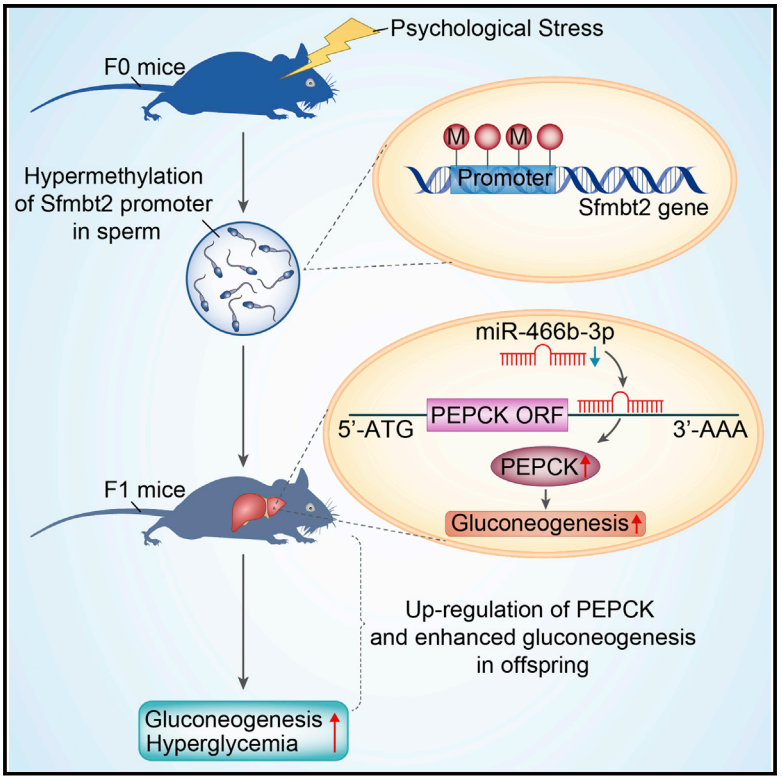
Short Article

Cell Metabolism

Paternal Psychological Stress Reprograms Hepatic Gluconeogenesis in Offspring

Graphical Abstract



Highlights

- Paternal psychological stress promotes hepatic gluconeogenesis in offspring
- Reduced expression of miR-466b-3p increases PEPCK protein contents in stress-F1 mice
- Promoter hypermethylation of the *Sfmbt2* gene leads to downregulation of miR-466b-3p
- Paternal elevated glucocorticoids contribute to hyperglycemia in offspring

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In Brief

Using a mouse model of restraint stress, Wu et al. uncover the intergenerational effects of paternal psychological stress on glucose metabolism in offspring. Paternal stress epigenetically downregulates miR-466b-3p expression, leading to increased PEPCK expression and hepatic gluconeogenesis in hyperglycemic F1 mice.

Accession Numbers

GSE73530

Wu et al., 2016, Cell Metabolism *23*, 735–743
April 12, 2016
http://dx.doi.org/10.1016/j.cmet.2016.01.014

CellPress

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Paternal Psychological Stress Reprograms Hepatic Gluconeogenesis in Offspring

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http://dx.doi.org/10.1016/j.cmet.2016.01.014

SUMMARY

Both epidemiologic and experimental animal studies demonstrate that chronic psychological stress exerts adverse effects on the initiation and/or progression of many diseases. However, intergenerational effects of this environmental information remains poorly understood. Here, using a C57BL/6 mouse model of restraint stress, we show that offspring of stressed fathers exhibit hyperglycemia due to enhanced hepatic gluconeogenesis and elevated expression of PEPCK. Mechanistically, we identify an epigenetic alteration at the promoter region of the *Sfmbt2* gene, a maternally imprinted polycomb gene, leading to a downregulation of intronic microRNA-466b-3p, which post-transcriptionally inhibits PEPCK expression. Importantly, hyperglycemia in F1 mice is reversed by RU486 treatment in fathers, and dexamethasone administration in F0 mice phenocopies the roles of restraint stress. Thus, we provide evidence showing the effects of paternal psychological stress on the regulation of glucose metabolism in offspring, which may have profound implications for our understanding of health and disease risk inherited from fathers.

INTRODUCTION

Type 2 diabetes has become a major public health concern worldwide. Although gene-environment interactions contribute to hyperglycemia, increasing evidence indicates that maternal and paternal inductions of intergenerational responses are also important (Somer and Thummel, 2014). Specifically, the effects of father-to-offspring transmission attract recent attention. It has been noted that changes in paternal diets, including food deprivation (Anderson et al., 2006), and the modulation of fat (Ng et al., 2010), protein (Carone et al., 2010), and sugar (Öst et al., 2014) are implicated in the metabolic programming of the resulting offspring. Mechanistically, altered DNA methylation and histone modifications, as well as changed expression of small non-coding RNAs, have been reported to mediate this inter/transgenerational effect (Carone et al., 2010; Radford et al., 2014; Martínez et al., 2014; Gapp et al., 2014a). However, it remains poorly understood whether epigenetic remodeling induced by paternal exposure to other types of environmental information can be inherited and define offspring metabolic states.

Psychological stress is common in our modern society (Oken et al., 2015). Epidemiologic and/or experimental animal studies demonstrate that prolonged stress increases risk for many health problems, including neuropsychiatric disorders (Lupien et al., 2009), tumorigenesis (Thaker et al., 2006), and type 2 diabetes (Chandola et al., 2006). Interestingly, it has been reported that maternal stress, such as stress experienced during pregnancy (Entringer et al., 2012), could alter body weight and glucose metabolism in offspring. However, it is unclear whether paternal stress could affect glucose metabolism in their offspring.

In the present study, using a mouse model of restraint stress (Bali and Jaggi, 2015), we address how paternal stress exerts an intergenerational effect in mammals, and we further explore the underlying mechanisms. We show that the offspring of stressed fathers have elevated blood glucose concentrations and hepatic gluconeogenesis with increased protein expression of phosphoenolpyruvate carboxykinase (PEPCK). Moreover, paternal stress modifies an epigenetic signature and downregulates expression of a specific microRNA (miRNA) that targets the 3′ UTR of PEPCK.

RESULTS

Metabolic Phenotypes in Offspring of Stressed Fathers

Eight-week-old C57BL/6 male mice were subjected to 14 days of daily restraint stress (stress-F0), as previously described (Uchida et al., 2012). These male mice were then mated with females for 2 days (Figure S1A). In agreement with previous observations (Depke et al., 2008; Uchida et al., 2012), stress-F0 mice exhibited a reduced body weight gain and increased blood

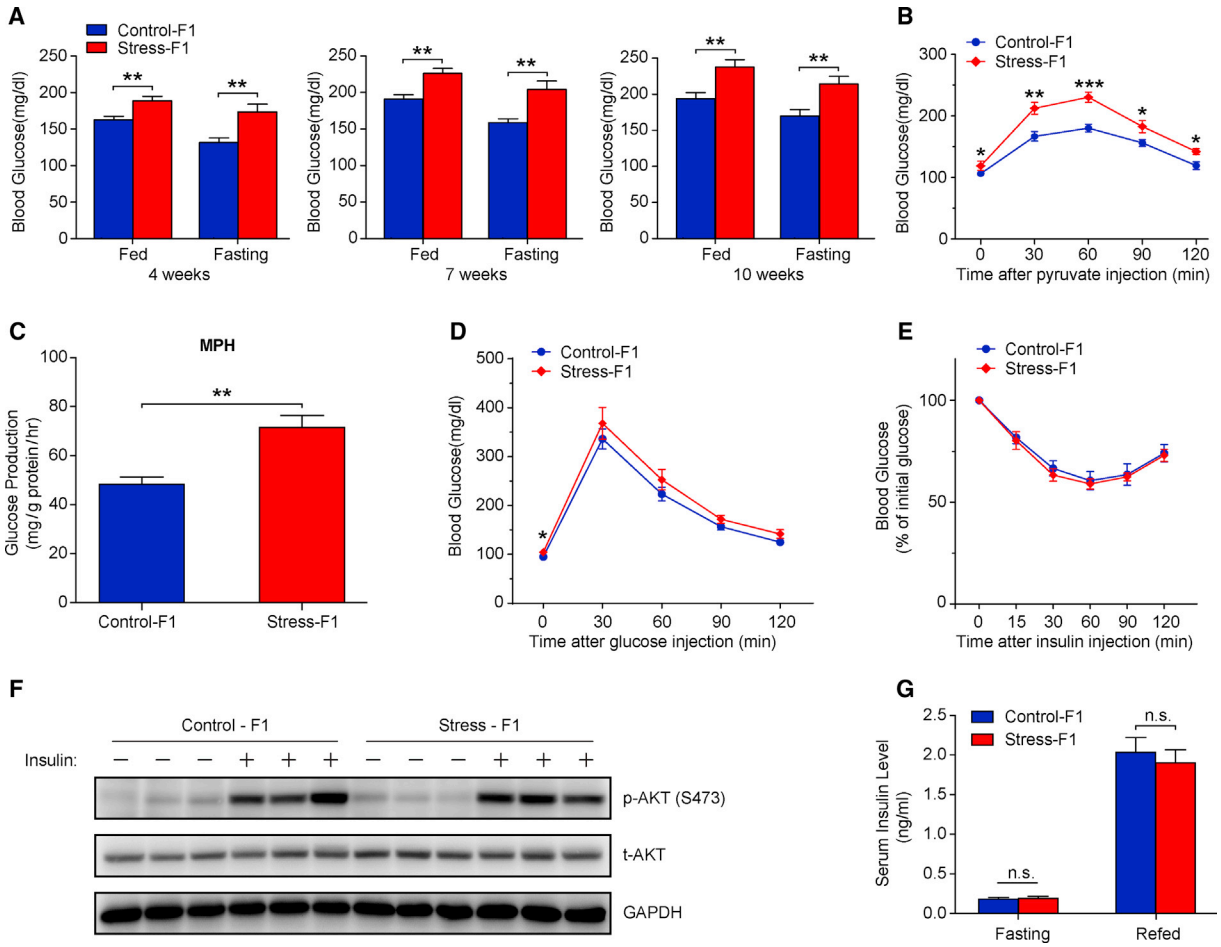

Figure 1. Increased Hepatic Gluconeogenesis in the Offspring of Stressed Fathers

(A) Fed or fasting (8 hr) blood glucose levels in 4-week, 7-week, and 10-week-old male control-F1 (n = 9) and stress-F1 (n = 6) mice.

(B) Intraperitoneal PTT (1.5 g pyruvate per kilogram of body weight) in male control-F1 (n = 9) and stress-F1 (n = 6) mice. Mice were fasted for 16 hr before assays.

(C) Glucose production in primary hepatocytes from control-F1 and stress-F1 male mice. n = 5 per group. MPH, mouse primary hepatocytes.

(D and E) An intraperitoneal glucose tolerance test (D) (2.0 g glucose per kilogram of body weight) and an insulin tolerance test (E) (0.75 U insulin/kg body weight) were performed in 8- and 9-week-old male mice. control-F1 (n = 9) and stress-F1 (n = 6) mice. Mice were fasted for 16 hr (D) or 6 hr (E) before assays.

(F) Representative protein levels of phosphorylated AKT (p-AKT) (serine 473) in livers after insulin stimulation (5 min, 0.75 U insulin per kilogram of body weight). Total AKT (t-AKT) and GAPDH were included as loading controls.

(G) Serum insulin levels in 10-week-old male mice in the fasted (16 hr) or refed (4 hr) states. n = 5 per group.

Data are presented as mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001; n.s., not statistically significant, versus control-F1.

glucose levels, compared with the control-F0 mice (Figures S1B–S1E). However, there were no significant differences in food intake, sperm density, motility, or the pregnancy rates of females between stress- and control-F0 mice (Figures S1F–S1I). No significant growth defects were observed in male offspring from stress-F0 fathers (stress-F1 mice) during their early lives, compared to offspring from control-F0 fathers (control-F1 mice) (Figures S2A–S2C). Notably, stress-F1 mice presented with elevated blood glucose in both fed and fasting conditions (Figure 1A). Stress-F1 mice also showed higher glucose levels during pyruvate tolerance tests (PTTs), pointing to hyperactive hepatic gluconeogenesis (Figure 1B). Glucose production was also higher in primary hepatocytes from stress-F1 mice (Figure 1C). However, insulin sensitivity was not changed in stress-F1 mice, as evidenced by glucose and insulin tolerance tests (Figures 1D and 1E), as well as insulin-stimulated phosphorylation of AKT in the liver (Figure 1F; Figure S2D) and serum insulin levels (Figure 1G). Besides, serum glucagon, leptin, and pro-inflammatory cytokines (tumor necrosis factor α [TNFα], interleukin-6 [IL-6]) were unaffected (Figures S2E–S2H). The body weight, food intake, locomotor activity, CO_2 production, O_2 consumption, and respiratory exchange ratios also remained unchanged (Figures S2I–S2N). In addition, liver weight, liver weight/body weight ratios, hepatic triglyceride content, and the histological phenotypes were also comparable between control-F1 and stress-F1 mice (Figures S2O–S2R). Hyperglycemia and enhanced hepatic gluconeogenesis were also detected in female offspring from stressed fathers (Figures S3A–S3D), although they were not as significant as those in male stress-F1 offspring. Therefore, male F1 mice were further assessed in

the following experiments. Taken together, these data demonstrate that mice in the stress-F1 group undergo hepatic gluconeogenesis at an elevated rate.

Decreased miR-466b-3p Expression Contributes to Upregulation of PEPCK in Stress-F1 Mice

Next, we investigated the expression levels of glucose-6-phosphatase (G6Pase) and PEPCK, two key enzymes involved in the regulation of gluconeogenesis (Hall and Granner, 1999). Strikingly, while mRNA levels of these two enzymes were normal (Figure 2A), protein levels of PEPCK were significantly increased in the stress-F1 mice (Figure 2B; Figure S3E), pointing to a posttranscriptional regulatory mechanism. Because miRNAs often play an important role in regulating gene expression at the posttranscriptional level (Ameres and Zamore, 2013), we investigated the possible role of miRNAs in the regulation of PEPCK. Therefore, we examined global miRNA expression in the livers of these mice, using a microarray analysis. Among 1,175 miRNAs, 128 miRNAs were increased and 206 miRNAs were reduced in stress-F1 mice (fold change > 1.5) (data not shown). Therefore, a $p < 0.05$ was set as an additional cutoff point to further narrow down the candidate miRNAs, although this p value did not reach the statistical significance level after Bonferroni correction. Our screening revealed a reduction of miRNA-466b-3p (miR-466b-3p) in the livers of stress-F1 mice (fold change = 0.559, $p = 0.025$) (Table S1). miR-466b-3p was selected because it is predicted to target the 3′ UTR of PEPCK by several bioinformatics programs (Table S2). We further confirmed that there was a reduction in miR-466b-3p by real-time qPCR in the livers of stress-F1 mice (Figure 2C). To assess whether miR-466b-3p is relevant to PEPCK expression, a luciferase reporter construct containing the PEPCK 3′-UTR was generated. miR-466b-3p mimics or antisense were co-transfected into mouse hepatoma Hep1-6 cells to overexpress or inhibit this miRNA. As expected, miR-466b-3p mimics dramatically reduced, while the antisense increased, PEPCK 3′ UTR activity (Figure 2D). Furthermore, a mutation in the miR-466b-3p-binding motif abrogated the effect of miR-466b-3p on luciferase activity (Figure 2D), suggesting a direct interaction of miR-466b-3p with this site. Ultimately, miR-466b-3p mimics reduced, while its antisense increased, endogenous PEPCK protein levels (Figures 2E and 2F; Figures S3F and S3G). Cellular glucose production was also affected as expected: high PEPCK levels led to a high rate of gluconeogenesis (Figures 2G and 2H).

Additionally, adenovirus-mediated overexpression of miR-466b-3p lowered blood glucose levels and hepatic glucose production in C57BL/6 male mice (Figures S3H and S3I). Protein levels of PEPCK were also downregulated by miR-466b-3p overexpression (Figure S3J). Moreover, restoration of miR-466b-3p in the livers of stress-F1 mice significantly counteracted the upregulation of PEPCK (Figures 2I and 2J; Figure S3K). At the same time, blood glucose levels and hepatic gluconeogenesis rates were markedly reduced (Figures 2K and 2L). Collectively, these data support a hypothesis that enhanced PEPCK protein levels in stress-F1 mice contribute to an elevated rate of gluconeogenesis, while the increased levels of PEPCK can be explained by a reduction in the abundance of miR-466b-3p.

Altered *Sfmbt2* Promoter Methylation Occurs in Livers from Stress-F1 Mice and Sperm from Stress-F0 Mice

Next, we examined whether the regulation of miR-466b-3p might be explained by an epigenetic modification. miR-466b-3p is encoded within intron 10 of the *Sfmbt2* gene (Dawes et al., 2015), an imprinted polycomb gene, which contains a large cluster of intronic miRNAs (Zheng et al., 2011). Our qPCR analysis showed that *Sfmbt2* and other two intronic miRNAs (miR-467a and miR-669a) were also downregulated in stress-F1 mice (Figures 3A and 3B). Therefore, miR-466b-3p and its host gene, *Sfmbt2*, were co-expressed and potentially regulated by common transcriptional events. Therefore, we dissected the DNA methylation patterns in the 5′-regulatory regions of *Sfmbt2*, in which a CpG island was identified using EMBOSS Cpgplot software (http://www.ebi.ac.uk/Tools/seqstats/emboss_cpgplot) (Figure 3C). Using methylated DNA immunoprecitation (MeDIP) followed by qPCR (MeDIP-qPCR) and pyrosequencing analysis, we found that the captured methylation in *Sfmbt2* promoter was enhanced in livers of stress-F1 mice (Figures 3D and 3E). These DNA methylation patterns and the altered expression of *Sfmbt2* and the relevant miRNAs were not present in the epididymal white adipose tissue (eWAT) and kidney of stress-F1 mice (Figures S4A–S4D). In agreement, protein levels of PEPCK in the eWAT and kidney were also comparable between control-F1 and stress-F1 mice (Figures S4E and S4F), suggesting that there is a tissue-specific epigenetic reprogramming of the *Sfmbt2* promoter. Moreover, treatment of 5-aza-cytidine (5-AZA), an inhibitor of DNA methylation (Kusaba et al., 1999), increased the expression of *Sfmbt2* and miR-466b-3p in a dose-dependent manner in Hep1-6 cells (Figure 3F). Together, these findings indicate that transcription of miR-466b-3p is negatively regulated by DNA methylation.

To delineate the mechanism responsible for the altered methylation pattern at the *Sfmbt2* promoter, we investigated its DNA methylation status in fetal livers (embryonic day 16.5) of stress-F1 mice and in sperm of stress-F0 mice. Strikingly, the epigenetic alterations at the *Sfmbt2* promoter were also observed between the two cohorts (Figures 3G–3J). By contrast, the methylation pattern and expression of miRNAs were not affected in the fetal brains of stress-F1 mice (Figures S4G and S4H).

In addition, to exclude the maternal oocyte and gestational effects, in vitro fertilization (IVF) was used. Sperm were collected from control-F0 or stress-F0 males at day 14 after the last restraint stress and co-incubated with oocytes from a common donor. Then, the embryos were evenly transferred into the bilateral uterine horns of the same recipient. As a result, the methylation changes at the *Sfmbt2* promoter were also detected in fetal livers of IVF-derived mice (Figures 3K and 3L). These data suggest that psychological stress could reprogram the epigenome of germline cells and be passed onto the offspring.

Promoter Methylation Alters Gene Expression through Interruption of NRF-2 Binding and Histone Modification

We further explored possible molecular mechanisms by which methylation at the promoter region alters the expression of the intronic miRNA. Changes in DNA methylation patterns has been shown to affect the binding of specific transcription factors and histones (Hu et al., 2013; Rönn et al., 2013). We identified a

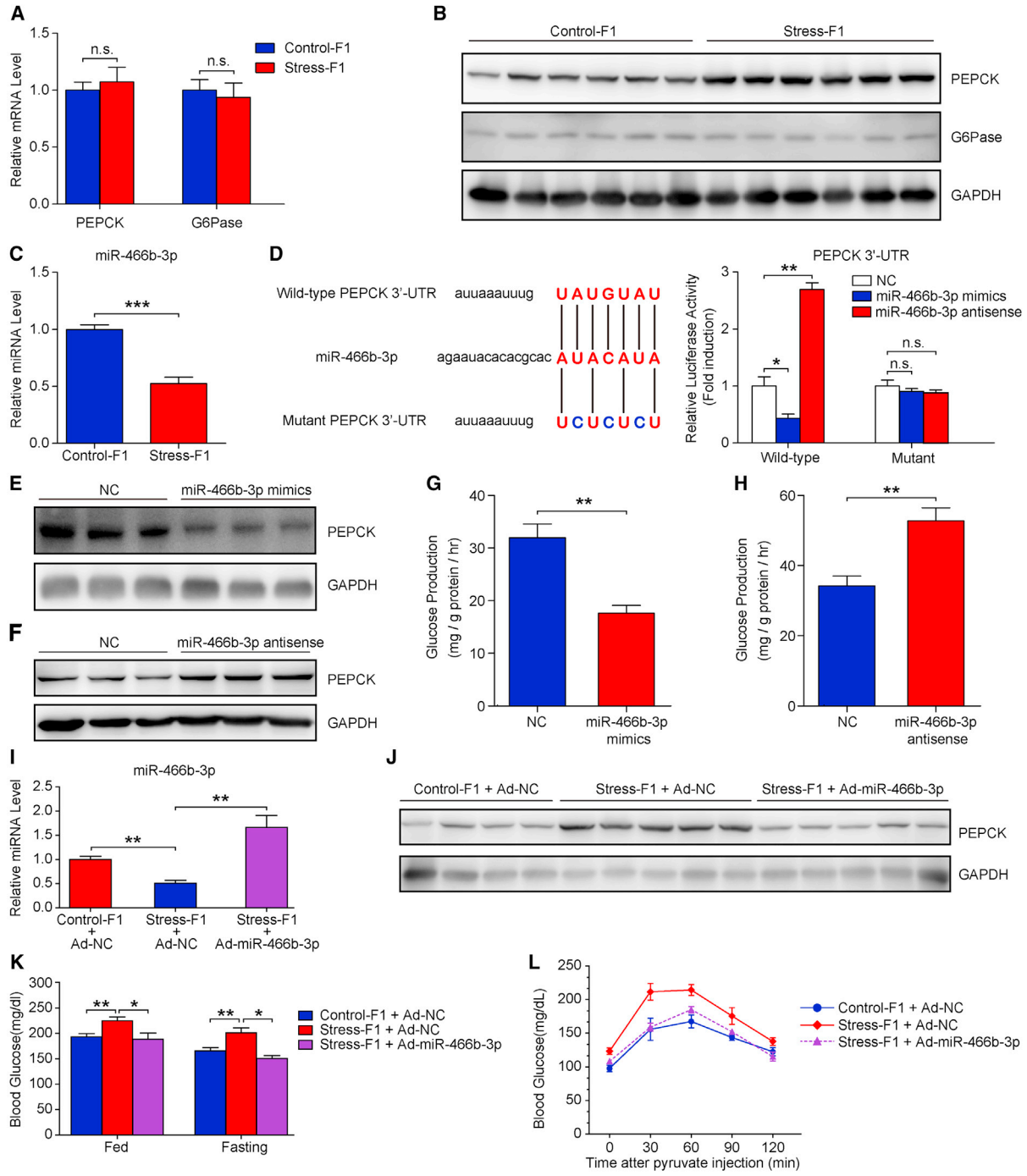

Figure 2. Reduction in miR-466b-3p Levels Leads to Upregulation of PEPCK in Stress-F1 Mice

(A and B) PEPCK and G6Pase mRNA expression (A) and protein levels (B) from 12-week-old male control-F1 (n = 9) and stress-F1 (n = 6) mice.

(C) Relative expression levels of miR-466b-3p from two groups of mice: control-F1 (n = 9) and stress-F1 (n = 6) mice.

(D) The reporter activity of the 3′ UTR of the mouse PEPCK gene in the presence of miR-466b-3p mimic or antisense sequences was determined in Hep1-6 cells. Reporter activity with a mutated sequence (highlighted in blue) was also analyzed. NC, negative control.

(E and F) Protein levels of PEPCK in Hep1-6 cells treated with miR-466b-3p mimics (E) or antisense (F).

(G and H) Glucose production in Hep1-6 cells overexpressing miR-466b-3p mimics (G) or antisense (H).

(I) Relative expression levels of miR-466b-3p in livers from male control-F1 mice (n = 4) and stress-F1 mice administered with adenovirus containing miR-466b-3p (n = 5) or negative control (n = 5).

(J) Representative protein levels of PEPCK in livers from mice as indicated in (I).

(K and L) Blood glucose levels (K) and PTT (L) (1.5 g pyruvate per kilogram of body weight) in mice as in (I). n = 4–5 per group.

Data are presented as mean ± SEM.*p < 0.05; **p < 0.01; ***p < 0.001; n.s., not statistically significant.

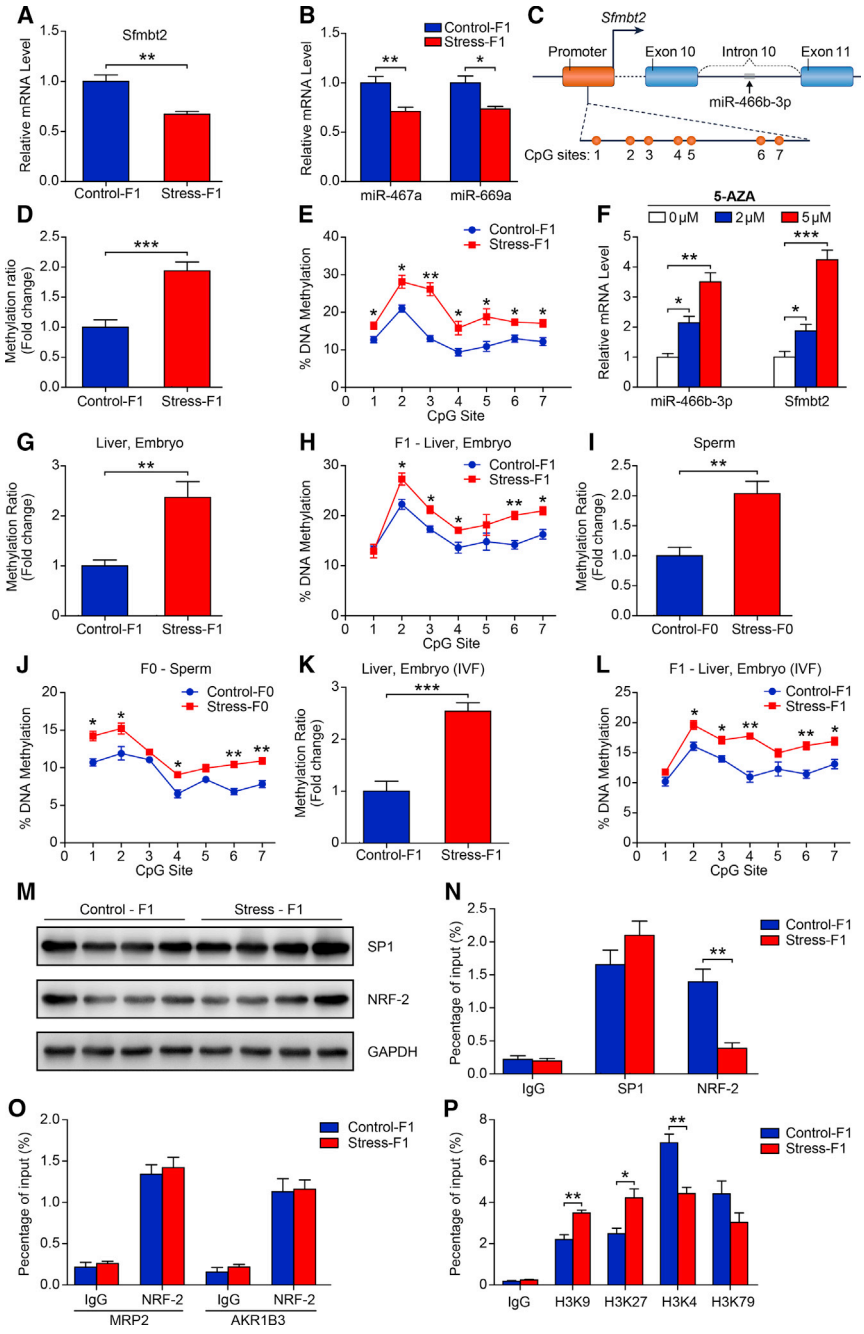

Figure 3. Altered *Sfmbt2* Promoter Methylation in Stress-F1 Livers and in Stress-F0 Sperm

(A and B) Relative expression levels of *Sfmbt2* (A) and of miR-467a and miR-669a (B) in livers from 12-week-old male control-F1 (n = 9) and stress-F1 (n = 6) mice.

(C) Structure of the *Sfmbt2* gene, detailing the CpG islands by dots.

(D) Promoter-specific methylation levels were analyzed by MeDIP-qPCR. The ratio of methylated DNA levels in livers from male control-F1 and stress-F1 mice is presented. n = 6 per group.

(E) The percentage of DNA methylation in individual CpG sites in livers from male control-F1 and stress-F1 mice was analyzed by pyrosequencing. n = 6 per group.

(F) Relative expression levels of miR-466b-3p and *Sfmbt2* in Hep1-6 cells after 36 hr treatment with 5-AZA or vehicle control.

(G–J) MeDIP-qPCR analysis and the percentage of DNA methylation in the *Sfmbt2* promoter in male embryonic livers (at embryonic day [E]16.5) (G and H) and paternal sperm (I and J). n = 6 per group.

(K and L) MeDIP-qPCR analysis (K) and the percentage of DNA methylation (L) in the *Sfmbt2* promoter in male embryonic livers (E16.5) from IVF-derived mice. n = 3 per group.

(M) Representative protein levels of SP1 and NRF-2 in livers from male control-F1 and stress-F1 mice.

(N) Chromatin immunoprecipitation (ChIP)-qPCR analysis for binding of SP1 and NRF-2 to the *Sfmbt2* promoter. n = 6 per group. IgG, immunoglobulin G.

(O) ChIP-qPCR analysis for binding of NRF-2 to the *MRP2* and *AKR1B3* promoters. n = 6 per group.

(P) ChIP-qPCR analysis for binding of histones H3K9me3, H3K27me3, H3K4me3, and H3K79me2 in the *Sfmbt2* promoter. n = 4 per group.

Data are presented as mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001.

consensus binding site for nuclear factor erythroid 2 (NF-E2)-related factor 2 (NRF-2) and specificity protein 1 (SP1) in the proximal promoter region of *Sfmbt2* using bioinformatics and luciferase reporter assays (Figures S4I–S4K). Although protein levels of NRF-2 were not changed in the livers of stress-F1 mice (Figure 3M), we found that the NRF-2 binding affinity for the *Sfmbt2* promoter was dramatically reduced (Figure 3N). However, its binding to downstream target genes (e.g., *MRP2* and *AKR1B3*) (Vollrath et al., 2006; Nishinaka and Yabe-Nishimura, 2005), was not changed (Figure 3O), and DNA methylation levels of these gene promoters were unaffected (Figure S4L). In contrast, SP1 protein levels and binding to the *Sfmbt2* promoter were similar between control-F1 and stress-F1 mice (Figures 3M and 3N). In addition, we found an enrichment of repressive histones (H3K9 and H3K27) and a reduction of active histones (H3K4) in the *Sfmbt2* promoter in stress-F1 mice (Figure 3P). Collectively, our data support the notion that DNA methylation in the *Sfmbt2* promoter might be associated with altered transcription factor binding and histone modifications, leading to the downregulation of miR-466b-3p expression.

Elevated Glucocorticoids in Stress-F0 Mice Contribute to Enhanced Gluconeogenesis in Offspring

It is well established that stressful stimuli can increase circulating glucocorticoid levels, which play a critical role in the stress response (Doom and Gunnar, 2013). We also observed elevated serum corticosterone levels and the upregulation of glucocorticoid receptor (GR) expression in the sperm of stress-F0 mice (Figure 4A; Figure S4M). Therefore, control-F0 and stress-F0

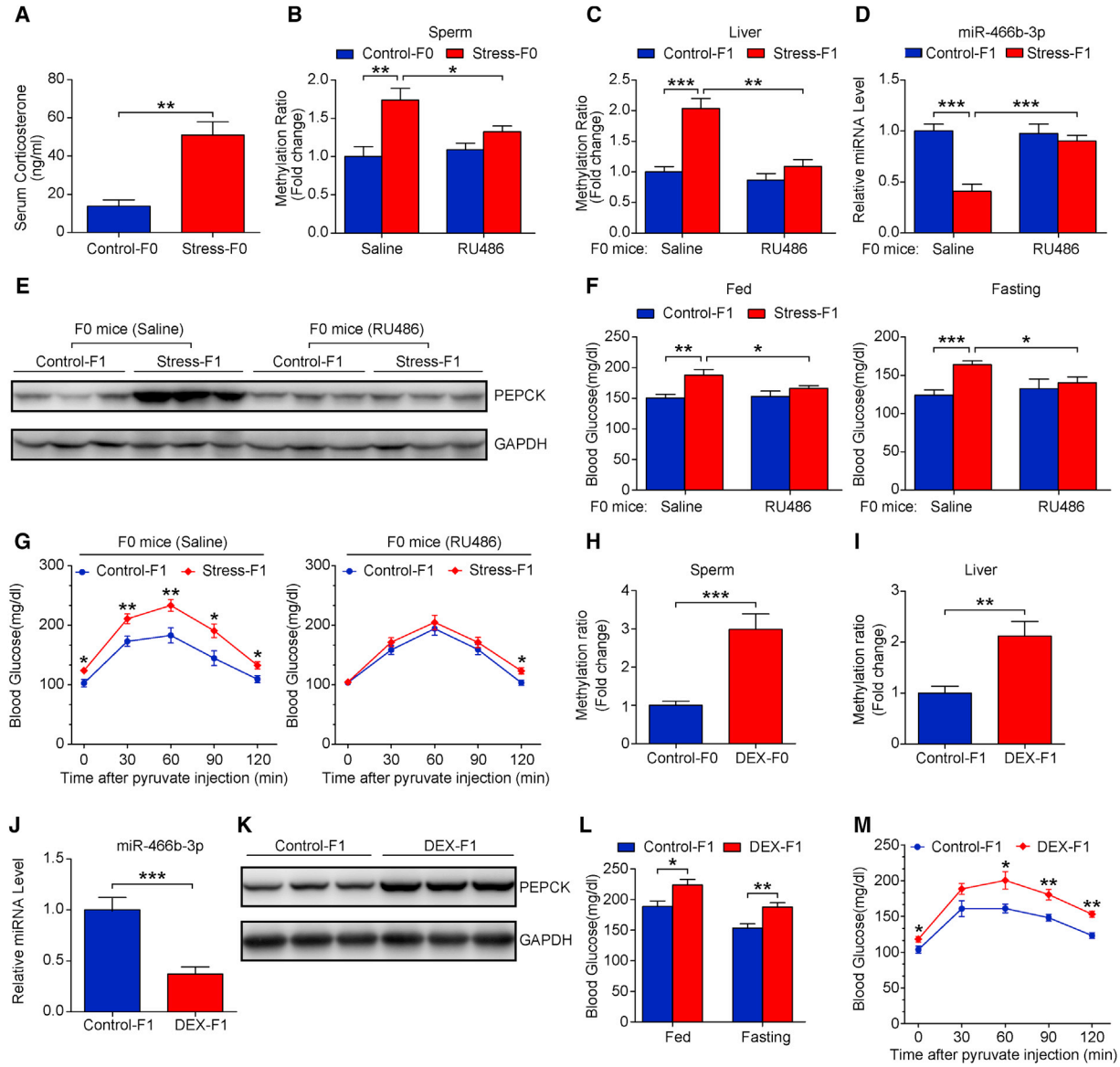

Figure 4. Paternal Glucocorticoids Contribute to the Enhanced Hepatic Gluconeogenesis in Stress-F1 Mice

(A) Serum corticosterone concentrations in male control-F0 and stress-F0 mice. n = 5 per group.

(B and C) Promoter-specific methylation levels were analyzed by MeDIP-qPCR. The ratio of methylated DNA levels in sperm from male F0 mice (B) (n = 5) and livers from male F1 mice (C) (n = 6) is presented. For RU486 treatment, control-F0 and stress-F0 mice received daily injections of RU486 (25 mg/kg) or vehicle control (Saline) for 14 days.

(D) Relative expression levels of miR-466b-3p from F1 mice from saline- or RU486-treated fathers. n = 8 per group.

(E) Representative protein levels of PEPCK from F1 mice as in (D).

(F and G) Fed or fasting (8 hr) blood glucose levels (F) and intraperitoneal PTTs (G) (1.5 g pyruvate per kilogram of body weight). Mice were fasted for 16 hr before PTTs. n = 8 per group.

(H and I) Promoter-specific methylation levels were analyzed by MeDIP-qPCR. The ratio of methylated DNA levels in sperm from F0 mice (H) (n = 6) and livers from F1 mice (I) (n = 6) is presented. For DEX treatment, F0 mice received daily injections of DEX (200 μg/kg) or vehicle control for 14 days.

(J–M) Analysis of control-F1 and DEX-F1 mice, including miR-466b-3p expression (J), PEPCK protein levels (K), blood glucose levels (L), and PTTs (M) (1.5 g pyruvate per kilogram of body weight). Mice were fasted for 16 hr before PTTs. n = 6 per group.

Data are presented as mean ± SEM. *p < 0.05; **p < 0.01; ***p < 0.001.

See also Figure S4N.

fathers received daily injections of vehicle control (saline) or RU486, a glucocorticoid antagonist (Figure S4N). Notably, RU486 blocked hyper-methylation patterns at the *Sfmbt2* promoter in stress-F0 sperm and stress-F1 livers (Figures 4B and 4C). RU486 also attenuated the downregulation of miR-466b-3p in the livers of stress-F1 mice (Figure 4D). Besides, PEPCK expression and the abnormal metabolic phenotypes, including elevated blood glucose and hepatic gluconeogenesis, in

stress-F1 mice were largely reversed by RU486 (Figures 4E–4G; Figure S4O).

To further clarify this issue, we treated C57BL/6 male mice with vehicle control (control-F0) or dexamethasone (DEX) (DEX-F0), a synthetic analog of glucocorticoids, for 14 days and then placed them with females for another 2 days. As a result, DEX administration resulted in an enhanced methylation at the *Sfmbt2* promoter of DEX-F0 sperm and DEX-F1 livers (Figures 4H and 4I). In addition, miR-466b-3p expression was reduced, while PEPCK protein levels were increased (Figures 4J and 4K; Figure S4P). Consistently, blood glucose levels and hepatic gluconeogenesis were increased (Figures 4L and 4M). Taken together, these results demonstrate that excess glucocorticoids play a crucial role in reprogramming gluconeogenesis in stress-F1 mice.

DISCUSSION

Here, we described a mouse model in which paternal exposure to psychological stress leads to hyperglycemia in the offspring, due to hepatic overproduction of PEPCK. Increased protein expression level or enzymatic activity of PEPCK has been shown to enhance hepatic gluconeogenesis in vitro and in vivo (Lin et al., 2009; Jiang et al., 2011), which is consistent with our observations, and also increases the risk for type 2 diabetes (Valera et al., 1994). Thus, our results support the notion that alterations in PEPCK expression have important impacts on hepatic glucose production and whole-body glucose homeostasis.

Mechanistically, the upregulation of PEPCK in offspring is attributed to increased DNA methylation patterns of the *Sfmbt2* gene promoter. Kuzmin et al. reported that *Sfmbt2* was expressed preferentially from the paternal allele in early embryos due to a methylated CpG island on the maternal allele (Kuzmin et al., 2008). Therefore, we speculate that maternal psychological stress may not affect the DNA methylation status of *Sfmbt2* promoter region or its expression in offspring. Interestingly, administration of valproate, a widely prescribed mood-stabilizing drug, in mice could upregulate *Sfmbt2* in brain tissues (Chetcuti et al., 2006), suggesting that stress might be a negative regulator of *Sfmbt2* expression. Altered DNA methylation was also mapped to the *Sfmbt2* region in mice as a result of fetal alcohol exposure (Laufer et al., 2013). Therefore, further studies would help clarify whether paternal exposure to other types of stress, such as smoking and toxicants, similarly influences the offspring's glucose metabolism through the epigenetic mechanisms involving *Sfmbt2* and miRNA-466b-3p. Moreover, the percentage of methylation in our study represents the level of DNA methylation in sperm, not the population or number or subset of sperm. The subtle changes of DNA methylation were also reported in recent studies (Martínez et al., 2014; Radford et al., 2014), suggesting that the change of DNA methylation levels in sperm could be stably inherited and maintained in somatic tissues of the offspring.

Notably, our present study showed that paternal-stress-induced metabolic effects in offspring could be largely reversed by RU486. Consistently, DEX administration in fathers also induced reprogramming of hepatic gluconeogenesis in offspring. A recent study demonstrated that administration of DEX to adult male mice altered DNA methylation in sperm and regulated the expression of several nuclear steroid receptors in the hippocampus and kidney of male F1 offspring (Petropoulos et al., 2014). In addition, fetal exposure to excessive maternal glucocorticoids during gestation resulted in metabolic disorders in the adulthood due to increased mRNA expression of hepatic GR and PEPCK (Nyirenda et al., 1998; de Vries et al., 2007). These results may be conceptually relevant to our observations, because the inheritance of both maternal and paternal exposure to glucocorticoids could be mediated by epigenetic mechanisms. However, the precise mechanisms underlying glucocorticoid-induced DNA methylation changes remain poorly understood (Moisiadis and Matthews, 2014).

In support of our findings, the role of miRNAs in the transmission of paternal or maternal information has recently been explored in the literature (Gapp et al., 2014a; Alejandro et al., 2014). Moreover, it has also been established that paternal experiences could affect behavior in the resulting offspring (Gapp et al., 2014a, 2014b; Dias and Ressler, 2014). These results are likely to be relevant for human disease, since intergenerational effects of paternal information on the growth of their future offspring have been observed in two general populations (Pembrey et al., 2006). Therefore, together with these studies, our data point to the need for evaluating the role of this non-Mendelian form of inheritance in non-communicable human diseases.

EXPERIMENTAL PROCEDURES

Animal Experiments

The animal protocol was reviewed and approved by the Animal Care Committee of Shanghai Jiao Tong University School of Medicine. The study design was presented as detailed in the Supplemental Experimental Procedures.

MeDIP

Four micrograms of purified genomic DNA was fragmented to a mean size of 300 bp using a Covaris machine, denatured, and immunoprecipitated with 5mC antibody (Eurogentec). Immunoprecipitated DNA was recovered with proteinase K digestion followed by column based-purification (DNA Wizard, Promega). Recovered DNA fractions were diluted 1/50 and measured using real-time PCR with an ABI PRISM 7000 sequence detector system and fluorescence-based SYBR Green technology (Applied Biosystems).

Biochemical Analysis

Blood glucose was determined using a portable blood glucose meter (LifeScan, Johnson & Johnson). Metabolic studies, including pyruvate, glucose, and insulin tolerance tests, were measured as detailed in the Supplemental Experimental Procedures.

Microarray analysis, real-time qPCR, and western blots were carried out as detailed in the Supplemental Experimental Procedures.

Statistical Analysis

All values are shown as mean ± SEM. Statistical differences were determined by two-tailed Student's t test. A p value < 0.05 was considered significant.

ACCESSION NUMBERS

The accession number for the datasets of microarray analysis, statistically analyzed using Student's t test and Bonferroni correction, reported in this paper is GEO: GSE73530.

SUPPLEMENTAL INFORMATION

Supplemental Information includes Supplemental Experimental Procedures, four figures, and two tables and can be found with this article online at http://dx.doi.org/10.1016/j.cmet.2016.01.014.

AUTHOR CONTRIBUTIONS

L.W., Y. Lu, Y.J., B.L., S.L., Y. Li, X. Liu, J.Z., and X.X. performed experiments. L.W. and Y.J. analyzed the metabolic phenotypes of mice. Y. Lu and B.L. performed epigenetic studies. F.X., D.C., Y.G., and J.L. provided technical assistance and reagents. L.W., Y. Lu, and X. Li wrote the manuscript. Y. Lu, X.C., and X. Li designed the project and coordinated the execution of the experimental plan.

ACKNOWLEDGMENTS

This study was supported by grants from the National Key Basic Research Program of China (973 Program) (no. 2012CB524902) and the China Natural Science Foundation (nos. 31530033, 81321001, 81300474, and 813111105). D.C. was a recipient of The Program for Professor of Special Appointment (Eastern Scholar) at Shanghai Institutions of Higher Learning.

Received: May 3, 2015
Revised: October 9, 2015
Accepted: January 22, 2016
Published: February 18, 2016

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