The effects of paternal high-fat diet exposure on offspring metabolism with epigenetic changes in the mouse adiponectin and leptin gene promoters

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Masuyama H, Mitsui T, Eguchi T, Tamada S, Hiramatsu Y. The effects of paternal high-fat diet exposure on offspring metabolism with epigenetic changes in the mouse adiponectin and leptin gene promoters. Am J Physiol Endocrinol Metab 311: E236–E245, 2016. First published May 31, 2016; doi:10.1152/ajpendo.00095.2016.—Recent studies have demonstrated that epigenetic changes resulting from malnutrition might play important roles in transgenerational links with metabolic diseases. Previously, we observed that exposure to a high-fat diet (HFD) in utero caused a metabolic syndrome-like phenomenon through epigenetic modifications of the adiponectin and leptin genes that persisted for multiple generations. Recent etiological studies indicated that paternal BMI had effects on offspring BMI that were independent of but additive to maternal BMI effects. Thus, we examined whether paternal HFD-induced obesity affected the metabolic status of offspring through epigenetic changes in the adiponectin and leptin genes. Additionally, we investigated whether a normal diet during subsequent generations abolished the epigenetic changes associated with paternal HFD exposure before conception. We observed the effects of paternal HFD exposure before conception over multiple generations on offspring metabolic traits, including weight and fat gain, glucose intolerance, hypertension, and adiponectin and leptin gene expression and epigenetic changes. Normal diet consumption by male offspring during the subsequent generation following paternal HFD exposure diminished whereas consumption for two generations completely abolished the effect of paternal HFD exposure on metabolic traits and adipokine promoter epigenetic changes in the offspring. The effects of paternal HFD exposure on offspring were relatively weaker than those following HFD exposure in utero. However, paternal HFD exposure had an additive metabolic effect for two generations, suggesting that both paternal and maternal nutrition might affect offspring metabolism through epigenetic modifications of adipokine genes for multiple generations.

Adipokines are hormones that have local and systemic biological effects and influence insulin sensitivity and metabolic disease development (15). Adiponectin is an adipocyte-derived hormone that acts as an antidiabetic, antiatherogenic, and anti-inflammatory adipokine. Decreased circulating adiponectin levels are associated with obesity, insulin resistance, and type 2 diabetes (11, 25, 39). Leptin plays important roles in modulating satiety and energy homeostasis (23, 32). Leptin levels during the perinatal period are important for the development of metabolic systems. Specifically, circulating leptin acts as a trophic factor for the development of hypothalamic circuits that control energy homeostasis and food-seeking and reward behaviors (23, 32, 33). Moreover, 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 (6). 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 (6).

In utero high-fat diet (HFD) exposure in mice can cause a metabolic syndrome-like phenomenon that can be transmitted to the progeny (16, 27, 38). Recent studies have demonstrated that epigenetic changes following malnutrition in utero might play important roles in transgenerational links with metabolic diseases (22). In addition, paternal obesity initiated metabolic disturbances in two generations of mice (13). Previously, we demonstrated that in utero HFD exposure caused a metabolic syndrome-like phenomenon through epigenetic modifications of the adiponectin and leptin genes that persisted for multiple generations (27, 28, 29). However, obesogenic and diabetogenic traits were abolished after a normal maternal diet for three generations (29). Recent studies have indicated that paternal BMI has effects on offspring BMI that are independent of but additive to the effects of maternal BMI (8). Thus, we examined whether paternal obesity induced by HFD expo-
Table 1. The compositions and caloric values of the experimental diets.

<table>
<thead>
<tr>
<th></th>
<th>CD (Composition, g)</th>
<th>Calories, kcal</th>
<th>HFD (Composition, g)</th>
<th>Calories, kcal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Protein</td>
<td>23.1</td>
<td>92.4 (25.6%)</td>
<td>23</td>
<td>91.6 (18.0%)</td>
</tr>
<tr>
<td>Carbohydrate</td>
<td>55.3</td>
<td>221.2 (61.6%)</td>
<td>25.3</td>
<td>100.7 (20.0%)</td>
</tr>
<tr>
<td>Fat</td>
<td>5.1</td>
<td>45.9 (12.8%)</td>
<td>35</td>
<td>313.7 (62.0%)</td>
</tr>
<tr>
<td>Total calories</td>
<td>359</td>
<td></td>
<td>313.7</td>
<td></td>
</tr>
</tbody>
</table>

CD, control diet; HFD, high-fat diet.

sure affected the metabolic status of offspring through epigenetic changes to the adiponectin and leptin genes. Furthermore, we investigated whether a normal diet for multiple generations abolished such epigenetic changes after paternal HFD exposure before conception.

MATERIALS AND METHODS

Materials and animal procedures. The Institute of Cancer Research (ICR) mouse is an outbred strain with genetic variability and is used for general purposes. Furthermore, this strain displayed good reproductive performance on a HFD. Thus, we chose the ICR mouse for this study. Both male and female 8-wk-old ICR mice were obtained from Charles River Laboratories (Tokyo, Japan). Male and female mice were placed on a HFD for 6 and 4 wk, respectively, to generate obese mice. After 4 wk of HFD (energy content: 62.0% fat from lard, 18.0% protein, and 20.0% carbohydrate; Oriental Yeast, Tokyo, Japan) or control diet (CD; 12.8% fat, 25.6% protein and 61.6% carbohydrate; Oriental Yeast) consumption for female mice and 6 wk of feeding for male mice, mice were weighed and mated. The compositions and caloric values of the experimental diets are presented in Table 1. All generations were mated between 12 and 16 wk of age after diet initiation between 8 and 10 wk of age. Females were checked daily for postcoital plugs, and the presence of a plug in the morning after mating was interpreted as day 0.5 of pregnancy. Each male mouse was used for mating once. Pregnant mice were housed singly with ad libitum access to food and water. Daily food consumption was estimated by weighing the remaining food every week. We used 12 litters of mice for each group of experiments. We generated four groups for the F1 generation: mating between male and female mice fed a CD (group A), mating between males fed a HFD and females fed a CD (group B), mating between males fed a CD and females fed a HFD (group C), and mating between males and females fed a HFD (group D) (Fig. 1A). Additionally, to investigate whether a CD for multiple generations after paternal HFD exposure affected offspring metabolic parameters, we examined four groups: offspring from males fed a CD after parental HFD exposure for 6 wk before conception after two generations (group II) and one (group III) generation, offspring from males fed a HFD for 6 wk before conception (group IV), and offspring exposed to a CD throughout all

Fig. 1. Effects of parental high-fat diet (HFD) exposure on body weight and fat mass gain in offspring. Experimental scheme for F0 (A), weight of females (B) and males (C), and body composition of females (D) and males (F) in groups A–D. Results are means ± SD (n = 12/group). *P < 0.01 vs. group A; #P < 0.01 vs. group B; $P < 0.01 vs. group C. CD, control diet.
generations (group J) (Fig. 4A). Moreover, to evaluate the effect of parental HFD exposure, in the absence of any further challenges, on offspring for multiple generations, we examined four groups: the F3 generation after mating between male and female mice fed a CD (group α), between males fed a HFD and females fed a CD (group β), between males fed a CD and females fed a HFD (group γ), and between males and females fed a HFD (group δ) at the F0 generation (Fig. 5A). Offspring weights were then measured every 4 wk. All offspring were weaned onto a CD at 3 wk of age. All F1, F2, and F3 generation offspring were also fed a CD. After weaning, the offspring had ad libitum access to food and water, and daily food consumption was estimated by weighing the remaining food. Body composition was analyzed in live mice using Echo MRI-100 (Echo Medical Systems, Houston, TX). Offspring systolic blood pressure was measured at 12 and 24 wk of age by the tail-cuff method using a Softtron BP98A tail-cuff hemodynamometer (Softtron, Tokyo, Japan) after mouse behavior and heart rate had stabilized. Blood pressure is reported as the mean of at least three measurements recorded during the same session, which had to vary by <5%. Most blood pressure values were within the required range once the mice had stabilized. At birth and at 24 wk of age, after the mice had been anesthetized with ether and euthanized by cervical dislocation, the white mesenteric adipose tissues were removed, frozen immediately, and stored at −70°C until analysis. Because the white mesenteric adipose tissues were sometimes unclear at birth, the mesenteric tissues, including adipose tissue and connective tissues, were removed from three or four male or female offspring at birth. The Institutional Animal Care and Use Committee of Okayama University approved all animal procedures.

Glucose, insulin, total triglyceride, adiponectin, and leptin level measurements. Blood samples at conception and in offspring at 24 wk of age after fasting for 16 h were obtained from the saphenous vein to measure total triglyceride, adiponectin, leptin, glucose, and insulin levels and for a homeostasis model assessment of insulin resistance (HOMA-IR). Blood glucose levels were measured by the glucose oxidase method using a Medisafe automated analyzer (Terumo, Tokyo, Japan). As described previously (27, 28), fasting insulin, total triglyceride, adiponectin, and leptin levels were determined using ELISA kits (insulin kit from Mornigana Institute of Biological Sciences, Yokohama, Japan; triglyceride kit from Abcam, Cambridge, UK; adiponectin and leptin kits from R & D Systems, Minneapolis, MN). The intra- and interassay coefficients of variation were <2.4 and 7.5%, respectively, for insulin, 3.0 and 7.1%, respectively, for total triglyceride, 2.5 and 6.4%, respectively, for leptin, and 3.2 and 8.0%, respectively, for adiponectin. Serum sample volumes for each measurement were 5 μl for insulin and triglycerides, 10 μl for adiponectin, and 20 μl for leptin. The total blood sample volume collected from each mouse was <100 μl, which was <5% of the total blood volume. HOMA-IR was calculated as the fasting insulin concentration (μU/ml) × fasting glucose concentration (mg/dl)/405 (18).

Real-time quantitative PCR. Total RNA from white mesenteric adipose tissues was extracted using TRIzol reagent (Life Technologies, Carlsbad, CA) according to the manufacturer’s instructions. Real-time quantitative PCR was performed to measure leptin and adiponectin mRNA levels using a StepOne Real-time PCR System and a TaqMan RNA-to-CT Gene Kit (Applied Biosystems, Carlsbad, CA). Specific primers for mouse leptin, adiponectin, and β-actin gene sequences were purchased from Applied Biosystems. Specific primers sequences and accession numbers were as described (14, 26, 43). RNA samples (25 ng) were assayed in triplicate using 1 μl of gene-specific primers and 5 μl of gene-specific probes. High throughput screening of several housekeeping genes, including glyceraldehyde-3-phosphate dehydrogenase, β-microglobulin, phosphoglycerate kinase, and β-globin, were used as control genes. TaqMan endogenous control assay (data not shown), confirmed that there were no significant differences in adipose tissue β-actin expression between groups A, B, C, and D, groups I, II, III, and IV, and groups α, β, γ, and δ. Thus, mouse β-actin mRNA levels were measured as an internal control using a predeveloped TaqMan primer and probe mixture (Applied Biosystems). Target gene mRNA levels were normalized against β-actin mRNA levels.

Chromatin immunoprecipitation assays. Chromatin immunoprecipitation (ChIP) assays were performed using a ChIP assay kit (Upstate Biotechnology, Lake Placid, NY) according to the manufacturer’s protocol. White mesenteric adipose tissue samples from groups A–D at birth and 24 wk of age and groups I–IV and A–D at the F3 generation (n = 12 male and female) at 24 wk of age were obtained for analysis. As described previously (27, 29), 20 μg of frozen samples were ground in liquid nitrogen using a mortar and pestle and then washed with PBS at room temperature. Because it was difficult to obtain sufficient tissue quantities from offspring at birth, we collected adipose tissue from three or four offspring at birth. The samples were resuspended in PBS and cross-linked in 1% formaldehyde for 10 min. After centrifugation, the pellet was resuspended in nucleus swelling buffer containing protease and phosphorylation inhibitors. Chromatin was sonicated to reduce DNA fragment lengths to 0.3–1.0 kb. Chromatin was preclared in the presence of 20 μl of normal serum, 2 μg of salmon sperm DNA, and 80 μl of 25% protein A-agarose slurry. Preclared 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, Bedford, MA), dimethyl histone H3 at lysine 9 (dimethyl H3K9; Abcam, Cambridge, MA), and mono- methyl histone H4 at lysine 20 (monomethyl H4K20; Abcam) or nonimmune rabbit IgG (Millipore). After the complex was collected by incubation with 60 μl of 25% protein A-sepharose slurry and centrifugation, the beads were washed five times and the chromatin-immune complex was eluted. After the cross-linking was reversed, DNA was purified and used as a template for PCR. PCR was performed using primer sets specific for the mouse adiponectin (positions −549 to −481) (34) and leptin (−181 to +20) promoters (45).

Statistical analysis. Statistical analyses were performed by two-way ANOVA test followed by Barlett test for comparisons of body weight, body composition, systolic blood pressure, HOMA-IR, triglyceride, adiponectin and leptin levels, and adiponectin and leptin mRNA expression and epigenetic modifications between groups A, B, C, and D. Statistical analyses were performed by Kruskal-Wallis test followed by Scheffé’s test for comparisons of body weight, HOMA-IR, adiponectin and leptin mRNA expression, and epigenetic modifications between groups I, II, III, and IV and between groups α, β, γ, and δ. All statistical analyses were performed with StatView software (Abacus Concepts, Berkeley, CA). Data are presented as means ± SD. A P value of <0.05 was considered statistically significant.

RESULTS

Effects of parental HFD exposure on body weight and fat mass gain in offspring. There were significant differences in mean body weight, HOMA-IR and triglyceride, and leptin and adiponectin levels at conception between CD- and HFD-fed male and female mice (Table 2). We examined four F1 generation groups: offspring from male and female mice fed a CD (group A), males fed a HFD and females fed a CD (group B), males fed a CD and females fed a HFD (group C), and males and females fed a HFD (group D) (Fig. 1A). There was no significant difference in mean litter size among groups A, B, C, and D (9.1 ± 0.5, 9.0 ± 0.8, 8.8 ± 0.8, and 9.2 ± 0.7, respectively). The mean weights of both male and female offspring in groups B, C, and D were significantly higher than that of group A from 8, 16, and 20 wk of age, respectively. Mean body weights in groups C and D were significantly higher than that of group B from 8 and 16 wk of age.
Table 2. Characteristics of CD-fed and HFD-fed male and female mice at conception

<table>
<thead>
<tr>
<th>Group</th>
<th>Body Weight, g</th>
<th>HOMA-IR</th>
<th>Triglyceride, mg/dl</th>
<th>Leptin, ng/dl</th>
<th>Adiponectin, μg/dl</th>
</tr>
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<tbody>
<tr>
<td>HFD-fed male</td>
<td>55.6 ± 4.1*#</td>
<td>5.21 ± 0.42*#</td>
<td>392 ± 29*#</td>
<td>8.3 ± 1.0*#</td>
<td>3.2 ± 0.5*#</td>
</tr>
<tr>
<td>CD-fed male</td>
<td>41.8 ± 3.7*</td>
<td>4.02 ± 0.33*</td>
<td>281 ± 36*</td>
<td>5.3 ± 0.9*</td>
<td>8.2 ± 1.1*</td>
</tr>
<tr>
<td>HFD-fed female</td>
<td>47.2 ± 2.8*</td>
<td>4.88 ± 0.24*</td>
<td>333 ± 27*</td>
<td>6.9 ± 0.7*</td>
<td>5.6 ± 0.7*</td>
</tr>
<tr>
<td>CD-fed female</td>
<td>35.0 ± 2.8</td>
<td>3.16 ± 0.29</td>
<td>226 ± 19</td>
<td>3.7 ± 0.5</td>
<td>11.5 ± 0.9</td>
</tr>
</tbody>
</table>

Values are means ± SE. CD, control diet; HFD, high-fat diet; HOMA-IR, homeostasis model assessment of insulin resistance. *P < 0.01 vs. group D; $P < 0.01 vs. group C; #$P < 0.01 vs. group B.

respectively, and group D body weight was significantly higher than that of group C from 8 wk of age (Fig. 1, B and C). The fat mass gains in group D from 4 wk of age, group C from 12 wk of age, and group B from 16 wk of age were significantly higher than that of group A. Furthermore, fat mass gains in groups D and C were significantly higher than that in group B from 8 and 12 wk of age, respectively, and the gain in group D was significantly higher than that in group C from 8 wk of age (Fig. 1, D and E).

Effects of parental HFD exposure on blood pressure, HOMA-IR, and serum triglyceride, adiponectin, and leptin levels in offspring. Systolic blood pressures of female offspring in groups C and D and of male offspring in groups B, C, and D were significantly elevated compared with that of group A at 12 wk of age. Additionally, systolic blood pressures in groups B, C, and D were significantly elevated compared with that in group A at 24 wk of age. Similarly, systolic blood pressures in groups C and D were significantly different from that in group B, and those in group D were also significantly elevated compared with that in group C at 24 wk of age in both female and male offspring (Fig. 2, A and B). HOMA-IR and glucose, insulin, total triglyceride, and leptin levels were significantly elevated, and adiponectin levels were decreased significantly in groups B, C, and D compared with those in group A. Similarly, triglyceride, leptin, and adiponectin levels in groups C and D were significantly different from those in group B, and those in group D were significantly different from those in group C at 24 wk of age (Fig. 2, C–F). However, these effects did not include adiponectin and leptin levels between groups B and C in male offspring only.

Fig. 2. Effects of parental HFD exposure on blood pressure, homeostasis model assessment of insulin resistance (HOMA-IR), and serum triglyceride, adiponectin, and leptin levels in offspring. Blood pressure of females (A) and males (B) at 12 and 24 wk of age. HOMA-IR (C) and triglyceride (D), adiponectin (E), and leptin (F) levels at 24 wk of age. Results are means ± SD (n = 12 litters/group). *P < 0.01 vs. group A; #P < 0.01 vs. group B; $P < 0.01 vs. group C.
Effects of parental HFD exposure on adiponectin and leptin gene expression and modifications to H3K9 and H4K20 in the adiponectin and leptin promoters in adipose tissue of offspring. Adiponectin gene expression was significantly decreased and leptin gene expression was significantly elevated in the white mesenteric adipose tissue of offspring in groups B, C, and D compared with those in group A. Furthermore, adiponectin and leptin gene expression in groups C and D were significantly different from those of group B, and those of group D were significantly different from those of group C at 24 wk of age (Fig. 3, A and B). However, these effects did not include adiponectin gene expression between groups B and C in male offspring. To investigate whether paternal HFD exposure would affect histone modifications in the adiponectin and leptin gene promoters in offspring adipose tissue, we performed ChIP assays using antibodies for acetyl and dimethyl H3K9 and monomethyl H4K20 at 24 wk of age. Acetyl H3K9 levels were significantly decreased and dimethyl H3K9 levels were significantly increased in the adiponectin promoter region of offspring in groups B, C, and D compared with those in group A. Similarly, those in groups C and D differed from those in group B, and those in group D differed from those in group C at 24 wk of age in both female and male offspring (Fig. 3C). However, these differences did not include either modification between groups B and C in male offspring (Fig. 3C). Furthermore, there were no significant differences in monomethyl H4K20 levels in the adiponectin promoter region among groups A, B, C, and D, and the levels in all groups were <0.1% relative to input (data not shown). Monomethyl H4K20 levels were significantly increased in the leptin promoter region of offspring in groups B, C, and D compared with those in group A. Furthermore, monomethyl H4K20 levels in groups C and D differed from those in group B, and those in group C were significantly elevated compared with those in group D at 24 wk of age in both female and male offspring (Fig. 3D). There were no significant differences in acetyl and dimethyl H3K9 levels in the leptin promoter region among groups A, B, C, and D, and the levels in all groups were <1% relative to input (data not shown). We also observed histone modification differences in the adiponectin gene of adipose tissues that were collected.

Fig. 3. Effects of parental HFD exposure on adiponectin and leptin gene expression and modifications to H3K9 and H4K20 in the adiponectin and leptin gene promoters in adipose tissue of female offspring. A: adiponectin expression. B: leptin expression. mRNA levels were normalized to β-actin mRNA levels. C–E: H3K9 and H4K20 modifications in the adiponectin (C) and leptin (D) promoter regions at 24 wk of age and H3K9 modifications in the adiponectin (E) promoter region at birth. Results are means ± SD (n = 12 litters/group at 24 wk of age, and n = 4 pools from 12 litters/group at birth). *P < 0.01 vs. group A; #P < 0.01 vs. group B; $P < 0.01 vs. group C.

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from three or four male or female offspring at birth (Fig. 3E). We confirmed that there were no effects of maternal diet on the association of IgG binding with adipose tissue leptin or adiponectin promoter regions, and the levels were <0.1% relative to input with nonimmune IgG (data not shown).

**Effect of paternal CD on offspring after HFD exposure.** There were no significant differences in body weights between groups I and II. However, group III and IV offspring body weights were significantly increased only at 24 wk and from 20 wk, respectively, in females and from 24 and 20 wk, respectively, in males compared with those of group I. The mean body weights of offspring in groups III and IV were significantly increased compared with those of group II at 28 wk and from 20 wk, respectively, in female offspring and from 24 and 20 wk, respectively, in male offspring. Moreover, there were significant differences between groups III and IV from 20 wk in both male and female offspring (Fig. 4). Adiponectin expression levels in groups III and IV were significantly decreased compared with those in groups I and II (Fig. 4E). In contrast, leptin expression levels in groups III and IV were significantly elevated compared with those in groups I and II in both male and female offspring (Fig. 4F). There were no significant differences in HOMA-IR or adiponectin or leptin expression between groups I and II or between groups III and IV. We also observed that serum adiponectin and leptin levels in groups I, II, III, and IV correlated with their respective mRNA expression in adipose tissue (data not shown). The acetyl H3K9 level in the adiponectin promoter region was significantly decreased, and the monomethyl H4K20 level in the leptin promoter region was significantly increased in groups III and IV compared with those in groups I and II. There were no significant differences in either histone modification between groups I and II or between groups III and IV in male or female offspring (Fig. 4G). There were no significant differences in monomethyl H4K20 levels in the adiponectin promoter region or acetyl and dimethyl H3K9 levels in the leptin promoter region among groups I, II, III, and IV. The levels in all groups were <0.1% relative to input (data not shown). From this experiment, we observed that there were parental and grandpaternal effects of HFD on offspring (i.e., groups III and IV) but no great-grandpaternal effect (i.e., group II). In addition, there were no significant differences compared with group B (Figs. 1 and 2), which displayed only a paternal effect,
indicating that there were no cumulative transgenerational effects on offspring.

Effect of CD on F3 offspring following paternal and/or maternal HFD exposure. We examined four groups: the F3 generation after mating between male and female mice fed a CD (group α), the F3 generation after mating between males fed a HFD and females fed a CD (group β), the F3 generation after mating between males fed a CD and females fed a HFD (group γ), and the F3 generation after mating between males and females fed a HFD (group δ) at the F0 generation (Fig. 5A). The mean body weight in group δ was significantly increased compared with that in groups α and β in both male and female offspring. Similarly, the group γ mean body weight was also elevated compared with those of groups α and β in male offspring only at 24 wk of age (Fig. 5B). We also observed that the HOMA-IRs in groups γ and δ were significantly elevated compared with those in groups α and β in both male and female offspring (Fig. 5C). Adiponectin expression levels in groups γ and δ were significantly decreased compared with those in groups α and β (Fig. 5D). In contrast, leptin expression levels in groups γ and δ were significantly elevated compared with those in groups α and β in both male and female offspring (Fig. 5E).

There were no significant differences in mean body weights, HOMA-IRs, or adiponectin and leptin expression between groups α and β or between groups γ and δ, with the exception of mean body weight between female group γ and δ offspring. We also observed that serum adiponectin and leptin levels in groups α, β, γ, and δ correlated with their respective mRNA expression in adipose tissue (data not shown). The acetyl H3K9 level in the adiponectin promoter region was decreased significantly, and the monomethyl H4K20 level in the leptin promoter region was increased significantly in groups γ and δ compared with those in groups α and β. There were no significant differences in either histone modification between groups α and β or between groups γ and δ in male or female offspring (Fig. 5F). Furthermore, we observed no significant differences in monomethyl H4K20 levels in the adiponectin promoter region or acetyl and dimethyl H3K9 levels in the leptin

![Diagram of experimental scheme for F3 generation after mating between male and female mice fed a control diet (CD) and high-fat diet (HFD).](http://ajpendo.physiology.org/)

Fig. 5. Effects of control diet (CD) consumption on F3 offspring after paternal and/or maternal high-fat diet (HFD) exposure. Experimental scheme for F3 (A), body weights (B), HOMA-IR (C), adiponectin (D) and leptin (E) gene mRNA expression, and H3K9 and H4K20 modifications in the adiponectin and leptin promoter regions (F) in the adipose tissue of offspring at 24 wk in groups α, β, γ, and δ. mRNA levels were normalized to β-actin mRNA levels. Results are means ± SD (n = 12 litters/group). *P < 0.01 vs. group α; #P < 0.01 vs. group β; $P < 0.01 vs. group γ.
promoter region among groups α, β, γ, and δ. The levels in all groups were <0.1% relative to input (data not shown).

**DISCUSSION**

In this study, we observed the effects of paternal HFD consumption before conception on offspring over multiple generations on metabolic syndrome-like phenomena, including weight and fat gain, glucose intolerance, hypertriglyceridemia, abnormal adipocytokine levels, hypertension, and adiponectin and leptin gene expression and epigenetic changes. The effect of paternal HFD consumption was weaker than that of HFD exposure in utero, but it had an additive effect on offspring for two generations. A CD in male offspring in the subsequent generation after paternal HFD exposure before conception diminished whereas a CD for two generations completely abolished the effects of paternal HFD consumption on offspring.

Offspring from pregnant female mice and rats fed a HFD displayed permanent detrimental effects to body composition and metabolism, predisposing them to metabolic diseases later in life, even after having been weaned onto standard chow (3, 40). Several studies have indicated that aberrant production of adipokines might play a role not only in the dysregulation of glucose and lipid metabolism but also in blood pressure elevation (4, 35, 41). We have demonstrated previously that HFD exposure in utero may cause a metabolic syndrome-like phenomenon through adiponectin and leptin gene epigenetic modifications that persist for multiple generations (27, 28, 29). Obesogenic and diabetogenic traits were abolished after a maternal CD for three generations (29). Additionally, several reports have analyzed the effect of HFD-induced paternal obesity on offspring metabolism. HFD feeding in male Sprague-Dawley rats resulted in β-cell dysfunction in CD-fed offspring (34). Hypomethylation of the interleukin 13 receptor-α2 gene, which displayed the highest fold difference in expression, was observed in pancreatic islets of female offspring from HFD-fed fathers (34). This provides important confirmation of an epigenetic mechanism of paternal dietary composition on offspring phenotype.

H3K9 methylation status is important for regulating the expression of metabolic genes in adipose tissue (19, 42). H3K9 methylation positively affected whereas H4K20 methylation negatively affected adipogenesis through the peroxisome proliferator-activated receptor γ-mediated pathway (44). Moreover, there have been several reports that H3K9 modifications in the adiponectin promoter region might play important roles in adipogenesis (31, 37). Thus, we evaluated histone modifications in the adiponectin and leptin promoter regions in this study. We also observed a grandpaternal effect of HFD on offspring but no great-grandpaternal effect on offspring. There was a paternal linkage for multiple generations with histone modification of adipose tissue adipocytokine genes in offspring at birth. In addition, the effect of paternal HFD exposure before conception was significantly weaker than that of HFD exposure in utero, with the exception of adiponectin gene expression and epigenetic changes. However, paternal HFD consumption had an additive effect on metabolism following in utero HFD exposure. Although pancreatic β-cell function is important for the transgenerational effect on glucose metabolism, as reported previously (34), we have not examined the effect of paternal diet on pancreatic islet mass and function in offspring. Further analysis will be required to investigate the mechanism underlying the transgenerational effect.

Previous reports demonstrated that HFD consumption for 10 wk by male rats programmed β-cell dysfunction in their female but not male offspring on regular chow (34). Overfeeding of male mice resulted in altered insulin and glucose metabolism in two subsequent generations in male offspring only (36). However, we demonstrated that there were no significant sex-based differences in metabolic traits following paternal and/or maternal HFD exposure in this study. We did not obtain data from a larger sample size and/or with a longer observation period. Thus, future studies will investigate whether there are any differences following HFD exposure in male and female offspring metabolism through paternal and maternal linkages.

We also demonstrated that paternal CD consumption in the subsequent generation following paternal HFD exposure before conception diminished whereas CD consumption for two generations completely abolished the effects of paternal HFD exposure on offspring. In contrast, it took three generations of CD feeding in utero to abolish the effects of maternal HFD exposure in utero on offspring (29). A recent report also indicated that undernutrition for 50 generations increased susceptibility to obesity and diabetes, and that was not reversed after dietary recuperation for two generations (17). The mechanisms underlying the maintenance and enhancement of epigenetic modifications of adipocytokine gene promoters following paternal overnutrition across multiple generations and the elimination of this effect following paternal CD consumption remain 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 that will form the F2 generation. Moreover, there was no cumulative effect on offspring through paternal lineages in this study. However, the effect of maternal HFD consumption on offspring over multiple generations was cumulative (29), although we observed that the epigenetic modifications of the adiponectin and leptin gene promoters were associated with both maternal and paternal linkages. We have not investigated differences in the transgenerational effect or mechanisms between paternal and maternal lineages. Further analysis is required to resolve these questions, including sex-based differences and mechanisms such as the potential role of noncoding RNA (9).

Taken together, our data suggest that paternal HFD exposure before conception might lead to a metabolic syndrome-like phenomenon through epigenetic modifications of the genes encoding adipocytokines adiponectin and leptin in the offspring that persist for multiple generations. In contrast, paternal CD consumption diminished the epigenetic effect caused by HFD exposure in utero and ultimately abolished the effect after a CD was consumed for two generations. The effects of paternal HFD exposure on offspring were relatively weaker than those of HFD exposure in utero. However, paternal HFD consumption had an additive effect on the metabolic syndrome-like phenomena in offspring across multiple genera-
tions. Understanding how epigenetic mechanisms contribute to the transgenerational transmission of obesity and metabolic dysfunction through paternal and maternal lineages is crucial for the development of novel early detection and prevention strategies for programmed metabolic syndrome.

GRANTS
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DISCLOSURES
No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS
H.M. conception and design of research; H.M., T.M., E.T., and S.T. performed experiments; H.M., T.M., E.T., and S.T. analyzed data; H.M. interpreted results of experiments; H.M. prepared figures; H.M. and Y.H. drafted manuscript; H.M. and Y.H. edited and revised manuscript; H.M., T.M., E.T., S.T., and Y.H. approved final version of manuscript.

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