Am J Physiol Endocrinol Metab 311: E236–E245, 2016.
First published May 31, 2016; doi:10.1152/ajpendo.00095.2016.

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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Submitted 14 March 2016; accepted in final form 26 May 2016

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, hypertriglyceridemia, abnormal adipocytokine levels, 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 adipocytokine 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 adipocytokine genes for multiple generations.

adiponectin; epigenetics; high-fat diet; leptin; metabolic syndrome

OBESITY IS A PUBLIC HEALTH CRISIS contributing to the global morbidity and mortality of metabolic diseases such as metabolic syndrome (20). Recent data demonstrated that ~40% of adults in the US are obese, and there is no difference in prevalence between males and females at any age (12). Maternal obesity during human pregnancy often results in fetal overgrowth (7, 21), which increases the risk of the offspring developing obesity and metabolic syndrome later in life, thereby contributing to increased type 2 diabetes incidence (1, 2, 5). Although obesity is associated with an increased risk of almost every common complication of pregnancy, obesity in the mother might also play a direct role in the transmission of obesogenic and diabetogenic traits across generations. A recent family cohort study reported a persisting association between mother and offspring BMI over three generations through the maternal line (30). In addition, recent studies indicated that paternal BMI had effects on offspring BMI that were independent of but additive to the effects of maternal BMI (8). Both maternal and paternal BMI were positively associated with offspring BMI at the age of 11 yr, and this effect did not diminish by 44–45 yr in either sex (24). Furthermore, in laboratory rodents, HFD-induced paternal obesity affected both body weight and glucose metabolism in offspring(13, 34).

Adipose tissue functions as a highly specialized endocrine and paracrine tissue, producing an array of adipocytokines, such as leptin and adiponectin. These factors 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 adipocytokine. 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-

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Table 1. *The compositions and caloric values of the experimental diets*

	CD		HFD	
	Composition, g	Calories, kcal	Composition, g	Calories, kcal
Protein	23.1	92.4 (25.6%)	23	91.6 (18.0%)
Carbohydrate	55.3	221.2 (61.6%)	25.3	100.7 (20.0%)
Fat	5.1	45.9 (12.8%)	35	313.7 (62.0%)
Total calories		359		506

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 postcopulatory 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. 1*A*). 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 paternal 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

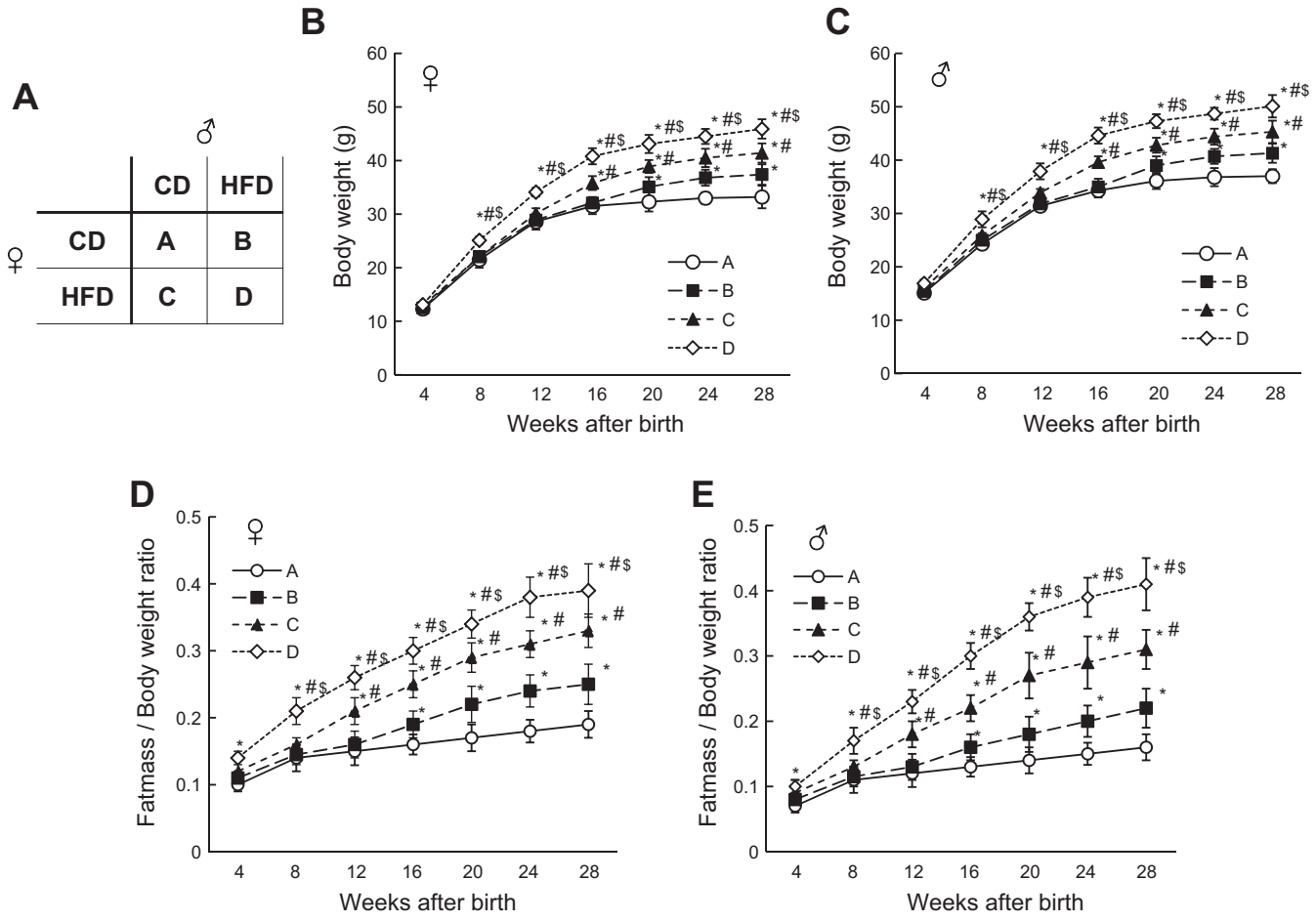

Fig. 1. Effects of parental high-fat diet (HFD) exposure on body weight and fat mass gain in offspring. Experimental scheme for F0 (*A*), weight of females (*B*) and males (*C*), and body composition of females (*D*) and males (*F*) in *groups A–D*. Results are means ± SD ($n = 12$/group). *$P < 0.01$ vs. *group A*; #$P < 0.01$ vs. *group B*; \$$P < 0.01$ vs. *group C*. CD, control diet.

generations (*group I*) (Fig. 4*A*). Moreover, to evaluate the effect of parental HFD exposure, in the absence of any further challenge, 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. 5*A*). 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 Softron BP98A tail-cuff hemodynamometer (Softron, 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 Morinaga 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 15 pmol of gene-specific primers and 5 pmol of gene-specific probes. High throughput screening of several housekeeping genes, including glyceraldehyde-3-phosphate dehydrogenase, β_2-microglobulin, phosphoglycerate kinase, and β-glucuronidase, using the 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 mg 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. 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, Bedford, MA), dimethyl histone H3 at lysine 9 (dimethyl H3K9; Abcam, Cambridge, MA), and monomethyl 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 Scheffe'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. 1*A*). 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*

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

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.

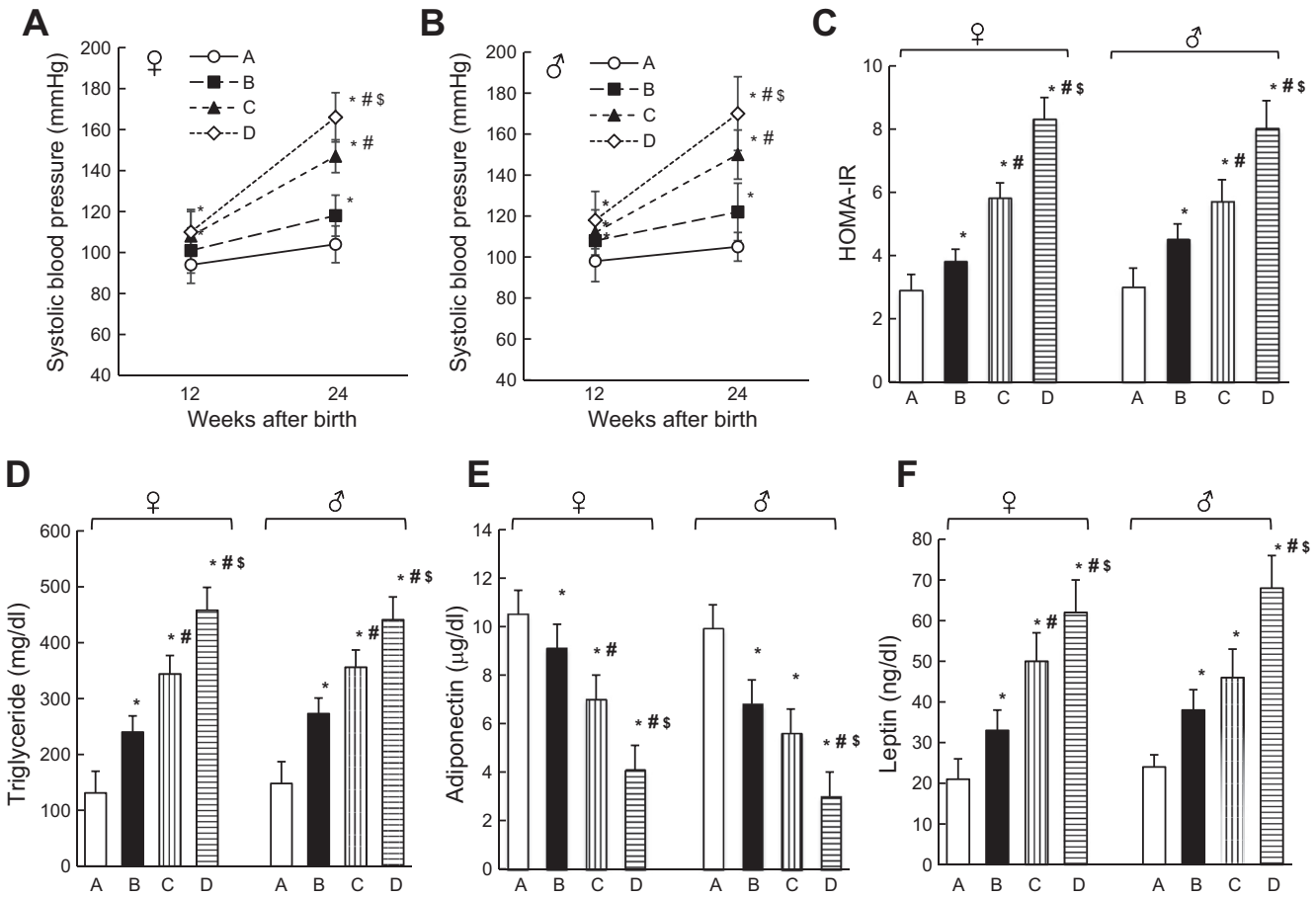

Fig. 2. Effects of parental HFD exposure on blood pressure, homeostatis model assessment of insulin resistance (HOMA-IR), and serum triglyceride, adiponectin, and leptin levels in offspring. Blood pressure of females (*A*) and males (*B*) at 12 and 24 wk of age. HOMA-IR (*C*) and triglyceride (*D*), 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. 3*C*). However, these differences did not include either modification between *groups B* and *C* in male offspring (Fig. 3*C*). 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. 3*D*). 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

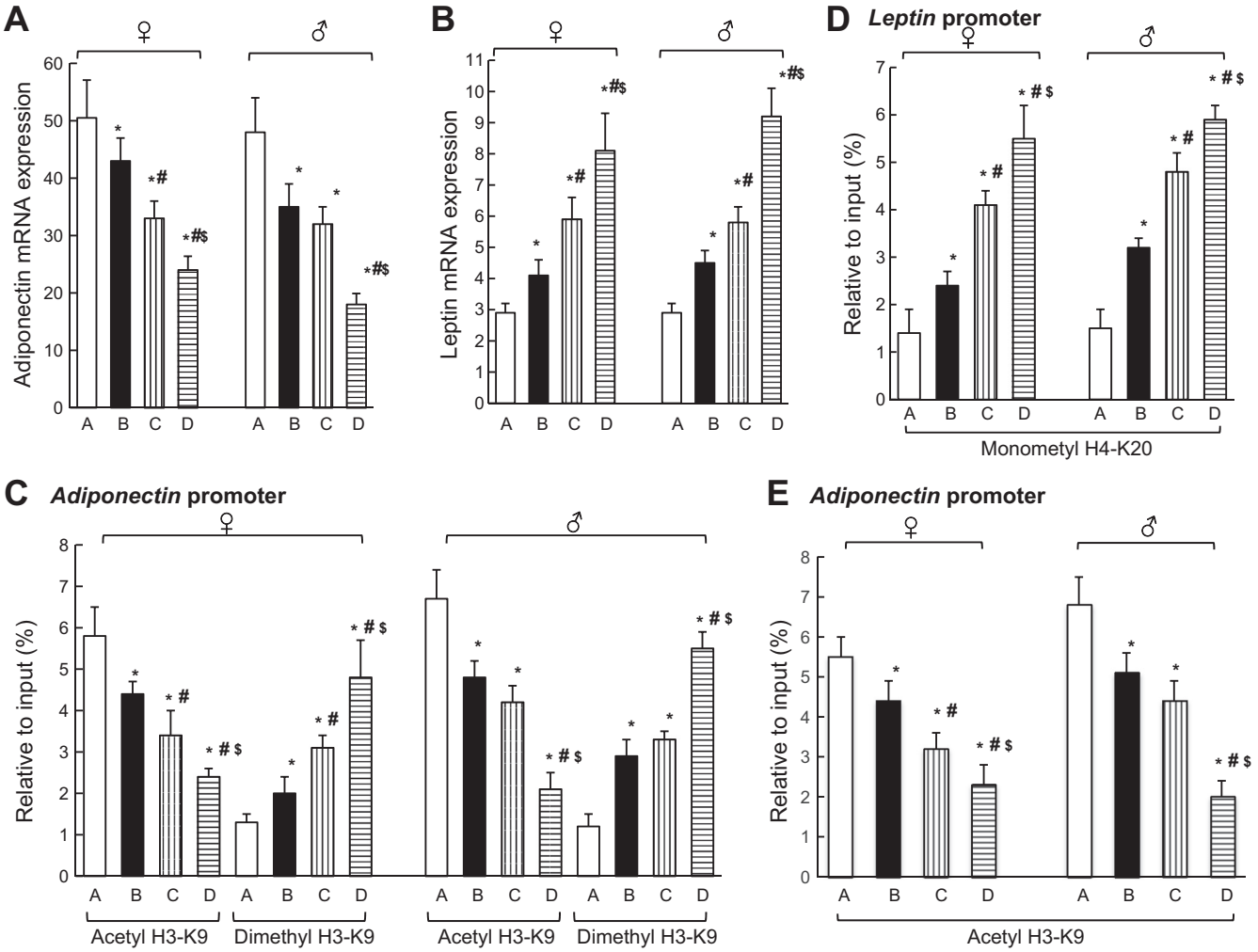

Fig. 3. Effects of parental HFD exposure on *adiponectin* and *leptin* gene expression and modifications to H3K9 and H4K20 in the *adiponectin* and *leptin* gene promoters in adipose tissue of female offspring. *A*: *adiponectin* exppression. *B*: *leptin* expression. mRNA levels were normalized to β-*actin* 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*.

from three or four male or female offspring at birth (Fig. 3*E*). 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, *B* and *C*). We also observed that the HOMA-IRs in *groups III* and *IV* were significantly elevated compared with those in *groups I* and *II* in both male and female offspring (Fig. 4*D*). *Adiponectin* expression levels in *groups III* and *IV* were significantly decreased compared with those in *groups I* and *II* (Fig. 4*E*). 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. 4*F*). 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. 4*G*). 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 paternal 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,

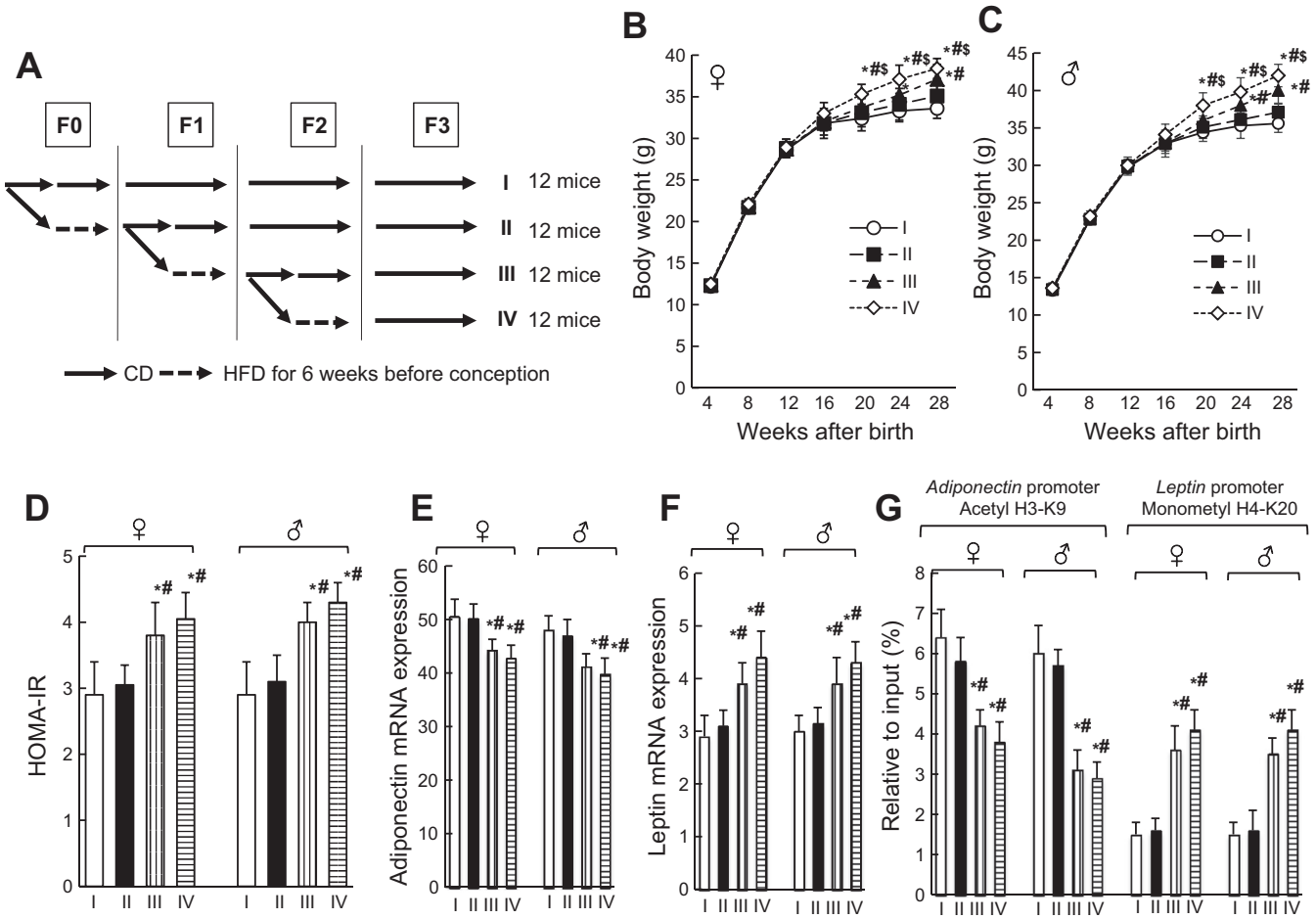

Fig. 4. Effects of a control diet (CD) on offspring after high-fat diet (HFD) exposure. Experimental scheme for F0-3 (*A*), body weights of females (*B*) and males (*C*), HOMA-IR (*D*), *adiponectin* (*E*) and *leptin* (*F*) gene mRNA expression, and H3K9 and H4K20 modifications in the *adiponectin* and *leptin* promoter regions (*G*) in the adipose tissue of offspring at 24 wk in *groups I–IV*. mRNA levels were normalized to *β-actin* mRNA levels. Results are means ± SD (n = 12 litters/group). *$P < 0.01$ vs. *group I*; #$P < 0.01$ vs. *group II*; \$$P < 0.01$ vs. *group III*.

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. 5*A*). 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. 5*B*). 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. 5*C*). *Adiponectin* expression levels in *groups* γ and δ were significantly decreased compared with those in *groups* α and β (Fig. 5*D*). 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. 5*E*). 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. 5*F*). Furthermore, we observed no significant differences in monomethyl H4K20 levels in the *adiponectin* promoter region or acetyl and dimethyl H3K9 levels in the *leptin*

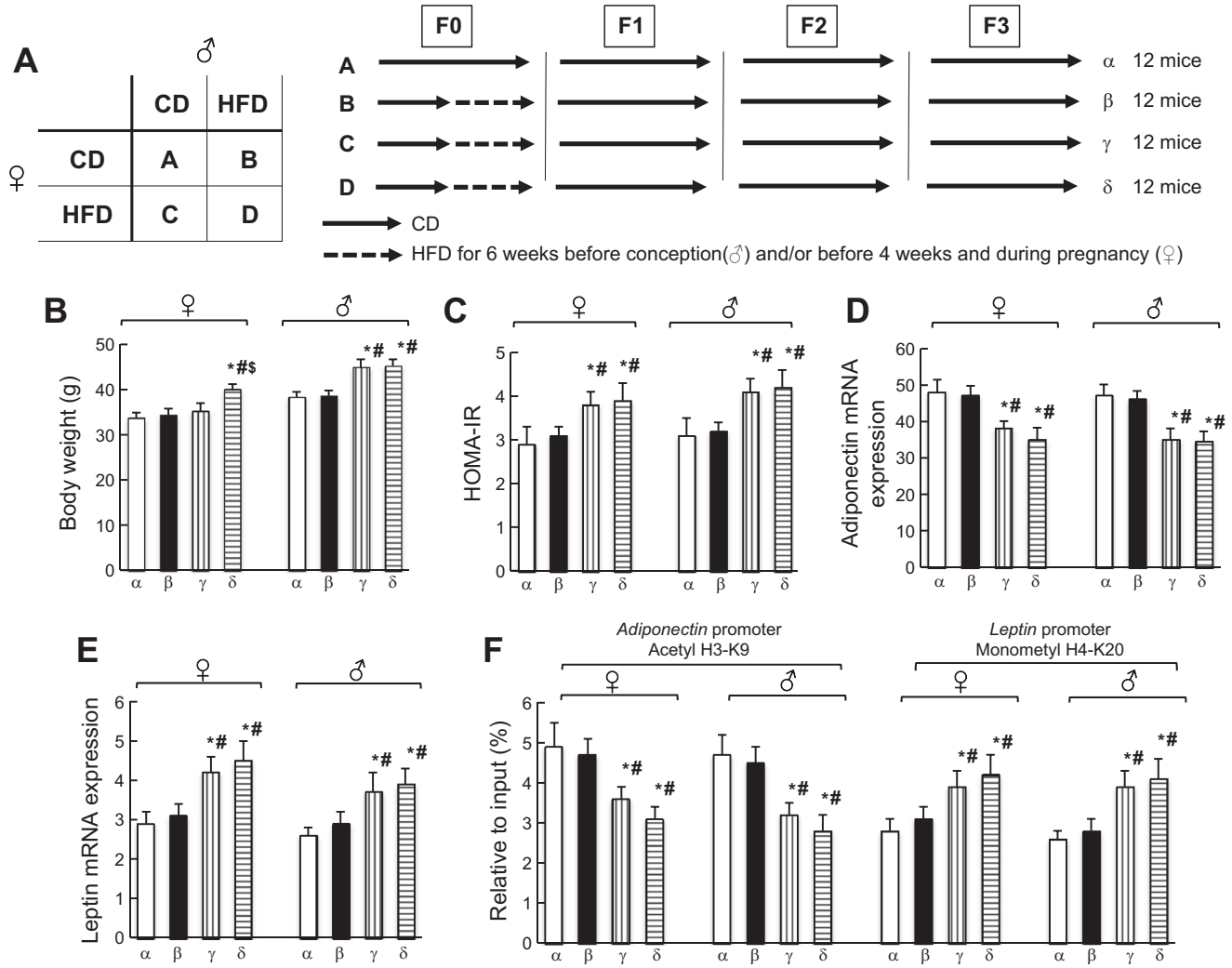

Fig. 5. Effects of control diet (CD) consumption on F3 offspring after paternal and/or maternal high-fat diet (HFD) exposure. Experimental scheme for F3 (*A*), body weights (*B*), HOMA-IR (*C*), *adiponectin* (*D*) and *leptin* (*E*) gene mRNA expression, and H3K9 and H4K20 modifications in the *adiponectin* 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 adipocytokines 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 (10). A recent report demonstrated that paternal obesity initiated metabolic disturbances in two generations of mice with incomplete penetrance to the F2 generation and altered the transcriptional profile of testis and sperm microRNA content (13). 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

This work was supported in part by a research grant (no. 25462558) from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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., T.E., and S.T. performed experiments; H.M., T.M., T.E., 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., T.E., S.T., and Y.H. approved final version of manuscript.

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