are associated with increased *de novo* lipogenesis, a process regulated by multiple transcription factors including SREBP-1c, LXRs, and key enzymes like FASN [14]. Recent studies have shown that LXRs directly regulate SREBP-1c to promote hepatic fatty acid synthesis [36]. The critical role of LXRs in the development of NAFLD is proven by the fact that the pharmacological activation of LXRs leads to severe hepatic steatosis in lean mice [37]. We observed progressively elevated LXRα and LXRβ protein expression levels over generations, which are correlated with the level of severity of hepatic steatosis scoring (Fig. 2 and 3D). These transgenerational elevated LXRs levels after continual HFD feeding may lead to gradually accumulated FASN levels through generations in the liver (Fig. 3A and B). Consistent with our results, increased expression levels of FASN along with diacylglycerol o-acyltransferase 1, the key enzyme that regulates triacylglycerol synthesis, were also found in the HF F1 generation compared to the HF F0 generation with an exacerbated form of NAFLD due to both developmental and adult HFD feeding stress [12].

Recent studies demonstrated a close relationship between ER stress and lipid metabolism, specifically, the development of liver steatosis [23,38,39]. SREBPs can be up-regulated upon ER stress [40]. A recent report shows that XBP-1, a transcription factor directly activated by the IRE1a branch of ER stress, positively regulates lipogenesis [39]. However, the manipulation of genes involved in the ER stress signaling pathway showed controversial results on the development of NAFLD. As an ER stress marker, both BIP overexpression and its heterozygosity seem to have beneficial effects on the liver [41,42]. Dephosphorylation of eIF2α by over expression of an eIF2α-specific phosphatase, GADD34, attenuates hepatic steatosis in mice [43], while homozygous mice with a mutated eIF2α phosphorylation site die immediately after birth due to β-cell dysfunction-induced hypoglycemia [44]. These controversial data imply that manipulations of gene expression involved in the ER stress pathway may have uncertain roles in the development of NAFLD. Presumably, only unresolved ER stress, characterized by prolonged CHOP expression and/or increased p-eIF2α levels, could induce the disruption of lipid homeostasis and the development of hepatic steatosis [23]. Consistent with this hypothesis, tunicamycin, an ER stress inducer, causes unresolved ER stress and leads to an increase of lipogenesis and NAFLD in multiple mouse models [38]. In our study, there was a gradual transgenerational increase in the levels of p-eIF2α/eIF2α and CHOP in HFD-fed mice (Fig. 3E and F), suggesting a progressive accumulation of unresolved ER stress in the liver over generations after continuous over-nutrition feeding.

It has been shown that maternal HFD feeding can epigenetically alter fetal gene expressions via hyperacetylation of histones in hepatic tissue, suggesting that the remodeling of chromatin structure in the intrauterine environment during fetal development might contribute to the development of NAFLD in offspring of maternal obesity [45]. Besides maternal obesity, HFD feeding in fathers also can cause epigenetic changes in their offspring. It has been recently reported that the daughters of obese male rats have an altered expression of multiple pancreatic islet genes with hypomethylation on the *Il13ra2* gene, suggesting that β-cell dysfunction can be epigenetically intergenerationally transmitted from obese fathers to daughters [46]. In the present study, gradual changes of histone methylation profiles were found in the HFD feeding male offspring over multiple generations (Fig. 4A and B), suggesting the accumulation of epigenetic changes in the liver. Downregulation of the transcriptional repressive epigenetic marker of H3K9Me2 is consistent with activation of lipogenesis and ER stress pathways in the present study. Hypomethylated histones on *LXRα* and *ERO1-α* promoters could be a good explanation of elevated LXRα and ERO1-α levels in the HF F2 mice. This could contribute to accumulated lipogenesis and ER stress as well as aggravated NAFLD in the HF F2 mice.

The huge health and economic burden of obesity has been reviewed recently [47,48]. If the presently observed feed-forward circle among generations does exist in the human society, we are facing an even worse situation of obesity-related diseases, including NAFLD in the near future. Therefore, it is critical to obtain statistical data on the correlation of transgenerational obesity and NAFLD in humans. It is also important to design possible intervention therapies that can break or slow down this feed-forward circle.

In summary, our data shows that in the feed-forward circle of continuous exposure to HFD feeding over generations, the HF F2 generation has a greater chance to develop obesity with NAFLD at an earlier age with a more severe phenotype than the HF F1 and HF F0 generations. Gradual and transgenerational changes of histone modifications lead to an epigenetically altered expression of genes involved in regulation of lipid homeostasis and ER stress. Accumulated unresolved ER stress and lipogenesis in the liver contribute to a gradual increase in severity of NAFLD after continual HFD-feeding over generations (Fig. 4D). Our study revealed some of the potential molecular mechanisms responsible for the world epidemic of childhood obesity and NAFLD.

Conflict of interest

The authors who have taken part in this study declared that do not have anything to disclose regarding funding of conflict of interest with respect to this manuscript.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2011.10.018.

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