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**ORIGINAL ARTICLE**

# Diet-induced obesity leads to metabolic dysregulation in offspring via endoplasmic reticulum stress in a sex-specific manner

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**BACKGROUND/OBJECTIVES:** Exposure to metabolic stress has been suggested to influence the susceptibility to metabolic disorders in offspring according to epidemiological and animal studies. Nevertheless, molecular mechanisms remain unclear. We investigated impacts of diet-induced paternal obesity on metabolic phenotypes in offspring and its underlying molecular mechanism.

**SUBJECTS/METHODS:** Male founder mice (F0), fed with control diet (CD) or high-fat diet (HFD), were mated with CD-fed females. F1 progenies were mated with outbred mice to generate F2 mice. All offspring were maintained on CD. Metabolic phenotypes, metabolism-related gene expression and endoplasmic reticulum (ER) stress markers were measured in serum or relevant tissues of F2 mice. DNA methylation in sperm and testis of the founder and in the liver of F2 mice was investigated.

**RESULTS:** Male founder obesity, instigated by HFD, led to glucose dysregulation transmitted down to F2. We found that F2 males to HFD founders were overweight and had a high fasting glucose relative to F2 to CD founders. F2 females to HFD founders, in contrast, had a reduced bodyweight relative to F2 to CD founders and exhibited an early onset of impaired glucose homeostasis. The sex-specific difference was associated with distinct transcriptional patterns in metabolism-related organs, showing altered hepatic glycolysis and decreased adipose *Glucose transporter 4 (Glut4)* in males and increased gluconeogenesis and lipid synthesis in females. Furthermore, the changes in females were linked to hepatic ER stress, leading to suppressed insulin signaling and non-obese hyperglycemic phenotypes. DNA methylation analysis revealed that the *Nr1h3* locus was sensitive to HFD at founder germ cells and the alteration was also detected in the liver of F2 female.

**CONCLUSION:** Our findings demonstrate that male founder obesity influences impaired glucose regulation in F2 progeny possibly via ER stress in a sex-specific manner and it is, in part, contributed by altered DNA methylation at the *Nr1h3* locus.

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## INTRODUCTION

In recent decades, diabetes has emerged as a major public health problem. In research efforts identifying obesogenic factors to its etiology, recent studies have reported that people with normal bodyweight also exhibited problems in glucose homeostasis,[1–3] emphasizing the complexity of its etiology. Accumulating evidence suggests that diverse metabolic conditions could be caused by transmission of environmentally acquired traits through non-genetic mechanisms.[4–10] Epidemiological studies including Dutch hunger and Overkalix study have revealed associations between prenatal exposure to famine and the later body mass index, as well as a sex-specific link between grandparents' food supply and third-generation mortality.[4,5] Animal studies have similarly reported that dietary stress *in utero* affects offspring bodyweight and glucose metabolism, which are further transmitted and elevated in subsequent generations.[6–8] Although researchers have heavily reported on maternal influence, surprisingly little has been reported for paternal diet stress on transgenerational metabolic disturbances. A few reports have suggested the possibility that paternal HFD also contributed to an early onset of hyperglycemic phenotypes in rodents,[9,10] although the underlying mechanism remains elusive. We therefore hypothesized that dietary-induced founder obesity in male mice may have an impact on congenital predisposition to diabetes in following generations. In the current study, we investigated the molecular features of metabolism-related organs to explain the susceptibility to diabetes in F2 offspring. We showed that obesity in male founder produced a sex-specific response that is centered around a high fasting glucose and altered hepatic glycolysis and adipose glucose transport in F2 male mice. In contrast, F2 female mice to the same dietary regiment had affected gluconeogenesis, with suppression of insulin signaling and non-obese hyperglycemic phenotypes via endoplasmic reticulum (ER) stress. Furthermore, we demonstrated the possibility that the transgenerational effect might have been mediated by altered DNA methylation such as the *Nr1h3* locus.

## MATERIALS AND METHODS

### Animals

Animal experiments were approved by the Institutional Animal Care and Use Committee of Ewha Womans University (2013-14-046). All animals were housed under specific pathogen-free conditions and 12:12-h light:

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dark cycle. Four-week-old C57/BL6 founder (F0) male mice were obtained from the Ewha Laboratory Animal Genomic Center and were fed with either a control diet (CD) or a HFD (Supplementary Table 1, Doo Yeol Biotech, Seoul, South Korea) for 20 weeks. F1 progenies were obtained by mating F0 males with 6-week-old control females and F2 progenies were obtained by mating 9-week-old F1 progenies with 6-week-old control mice. All mice were fed with *ad libitum* access to water and food and the mice, except F0 HFD, were maintained with CD. Body weights and food intakes were monitored every week. F0 males and F2 progenies were killed at 25 and 27 week of age, respectively.

### Intraperitoneal glucose tolerance test

In the intraperitoneal glucose tolerance tests, 2 g $kg^{-1}$ of 25% glucose solution (Sigma, St Louis, MO, USA) were injected into the intraperitoneal spaces after 12 h fasting. Tail blood glucose concentrations were measured at fasting (0), 15, 30, 60 and 120 min using Accu-Chek Performa (Roche, Indianapolis, IN, USA).

### Analysis of triglyceride and insulin levels

Hepatic lipids were extracted by the Bligh and Dyer methods.[11] Briefly, frozen liver tissues were homogenized (IKA, Staufen, Germany) and lipid layers were extracted with chloroform: methanol (2:1, v-v). The lipid was dried and resuspended in isopropyl alcohol and its triglyceride (TG) level was measured by commercial kits (Asan Pharm Co, Seoul, Korea). In serum, the levels of TG and insulin were measured by commercial kits (Asan Pharm Co and Crystal Chem, Chicago, IL, USA, respectively). All parameters were measured in duplicate for each sample.

### RNA isolation and quantitative real-time PCR

Total RNAs were extracted after tissue homogenizations (IKA) by RNAzol B reagent (Tel-test, Friendswood, TX, USA), followed by DNase I treatment (Thermo Scientific, Waltham, MA, USA). Total RNA (500 ng) was transcribed into complementary DNAs using oligo dT and RevertAid reverse transcriptase (Thermo Scientific). Quantitative PCRs were performed using SYBR green mix on Rotor-Gene Q (Qiagen, Hilden, Germany) and primers (Supplementary Table 2).

### DNA isolation and DNA methylation analysis

Genomic DNA was isolated using DNeasy blood and tissue kit (Qiagen) according to the manufacturers' instructions. DNA methylation was quantitatively measured using the matrix assisted laser desorption/ionization-time of flight mass spectrometry assay. Briefly, 200 ng sperm genomic DNA and 500 ng liver and testis DNA were bisulfite-treated using the Zymo EZ DNA methylation kit (Zymo Research, Irvine, CA, USA) and amplified using taq polymerase (Qiagen) and primer including T7-promoter tag to amplify bisulfite-treated template in a specific locus of the genome. PCR products were *in vitro* transcribed by T7 polymerase after Shrimp Alkaline Phosphatase (Sequenom, San Diego, CA, USA) treatment and T-specific RNAaseA cleavage (Sequenom). The cleaved fragments were quantified using MassArray EpiTYPER (Sequenom).

### Western blots

Frozen liver tissues were homogenized (IKA) with RIPA lysis buffer (20 mM HEPES (pH 7.0), 150 mM NaCl, 10% glycerol, 1% Triton X-100, 1 mM EDTA, 1 mM EGTA, 10 mM β-Glycerophosphate, 1 mM $Na_3VO_4$, 5 mM NaF). Hepatic lysates containing 100 μg proteins were separated on 6% SDS-PAGE. Proteins were transferred to polyvinylidene fluoride membranes and blotted with anti-pIRS1 (Ser307; Cell Signaling, Beverly, MA, USA), anti-IRS1 (Cell Signaling) and anti-α-tubulin (Sigma). Band intensities were quantified with the Image J software (NIH, Bethesda, MD, USA).

### Statistical analysis

Results are presented as the means ± s.e.'s. Statistical analysis was performed using two-tailed Student's *t*-tests (Microsoft excel 2013). *P*-value was considered significant at $< 0.05$ and highly significant at $< 0.01$.

## RESULTS

### Altered metabolic phenotypes in F2 offspring from obese male founder

To investigate whether paternal metabolic stress is transmitted to next generations, we developed obese male founders using HFD and observed various metabolic phenotypes in F2 offspring, especially born to F1 females. The diet-induced obese founders showed higher body weights, impairment of glucose homeostasis during intraperitoneal glucose tolerance test and increased hepatic TG content, as expected (Figures 1a–c). The obese male founders led to sex-specific patterns of bodyweight and glucose regulation in their F2 progeny. F2 male mice born to F1 females exhibited obese hyperglycemic phenotypes relative to F2 male of CD-fed founders. More specifically, they were overweight from 10 weeks of ages but the difference became marginal from 21 weeks of age and was faded away (Figure 2a). In line with the bodyweight change, intraperitoneal glucose tolerance test-based glucose homeostasis at 9 weeks of age was not different regardless of diets to the founders, whereas fasting glucose levels at 20 weeks of age were significantly higher in the male progeny of the obese founder compared with control, without glucose intolerance (Figures 2b and c). Serum insulin and hepatic TG content did not differ in F2 male progeny regardless of diets to the founders (Figures 2g and h).

F2 female offspring of obese founders showed significantly lower bodyweight than F2 female of CD-fed founders at the early period (from the weaning to 11 weeks of age) and a marginal underweight pattern was also observed around 20 and 21 weeks of age (Figure 2d). Impaired glucose homeostasis carrying high fasting glucose as well as high glucose intolerance was observed in F2 female offspring of obese founders at the early period (Figure 2e). The impairment of glucose homeostasis was

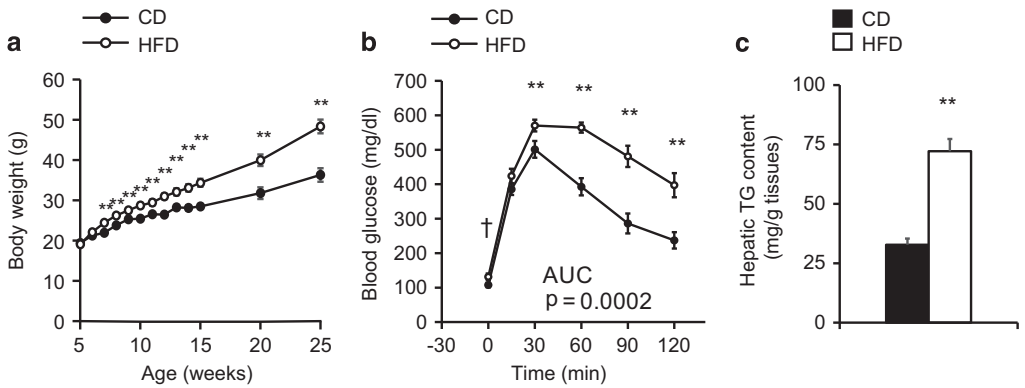


**Figure 1.** Metabolic phenotypes of HFD-induced obese founders. C57BL/6 male mice were fed with CD or HFD from 5 weeks of age. Body weights (**a**) and glucose levels upon intraperitoneal glucose tolerance tests (**b**) at 17 weeks of age, hepatic TG contents (**c**) at 25 week of age were presented. Blood glucose and hepatic TG levels were replicated twice per sample. (Black and white bars/circles indicate F0 CD founder and F0 HFD founder, respectively; $n = 6–7$ per group). Data presented as mean ± s.e. Student's *t*-test indications, $^{**}P < 0.01$, $^{\dagger}P < 0.1$.

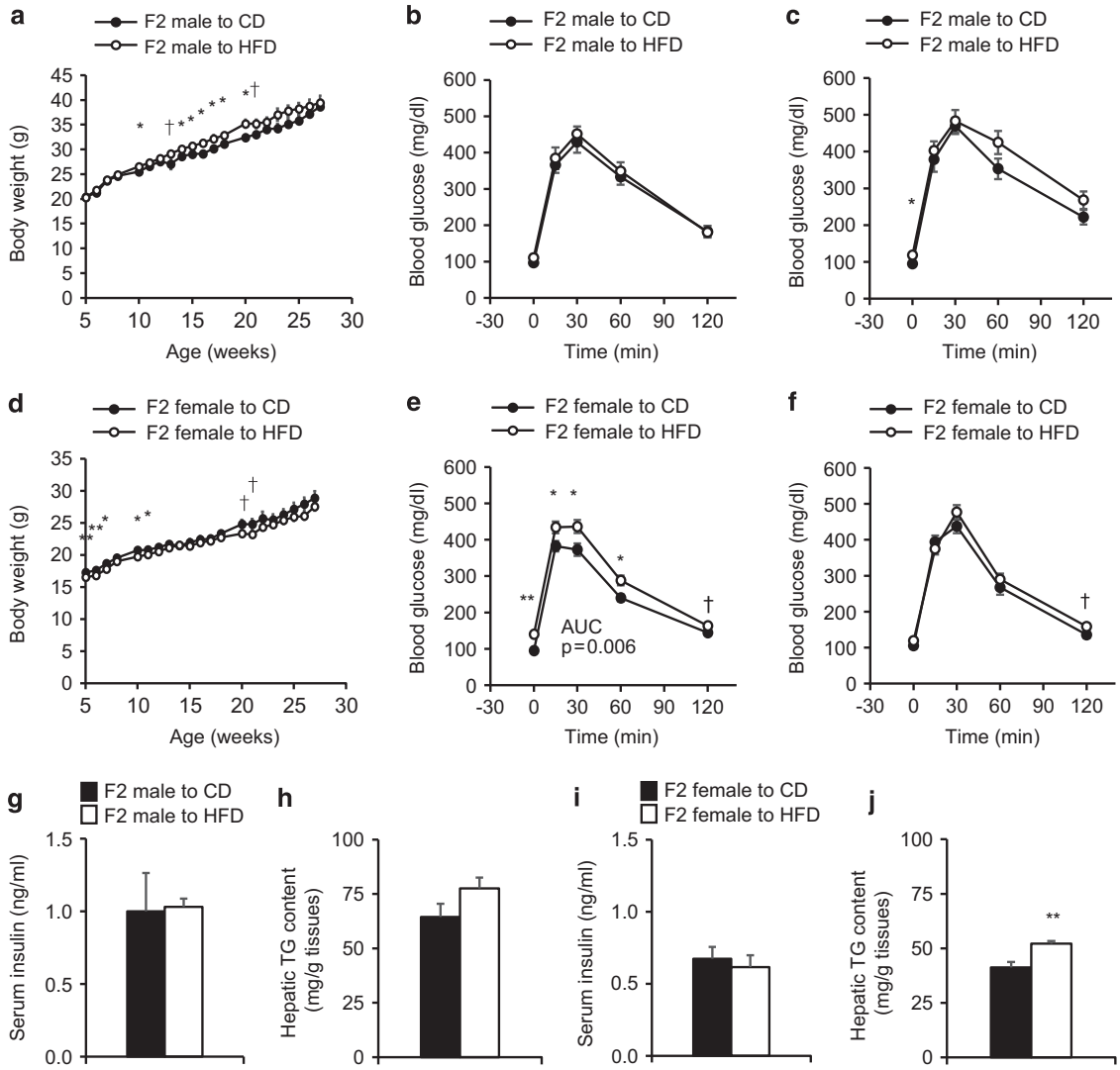


**Figure 2.** Metabolic phenotypes of F2 offspring born to F1 females, depending on diets to founders. (**a–c**) Body weights (**a**) and glucose levels after intraperitoneal glucose tolerance test (IPGTT) treatment at 9 weeks of age (**b**) and 20 weeks of age (**c**) were measured in F2 male. (**d–f**) Body weights (**d**) and glucose levels after IPGTT treatment at 9 weeks of age (**e**) and 20 weeks of age (**f**) were measured in F2 female. (**g–j**) Serum insulin and hepatic TG content were measured after being killed at 27 weeks of age of F2 male (**g**, **h**) and in F2 female (**i**, **j**). Blood glucose, serum insulin and hepatic TG levels were replicated twice per sample. (Black and white bars/circles indicate F2 offspring of F0 CD founder and those of F0 HFD founder, respectively; $n = 5–12$ per group). Data presented as mean ± s.e. Student's *t*-test indications $^{**}P < 0.01$, $^{*}P < 0.05$, $^{\dagger}P < 0.1$.

recovered at 20 weeks, but glucose levels at 120 min after the injection remained slightly high (Figure 2f). Glucose dysregulation in F2 female offspring of obese founders was accompanied by increased hepatic TG content, but no alteration in serum insulin level (Figures 2i and j). In addition, similar phenotypes were observed in F2 male and female offspring born to F1 males but to a lesser extent (Supplementary Figure S1). These results indicate that obese male founders transmitted metabolic stress as obese phenotypes in F2 males and lean hyperglycemic phenotypes in F2 females with increased hepatic TG content.

### Transcriptional changes of metabolism-related genes in the liver and adipose tissues in F2 progeny

To assess the underlying mechanisms for altered glucose homeostasis and lipid metabolism, we investigated relative mRNA levels of metabolism-related genes in the liver and adipose tissues. The liver of F2 males from obese founders had upregulated expression of the *Glucokinase* (*Gck*) and the *Carnitine palmitoyltransferase 1* (*Cpt1a*) genes (Figure 3a) compared with F2 males from CD-fed founders. In contrast, F2 females from obese founders showed upregulated gene expression of a gluconeogenic gene, *phosphoenol-pyruvate carboxylase 1* (*Pck1*), the *lipid synthesis-related the liver X receptor-alpha (Lxra, encoded by the gene Nr1h3),* the *sterol regulatory element binding transcription factor 1* (*Srebf1*) and the *ATP citrate lyase* (*Acl*; Figure 3b). The *peroxisome proliferator-activated receptor-alpha* (*Ppara*) gene, which is a major regulator of fatty-acid catabolism, was also upregulated in F2 females (Figure 3b). In the inguinal adipose tissues, founder obesity led to downregulated expression of the *glucose transporter 4* (*Glut4)* gene in F2 males (Figure 3c) and upregulated expression of the *hormone sensitive lipase* (*Hsl*) gene in F2 females (Figure 3d). Together, these results indicate that F2 males from the obese founder upregulated glycolysis and fatty-acid oxidation-related genes in the liver and downregulated glucose uptake-related genes in adipose tissue. In contrast, F2 females from founder obese males facilitated gluconeogenesis and lipid synthesis in the liver and lipolysis in adipose tissue.

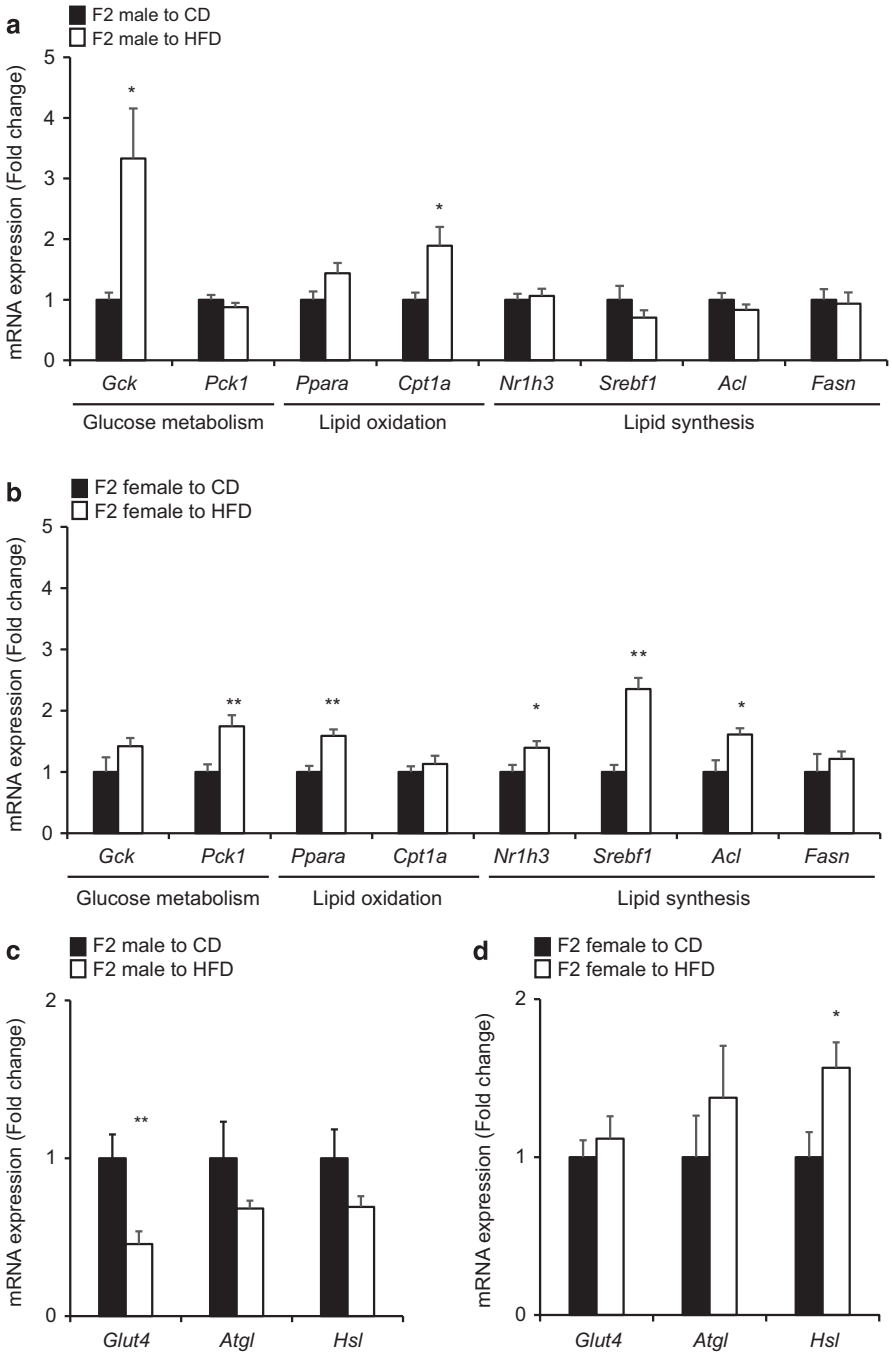


**Figure 3.** Differential gene expression of metabolism-related genes in the liver and inguinal white adipose tissues in F2 offspring born to F1 females depending on diets to founders. mRNA expression measured by qPCR was normalized against the average of *Actinb* and *Gapdh*. (**a**, **b**) Genes related with glucose and lipid metabolism were measured in the liver of F2 males (**a**) and F2 females (**b**). (**c**, **d**) Genes related with lipid metabolism were measured in adipose tissues of F2 male (**c**) and F2 female (**d**). (Black and white bars indicate F2 offspring of F0 CD founder and those of F0 HFD founder, respectively; $n = 6–8$ per group). Data presented as mean ± s.e. Student's *t*-test indications $^{**}P < 0.01$, $^{*}P < 0.05$.

Altered hepatic ER stress and insulin signaling in F2 offspring from obese founder

Next, molecular markers for hepatic ER stress were analyzed to investigate a possible mechanism connecting aberrant hepatic lipid metabolism and glucose dysregulation. We analyzed gene expression relevant to ER stress in both male and female F2 progenies, including the *heat shock protein 5* (*Hspa5*) encoding GRP78 and the *DNA-damage inducible transcript 3 (Ddit3)* encoding CHOP. Expression levels of *Hspa5* and *Ddit3* genes were upregulated in F2 males and females, respectively (Figures 4a and b). In addition, serine-307 phosphorylation of insulin receptor substrate 1 (IRS1), a downstream target of ER stress, was increased in F2 females, but not in F2 males (Figures 4c and d). These data suggest the possibility that lean diabetes observed in female progeny may be related with metabolic overload-induced ER stress and the resulting glucose dysregulation.

DNA methylation in F0 sperm and testis and F2 female liver at the *Nr1h3* locus

Next, we investigated a possible mechanism by which the effects of founder obesity are transmitted to the F2 generation. We

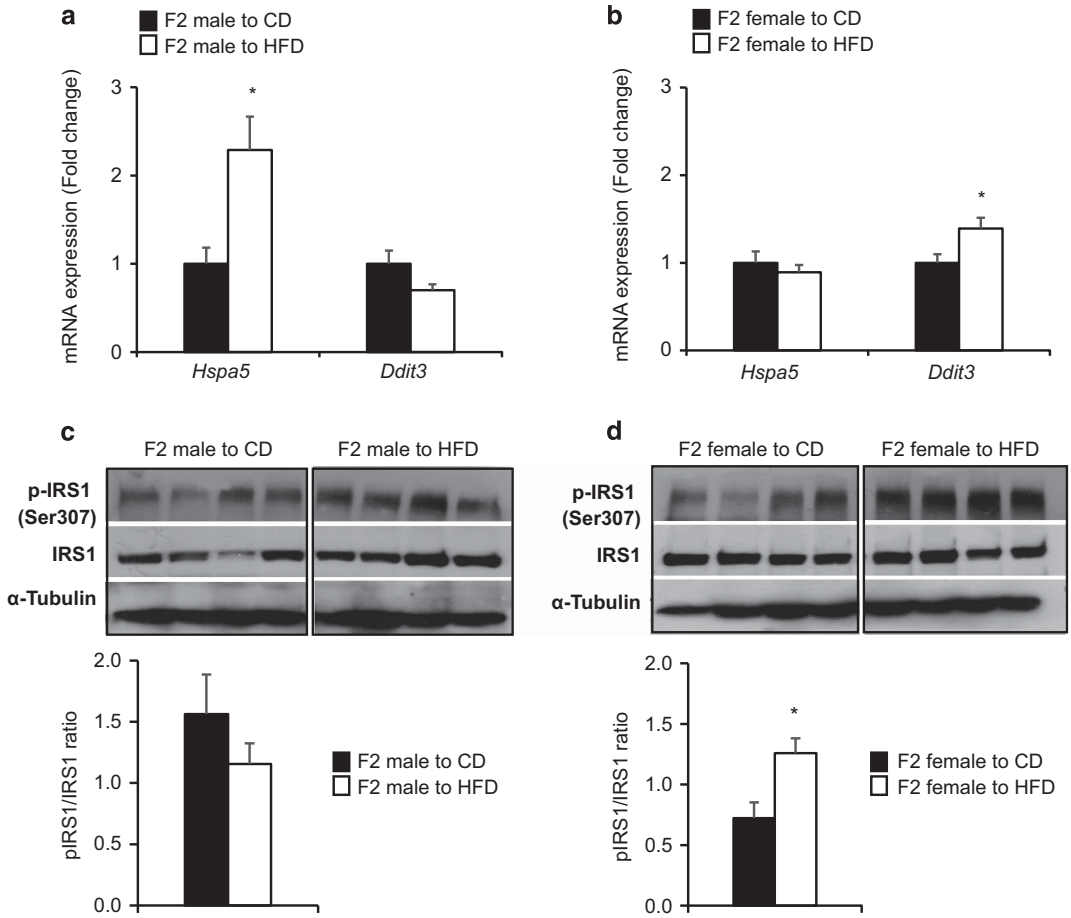


**Figure 4.** Hepatic ER stress and insulin signaling in F2 offspring born to F1 females depending on diets to founders. (**a**, **b**) *Hspa5* and *Ddit3* mRNA expression in livers of F2 male (**a**) and F2 female (**b**) was measured by qPCR and was normalized against the average of *Actinb and Gapdh*. (**c**, **d**) Hepatic IRS-1 and its phosphorylation levels at serine-307 (pIRS1) were examined by immunoblotting. α-tubulin was used as loading control in F2 males (**c**) and in F2 females (**d**). Lower panels show band intensity ratios of IRS1 phosphorylation at serine-307 compared with total IRS1. (Black and white bars indicate F2 offspring of F0 CD founder and those of F0 HFD founder, respectively; $n = 4–7$ per group). Data presented as mean ± s.e. Student's *t*-test indications $^*P < 0.05$.

hypothesized that epigenetic changes by HFD-induced metabolic stress in the founder gamete might be inherited into next generations. We focused DNA methylation changes in metabolism-related loci. Among the hepatic genes whose expression levels we showed in Figure 3, only the *Nr1h3* gene showed alterations in DNA methylation in F0 sperms and testis as well as in F2 female livers (Figures 5a–c), but the others did not show any difference depending on ancestral diets (data not shown). Hypermethylation at CpG6/7 and hypomethylation at CpG15/16 were detected in sperms and hypermethylation at CpG1, CpG2 and CpG13/14 was detected in testis of obese founder (Figures 5a and b, Supplementary Figure S2). In the liver of F2 female, CpG17/18 was slightly hypermethylated (Figure 5c). Although we could not find significant changes at the *Nr1h3* locus, consistent both in F0 sperms or testis and F2 female liver, several CpG units were sensitive to DNA methylation changes relatively to other metabolism-related loci.

In addition, we measured DNA methylation levels in repetitive elements and differentially methylated regions at the locus of *H19* and insulin-like growth factor 2 (*Igf2)* to find global methylation and imprinting gene methylation, respectively. Ancestral HFD-induced obesity did not affect global changes in DNA methylation in sperm and testis (Supplementary Figures S3A, B). In the *H19* and *Igf2* locus, hypermethylated CpG sites were detected in testis DNA of obese founder, but not in hepatic DNA of F2 female (Supplementary Figures S3C–F).

## DISCUSSION

Here, we demonstrate that obesity of male founder induces hyperglycemic phenotypes in the third generation, even though all progenies consumed CD. Our data suggest that sex-specific phenotypes in the offspring are related to different programming of the molecular parameters, mainly in hepatic energy metabolism and ER stress. We focused on a paternal dietary stress model to exclude *in utero* effects, in which the transmitted gamete information may be a sole contributor of altered phenotypes of the following generations. In our model, transmitted effects were robust in F2 animals and more obvious in progeny born to F1 females than progenies born to F1 males, although the trends were similar (Figure 1 and Supplementary Figure 1). Although all F2 progenies born to F1 females of obese founders showed a range of glucose dysregulation relative to control, there was a sex discrepancy in the onset of hyperglycemic phenotypes and bodyweight patterns. Distinct impairment of glucose tolerance with underweight was observed in the early age of F2 females, whereas F2 males exhibited a high fasting glucose with overweight in the later ages. In close agreement with a previous paternal transgenerational model, our study also showed early onset of diabetes with lean bodyweight in female progenies.[9] Impaired glucose regulation was diminished at later age in founder obese F2 females, which may be the result of the relatively low metabolic challenge by CD. There is a possibility that the susceptible groups might maintain the aberrant glucose

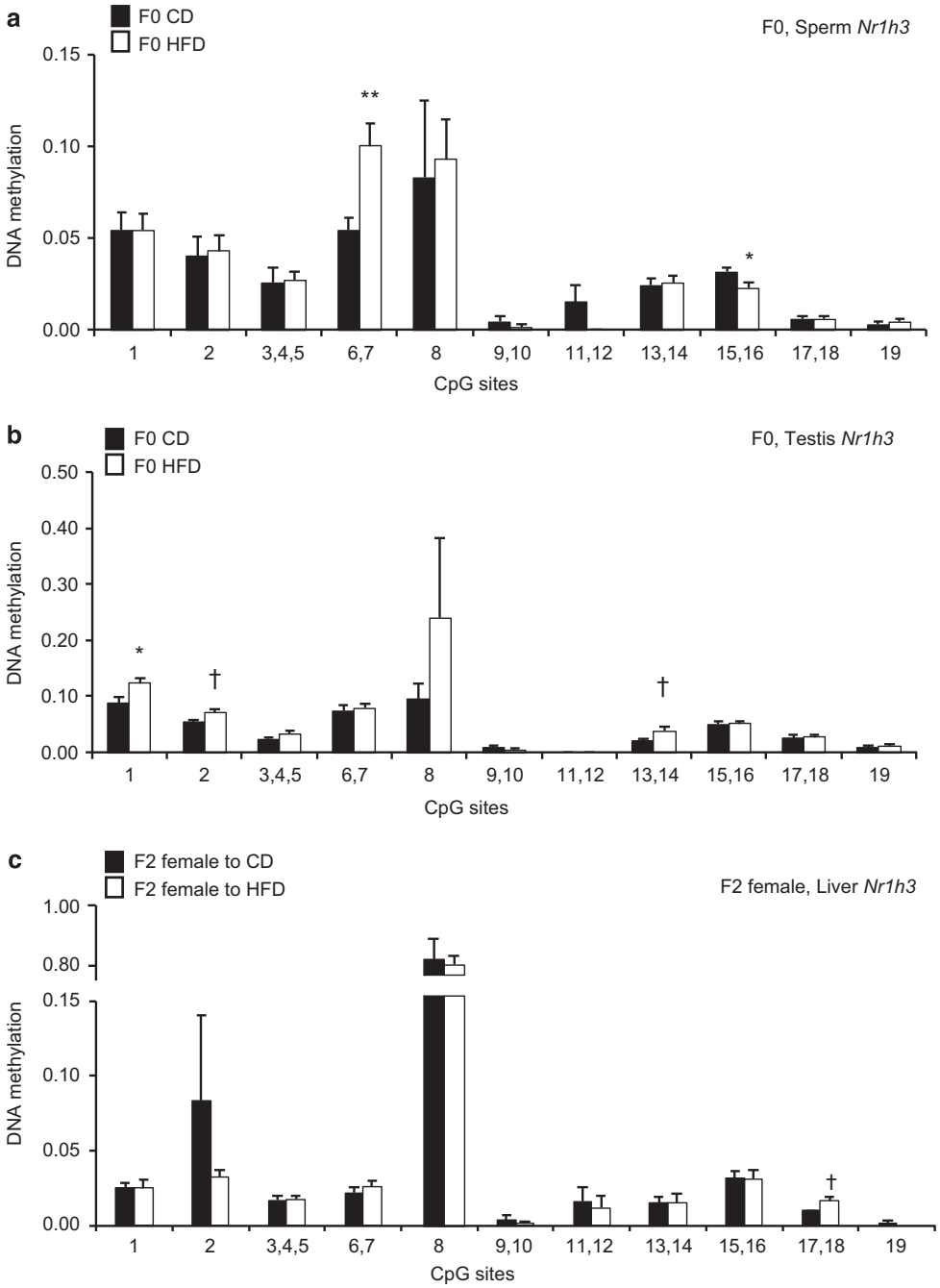


**Figure 5.** DNA methylation at the Nr1h3 locus in sperm and testis of founders and in the liver of F2 female. DNA methylation levels at the *Nr1h3* locus were quantitatively measured using MassArray in sperm (**a**) and testis (**b**) of founder and in the liver of F2 female (**c**). (Black and white bars indicate F2 offspring of F0 CD founder and those of F0 HFD founder, respectively; $n = 6–8$ per group). Data presented as mean ± s.e. Student's *t*-test indications $^{**}P < 0.01$ $^{*}P < 0.05$, $^{\dagger}P < 0.1$.

regulation and exhibit more obvious metabolic problems if the progeny was exposed to metabolic stress.[12,13]

Previous publications have reported inconsistencies regarding bodyweight patterns, which displayed hyperglycemic offspring without significant change in F2 bodyweight in their transgenerational models.[9,10,13] In our study, we showed significantly underweight with hyperglycemic phenotypes in the female offspring. In agreement, in rat model, when F1 and F2 female progenies were fed with high-fat diet, obese founder led to impaired glucose regulation with decreased bodyweight compared with progenies of normal founder.[13] Collectively, the results indicate that female progeny is more prone to lean diabetes-like phenotypes in a paternally induced transgenerational model.

Our molecular evidence supports the sex-specific hyperglycemic phenotypes of F2 progeny of obese founders. Upregulated hepatic *Gck* and downregulated adipose *Glut4* in F2 males indicate typical hyperglycemic phenotypes accompanying obesity, because the former is associated with obesity via the reduction of energy expenditure[14] and the latter is associated with the insulin resistance and ectopic fat metabolism.[15] By contrast, upregulation of the *Hsl* gene in F2 female reflects the increased lipolysis of adipose tissues, which might have an impact on underweight phenotypes. The F2 females also showed

upregulation of the *Pck1* gene and lipogenic genes, possibly causing gluconeogenesis and lipid overload in the liver and, moreover, leading to lipid-induced insulin resistance.[16] The upregulation of lipogenic genes was linked to a significantly increased hepatic TG level and a marginally increased serum TG levels ($P = 0.08$, data not shown). We further investigated hepatic ER stress status as a possible mechanism connecting lipid overload and glucose dysregulation because ER stress has emerged as a possible mechanism for diabetes and other dysregulation of metabolic homeostasis.[17] During HFD metabolic overload, ER develops an adaptive pathway in which hepatic ER stress-related genes such as *Hspa5* and *Ddit3* become upregulated.[17,18] Although it is not clear why the expression of two markers was differently responsive in males and females, there was a previous report demonstrating that *Hspa5* expression differently responded in obese mice versus streptozotocin-induced diabetic mice.[17] In consistent with the results, we observed that expression of the *Hspa5* gene was upregulated in F2 males, but not in F2 females, whose phenotype was lean diabetes. F2 females showed rather upregulated of the *Ddit3* gene. ER stress has been reported to mediate JNK-dependent IRS1 phosphorylation.[19] IRS1 is a substrate of insulin receptor kinase that functions as an essential mediator of hepatic insulin signaling by suppression of gluconeogenesis.[20] Intriguingly, our observation on increased serine-307 phosphorylation of IRS1 in F2 females of obese founders indeed links between ER stress and aberrantly stimulated gluconeogenesis such as upregulated expression of the *Pck1* gene. JNK overexpression, which leads to IRS1 hyperphosphorylation, is known to decrease insulin sensitivity[21] and to enhance gluconeogenesis in the liver.[22] Our data suggest that F2 females of obese founder carry an aberrant hepatic lipid overload, which causes ER stress and a stress-induced JNK kinase response, subsequently promoting gluconeogenesis.

It is not fully understood how ancestral metabolic stress is transmitted to the next generations. Several publications have suggested that metabolic stress-induced epigenetic alterations could be transmissible to the offspring.[23–27] We demonstrated that DNA methylation at the *Nr1h3* gene in testis and sperms was prone to change in response to HFD-induced metabolic stress. DNA methylation at the locus was also hypermethylated and transcriptionally upregulated in the liver of F2 females from HFD-fed founder, compared with F2 females from CD-fed founder. A previous study reported that DNA methylation of the *Nr1h3* locus in the fetus is sensitive to *in utero* undernutrition, leading to hypomethylation, and the altered epigenetic marks in the gamete of fetus can be transmitted to further generations, resulting in altered hepatic expression of the *Nr1h3* gene.[23] In consistence with the result with the hypomethylation associated with transcriptional repression in the liver, we observed that hypermethylation at the locus was associated with transcriptional activation in F2 females. Although the range of the alteration was lesser compared with that of the previous study, it might be functionally connected with metabolic changes in F2 females, as *Nr1h* has been suggested to contribute to regulation of hepatic ER stress through phospholipid metabolism.[28] In addition to *Nr1h3*, we found that *H19* and *Igf2* differentially methylated regions were hypermethylated in testis DNA of the obese founder, although hepatic methylation of F2 females was not changed. Further studies are required to investigate which genomic loci are associated with such germline transmissions.

Taken together, we demonstrated that dietary-induced obesity in male founder predisposed hyperglycemic phenotypes in F2 progeny in a sex-specific manner, owing to metabolic reprogramming of nutrient homeostasis. Our findings indicate that transgenerational inheritance of metabolic stress contributes to the diverse hyperglycemic phenotypes as well as metabolic diseases possibly through ER stress, emphasizing the significance of non-genetic predisposition by environmental factors.

## CONFLICT OF INTEREST

The authors declare no conflict of interest.

## ACKNOWLEDGEMENTS

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## AUTHOR CONTRIBUTIONS

YJP conceived the study, and YJP and AML designed the experiments; JHP and YY performed all animal experiments; JHP, YY, MC, JL and AML did the biochemical and molecular analyses; JHP and YJP interpreted the data; JHP, YJP and AML discussed and wrote the paper.

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