The Effects of High-Fat Diet Exposure In Utero on the Obesogenic and Diabetogenic Traits Through Epigenetic Changes in *Adiponectin* and *Leptin* Gene Expression for Multiple Generations in Female Mice

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Recent studies demonstrate that epigenetic changes under malnutrition in utero might play important roles in transgenerational links with metabolic diseases. We have previously shown that exposure to a high-fat diet (HFD) in utero may cause a metabolic syndrome-like phenomenon through epigenetic modifications of *Adiponectin* and *Leptin* genes. Because an association of obesity between mother and offspring endured in multiple generations, we examined whether HFD exposure in utero might affect the metabolic status of female offspring through multigenerational epigenetic changes of *Adiponectin* and *Leptin* genes and whether a normal diet in utero for multiple generations might abolish such epigenetic changes after exposure to a HFD in utero using ICR mice. We observed that the effect of maternal HFD on offspring over multiple generations in metabolic syndrome-like phenomenon such as weight and fat mass gain, glucose intolerance, hypertriglyceridemia, abnormal adiponectin and leptin levels, and hypertension, were accumulated with expression and epigenetic changes in *Adiponectin* and *Leptin* genes. A normal diet in utero in the subsequent generations after HFD exposure in utero diminished, and a normal diet in utero for 3 generations completely abolished, the effect of HFD in utero on weight and fat mass gain, insulin resistance, serum triglyceride, adiponectin, and leptin levels, with epigenetic changes of *Adiponectin* and *Leptin* genes. Exposure to a HFD in utero might affect glucose and lipid metabolism of female offspring through epigenetic modifications to *Adiponectin* and *Leptin* genes for multiple generations. Obesogenic and diabetogenic traits were abolished after a maternal normal diet for 3 generations. **(*Endocrinology* 156: 2482–2491, 2015)**

Maternal obesity in human pregnancy often results in fetal overgrowth (1, 2), which increases the risk of offspring developing obesity and metabolic syndrome later in life, thereby contributing to the increased incidence of diabetes (3–5). Although obesity is associated with an increased risk of almost every common complication of pregnancy, obesity in the mother might play a direct role in the transmission of an obesogenic and diabetogenic trait from generation to generation. A recent family cohort study has reported an enduring association between mother and offspring BMI over 3 generations through the maternal line (6).

Adipose tissue functions as a highly specialized endocrine and paracrine tissue, producing an array of adipocytokines, such as leptin and adiponectin, with such factors having local and systemic biological effects and an influence on insulin sensitivity and the development of metabolic diseases (7). Adiponectin is an adipocyte-derived hormone that acts as an antidiabetic, antiatherogenic, and antiinflammatory adipocytokine. Decreased circulating adiponectin levels are associated with obesity, insulin resistance, and type 2 diabetes (8–10). Leptin plays important roles in modulating satiety and energy homeostasis (11, 12).

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Abbreviations: acetyl H3K9, acetyl-histone H3 at lysine 9; BP, blood pressure; CD, control diet; ChIP, chromatin immunoprecipitation; dimethyl H3K9, dimethyl histone H3 at lysine 9; GTT, glucose tolerance test; HFD, high-fat diet; HOMA-IR, homeostasis model assessment of insulin resistance; ITT, insulin tolerance test; monomethyl H4K20, monomethyl histone H4 at lysine 20.

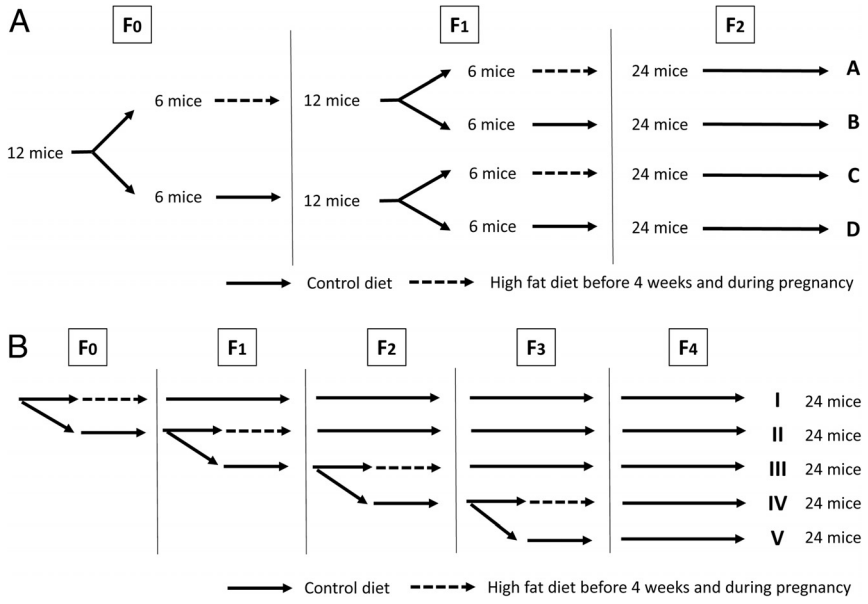

Figure 1. Experimental scheme for F0–2 in groups A–D (A) and for F0–4 in groups I–V (B).

Epigenetics can be defined as somatically heritable states of gene expression resulting from changes in chromatin structure without alterations in the DNA sequence, including DNA methylation, histone modifications, and chromatin remodeling (13). Nutrients can affect epigenetic phenomena such as DNA methylation and histone modification, thereby changing the expression of critical genes associated with physiological and pathological processes, including embryonic development (14). In recent years, epigenetics has emerged as a tool for understanding a broad range of human diseases, such as type 2 diabetes mellitus, obesity, inflammation, and neurocognitive disorders (14).

Exposure to a high-fat diet (HFD) in utero in mice can cause a metabolic syndrome-like phenomenon that can be transmitted to the progeny (15–17). Recent studies demonstrated that epigenetic changes under malnutrition in utero might play some important roles in transgenerational links with metabolic diseases (18). We have previously shown that exposure to a HFD in utero may cause a metabolic syndrome-like phenomenon through epigenetic modifications of *Adiponectin* and *Leptin* genes (17, 19). Because an association of BMI between mother and offspring endured over 3 generations through the maternal line (6), we examined whether HFD exposure in utero affected the metabolic status of offspring through multigenerational epigenetic changes in *Adiponectin* and *Leptin* genes and whether a normal diet in utero for multiple generations abolished such epigenetic changes after HFD exposure in utero.

Materials and Methods

Materials and animal procedures

Female 8-week-old ICR strain mice were obtained from Charles River Co Ltd. After 4 weeks of feeding with the HFD (energy content: 62% fat, 18% protein, and 20% carbohydrate) or a control diet (CD) (12% fat, 28% protein, and 60% carbohydrate) purchased from Oriental Yeast Co, mice were weighed and mated. All generations were mated at 12 weeks of age after HFD or CD for 4 weeks from 8 weeks of age. Females were checked daily for postcopulatory plugs and the presence of a plug in the morning after mating was taken as day 0.5 of pregnancy. Eight-week-old male mice for mating were fed with the CD for 4 weeks before experiments or mating. And male mice were fed with HFD only at mating with HFD-fed female mice. We used each male mice for the mating once. We employed 4 female groups of F2 generation: maternal HFD before 4 weeks and during pregnancy in both F0 and F1 (A), in F0 (B), in F1 (C), and no HFD in F0 and F1 generations (D) (Figure 1A). And maternal HFD before 4 weeks and during pregnancy in F0 (I), F1 (II), F2 (III), and F3 (IV) and CD in all generations (V) were also examined (Figure 1B). Pregnant mice had free access to food and water, and their daily food consumption was estimated by weighing the remaining food every weeks. The maternal weight on day 20 of gestation and the neonatal weight on day 0.5 after birth were measured. Female offspring weights were then measured every 2 weeks. All offspring were weaned onto CD at 3 weeks of age. After weaning, the offspring had free access to food and water, and their daily food consumption was estimated by weighing the remaining food. Body composition was analyzed in live mice using Echo MRI-100 (Echo Medical Systems). Systolic blood pressure (BP) of offspring was measured at 12 and 24 weeks of age by the tail-cuff method using a Softron BP98A tail-cuff hemodynamometer (Softron) after the behavior and heart rate of the mice had stabilized. BP is reported as the mean of at least 3 measurements recorded during the same session, which had to vary by less than 5%. Most BP values were within the required range once the mice had stabilized. At 2, 12, and 24 weeks of age, after the mice had been anesthetized with ether, the white mesenteric adipose tissues were removed, frozen immediately, and stored at −70°C until analysis. Mice were kept in a temperature- and light-controlled room with free access to food and water except during glucose tolerance tests (GTTs) and insulin tolerance tests (ITTs). The Institutional Animal Care and Use Committee of Okayama University approved all animal procedures.

GTT, ITT, and measurements of insulin, total triglyceride, adiponectin, and leptin levels

D-glucose and human insulin were purchased from Sigma-Aldrich. Blood samples from pregnant mice after fasting for 16 hours on day 20 of gestational age were taken for measurements

of total triglyceride, adiponectin, leptin, glucose, and insulin levels and for a homeostasis model assessment of insulin resistance (HOMA-IR). Offspring at 12 or 24 weeks of age were fasted for 16 hours before receiving an ip injection of D-glucose (2 g/kg body weight) for the GTT or for 4 hours before receiving an ip injection of human insulin (1.0 U/kg body weight) for the ITT. Blood samples were taken from saphenous vein before and at 30, 60, 90, and 120 minutes after the injections of glucose or insulin. Blood glucose levels were measured by the glucose oxidase method using a Medisafe automated analyzer (Terumo). As described previously (17, 19), fasting insulin, total triglycerides, adiponectin, and leptin levels were determined using ELISA kits (insulin kit from Morinaga Institute of Biological Sciences, Inc; triglyceride kit from Abcam Plc; and adiponectin and leptin kits from R&D Systems, Inc). Serum sample volumes for each measurement were 5 μL for insulin and triglycerides, 10 μL for adiponectin, and 20 μL for leptin, and total blood sample volume collected from each mouse was less than 200 μL, which was less than 5% of total blood volume. HOMA-IR was calculated as the fasting insulin concentration (μU/mL) $\times$ fasting glucose concentration (mg/dL)/405 (20).

Real-time quantitative PCR

Total RNA from white mesenteric adipose tissues was extracted using TRIzol reagent (Life Technologies, Inc), according to the manufacturer's instructions. Real-time quantitative PCR was performed to measure the mRNA levels of the *Leptin* and *Adiponectin* genes using a StepOne Real-time PCR System and a TaqMan RNA-to-CT Gene kit (Applied Biosystems). Specific primers for mouse *Leptin*, *Adiponectin*, and *β-actin* gene sequences were purchased from Applied Biosystems. Sequences of specific primers and accession numbers were as described (21–23). RNA samples (25 ng) were assayed in triplicate using 15 pmol of gene-specific primers and 5 pmol of gene-specific probes. Because we confirmed that there were no significant differences of β-actin expression in adipose tissues of group A–D and group I–V after high throughput screening of several housekeeping genes using TaqMan endogenous control assay (data not shown), mouse *β-actin* mRNA levels were measured as an internal control using a predeveloped TaqMan primer and a probe mixture (Applied Biosystems). mRNA levels of the target genes were normalized against *β-actin* mRNA levels.

Chromatin immunoprecipitation (ChIP) assays

ChIP assays were performed using a ChIP assay kit (Upstate Biotechnology) according to the manufacturer's protocol. White mesenteric adipose tissues from groups A–D and groups I–V (n = 12 female) at 2 and 24 weeks of age were taken for sampling. Briefly, 20 mg of frozen samples were ground in liquid nitrogen using a mortar and pestle and then washed with PBS at room temperature. The samples were resuspended in PBS and cross-linked in 1% formaldehyde for 10 minutes. After centrifugation, the pellet was resuspended in nucleus swelling buffer containing protease and phosphorylation inhibitors. The nuclei were lysed in SDS lysis buffer containing protease and phosphorylation inhibitors. Chromatin was sonicated to reduce DNA fragment lengths to 0.3–1.0 kb. Chromatin was precleared in the presence of 20 μL of normal serum, 2 μg of salmon sperm DNA, and 80 μL of 25% protein A-agarose slurry. Precleared chromatin samples were subjected to immunoprecipitation at 4°C overnight in the presence of 2 μg of rabbit polyclonal antibody for acetyl-histone H3 at lysine 9 (acetyl H3K9) (Millipore), dimethyl histone H3 at lysine 9 (dimethyl H3K9) (Abcam, Inc), and monomethyl histone H4 at lysine 20 (monomethyl H4K20) (Abcam, Inc) or nonimmune rabbit IgG (Millipore). After collecting the complex by incubation with 60 μL of 25% protein A-sepharose slurry and centrifugation, the beads were washed 5 times and the chromatin-immune complex was eluted. After reversing the cross-linking, DNA was purified and used as a template for PCR. PCR was performed using primer sets specific for the promoter region of the mouse *Adiponectin* gene (positions −549 to −481) (24) and the promoter region of the mouse *Leptin* gene (−181 to +20) (25).

Statistical analysis

Statistical analyses were performed by two-way and multiple ANOVA for comparison between group A, B, C, and D and between group I, II, III, IV, and V and repeated measures ANOVA for GTT and ITT values followed by the Dunnett's test. All statistical analyses were performed with StatView software (Abacus Concepts). Data are presented as mean ± SD. $P < .05$ was considered statistically significant.

Results

Multigenerational effect of HFD in utero on weight, caloric intake, gain of fat mass, and BP of female offspring

There were significant differences in mean maternal weight on day 20 of gestation and mean weights of the neonates on day 0.5 after birth among the 4 groups, A–D. There was no significant difference in mean litter size among the 4 groups (8.9 ± 0.6, 9.2 ± 0.8, 9.0 ± 0.8, and 8.9 ± 0.7, respectively). We also observed significant increases in maternal weight, HOMA-IR, triglyceride, and leptin levels and a decrease in the adiponectin level in group A–C compared with those in group D, significant increases in maternal weight, triglyceride and leptin levels and a decrease in the adiponectin level in group A and B and HOMA-IR in group B compared with those in group C, and significant increases in maternal weight, HOMA-IR, triglyceride, and leptin levels and a decrease in the adiponectin level in group A compared with those in group B in F1 generation (Table 1). In F2 generation, the mean weights of offspring in groups A–C were significantly higher than group D from 6 weeks, 14 weeks or 18 weeks of age, those in groups A and C were significantly higher than group B from 6 weeks or 16 weeks of age, and that in group A was significantly higher than group C from 6 weeks of age (Figure 2A). The caloric intakes of offspring in groups A–C were greater than group D from 4, 12, or 16 weeks of age, before a significant increase in body weight, those in groups A and C were significantly higher than group B from 8 weeks of age, and that in group A was

Table 1. Maternal Characteristics and Neonatal Weights in F1 Generation

Group	Maternal Weight (g)	HOMA-IR	Triglyceride (mg/dL)	Leptin (ng/dL)	Adiponectin (μg/dL)	Neonatal Weight (g)
A	55.6 ± 4.1[a,b,c]	5.21 ± 0.42[a,c]	392 ± 29[a,b,c]	8.3 ± 1.0[a,b,c]	3.2 ± 0.5[a,b,c]	1.81 ± 0.21[a,c]
B	41.8 ± 3.7[a,b]	4.02 ± 0.33[a,b]	281 ± 36[a,b]	5.3 ± 0.9[a,b]	8.2 ± 1.1[a,b]	1.44 ± 0.15[a,b]
C	47.2 ± 2.8[a]	4.88 ± 0.24[a]	333 ± 27[a]	6.9 ± 0.7[a]	5.6 ± 0.7[a]	1.66 ± 0.11[a]
D	35.0 ± 2.8	3.16 ± 0.29	226 ± 19	3.7 ± 0.5	11.5 ± 0.9	1.15 ± 0.08

Mean ± SEM.
[a] $P < .01$ vs group D.
[b] $P < .01$ vs group C.
[c] $P < .01$ vs group B.

significantly higher than group C from 8 weeks of age (Figure 2B). Furthermore, gains of fat mass in group A from 4 weeks of age, group B from 16 weeks of age and group C from 12 weeks of age were significantly higher than that of group D, those in groups A and C were significantly higher than group B from 8 weeks or 12 weeks of age, and that in group A was significantly higher than group C from 8 weeks of age (Figure 2C). Systolic BPs of offspring in groups A and C were significantly elevated compared with group D at 12 weeks of age, whereas those in group A–C were significantly elevated compared with that in group D, that in group A was significantly different from group C, and those in group A and C were also significantly elevated compared with group B at 24 weeks of age (Figure 2D).

Multigenerational effect of HFD exposure in utero on glucose intolerance and insulin resistance

We performed GTT and ITT, measured serum insulin levels and calculated HOMA-IR in groups A–D at 12 and 24 weeks of age to examine the multigenerational effects of HFD exposure in utero on glucose metabolism in offspring. There were significant differences in HOMA-IR for group A compared with group B–D at 12 weeks of age, whereas those in group A–C were significantly elevated compared with that in group D, that in group A was sig-

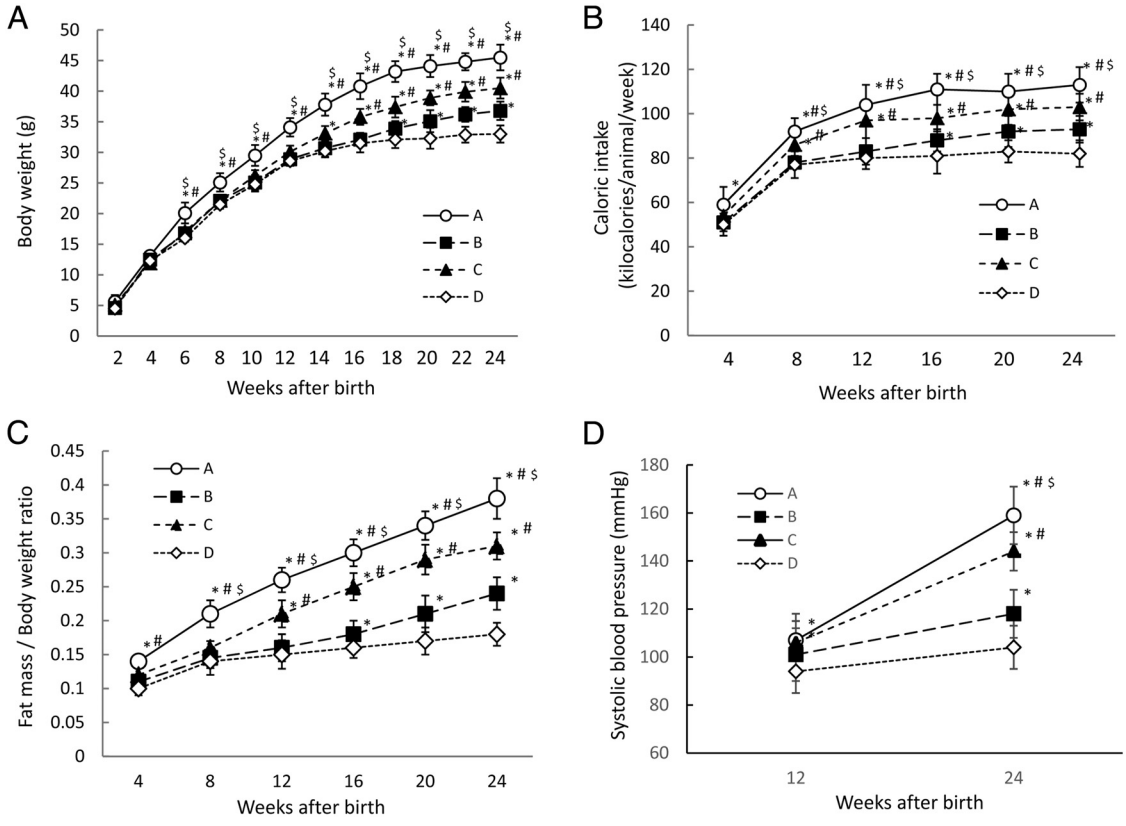

Figure 2. Multigenerational effects of HFD exposure in utero on weight, caloric intake, gain of fat mass, and BP of offspring. W (A), caloric intake (B), and body composition (C) in groups A–D. D, Systolic BP in groups A–D at 12 and 24 weeks of age. Results are mean ± SD (n = 12 female per group; *, $P < .01$ vs group D; $, $P < .01$ vs group C; #, $P < .01$ vs group B).

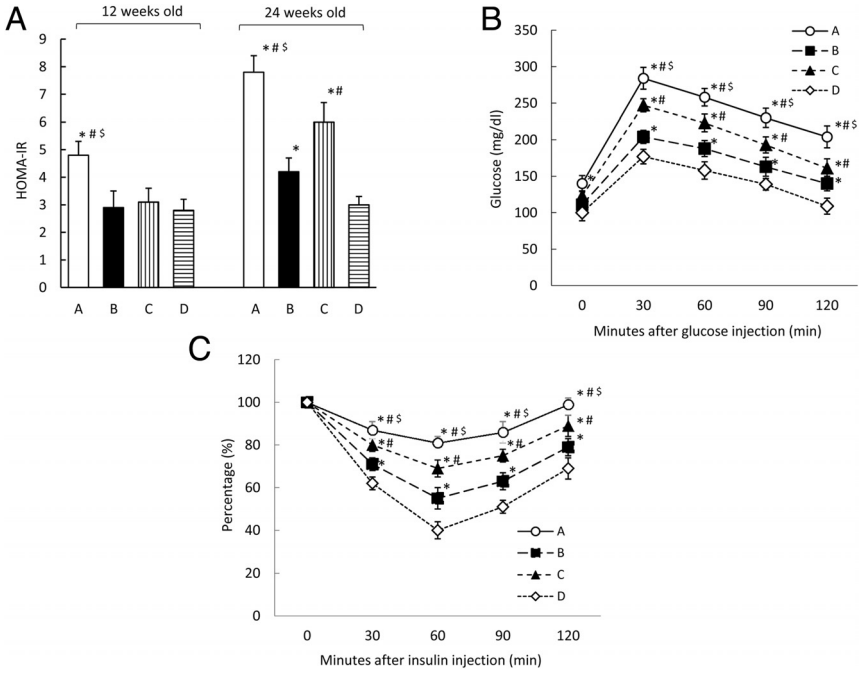

Figure 3. Multigenerational effects of HFD exposure in utero on glucose intolerance and insulin resistance of offspring. HOMA-IR (A), GTT (B), and ITT (C) at 24 weeks of age. Mice at 24 weeks of age were fasted for 16 hours before receiving an ip injection of D-glucose (2 g/kg body weight) for the GTT or for 4 hours before receiving an ip injection of human insulin (1.0 U/kg body weight) for the ITT. Blood samples were taken before and at 30, 60, 90, and 120 minutes after injections of glucose or insulin. Results are mean ± SD (n = 12 female per group; *, $P <$ .01 vs group D; $, $P <$ .01 vs group C; #, $P <$ .01 vs group B).

nificantly different from group C, and those in group A and C were also significantly increased compared with group B at 24 weeks of age (Figure 3A). Glucose intolerance and insulin resistance in group A–C were significantly elevated compared with those in group D, those in group A was significantly different from group C, and those in group A and C significantly worsened compared with group B at 24 weeks of age (Figure 3, B and C).

Multigenerational effect of HFD exposure in utero on serum triglyceride, leptin and adiponectin levels, and *Leptin* and *Adiponectin* gene expression in adipose tissue of female offspring

To clarify whether HFD exposure in utero during pregnancy would affect lipid metabolism and adipocytokine levels in the offspring of multiple generations, serum triglyceride, leptin, and adiponectin levels were examined. Total triglyceride and leptin levels were significantly elevated and adiponectin level were significantly decreased in group A–C compared with those in group D, those in group A were significantly different from group C, and those in group A and C were also significantly different from group B at 12 and 24 weeks of age, excluding the adiponectin and leptin levels between group B and D only at 12 weeks of age (Figure 4, A–C). The *Leptin* gene expression were significantly elevated and *Adiponectin* gene expression were significantly decreased in the white mesenteric adipose tissue of offspring in group A–C compared with those in group D, those in group A were significantly different from group C, and those in group A and C were also significantly different from group B at 2, 12, and 24 weeks of age, excluding the *Adiponectin* gene expression between group B and C at 12 weeks of age (Figure 4, D and E).

Multigenerational effect of HFD exposure in utero on modifications to H3K9 and H4K20 in the promoter regions of the *Adiponectin* and *Leptin* genes in adipose tissue of female offspring

To investigate whether HFD exposure in utero would affect histone modifications in the promoter regions of *Adiponectin* and *Leptin* genes in the adipose tissue of female offspring in multiple generations, we performed ChIP assays using antibodies for acetyl and dimethyl H3K9 and monomethyl H4K20 at 2 and 24 weeks of age (Table 2). The acetyl H3K9 level were significantly decreased and the dimethyl H3K9 level were significantly increased in the *Adiponectin* promoter region of offspring in group A–C compared with those in group D, those in group A were significantly different from group C, and those in group A and C were also significantly different from group B at 2 and 24 weeks of age (Figure 5A). And the monomethyl H4K20 level was significantly increased in the *Leptin* promoter region of offspring in group A–C compared with those in group D, that in group A was significantly different from group C, and those in group A and C were also significantly elevated compared with group B at 2 and 24 weeks of age (Figure 5B). There were no effects of the maternal diet on the association of IgG binding with the promoter regions of *Leptin* or *Adiponectin* genes in adipose tissues (data not shown).

Effect of CD during pregnancy on female offspring after HFD exposure in utero

To investigate whether a CD before and during pregnancy in multiple generations affected the female offspring after HFD exposure in utero, we examined 5 groups: female offspring from dams fed with CD before and during

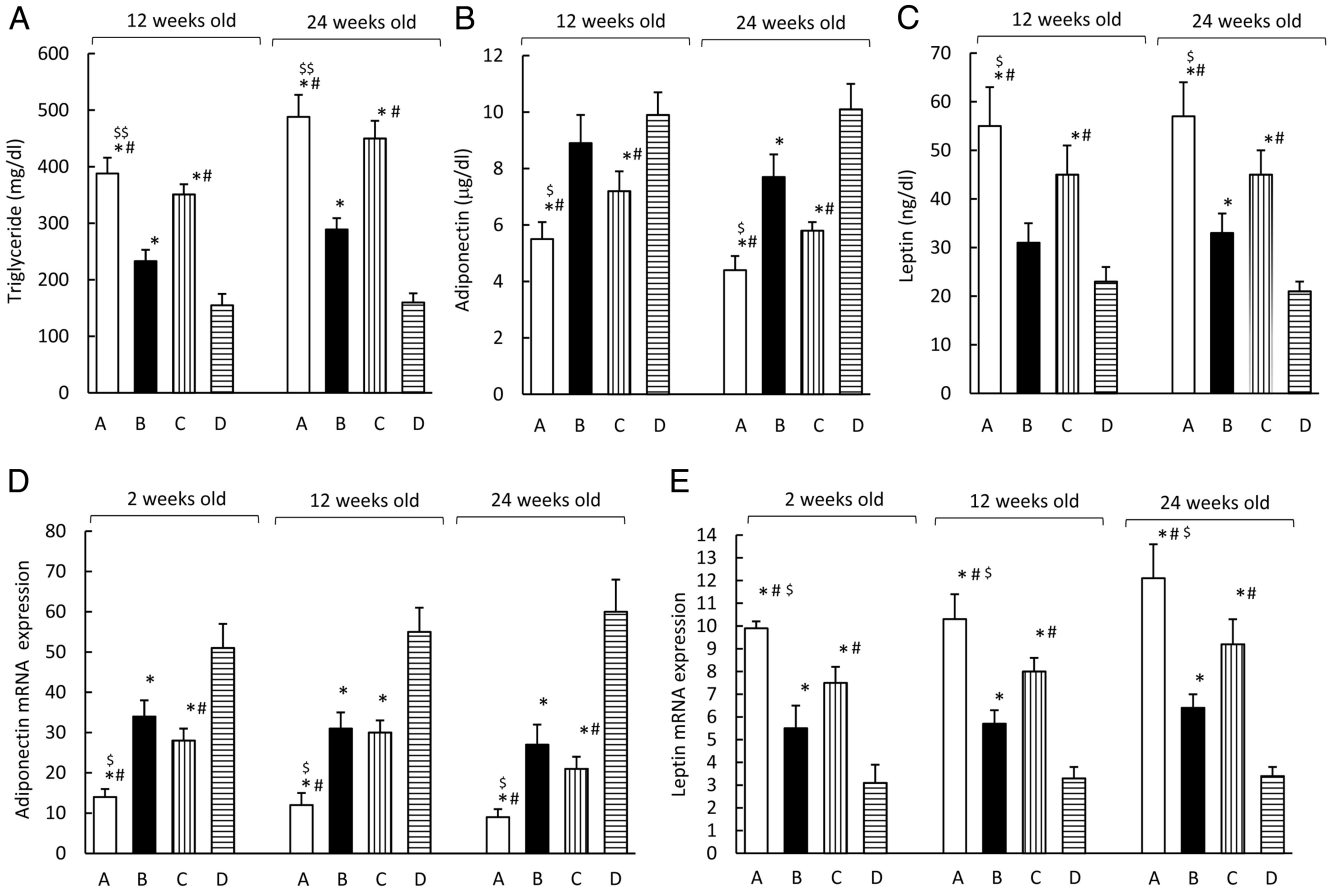

Figure 4. Multigenerational effects of HFD exposure in utero on serum triglyceride, adiponectin and leptin levels, and adiponectin and leptin expression of white adipose tissues in offspring. Mice at 12 and 24 weeks of age were fasted for 16 hours, and blood samples were taken. Serum total triglycerides (A), adiponectin (B), and leptin (C) levels were determined using ELISA kits. White adipose tissues were removed from offspring at 2, 12, and 24 weeks of age, frozen immediately, and stored at −70°C until analysis. Total RNA was extracted using TRIzol reagent, according to the manufacturer's instructions. Real-time quantitative PCR was performed using total RNA to determine the expression levels of *Adiponectin* (D) and *Leptin* (E). mRNA levels were normalized for *β-actin* mRNA levels. Results are mean ± SD (n = 12 female per group; *, $P < .01$ vs group D; $, $P < .01$ and $$, $P < .05$ vs group C; #, $P < .01$ vs group B).

pregnancy after HFD exposure in utero in 3 (I), 2 (II), and 1 (III) generations; from dams fed with HFD (IV); and from CD in all generations (V). There were no significant differences in body weights between groups I and V, and offspring in group II showed a significant increase only at 24 weeks compared with group I and V. And the mean body weight of offspring in group III was significantly increased compared with group I and V at 18 weeks and group II at 20 weeks and that in group IV compared with group I, II, III, and V at 14 weeks (Figure 6A). We also observed that HOMA-IR and triglyceride level in group IV were significantly elevated compared with those in group I, II, III, and V, those in group III were significantly different from group I, II, and V, and that triglyceride level in group II was also significantly increased compared with group I and V (Figure 6, B and C). The *Adiponectin* expression in group IV was significantly decreased compared with that in group I, II, and V, that in group II and III were significantly different from group I and V (Figure 6D), whereas the *Leptin* expression in group IV was significantly elevated compared with that in group I, II, III, and V, that in group III was significantly different from group I, II, and V, and that in group II was also increased compared with group I and V (Figure 6E). The acetyl H3K9

Table 2. Antibody Table

Peptide/Protein Target	Antigen Sequence (if known)	Name of Antibody	Manufacturer, Catalog Number, and/or Name of Individual Providing the Antibody	Species Raised in; Monoclonal or Polyclonal	Dilution Used
Acetyl-histone H3 at lysine 9		Acetyl H3K9	Millipore, 06-942	Rabbit polyclonal antibody	ChIP assay
Dimethyl histone H3 at lysine 9		Dimethyl H3K9	Abcam, Inc; ab1220	Rabbit polyclonal antibody	ChIP assay
Monomethyl histone H4 at lysine 20		Monomethyl H4K20	Abcam, Inc; ab9051	Rabbit polyclonal antibody	ChIP assay

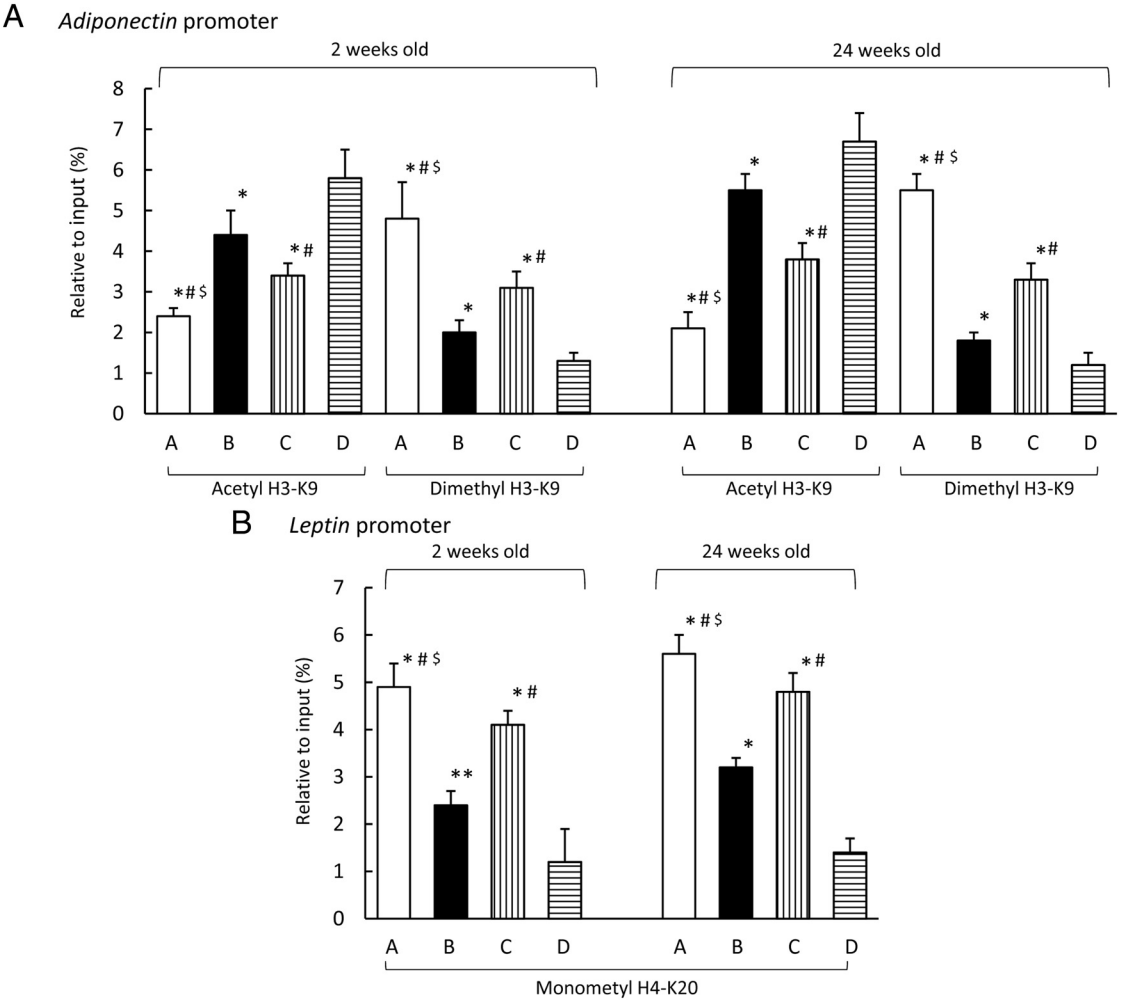

Figure 5. Multigenerational effects of HFD exposure in utero on the modifications of H3K9 and H4K20 in the promoter regions of *Adiponectin* and *Leptin* in adipose tissue of offspring. ChIP assays were performed on chromatin extracts from white mesenteric adipose tissue of mice to examine the effect of HFD exposure in utero on H3K9 and H4K20 modifications in the promoter regions of *Adiponectin* (A) and *Leptin* (B) at 2 and 24 weeks of age using antiacetyl and antidimethyl H3K9 and antimonomethyl H4K20. Results are mean ± SD (n = 12 female per group; *, $P <$ .01 and **, $P <$ .05 vs group D; $, $P <$ .01 vs group C; #, $P <$ .01 vs group B).

level in the *Adiponectin* promoter region was significantly decreased and the dimethyl H3K9 level in the *Adiponectin* promoter region and the monomethyl H4K20 level in the *Leptin* promoter region were significantly increased in group IV compared with those in group I, II, III, and V and those in group II and III were significantly different from those in group I and V (Figure 6F).

Discussion

In this study, we observed an accumulation of the effects of maternal HFD on offspring over multiple generations in metabolic-like phenomenon such as weight and fat gain, glucose intolerance, hypertriglyceridemia, abnormal adipocytokine levels, and hypertension, and the expression and epigenetic changes in *Adipocytokine*, *Adiponectin*, and *Leptin* genes. A normal diet in utero in next generations after HFD exposure in utero diminished, and a normal diet in utero for 3 generations completely abolished, the effect of HFD in utero on weight and fat mass gain, insulin resistance, serum triglyceride, and adipocytokine levels with the epigenetic changes in offspring's promoters of adipocytokine genes.

Recent animal models of maternal overnutrition with a HFD have been developed for investigation of offspring development (17, 19, 26–29). These data suggest that pregnant female mice and rats fed a HFD show permanent detrimental effects in body composition and metabolism in their offspring, predisposing them to metabolic syndromes later in life, even after having been weaned onto standard chow (26, 30). Several studies indicated that aberrant production of adipocytokines might play some roles in not only dysregulation of glucose and lipid metabolism but elevated BP (31–33). In this study, we also

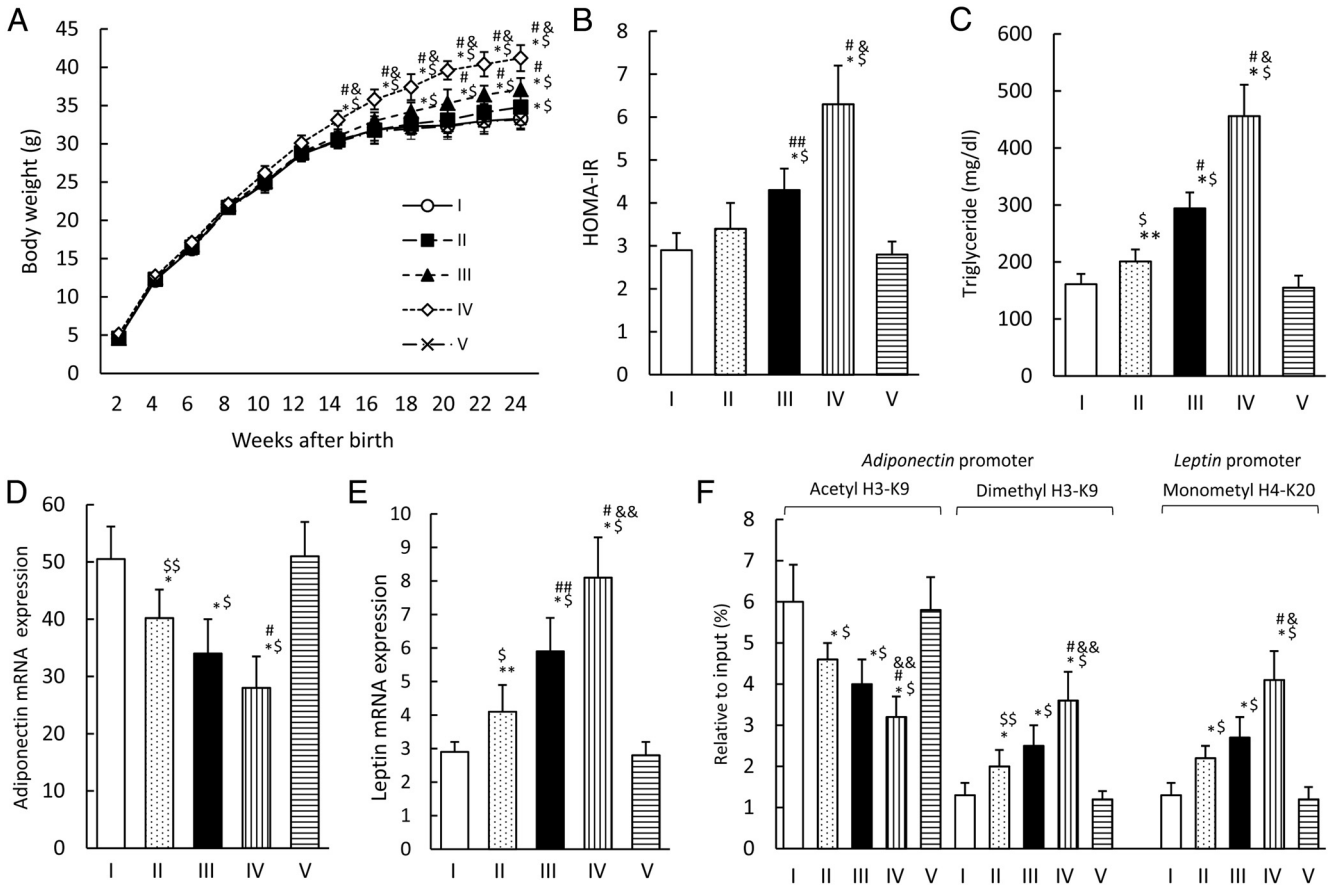

Figure 6. The effects of CD before and during pregnancy on offspring after HFD exposure in utero. W (A), HOMA-IR (B), serum level of triglyceride (C), mRNA expression of adiponectin (D) and leptin (F), and the modifications of H3K9 and H4K20 in the promoter regions of *Adiponectin* and *Leptin* (F) in the adipose tissue of offspring in groups I–V. Results are mean ± SD (n = 12 female per group; *, $P < .01$ and **, $P < .05$ vs group V; $, $P < .01$ and $$, $P < .05$ vs group I; #, $P < .01$ and ##, $P < .05$ vs group II; &, $P < .01$ vs group III).

observed the dysregulation of triglyceride and adipocytokine levels ahead of worsening glucose metabolism and elevation of BP. In addition, we also observed that exposure to HFD in utero modified H3K9 from methylation to acetylation in the *Adiponectin* promoter region and methylation of H4K20 in the *Leptin* promoter region of adipose tissue, which were consistent with our previous studies (17, 19). However, because we focused the effect of HFD in utero on the glucose and lipid metabolism of female offspring in this study, we will require to investigate the paternal effect and other traits like the behavior dominance for food seeking.

An etiological report indicated an enduring association between mother and offspring BMI in not only single but also multiple generations through the maternal line (6), thus we examined whether exposure to a HFD in utero affected the metabolic status of the offspring with epigenetic modifications of adipocytokine genes in multiple generations. Our data in this study demonstrated that exposure to a HFD in utero in the grandmother had an additive effect on offspring from the dam's mother fed a HFD, and weaker but significant effect on offspring in the grandchild's generation with similar changes of epigenetic modifications to *Adiponectin* and *Leptin* genes. A recent report demonstrated that obesity occurred earlier and became severe and with a high degree of hepatic steatosis and a transgenerational trend to up-regulate lipogenic genes after nutritional intervention with HFD for 3 generations (34). These data suggest that the transgenerational accumulation of epigenetic modifications might lead to accumulation of metabolic abnormality in the grandchild's generation. We observed the epigenetic modifications in offspring at 2 weeks after birth, but there was no fetal data of epigenetic modifications. And other adipocytokines, including resistin and inflammatory mediators such as TNF-α, might play some roles in glucose and lipid metabolism of offspring. We will do additional experiments about the fetal epigenetic modifications to investigate whether the epigenetic modifications could be heritable or not and to clarify the potential role of other adipocytokines in the obesogenic and diabetogenic traits.

We also demonstrated that normal diet in utero in next generations after HFD exposure in utero diminished, and that normal diet in utero during 3 generations completely

abolished, the effect of HFD in utero on offspring. The mechanism to maintain and enhance the epigenetic marks in the promoters of adipocytokine genes under maternal overnutrition over multiple generations and diminish this effect under maternal normal nutrition over multiple generations remains unclear. Environmental exposure might affect the developing F1 fetus but might also have direct effects on the developing germ cells, which form the F2 generation. Alternatively, effects induced in the developing F1 fetus can be transmitted to the germ cells which will form the F2 generation (35). Further analysis is required to resolve this question, including the potential role of noncoding RNA (36).

Taken together, our data suggest that exposure to a HFD in utero might lead to a metabolic syndrome-like phenomenon through epigenetic modifications of the genes encoding adipocytokines, adiponectin, and leptin in the offspring, and the effect will become much stronger if the HFD in utero continues for multiple generations. In contrast, a normal diet in utero will diminish the epigenetic effect caused by the HFD in utero and finally abolish the effect after a normal diet is taken for 3 generations.

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