Maternal High-Fat Diet Promotes Body Length Increases and Insulin Insensitivity in Second-Generation Mice

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Maternal obesity and diet consumption during pregnancy have been linked to offspring adiposity, cardiovascular disease, and impaired glucose metabolism. Furthermore, nutrition during development is clearly linked to somatic growth. However, few studies have examined whether phenotypes derived from maternal high-fat diet exposure can be passed to subsequent generations and by what mechanisms this may occur. Here we report the novel finding of a significant body length increase that persisted across at least two generations of offspring in response to maternal high-fat diet exposure. This phenotype is not attributable to altered intrauterine conditions or maternal feeding behavior because maternal and paternal lineages were able to transmit the effect, supporting a true epigenetic manner of inheritance. We also detected a heritable feature of reduced insulin sensitivity across two generations. Alterations in the GH secretagogue receptor (GHSR), the GHSR transcriptional repressor AF5q31, plasma IGF-I concentrations, and IGF-binding protein-3 (IGFBP3) suggest a contribution of the GH axis. These studies provide evidence that the heritability of body length and glucose homeostasis are modulated by maternal diet across multiple generations, providing a mechanism where length can increase rapidly in concert with caloric availability. **(*Endocrinology* 150: 4999–5009, 2009)**

Obesity and its associated metabolic complications such as hyperinsulinemia, hyperglycemia, and cardiovascular disease have increased to epidemic proportions within the past several decades (1). Currently, nearly a third of the population of the United States is now classified as obese (2). Obesity and its complications decrease overall quality of life and often result in premature death (3–5). Therefore, elucidation of the potential underlying causes and determination of disease prevention is critically important. Although evidence clearly supports decreased physical activity and/or increased caloric consumption as key components in the development of obesity, examination of the effects of maternal diet and obesity during pregnancy on long-term offspring consequences has been a recent focus in both animal models and human clinical assessment (6–13).

Early studies assessing the impact of maternal diet on offspring outcome focused on the impact of maternal undernutrition and intrauterine growth restriction in the development of hypertension, dyslipidemia, insulin resistance, vascular function, and cardiovascular disease (14–24). However, as obesity rates have increased, the emphasis has shifted toward determining the role of maternal obesity and/or high-fat diet consumption on offspring outcome. In general, studies in a range of organisms have linked maternal overnutrition with offspring obesity, metabolic syndrome, liver dysfunction, and cardiovascular disease (25–40). Furthermore, epidemiological reports have detected positive correlations between parental and offspring body mass index (41–44). Not surprisingly, phenotypes vary depending on the exact organism, strain, and dietary manipulation, because a divergent set of maternal contributing factors such as preexisting obesity, hyperinsulinemia, or gestational diabetes alter specific phenotypic elements including offspring insulin resistance or body composition (45–49).

ISSN Print 0013-7227 ISSN Online 1945-7170
Printed in U.S.A.

doi: 10.1210/en.2009-0500 Received April 29, 2009. Accepted July 29, 2009.
First Published Online October 9, 2009

Abbreviations: E17, Embryonic d 17; GHSR, GH secretagogue receptor; GTT, glucose tolerance test; IGFBP, IGF-binding protein; ITT, insulin tolerance test.

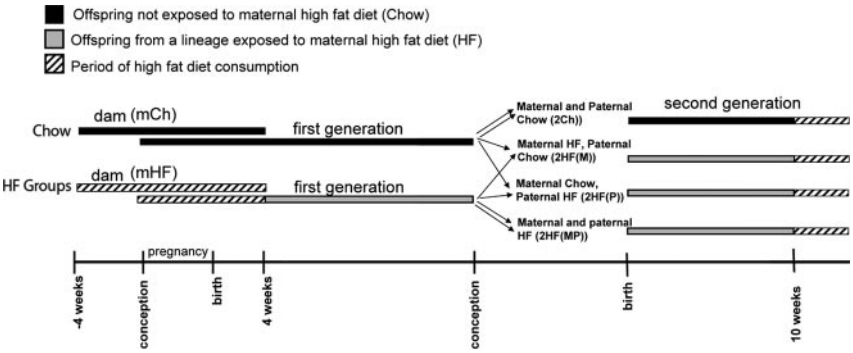

FIG. 1. Breeding scheme for first- and second-generation offspring. C57BL/6:129 dams (*top lines*) from the chow (mCh) group consumed a 12% fat house chow diet from 4 wk before conception and throughout pregnancy and the nursing period, whereas dams from the high-fat (mHF) group consumed a 45% fat high-fat diet during this time. All first-generation offspring were weaned onto chow. Approximately two thirds of first-generation mice were fed a high-fat diet for 8 wk starting at 10 wk of age to examine physiological responses, but these mice were not used to produce the second generation. The remaining mice (naive to high-fat diet as adults) were bred to produce second-generation offspring, one where the maternal [2HF(M)], the paternal [2HF(P)], or both lineages [2HF(MP)] had been exposed to maternal high-fat diet. All second-generation mice consumed a high-fat diet as adults, commencing at 10 wk.

The recent interest in the epigenetic programming of disease has provided a means to mechanistically examine the genes and pathways involved in the heritability of traits affected through maternal diet. Recent studies demonstrating the perpetuation of type 2 diabetes into second-generation offspring in response to maternal undernutrition support these long-term programming effects (50–58). However, the heritable features of maternal high-fat diet or overnutrition exposure on offspring obesity, body size, glucose regulation, or disruption of genetic networks have not been well characterized. Therefore, we have examined the first- and second-generation offspring of dams exposed to a chronic high-fat diet to determine the potential for epigenetic programming by both maternal and paternal lineages of body weight, body size, adiposity, glucose regulation, and caloric intake on both chow and high-fat diets. In addition, we conducted a mechanistic examination of the genes and pathways involved in this transgenerational phenotype.

Materials and Methods

Animals

C57BL/6:129 hybrid mice were bred in our colony and sustained on a 12-h light, 12-h dark cycle (lights on at 0700 h). In total, 12 chow-fed (mCh) and 20 high-fat-fed (mHF) dams were bred to produce 65 1Ch and 128 1HF first-generation offspring [supplemental methods (published as supplemental data on The Endocrine Society's Journals Online web site at http://endo.endojournals.org); five 1Ch and eight 1HF litters were killed during gestation for analysis]. These offspring were split into two groups, one of which received high-fat diet as adults and were subsequently killed and those who remained naive to high-fat diet as adults and who became breeding pairs for the second generation. Five chow (2Ch, produced from 1Ch males crossed with 1Ch females, five litters producing 35 total offspring), six first-generation HF (1HF) females crossed with first-generation Chow (1Ch) males [2HF(M), six litters producing 45 total offspring], six 1HF males crossed with six 1Ch females [2HF(P), six litters producing 47 total offspring], and three 1HF males crossed with three 1HF females [2HF(MP), three litters producing 22 total offspring] were used to produce the second generation (Fig. 1). Litters between five and nine were included in our analyses. Pups were weaned from their mothers at 4 wk of age and were singly housed beginning at 6 wk. The room was kept at a temperature of 22 C and relative humidity of 42%. Food and water was provided throughout the study *ad libitum*. All studies were done according to experimental protocols approved by the University of Pennsylvania Institutional Animal Care and Use Committee.

Diet

The 4.73-kcal/g high-fat diet used in these studies was obtained from Research Diets, Inc. (New Brunswick, NJ) and consists of (by kilocalories): 7% corn starch, 10% maltodextrin, 17% sucrose, 39% lard, 20% casein, 0.3% L-cystine, 6% soybean oil, and essential vitamins and minerals. The 4.00-kcal/g house chow diet was obtained from Purina Lab Diets (St. Louis, MO) and consisted of 28% protein, 12% fat, and 60% carbohydrate.

Body weight

Total n (from n unique litters) contributing to body weight measurements are 18–26 for first and 9–13 for second generation offspring.

Food intake

Measurements were collected from 8–10 wk when animals consumed a chow diet and between 12 and 14 wk when animals consumed a high-fat diet. Caloric intake was calculated as the average of five measurements of 24 h intake over 2 wk, normalized to average body weight over that period. Mice were allowed to habituate to high-fat diet for 1 wk (10–11 wk) before high-fat diet intake measurements were performed. For chow intake, first generation n = 18–26, and second generation n = 9–14. For high-fat diet intake, first- and second-generation n = 8–14.

Body length

Measurements were performed dorsally on adults from the tip of the nose to the base of the tail while mice were under anesthesia. To measure embryonic d 17 (E17) animals, fetuses were gently stretched out and then allowed to curl back to their normal position on a paper towel. Measurements were then taken from the highest point on the top of the head to the lowest point on the rump. Total n (from n unique litters) for first generation at E17 = 4–7, for first-generation at 18 wk = 4–6, and for second generation at 20–22 wk = 4–11.

Leptin

Mice had been on high-fat diet for 8 wk as adults at the time of killing. Trunk blood was collected, mixed with 15 μl 50 mM EDTA, and centrifuged at 5000 rpm for 5 min. Plasma was collected and frozen at −80 C. Plasma leptin levels were quantified by RIA (mouse leptin RIA kit; Linco Research, St. Charles, MO). Total n = 6–10.

Insulin

Blood was collected as described above for leptin. Plasma insulin levels were quantified by ELISA (ultrasensitive mouse ELISA kit; Crystal Chem, Downers Grove, IL). Total n (from n unique litters) for dams (killed at E14–17) = 4–8 and for first- and second-generation offspring = 5–8.

IGF-I

Blood was collected as described above for leptin. IGF-I levels were determined using ELISA (Quantikine mouse IGF-I; R&D Systems, Minneapolis, MN) in accordance with the manufacturer's instructions. Total n = 3–12.

Glucose tolerance

Mice were fasted overnight. A solution of sterile 0.3 g/ml glucose was freshly prepared in a 0.45% saline. Beginning at 0730 h, baseline glucose levels from whole blood removed from tail snips were analyzed using the OneTouch Ultra (Johnson & Johnson, New Brunswick, NJ) system. Animals were then given an ip glucose injection of 2 mg/g body weight. Glucose measurements were performed at 0, 30, 60, and 120 min after injection from tail blood. Glucose tolerance tests (GTTs) were performed within 3 d of the final food intake measurement (10 wk for chow, 14 wk for high-fat diet). Total n = 3–11.

Insulin tolerance

Mice were fasted for 4 h before the experiment. Baseline glucose measurements were performed from tail blood before ip injection of insulin (Humulin R; Lilly, Indianapolis, IN) diluted to 0.01 mU/μl in sterile saline for a final delivery of 1 mU/g body weight. Blood glucose measurements were performed at 0, 15, 30, 60, and 120 min after injection, and readings at all time points were normalized to the 0-min baseline reading. If blood glucose were to fall below 30 mg/dl, animals were rescued with ip-injected 4.5 mg/g glucose and were removed from the experiment. Total n = 3–10.

Quantitative RT-PCR

Specified tissues were dissected (arcuate nuclei, whole brain, or liver) and flash frozen in liquid nitrogen. Seven 1-mm micropunches of arcuate nuclei were performed on 300-μm cryostat sections of whole brains beginning at −1.22 mm and ending at −2.54 mm relative to bregma as designated by the Paxinos and Franklin mouse atlas. Tissues were expelled into Trizol (Invitrogen, Carlsbad, CA), and total RNA isolation was performed with chloroform followed by isopropanol precipitation. RNA was then converted to cDNA using a reverse transcriptase synthesis kit (Invitrogen; Superscript III reverse transcriptase). Quantitative PCR was performed using TaqMan probes and analysis equipment (Applied Biosystems, Foster City, CA). The following TaqMan primer and probe sets were used: *IGFBP-3* (NM_008343.2, Mm00515156_m1), *GHSR* (NM_177330, Mm00616415_m1), and *AF5q31* (NM_033565.1, Mm00466683_m1). n = 4.

PCR array

RNA was prepared from PN1 whole brains and then converted to cDNA as outlined above under *Quantitative RT-PCR*. These samples (n = 4 for 1Ch and 1HF males and females) were run on a quantitative RT-PCR-based array containing 84 genes focused on growth factors (SABiosciences, Frederick, MD; no. PAMM-041). Plates were run and data analyzed according to the manufacturer's instructions. Expression levels were normalized to β-actin internal controls. Fold expression change was calculated by dividing grouped values for 1HF by grouped 1Ch controls for each gene.

Bisulfite sequencing of GH secretagogue receptor (GHSR)

Genomic DNA was isolated from arcuate nucleus punches from the same samples used to isolate RNA. DNA was extracted from the interphase layer of the Trizol/chloroform RNA extraction using buffer QG from a column-based DNA isolation kit (QIAGEN, Valencia, CA) and further processed in accordance with manufacturer's instructions. Bisulfite conversion and pyrosequencing were performed by EpigenDX as previously described (59, 60). Completely methylated and unmethylated DNA samples were used as controls for analysis. n = 4–6.

Statistical measures

Results for first-generation animals were calculated by multifactor ANOVA using maternal diet as a factor and separating by sex. Second-generation results were analyzed by one-way ANOVA by combining 2HF(M), 2HF(P), and 2HF(MP) groups and comparing against 1Ch/2Ch controls. Individual group statistics were then calculated upon significance of the ANOVA using *post hoc* Student's two-tailed *t* tests. Measures for longitudinal weights and GTT and insulin tolerance tests (ITTs) were analyzed by repeated-measures ANOVA over time followed by *post hoc t* tests. Methylation data were processed with one-way ANOVA and subsequent Student's *t* tests within each individual CpG of the total island. For the PCR array, genes were selected for statistical analysis based on whether they exceeded a 2-fold expression change in either male or female HF groups relative to chow. Nine genes met these criteria and were analyzed by two-way ANOVA for sex and diet, and subsequent *post hoc* Student's two-tailed *t* tests were performed (see Fig. 4). All analyses were carried out using JMP statistical software (SAS, Cary, NC).

Results

Breeding scheme and maternal measures

To examine the heritable contribution of maternal high-fat diet on offspring outcome, female mice were exposed to a high-fat diet (mHF, 45% fat) for 4 wk before pregnancy through weaning at 4 wk. Littermate female mice on a chow diet (mCh, 12% fat) were used as controls. As expected, dams on the high-fat diet had elevated body weight, blood glucose, leptin, and insulin levels compared with chow control dams during pregnancy (Table 1). First-

TABLE 1. Dams on a high-fat diet have increased body weight, glucose, and leptin levels

	Chow (mCh)	High-fat diet (mHF)
Body wt (g)	18.303 ± 0.435	20.05 ± 0.584 ($P = 0.0164$)
Blood glc (mg/dl)	124.1 ± 4.11	147.4 ± 4.62 ($P = 0.0003$)
Leptin (ng/dl)	11.385 ± 1.562	50.847 ± 6.6147 ($P < 0.0001$)
Insulin (ng/dl)	0.463 ± 0.233	1.67 ± 0.441 ($P = 0.095$)

Body weight and blood glucose measurements were obtained before pregnancy after 4 wk on a chow diet (mCh, n = 17) or a high-fat diet (mHF, n = 20). Leptin (E17) and insulin (E14–17) levels are after 6 wk on a high-fat diet (leptin: mCh n = 5, and mHF n = 8; insulin: mCh n = 4, and mHF n = 8). Data are mean ± SEM. glc, Glucose; wt, weight.

and second-generation offspring of these dams were then analyzed for phenotype heritability to distinguish traits due to the direct effect of a high-fat intrauterine environment or maternal behaviors from those that persist across a generation. The second-generation mice were produced by breeding in three combinations wherein the maternal, paternal, or both lineages could be studied for potential transmission of an effect (Fig. 1).

First- and second-generation offspring exhibit increased body length

In examination of first- and second-generation offspring body size, we found that the major heritable trait transmitted to the second generation was that of body length, not adiposity [Fig. 2A, $F_{(1,18)} = 6.87$; Fig. 2B, $F_{(3,19)} = 4.989$; Fig. 2C, $F_{(7,65)} = 22.25$]. First-generation E17 embryos and both first- and second-generation male and female offspring were longer than control offspring as adults, a phenotype transmitted by either maternal or paternal lineage. Furthermore, exposure of both the maternal and paternal lines to developmental high-fat diet significantly augmented second-generation offspring length compared with either maternal or paternal exposure alone.

First- but not second-generation offspring display increased body weight

Previous studies examining effects of maternal overweight or obesity have reported increased body weight and adiposity in the first-generation offspring (26, 37, 61, 62). Our first-generation male and female offspring weighed more than controls when on a chow or high-fat diet as adults [Fig. 2D, $F_{(1,44)} = 14.29$; Fig. 2E, $F_{(1,40)} = 40.05$]. However, second-generation offspring did not show a continuation of this phenotype while on either a chow or high-fat diet (Fig. 2, F and G). Despite increased body weights in the first-generation offspring, no differences were detected in caloric intake on chow or high-fat diet for male or female first-generation (supplemental Fig. 1, a and b) or second-generation (supplemental Fig. 1, c and d) offspring.

First- and second-generation offspring show reductions in insulin sensitivity

Prior investigations have described the possible heritable links between maternal obesity and offspring predisposition toward diabetes due to insulin insensitivity (29, 34, 63). In our analyses of adult first- and second-generation offspring, we found no differences in responses to a GTT while on a chow (Fig. 3, A–D) or high-fat diet (supplemental Fig. 2, a–d). However, we detected a reduction in insulin sensitivity during ITTs in both first- and second-generation male and female offspring on a chow diet (Fig. 3, E–H: E, $F_{(1,10)} = 5.63$ (30 min); H, $F_{(3,23)} = 4.04$, 3.66, and 4.02]. Group differences in insulin sensitivity were not significant after adult offspring were exposed to 4 wk of a high-fat diet (supplemental Fig. 2, e–h). We did not detect differences in adult plasma insulin levels in either first- or second-generation offspring from the maternal high-fat lineage (Fig. 4A).

Perpetuation of body size to future-generation offspring has been proposed to stem from enhanced energy storage as adipose tissue (29, 64). However, serum leptin levels indicate a reduction in overall adipose storage in first- and second-generation males and females [Fig. 4B, $F_{(9,75)} = 8.40$].

First- and second-generation females exhibit growth-promoting alterations in the GH pathway

To examine the contribution of the GH pathway to the phenotype, we analyzed a critical downstream effector of GH activity, IGF-I. IGF-I impacts long bone growth, lean body mass, glucose homeostasis, and adiposity, all traits affected in our second-generation offspring (65, 66). First- and second-generation females but not males had significantly elevated plasma levels of IGF-I [Fig. 4C, females $F_{(4,36)} = 4.23$]. To determine whether the biological availability of IGF-I is potentially modulated by altered expression of IGF-I-binding proteins (IGFBPs), we analyzed expression levels of IGFBP-3 in liver (67, 68). Liver-synthesized IGFBP-3 transcript is elevated at postnatal d 1 in first-generation males and females [Fig. 4D, $F_{(1,15)} = 6.679$].

To identify potential gene candidates important in regulating heritable somatic growth upstream of IGF-I concerning GH secretion, we analyzed expression levels of the GHSR (69). Our results demonstrated a similar pattern of hypothalamic GHSR as was found for IGF-I, where first- and second-generation females but not males had higher levels compared with controls (Fig. 4, E and F).

GHSR expression is negatively regulated by the RNA polymerase II transcriptional repressor AF5q31 (also known as

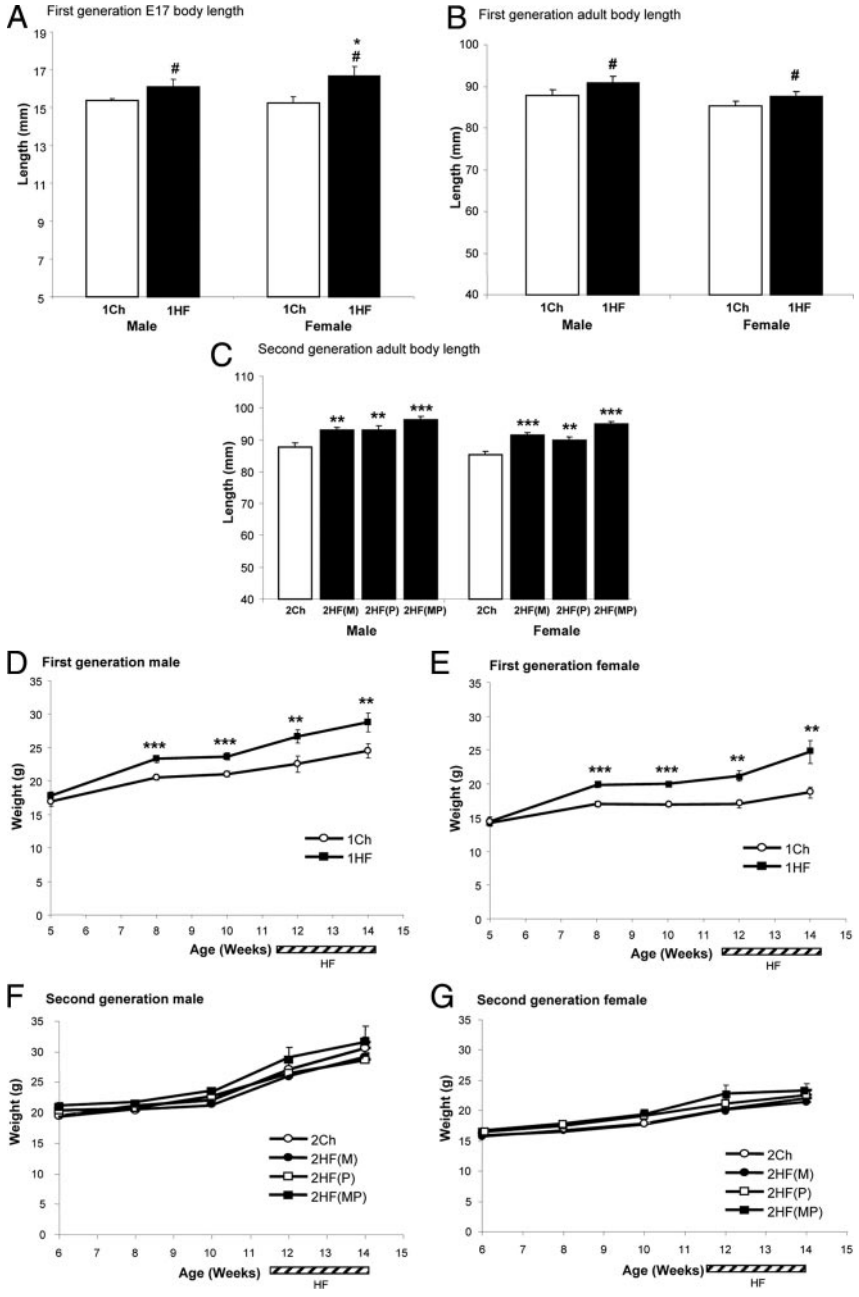

FIG. 2. Exposure to a maternal high-fat diet results in increased body weight and length in first-generation offspring with increased body length transmitted to the second generation through maternal or paternal lineages. A, First-generation E17 embryos exhibited increased body length after exposure to maternal high-fat diet (1HF, 45% fat) relative to those exposed to chow (1Ch, 12% fat) *, $P = 0.05$; #, $P = 0.018$, main effect of diet. B, These body length differences persisted into adulthood in 1HF male and female offspring. #, $P = 0.041$, main effect of diet. C, Body length increases persisted in second-generation offspring from the maternal lineage [2HF(M); **, $P = 0.0016$; ***, $P = 0.0002$], the paternal lineage [2HF(P); **, $P < 0.005$], and in the maternal/paternal lineage [2HF(MP); ***, $P < 0.0001$] compared with second-generation controls (2Ch). 1HF male (D) and female (E) offspring exhibit increased body weights (**, $P < 0.05$; ***, $P < 0.0005$) relative to 1Ch controls. This increase does not persist in 2HF males (F) and females (G). *Hatched bars* indicate time of high-fat diet consumption. Data are mean ± SEM.

MCEF) (70). In our study, first-generation females but not males exhibited decreased AF5q31 expression on postnatal d 1 [Fig. 4G, $F_{(3,3)} = 3.53$].

To identify other possible mediators of sex-specific mechanisms of inheritance, we performed a quantitative PCR-based array of 84 growth factors on first-generation postnatal d 1 whole-brain homogenates. After normalizing to chow controls for each gene to determine fold changes within each sex, we compared 1HF males and females and detected significant differences between the sexes in expression levels of four genes [Fig. 4H, $F_{(3,15)} = 4.108$ (Il7), 9.44 (Il3), 7.47 (Fgf6), and 9.89 (Fgf3)].

Second-generation animals have reduced DNA methylation at the GHSR promoter

We performed bisulfite sequencing analysis to examine whether changes in DNA methylation at the GHSR promoter are associated with enhanced GHSR transcription in second-generation males and females. We detected significantly decreased methylation in maternal and paternal lineages at position +72 relative to the start site of transcription in males and in all three second-generation lineages at position −31 in females [Fig. 5A, males $F_{(3,21)} = 3.03$; Fig. 5B, females $F_{(3,20)} = 4.83$].

Discussion

Global obesity rates have risen dramatically in the past 30 yr. Interestingly, industrialized nations have also witnessed a profound height increase of 3–5 in. in their human populations since the late 19th century (71, 72). Augmentation of these traits is driven in part by enhanced caloric availability, improved quality of life, and advances in health care (73). In addition, the impact of maternal diet and intrauterine conditions on the development of obesity has been a recent prominent focus of inquiry. However, the heritable features of maternal high-fat diet or overnutrition exposure on second-generation offspring obesity, body size, glucose regulation, or disruption of genetic networks have not been well characterized. To address this possibility, we examined whether maternal high-fat diet can lead to a heritable feature of increased body size in first- and second-generation offspring.

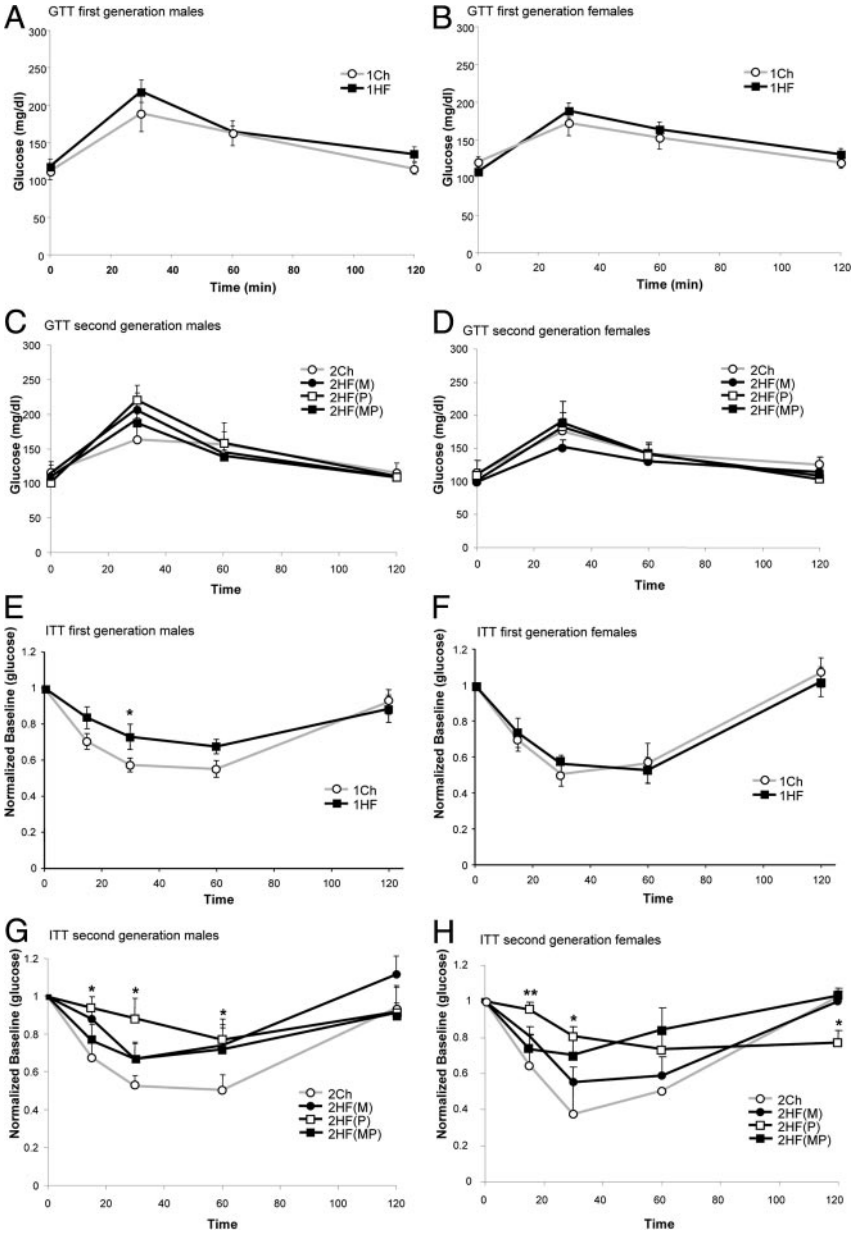

FIG. 3. Reduced insulin sensitivity during house chow diet consumption is inherited by the second generation from maternal or paternal first-generation high-fat offspring. GTTs are not significantly different between chow (1Ch) and high-fat diet (1HF) lines in either first-generation male (A) or female (B) adult offspring. Second-generation male (C) and female (D) offspring from HF maternal [2HF(M)], paternal [2HF(P)], or maternal and paternal lineages [2HF(MP)] have normal glucose tolerance relative to controls (2Ch). ITTs reveal impaired insulin sensitivity in 1HF male (E) but not female (F) offspring. *, $P = 0.041$. Insulin sensitivity is significantly impaired in 2HF(P) males (G) (*, $P < 0.05$) and 2HF(P) female (H) offspring (30 min; *, $P < 0.05$; **, $P < 0.005$). ITT points are normalized to baseline glucose levels at 0 min. Data are mean ± SEM.

Our studies revealed an increase in body length that persisted in both first- and second-generation offspring. Furthermore, this augmented length was conveyed into the second generation by either maternal or paternal lineages. That males are capable of passing on this phenotype provides evidence for an epigenetic mechanism underlying the nutritional determination of body length. Interestingly, length was augmented when both paternal and maternal lineages passed on the trait, suggesting the possibility that a dosage-dependent common pathway or divergent mechanisms contribute to the observed effects. These results provide novel insight into the epigenetic transmission of factors regulating body length using the advantage of independently examining maternal and paternal contributions.

Because previous studies have noted the impact of developmental high-fat diet on offspring body weight and caloric consumption, we analyzed these traits in first- and second-generation offspring. In agreement with previous studies, first-generation offspring were significantly heavier than controls (29, 33, 37, 39, 63). Surprisingly, this phenotype did not extend to the second generation. This result suggests the possibility that separate mechanisms govern the individual components of the overall phenotype because some traits manifest as nonheritable aspects due to acute developmental high-fat diet exposure, whereas others endure across generations. These body weight changes do not correlate with caloric consumption, because neither first- nor second-generation animals were hyperphagic on chow or high-fat diets as adults. First-generation offspring are heavier without being hyperphagic and are thus more calorically efficient than controls, possibly due to impaired glucose metabolism.

Transgenerational links between diet and glucose homeostasis have been reported previously (29, 34, 50, 56, 58, 64). To investigate the heritable aspects of impairments in glucose metabolism or insulin sensitivity, we examined first- and second-generation adult offspring in glucose and insulin tolerance tests during chow or high-fat diet exposure. We detected reduced insulin sensitivity in first-generation male and second-generation male and female offspring on a chow diet in an ITT. This insensitivity was not statistically significant in adult HF offspring during acute high-fat diet exposure as adults, perhaps indicating that baseline insulin sensitivity in controls is altered by acute high-fat diet and thus masks insensitivity effects in HF offspring. Our data indicating equivalent

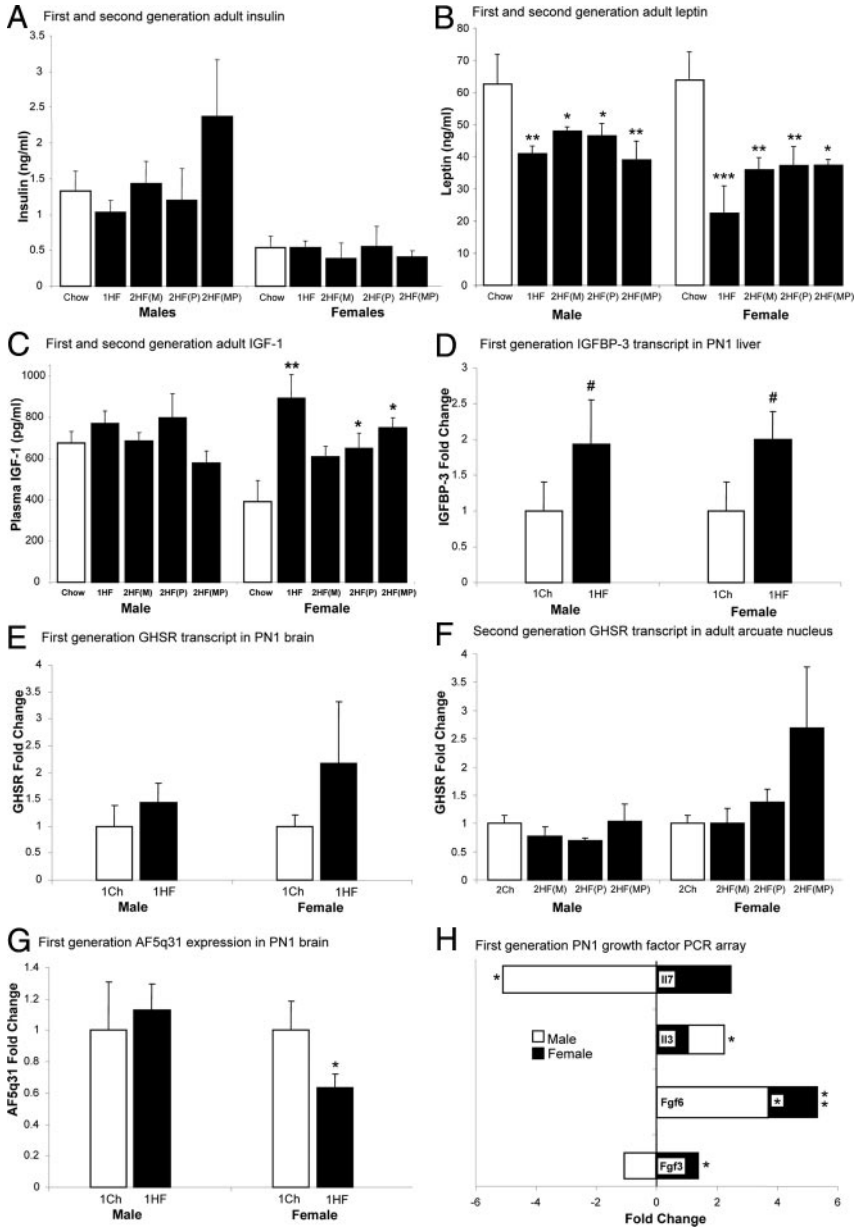

FIG. 4. Sex-dependent programming of the GH pathway through changes in IGF-I, IGFBP-3, GHSR, and growth factors by maternal high-fat diet. A, Plasma insulin is not changed in adult first-generation (1HF, *black bars*) or second-generation [2HF(M, P, and MP), *black bars*] males or females from the high-fat lineage relative to controls [Chow, *white bars*, combined from first-generation (1Ch) and second-generation (2Ch) mice]. B, Adult plasma leptin levels are reduced in 1HF (**, $P = 0.001$; ***, $P = 0.0002$) and 2HF offspring from the maternal [2HF(M); *, $P = 0.019$; **, $P = 0.0035$], paternal [2HF(P); *, $P = 0.017$; **, $P = 0.0025$], and maternal/paternal lines [2HF(MP); *, $P = 0.0065$; **, $P = 0.0006$] relative to controls. C, IGF-I protein levels are significantly increased in 1HF, 2HF(P), and 2HF(MP) females. *, $P < 0.05$; **, $P < 0.005$. D, IGFBP-3 transcript levels are significantly increased at postnatal d 1 (PN1) in 1HF male and female offspring. #, $P = 0.021$, overall effect of diet. E, Trend toward increased GHSR expression in 1HF females in PN1 brain homogenates by quantitative RT-PCR. F, Trend toward increased GHSR expression in 2HF(MP) adult females by quantitative RT-PCR in micropunches from the arcuate nucleus of the hypothalamus. G, Significant reduction in transcript levels of a GHSR transcriptional repressor (AF5q31) in 1HF female PN1 brains. *, $P = 0.05$. H, Significantly altered growth factor expression from a PCR array illustrate sex differences in response to maternal high-fat diet exposure in 1HF male and female PN1 brains. *, $P < 0.05$; **, $P < 0.005$. Data are presented as fold change in 1HF males and females relative to their respective 1Ch controls. All data are mean ± SEM.

insulin levels in adult chow and high-fat offspring consuming acute high-fat diet further supports this hypothesis, because the lack of hyperinsulinemia reflects the normalized insulin sensitivities at this time point. Overall, our observed impairments in insulin sensitivity are in agreement with previous studies that support epigenetic-based impairments in glucose homeostasis (50, 55, 57, 58).

In our examination of first- and second-generation offspring leptin levels, we found that male and female offspring had reduced leptin. Because leptin levels typically positively correlate with total adiposity, these results suggest that high-fat diet exposure during early development may be protective against future deposition of fat across multiple generations in mice. The combination of reduced adiposity and enhanced body length in the second generation may offset one another to yield the normal body weights detected in these animals. These surprising results are contrary to previous studies in rat models of maternal overweight and obesity where increased adiposity was reported in first-generation offspring (33, 37, 74). Differences in adiposity may relate to species-specific metabolic rates and energy utilization or to differences in fat compositions of diets used in the study.

Our results demonstrate a heritable aspect of somatic growth and reduced insulin sensitivity, both measures that support an epigenetic influence of the GH/IGF-I axis. GH is released in a pulsatile fashion from the anterior pituitary, stimulating the synthesis and release of IGF-I from the liver (66). Although increased body length was detected in both male and female offspring, we observed increased IGF-I levels and associated molecular events predominantly in first- and second-generation females and increased liver IGFBP-3 expression in first-generation males and females. Sex differences in IGF-I levels have previously been reported in response to maternal high-fat diet and in disruptions of the GH axis by

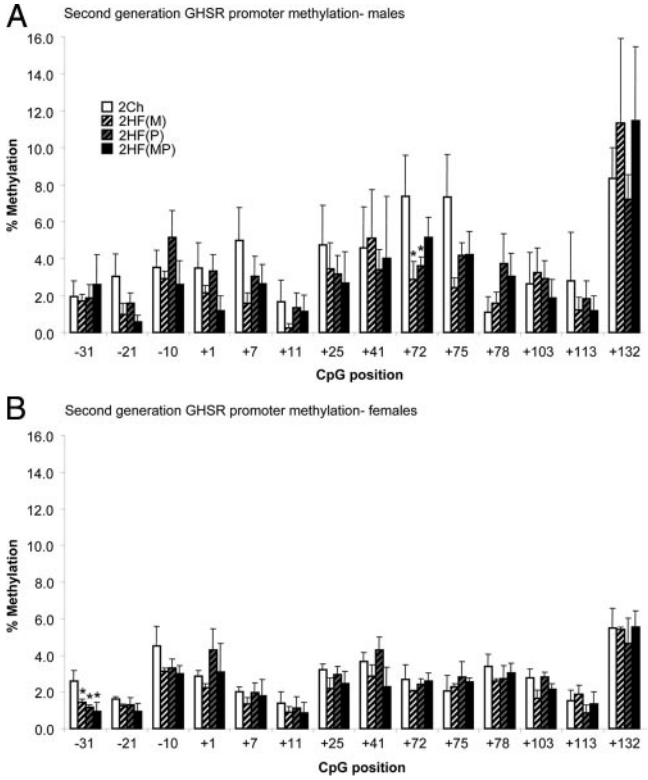

FIG. 5. Second-generation male and female adult offspring exhibit decreased methylation at the GHSR promoter. Percent methylation assayed by bisulfite conversion and pyrosequencing at 14 cytosines of the GHSR promoter-associated CpG island in microdissected arcuate nuclei in second-generation adult male (A) and female (B) offspring is significantly altered between the chow (2Ch) or high-fat groups from the maternal [2HF(M), *black and white hatched bars*], paternal [2HF(P); *gray and black hatched bars*], or maternal/paternal lineages [2HF(MP); *black bars*]. *, $P < 0.05$. Data are mean ± SEM.

deletion of somatostatin (74–76). However, it is interesting to note that studies investigating longitudinal bone growth find an additional 30% reduction of tibia length in GH compared with IGF-I knockout animals, supporting that IGF-I is not the only downstream effector of GH action and that multiple mechanisms contribute to overall growth (77). IGF-I biological activity is regulated through its complexing with IGFBPs in circulation and during development (65). In particular, studies have demonstrated a positive correlation between IGF-I and IGFBP-3 levels in umbilical cord blood and amniotic fluid with large-for-gestational-age births in humans (78, 79). Furthermore, IGFBP-3 can enhance the biological efficacy of IGF-I when complexed, and although our data reflect only mRNA expression levels of IGFBP-3, increased transcription of this gene may result in increased circulating protein (78, 79). Additional studies are necessary to measure circulating concentrations in these mice to confirm differences. Taken together, these results suggest sex-specific mechanisms underlying the body length phenotype, including potential differential effects of sex hormone-mediated input to the GH/IGF-I pathway. Furthermore, our identification of sex-specific expression changes in four growth-related genes at postnatal d 1 in first-generation offspring (Il7, Il3, Fgf3, and Fgf6) in response to maternal high-fat diet exposure provides further evidence that sex differences in the mechanisms of phenotype expression begin at early developmental time points.

In our study, first- and second-generation females appeared to masculinize their IGF-I levels in response to maternal high-fat diet, suggesting that females may be recruiting a mechanism already present in males that contributes to the typically enhanced body length in males relative to females. We propose that changes in the GH axis in females originate with developmental events surrounding the expression of GHSR and an associated transcriptional repressor, AF5q31, in response to an altered nutritional profile (70). We observed a nonsignificant trend for increased GHSR expression in our first- and second-generation adult female offspring that may be responsible for augmented GH release and subsequent IGF-I secretion (80). Because mRNA analysis gives only a partial indication of GHSR functionality, future analysis of GHSR protein distribution, receptor dynamics, and stimulation of GH release is necessary to characterize the role of this receptor in the body length phenotype. GHSR mRNA alterations negatively correlate with significant decreases in expression levels of a GHSR transcriptional repressor, AF5q31. Interestingly, mice hemizygous for AF5q31 have recently been shown to express increased levels of GHSR and display a similar phenotype of increased body length and reduced adiposity to mice in our study (70). Early developmental alterations in AF5q31 expression may provide a fundamental signal that results in increased GHSR expression leading to enhanced GH and IGF-I secretion during critical developmental windows, thus leading to augmented linear growth.

The increased GHSR expression found in second-generation mice suggests that this gene may be stably modulated through differential methylation of its promoter (81). In our methylation analyses, we detected several significant points within the GHSR-associated CpG island that may correlate with the epigenetic regulation of somatic growth. Hypermethylation of the GHSR promoter has been strongly implicated as a marker in breast carcinoma, demonstrating the potential for methylation to regulate this locus (82). Although statistically significant effects were detected in the GHSR promoter, these changes were not at the magnitude as would have been predicted. Because our analyses are from micropunches containing heterogeneous cell populations of the arcuate nucleus, the relevant signal from GHSR-expressing neurons may be overwhelmed by that of the vast numbers of glia and non-GHSR-expressing neurons. Analyzing methylation in a

pure population of GHSR neurons may reveal more profound effects as previously observed in a more homogeneous sample type such as breast carcinoma (82). Transcriptional augmentation may also occur at the level of histone modifications of the GHSR promoter or at the AF5q31 locus, mechanisms that remain to be explored.

To our knowledge, the links between height and grandparental diet have not yet been determined. From the perspective of natural selection, intergenerationally heritable flexibility in body size provides an excellent mechanism for rapidly improved organismal adaptation. In a general sense, appropriately modifying body size in response to changing factors such as food availability and natural or sexual selective forces is advantageous (83). It may be of further benefit to ensure the prolonged competitiveness of a bloodline by the rapid conversion of high calorie availability into a larger body size that can persist through multiple generations. Because grandparental nutrition has been epigenetically linked in humans to metabolic and cardiovascular health in the second generation, the similar epigenetic mechanisms demonstrated by our data may also explain phenomena such as the swift height increases detected in human populations in times of plentiful resources (50, 51, 53, 71, 72).

Acknowledgments

We thank K. Carlin and Y. Xiong for technical assistance.

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This work was supported by PubMed Central-National Institutes of Health Grant DK019525.

Disclosure Summary: The authors declare no conflict of interest.

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