

Abdelnaby Khalyfa, Vesco Mutskov, Alba Carreras, Ahamed A. Khalyfa, Fahed Hakim, and David Gozal



Sleep Fragmentation During Late Gestation Induces Metabolic Perturbations and Epigenetic Changes in Adiponectin Gene Expression in Male Adult Offspring Mice



Diabetes 2014;63:3230–3241 | DOI: 10.2337/db14-0202

Sleep fragmentation (SF) is a common condition among pregnant women, particularly during late gestation. Gestational perturbations promote the emergence of adiposity and metabolic disease risk in offspring, most likely through epigenetic modifications. Adiponectin (*AdipoQ*) expression inversely correlates with obesity and insulin resistance. The effects of SF during late gestation on metabolic function and *AdipoQ* expression in visceral white adipose tissue (VWAT) of offspring mice are unknown. Male offspring mice were assessed at 24 weeks after dams were exposed to SF or control sleep during late gestation. Increased food intake, body weight, VWAT mass, and insulin resistance, with reductions in *AdipoQ* expression in VWAT, emerged in SF offspring. Increased *DNMT3a* and *-b* and global DNA methylation and reduced histone acetyltransferase activity and *TET1*, *-2*, and *-3* expression were detected in VWAT of SF offspring. Reductions in 5-hydroxymethylcytosine and H3K4m3 and an increase in DNA 5-methylcytosine and H3K9m2 in the promoter and enhancer regions of *AdipoQ* emerged in adipocytes from VWAT and correlated with *AdipoQ* expression. SF during late gestation induces epigenetic modifications in *AdipoQ* in male offspring mouse VWAT adipocytes along with a metabolic syndrome-like phenotype. Thus, altered gestational environments elicited by SF impose the emergence of adverse, long-lasting metabolic consequences in the next generation.

The prevalence of chronic or noncommunicable diseases is increasing rapidly worldwide. Among the chronic diseases, obesity is a major concern because it significantly reduces life expectancy through increased risk and severity of cardiovascular and metabolic diseases and cancer (1,2). The fetal origins of the adult disease hypothesis suggests that intrauterine environmental exposures affect embryonic development and increase the risk of specific diseases in adult life (3–5). Epidemiological and experimental studies have revealed that imbalanced nutrition during gestation or early postnatal life promote the risk of type 2 diabetes and hypertension (6) possibly through metabolic reprogramming of the fetus and neonate (7,8). Furthermore, emerging evidence suggests that perturbations during gestation can increase the risk of morbidity and mortality from cardiovascular and metabolic diseases in adult life (4,9).

Epigenetic modifications play a critical role in the regulation of gene expression and are major contributors to the determination of the phenotype in multicellular organisms. Growing evidence suggests that epigenetic modifications, such as DNA methylation, histone modifications, and noncoding RNAs (e.g., microRNAs [miRNAs]), will alter gene expression without changing DNA sequence and are important effectors of developmental programming. The sensitivity of the epigenetic system to environmental factors occurs mainly during the period of

Section of Pediatric Sleep Medicine, Department of Pediatrics, Comer Children's Hospital, Biological Sciences Division, The University of Chicago, Chicago, IL

Corresponding author: David Gozal, dgozal@uchicago.edu.

Received 8 February 2014 and accepted 28 April 2014.

This article contains Supplementary Data online at <http://diabetes.diabetesjournals.org/lookup/suppl/doi:10.2337/db14-0202/-/DC1>.

© 2014 by the American Diabetes Association. Readers may use this article as long as the work is properly cited, the use is educational and not for profit, and the work is not altered.

developmental plasticity (i.e., gestation, early postnatal life) (10). Epigenetic marks offer plausible explanations of how environmental exposures can affect more than one subsequent generation and elicit the emergence of obesity and metabolic diseases (11).

Pregnancy is associated with significant hormonal, biochemical, and physical alterations that among others, affect sleep duration and continuity, particularly during the third trimester (12), and increase the risk of sleep-disordered breathing (13,14). During the third trimester of human pregnancy, the prevalence of habitual snoring and attendant sleep fragmentation (SF) has been estimated to affect 10–27% of pregnant women (15) and can impose significant increases in the risk for gestational diabetes and other complications (14,16). However, the long-term impact of sleep perturbations on metabolic function in the offspring has not been investigated.

In obesity, different fat depots are associated with differential metabolic risk (17). Visceral white adipose tissue (VWAT), which exerts important endocrine functions involving complex crosstalk with other organs and tissues, is a central player in metabolic regulation through the production and release of multiple adipokines that coordinate energy balance, glucose homeostasis, insulin sensitivity, and lipid pathways (18). Among the adipokines, adiponectin (*AdipoQ*) is a circulating hormone produced exclusively by adipose tissue in adult humans and rodents that modulates lipid and glucose metabolism, including promotion of fatty acid oxidation and glucose utilization and repression of hepatic gluconeogenesis (19). In mice, reductions in *AdipoQ* bioavailability lead to impaired glucose tolerance and to elevated hepatic glucose production (20). In humans, decreased *AdipoQ* levels are present in obese individuals and elevate the risk for insulin resistance and cardiovascular disease (21). On the basis of the aforementioned considerations, we hypothesized that excessively fragmented sleep during late gestation would induce metabolic perturbations in offspring mice and lead to epigenetic modifications of *AdipoQ* in VWAT.

RESEARCH DESIGN AND METHODS

All experiments were approved by The University of Chicago Institutional Animal Care and Use Committee. Male and female C57BL/6J mice were purchased from The Jackson Laboratory (Bar Harbor, ME) for breeding. After arrival, all animals were allowed to recover within the animal care facility for 7 days. Animals were fed a normal chow diet and housed in a controlled environment with 12-h light-dark cycles (7:00 A.M.–7:00 P.M.) in constant temperature ($24 \pm 0.2^\circ\text{C}$) with ad libitum access to food and water. For all measurements and assessments, experimenters were blinded to the identity of the mice being tested.

Pregnant Female Mice

Individual 3-month-old male and virgin female mice were used for breeding to generate only one litter. Male mice

were removed once inspection of the female revealed the presence of a copulation plug (day 1 of gestation). After 14 days of pregnancy, the mice were divided into two groups: control sleep (SC) mice were housed in standard housing conditions, and sleep fragmented mice were exposed to an SF paradigm for 5 days (days 14–19 of gestation). Upon delivery, all litters were immediately culled to six pups, and culling was always performed around 12:00 P.M. for both experimental groups. During lactation, all mothers were fed regular low-fat chow diet, and pups were kept with their mothers until weaning. Once weaned, offspring male mice were placed in individual cages, had unrestricted access to water and regular chow diet, and were housed in standard conditions in a temperature-controlled ($24 \pm 2^\circ\text{C}$) room with 12-h light-dark cycles (lights on at 7:00 A.M.).

Food Consumption, Body Weight, and Indirect Calorimetry in Offspring

Food consumption was registered daily, and body weight of each male offspring mouse was registered weekly for the 24-week period, always at the same time of the day during the middle of the light period. Indirect calorimetric measurements were carried out with the LabMaster system (TSE Systems, Midland, MI) in a separate subset of SF offspring and SC offspring at the Mouse Metabolic Core Facility to allow monitoring of VO_2 , VCO_2 , and respiratory exchange ratio. VO_2 , VCO_2 , and respiratory exchange ratio were recorded for 7 days during the 7th week of life, and gain was recorded during the 23rd to 24th weeks of life.

Sleep Fragmentation

The device used to induce SF in rodents has been previously described (22,23). The device induced moderate to severe SF with a 2-min interval that was implemented during the light period (7:00 A.M.–7:00 P.M.) for 5 days during the last one-third of the gestation period (days 14–19). This experimental procedure is not associated with any measurable increases in stress hormones (22,23). Furthermore, the SF paradigm does not reduce the overall duration of sleep or affect the macro- and microstructures of sleep state distribution (22,23). In addition, each pregnant mouse, whether SF or SC, was placed in a cage that included the SF device for the duration of the exposure, with all cages being located in the same room in the vivarium facility.

Glucose Tolerance Test

Intraperitoneal glucose tolerance test (GTT) was performed at 24 weeks of age. Mice were injected with glucose 2 mg/g body weight i.p. after 3 h of fasting. Water was available during the fasting period. Blood samples for glucose determination were collected from the tail vein in heparin-coated capillary tubes at 0, 4, 15, 30, 60, 90, and 120 min. Glucose response during GTT was evaluated by estimating the total area under the glycemia versus time curves.

Insulin Tolerance Test

Mice were injected intraperitoneally with Humulin 0.25 units/kg of body weight after 3 h of fasting at age 24 weeks. Blood was collected from the tail vein of each mouse, and blood glucose was measured using a OneTouch Ultra2 glucometer (LifeScan, Milpitas, CA). Blood samples for insulin determination were obtained from the cut tip of the tail at 0, 4, 15, 30, 60, and 120 min following injection. Insulin resistance before insulin injection was assessed using the HOMA-IR equation (fasting insulin \times fasting glucose)/22.5. Insulin tolerance test (ITT) glycemic trajectories were also analyzed for differences in insulin sensitivity as previously described (24). Of note, mice undergoing ITT did not undergo GTT and vice versa.

Lipid Profiles

Fasting plasma samples were assessed after 3 h of fasting for total cholesterol and triglyceride levels with Infinity kits (Thermo Scientific, Pittsburg, PA).

ELISAs

Mice were fasted for 3 h, and blood samples were collected in vacutainer tubes containing EDTA (Becton Dickinson, Franklin Lakes, NJ). The collected fresh blood was centrifuged at 2,000g for 20 min at 4°C; subsequently, plasma samples were centrifuged for 5 min at 13,000 rpm to remove remaining cells and platelets and immediately frozen at -80°C until further analysis. Plasma insulin and AdipoQ assays were carried out with ELISA kits (EMD Millipore, Billerica, MA) according to the manufacturer's protocol. For the insulin assay, the appropriate range of the assay was 0.2–10 ng/mL, with the limit of sensitivity at 0.2 ng/mL and intra- and interassay variations at 3.73% and 10.52%, respectively, within the assay range. For the AdipoQ assay, the appropriate range was 1–50 ng/mL, with the sensitivity threshold at 0.2 ng/mL, and intra- and interassay variations at 5.75% and 5.98%, respectively, within the assay range.

Preparation of Nuclear Extracts

For this and all other experiments, epididymal VWAT were used. Nuclear extracts of VWAT were isolated using Active Motif (Carlsbad, CA) kits. Concentrations of soluble nuclear proteins were determined in the supernatants using the Pierce BCA Protein Assay kit (Thermo Scientific, Rockford, IL).

Total RNA and miRNA Isolation

Total RNA and miRNA were isolated from the same VWAT of each animal. Total RNA was isolated through automated RNA extraction (Promega, Madison, WI), and DNase was treated according to the manufacturer's protocol. miRNA was isolated by using the miRNeasy Mini kit (Qiagen, Valencia, CA). The RNA quality and integrity were determined by Eukaryote Total RNA Nano 6000 LabChip assay (Agilent Technologies) on the Agilent 2100 Bioanalyzer. The quality of miRNA was determined with the Agilent Small RNA Kit according to the manufacturer's protocol. Both total RNA and miRNA samples

were quantified on a Nanodrop 2000 (Ambion, Austin, TX).

DNA Isolation

DNA was isolated from VWAT using automated tissue DNA extraction kits (Promega) and quantified on a Nanodrop 2000. DNA was isolated from adipocytes by collagenase digestion and flotation centrifugation. Briefly, fat pads were digested in Krebs-Ringer buffer containing 1 mg/mL type II collagenase (Sigma-Aldrich, St. Louis, MO) and incubated in a shaking water bath at 37°C for 30 min. Adipocytes were isolated from stromal elements by gentle centrifugation at ~ 150 rpm for 1 min. DNA was isolated by using a DNeasy Blood and Tissue kit (Qiagen).

Global DNA Methylation and Histone Acetyltransferase Activity Assay

DNA was extracted from VWAT by using the Maxwell 16 System and Maxwell 16 Blood DNA Purification kit (Promega) and analyzed for global methylation with the Methylamp Global DNA Methylation Quantification Kit (Epigentek, Farmingdale, NY). Histone acetyltransferase (HAT) activity using VWAT nuclear extracts was quantified using the HAT Activity Colorimetric Assay Kit (BioVision, Mountain View, CA).

Epigenetic Analyses for DNA Modifications

Epigenetic modifications in DNA isolated from adipocytes derived from VWAT samples (Supplementary Fig. 1) were assessed using methylated DNA immunoprecipitation (MeDIP) as previously described (25). In brief, genomic DNA was isolated and sonicated to produce random fragments ranging in size from 200 to 1,000 base pairs (bp). Denatured DNA samples were immunoprecipitated with antibodies against 5-methylcytosine (5-mC) (Diagenode, Denville, NJ) or 5-hydroxymethylcytosine (5-hmC) (EMD Millipore). These sonicated input DNA and immunoprecipitated DNA were analyzed in parallel by quantitative RT-PCR (qRT-PCR) using specific primers at the region of interest.

Epigenetic Analyses of Histone Modifications

Analyses of histone modifications of DNA from purified adipocytes were performed as previously described (26). Cell populations of individual purified adipocytes from VWAT tissues were cross-linked with low concentrations of formaldehyde. Cross-linked samples were sonicated to produce chromatin fragments ranging in size from 200 to 500 bp. Chromatin fragments were immunoprecipitated (chromatin immunoprecipitation [ChIP] assay) using antibodies specific for H3K4m3 and H3K9m2 (EMD Millipore). Purified input and immunoprecipitated DNA were analyzed by quantitative PCR (qPCR) for gene-specific analyses.

Quantitative Real-Time PCR Data Analysis for ChIP and MeDIP

DNA samples from input and antibody-bound fractions were analyzed by qPCR using SYBR Green PCR Master

Mix and 7500 Real-Time PCR system (Life Technologies–Applied Biosystems, Foster City, CA) according to the manufacturer's protocols. Individual qPCRs were carried out in triplicate. The primer sequences used in these experiments are listed in Supplementary Table 1. Quantifications were performed by applying the comparative cycle number (C_t) method as previously described (27). The fold differences between the antibody-bound and input fractions were calculated and corrected by subtraction of the nonspecific signal derived from the nonimmune rabbit IgG. In parallel, DNA samples were amplified with primers for an internal control gene.

Western Blot Analysis

VWAT samples were homogenized in lysis buffer (Sigma–Aldrich) with protease and phosphatase inhibitor cocktails (BD Biosciences, San Jose, CA). Protein content was measured in each soluble fraction using the Pierce BCA Protein Assay kit (Thermo Scientific). Homogenate proteins (20 μ g) were buffered with 4 \times Laemmli buffer and heated for 5 min at 95°C. Protein was electrophoresed through a 12% SDS–polyacrylamide gel and transferred to a nitrocellulose membrane. Membranes were processed according to standard Western blotting procedures. Immunoreactive bands were visualized using an enhanced chemiluminescence detection system (Chemidoc XRS+; Bio-Rad, Hercules, CA). The intensity of caveolin-1 (CAV1) was normalized to β -actin as a control.

Quantitative RT-PCR

qRT-PCR was performed for mRNAs and miRNAs using the ABI PRISM 7500 System (Applied Biosystems). cDNA was synthesized from 500 ng of total RNA from visceral fat by using a High-Capacity cDNA Archive Kit (Applied Biosystems). As a reference gene, 18S rRNA was used to normalize the expression ratios. All experiments were performed in triplicate. The mean cycle number (C_t) values of the 18S C_t and the gene of interest C_t were calculated. The relative expression of the gene of interest was calculated by the $2^{-\Delta\Delta C_t}$ method.

miRNAs were reverse transcribed with looped miRNA-specific reverse transcriptase primers (Applied Biosystems) by using the TaqMan miRNA mouse assay according to the manufacturer's protocol. All TaqMan assays were run in triplicate. The qPCR results were normalized against an internal control (RNU6) and expressed as fold changes.

Statistical Analysis

All data are mean \pm SE unless stated otherwise. Two-way ANOVA for repeated measures followed by post hoc Bonferroni corrections or unpaired t tests were used to compare quantitative data between SF and SC mice as appropriate. Nonnormally distributed data were analyzed with the Mann-Whitney U test. All analyses were conducted with SPSS version 18 software (IBM Corporation, Chicago, IL). A two-tailed $P < 0.05$ was considered statistically significant.

RESULTS

SF Exposure In Utero Increases Offspring Body Weight and Food Intake

At birth, there were no significant differences in the mean body weights of SF and SC male offspring (Fig. 1), and the litter sizes were similar between the two groups. However, starting at 16–18 weeks of age, the mean body weight of the SF offspring mice was significantly higher than SC offspring mice ($n = 8$, $P < 0.0004$) (Fig. 1A). The caloric intake of SF mice was higher than that of the SC mice ($P < 0.0002$) (Fig. 1B and C). In addition, the fat mass at 24 weeks of age was increased in SF mice (Fig. 1D). The adiposity index, which is calculated as the sum of the weight of various fat depots (visceral or epididymal, subcutaneous, perirenal, and mesenteric fats) expressed as a percentage of body weight was also significantly increased in SF mice (2.5 ± 0.21) compared with SC mice (1.7 ± 0.14 , $P < 0.01$). Measurements of energy expenditure in a subset of mice ($n = 6$ /experimental group) revealed no differences in total daily VO_2 at either 7 or 23 weeks of age ($3,225 \pm 365$ vs. $3,382 \pm 421$ mL/h/kg in SF vs. SC mice, respectively, $P > 0.05$).

SF Exposure During Gestation Alters Offspring Glucose Homeostasis

GTT and ITT were performed at 24 weeks of age. SF mice exhibited significantly higher glycemic levels during both GTT and ITT and increased HOMA-IR values during fasting conditions compared with SC mice ($P < 0.0001$) (Fig. 2). Compared with SC mice, SF mice also had significantly increased incremental glucose area under the curve ($P < 0.01$). Thus, evidence of systemic insulin resistance is present in SF offspring mice.

SF Exposure In Utero Alters Offspring Lipid Profiles and AdipoQ Expression

Fasting total triglyceride and cholesterol plasma levels were significantly higher in SF mice than in SC mice at 24 weeks of age ($P < 0.01$) (Table 1). In addition, *AdipoQ* gene expression in VWAT and AdipoQ systemic plasma levels were reduced in mice at 24 weeks of age ($P < 0.0009$) (Table 1).

SF Exposure In Utero Alters Global DNA Methylation and HAT Activity

Assessment of global DNA methylation in VWAT revealed higher levels in SF mice ($P < 0.05$) (Fig. 3A). This increase of global DNA methylation was significantly correlated with CpG DNA methyltransferases *Dnmt3a* and *Dnmt3b* gene expression levels in VWAT (Fig. 3C). HAT activity in VWAT nuclear extracts was significantly lower in SF mice ($P < 0.01$) (Fig. 3B).

Effects of SF Exposure In Utero on Epigenetic Modifications

We investigated the effects of SF exposure in utero on DNA and histone modifications by MeDIP and ChIP analyses. We validated the procedures for epigenetic analysis in adipocytes obtained from SC and SF mice

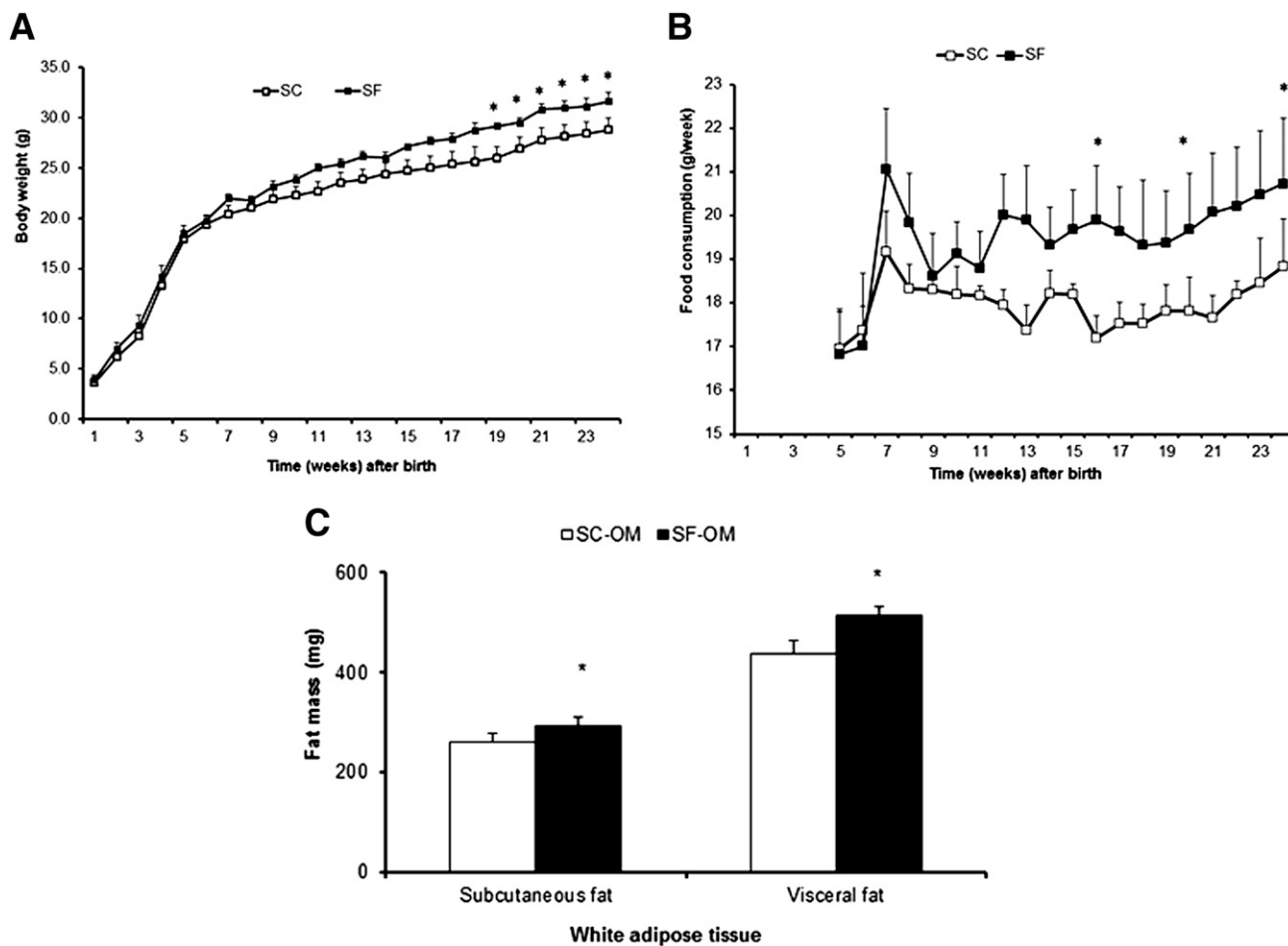


Figure 1—SF during late gestation leads to increased body weight, food intake, and mass fat distribution in offspring mice (OM). Body weight from birth to 24 weeks of age (A) and food consumption (B) in male OM exposed to SF or SC. $n = 24$ /experimental group. $*P < 0.0004$ and $P < 0.0001$ for body weight and food intake (g), respectively. The mass of subcutaneous and visceral adipose tissue distributions is significantly higher in SF than in SC OM (C). Data are mean \pm SE ($n = 24$ /experimental group). $*P < 0.01$.

using GAPDH gene (active chromatin) and TSHTB (inactive chromatin) (Supplementary Fig. 1). The epigenetic modifications of the *AdipoQ* gene promoter and its previously characterized enhancer region (28) were assessed in offspring adipocytes from VWAT at 24 weeks of age. Active epigenetic marks (histone H3K4m3 and DNA 5hmC) were significantly decreased in the promoter and enhancer regions of the *AdipoQ* gene (Fig. 4A and B) in 24-week-old offspring from SF-exposed mothers. In contrast, the inactive epigenetic marks in the *AdipoQ* gene (5-mC and histone H3K9m2) were significantly higher in SF offspring adipocytes as shown in Fig. 4C and D. The epigenetic alterations observed in VWAT adipocytes were correlated with *AdipoQ* mRNA expression in the same VWAT (Table 1 and Fig. 4A).

Alterations of the epigenome, including DNA methylation and histone modifications, usually lead to misregulation of gene expression (29). 5-mC can be converted to 5-hmC by the ten-eleven translocation (Tet) family of proteins (1–3) as a step of active demethylation (30,31).

Reduced mRNA expression levels of *Tet 1–3* genes (Fig. 5), particularly *Tet-1*, was found in VWAT of SF offspring.

miRNAs 103 and 107 and CAV1 Protein

Expression of miRNAs 103 and 107 (miR-103/107), which regulate the expression of the *Cav1* gene and affect insulin sensitivity in adipose tissues (32,33), was increased in SF offspring mice (Fig. 6A). In concordance with such assumption, we also found reduced CAV1 protein expression in VWAT (Fig. 6B and C).

DISCUSSION

We found that adult male offspring of pregnant mice subjected to late gestational SF exposures exhibited an altered phenotype during adulthood characterized by metabolic dysfunction, which included mild, albeit significant increases in food consumption, higher body weight and fat mass, increased insulin resistance, and systemic elevation of lipid levels. In addition to increased global DNA methylation and reduced HAT activity in VWAT,

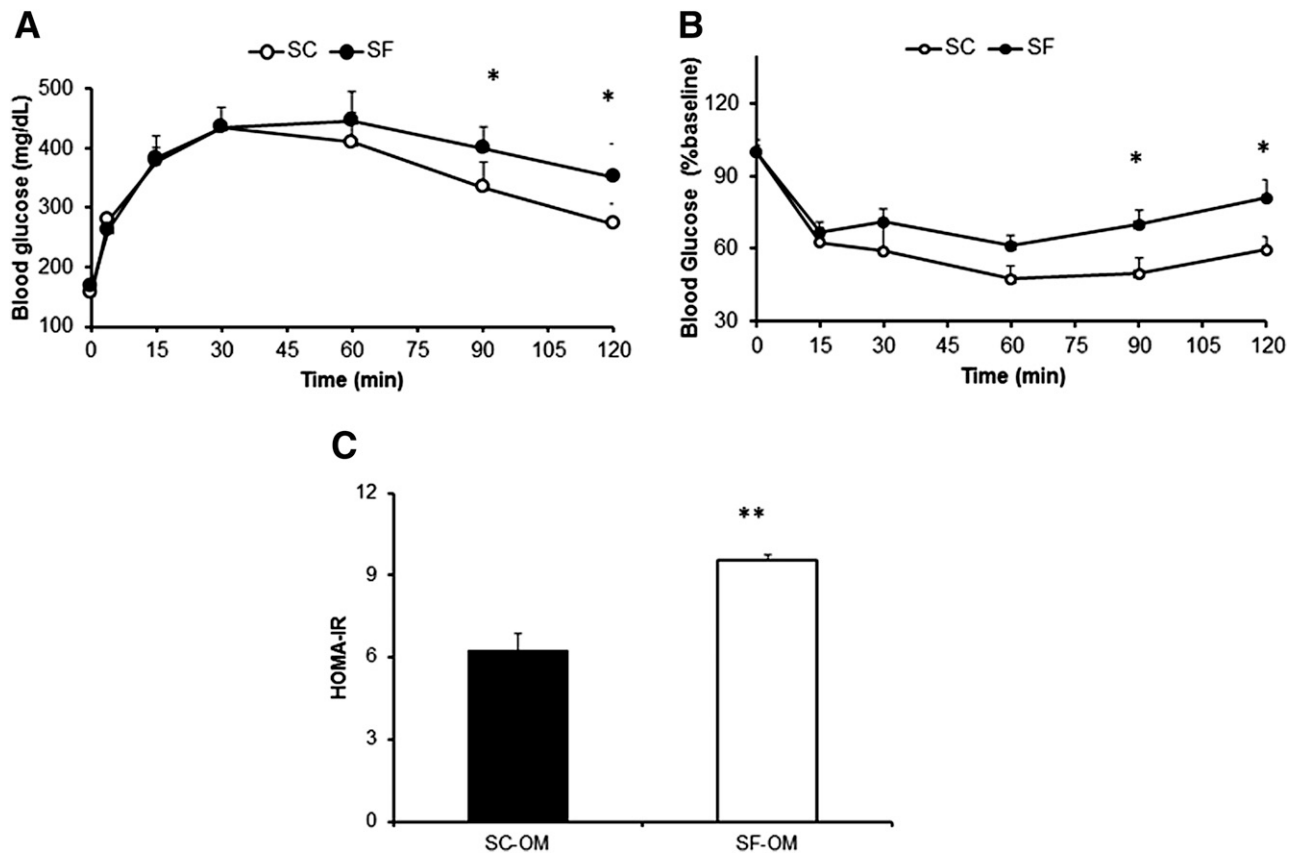


Figure 2—SF exposure in utero increases insulin resistance in offspring mice. GTT (A), ITT (B), and HOMA-IR (C) values at 24 weeks of age in SC and SF were determined. Data are mean ± SE (n = 8/experimental group). *P < 0.01 for SF vs. SC, **P < 0.001.

significant decreases in *AdipoQ* at the mRNA level were present, along with reduction in active 5-hmc and H3K4m3 epigenetic marks and increases in 5-mc and H3K9m2 inactive epigenetic marks, all of which correlated with *AdipoQ* expression levels. We also found increased miR-103/107 expression that was inversely associated with decreased CAV1 protein expression in VWAT, a finding that has been previously implicated in insulin resistance (33). The present findings provide a robust line of evidence linking sleep perturbations during intrauterine life and metabolic fate in adult male offspring

(Fig. 7). Accordingly, current findings provide initial observations on the adverse epigenetic and metabolic consequences associated with disrupted sleep during pregnancy and expand on the potential significance of gestational sleep disorders in human health.

There is now a vast body of epidemiological evidence indicating that the intrauterine and early postnatal environments have a significant long-term influence on body weight and energy homeostasis in the offspring (34). Changes in maternal diet can lead to offspring adiposity and elevation of plasma insulin and glucose levels by 6 months of age (35,36). We found that SF during late gestation induced changes in *AdipoQ* expression in VWAT of adult offspring, which correlated with both epigenetic misregulatory changes in the *AdipoQ* gene as well as with reduced *AdipoQ* plasma levels. It is well known that *AdipoQ* abundance is inversely associated with obesity, insulin resistance and type 2 diabetes, inflammatory markers, and endothelial dysfunction as well as with the development of cardiovascular disease (37). The production of *AdipoQ* by adipocytes is controlled at the level of gene expression through specific promoter regions (38) as well as by epigenetic mechanisms (28). Histone modifications of the *AdipoQ* gene were examined in mice exposed to a maternal high-fat diet (HFD) (39), and increased

Table 1—SF exposures in utero alter plasma lipid profiles and *AdipoQ* levels as well as *AdipoQ* mRNA expression in VWAT of male offspring at 24 weeks of age

	SC	SF	P value
Triglycerides (mg/dL)	127.0 ± 6.12	150.0 ± 7.75	0.003
Total cholesterol (mg/dL)	86.81 ± 2.69	110.64 ± 4.89	0.001
<i>AdipoQ</i> in plasma (µg/mL)	29.22 ± 0.91	23.15 ± 0.58	0.007
<i>AdipoQ</i> mRNA in VWAT	1 ± 0.12	0.68 ± 0.03	0.001

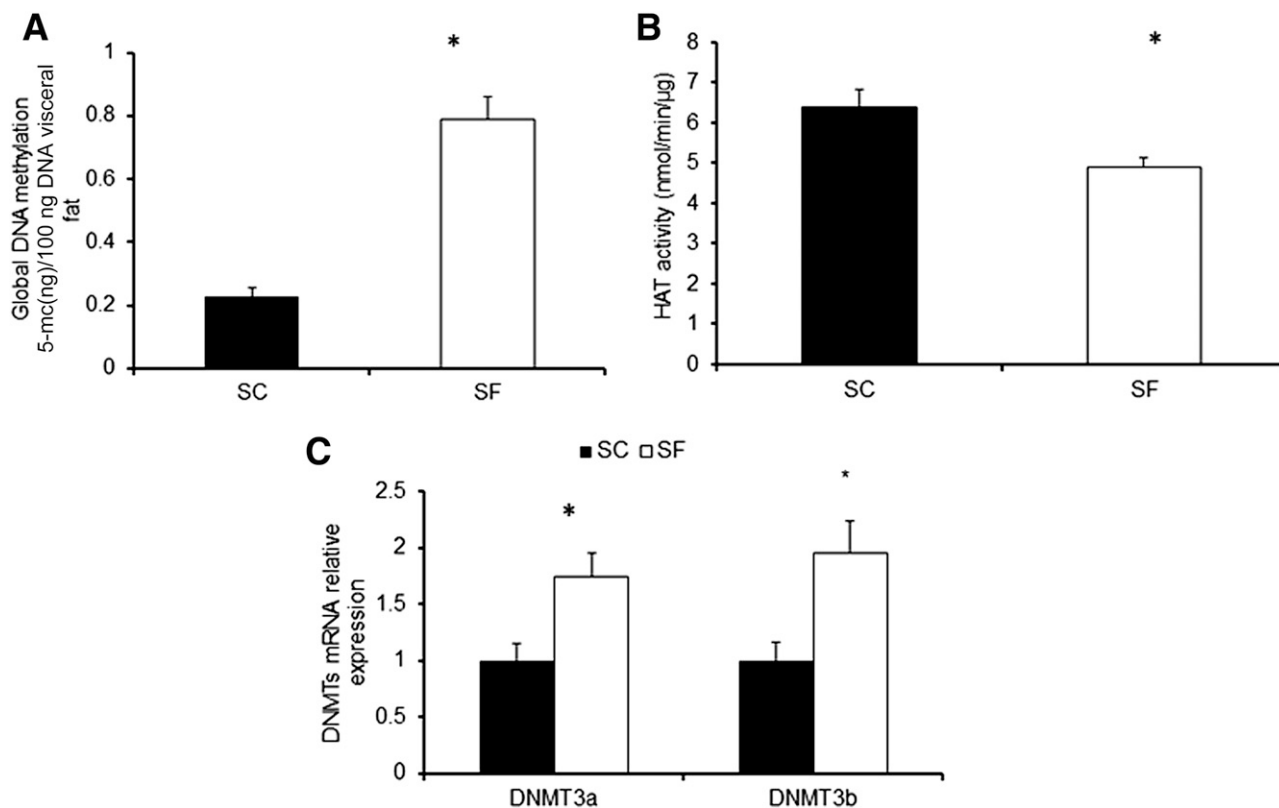


Figure 3—SF exposures in utero alter global DNA methylation (A), HAT activity (B), and DNMT mRNA expression (C) in visceral adipose tissue of offspring mice. $n = 12$ /experimental group. * $P < 0.003$ for global DNA methylation, $P < 0.0001$ for HAT activity, and $P < 0.01$ for DNMT expression.

DNA methylation in the *AdipoQ* promoter region was reported in mice fed an HFD during late gestation (35). In these studies, the acetyl H3K9m2 level in the *AdipoQ* promoter region was lower and the dimethyl H3K9m2 higher in adipose tissue of offspring mice exposed to gestational HFD compared with control mice (39). Of note, H3K9 demethylase is a crucial regulator of genes involved in energy expenditure and fat storage (40,41), and histone modifications of H3K4m3 and H3K9m2 in the promoter region of *AdipoQ* seem to play important roles in adipogenesis (42,43). These epigenetic marks may serve as a memory of exposure, in early life, to inappropriate environments, and these marks may ultimately induce long-term changes in gene expression, potentially leading to disease in later life (44). The overall epigenetic marks identified in the current study as induced by gestational SF are in concurrence with such previously reported findings. Thus, the current study provides biological plausibility to the concept that sleep disorders during pregnancy in general, and more specifically those associated with SF, may induce epigenetic modifications that ultimately promote the risk of a metabolically altered phenotype in the offspring. Notwithstanding such considerations, we should also emphasize that the changes in *AdipoQ* represent a proof of concept that SF during late gestation promotes changes in a cluster of metabolic gene pathways

but in no way exclusively implicates *AdipoQ* as the sole mechanism for the metabolic phenotype described here.

DNA methylation is a key epigenetic contributor to the maintenance of gene silencing and is reprogrammed during development through DNMTs(27). These DNMTs control gene expression by cytosine methylation. Furthermore, the regulation of the expression of DNMTs represents an additional mechanism of epigenetic control (45). In mammals, three *Dnmts* (*Dnmt1*, *Dnmt3a*, and *Dnmt3b*) coordinate the regulation of DNA methylation in the genome. *Dnmt1* promotes DNA methylation after DNA replication and plays a major role in the maintenance of methylation. *Dnmt3a* and *Dnmt3b* are developmentally regulated enzymes required for the initiation of de novo methylation during embryogenesis (46). The present data show that *Dnmt3a* and *Dnmt3b* were significantly upregulated in VWAT in gestational SF-exposed offspring mice compared with SC offspring mice along with reduced HAT activity, both of which are compatible with reduced transcription of the *AdipoQ* gene as illustrated by increased expression of DNMT3a in obese adipose tissue (47).

Developments in epigenetic technologies are showing promising results of DNA methylation levels at a single-base resolution and provide the ability to differentiate between 5-mC and other nucleotide modifications, such as 5-hmC. In addition, the global balance between 5-mC

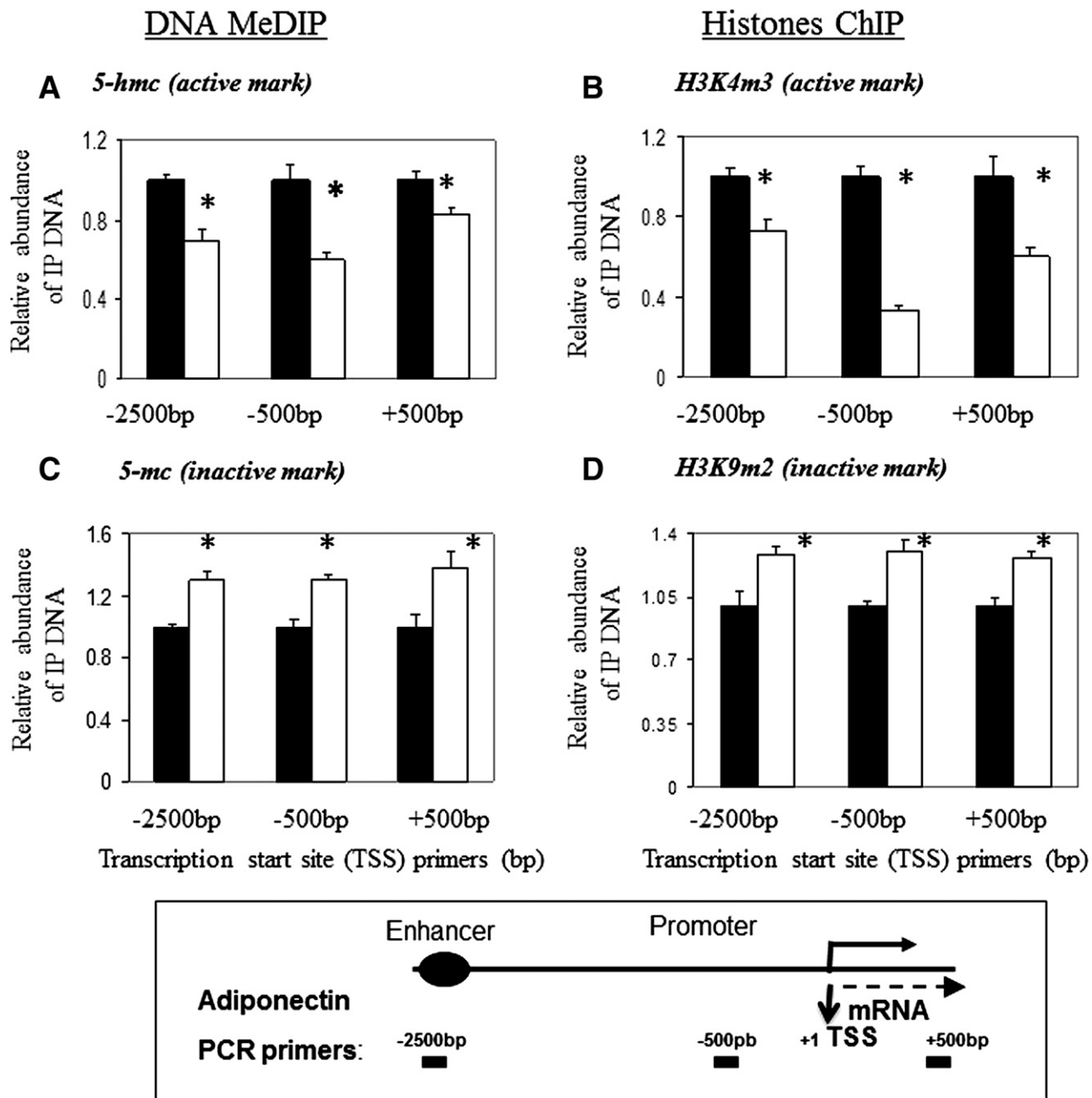


Figure 4—SF exposures during late gestation alter epigenetic markers in the *AdipoQ* gene promoter and enhancer regions in adipocytes from VWAT. Active (5-hmC and H3K4me3) and inactive (5-mC and H3K9me2) marks were mapped to the enhancer (–2,500 bp) and promoter (–500 bp) regions and downstream to the transcription start site (+500 bp) region, respectively, using ChIP assays. **A:** DNA MeDIP of 5-hmC (active mark). **B:** Histones ChIP for H3K4m2 (active mark). **C:** DNA MeDIP for 5-mC (inactive mark). **D:** Histones ChIP for H3K9m2 (inactive mark). Data are mean ± SD (*n* = 8/experimental group). **P* < 0.01. IP, immunoprecipitated.

and 5-hmC in the genome has been described as a critical step for regulating gene expression to maintain cellular functions (48,49). Furthermore, there are three TET enzymes that participate in the conversion of 5-mc to 5-hmc, and these can be further converted into 5-formylcytosine (5-fc) and 5-carboxylcytosine (5-caC) through successive oxidation steps (49,50), raising the possibility that 5-mC distribution can be dynamically regulated by the Tet family of DNA hydroxylases. The decreases in *Tet-1* expression

reported in the present study are therefore in accordance with the reduced *AdipoQ* expression found in VWAT (Table 1).

miRNAs are small, noncoding RNAs that provide posttranscriptional regulation of gene expression and control many metabolic processes and lipid metabolism. They combine with the 3' end of targeted mRNA to regulate the gene expression by degrading mRNA or inhibiting its translation. miRNAs exhibit tissue specificity, and

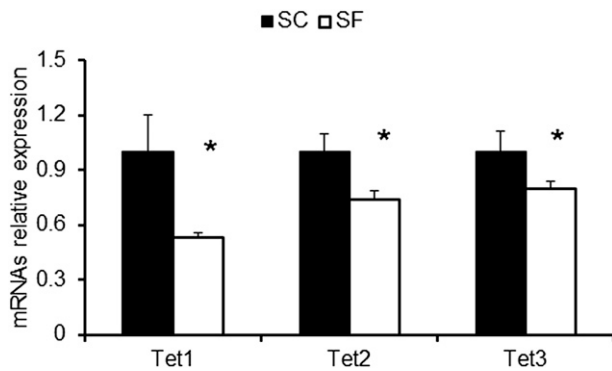


Figure 5—Late gestational SF alters TETs (TET1, TET2, and TET3) family gene expression levels in visceral fat tissues of offspring mice. mRNA levels were normalized to 18S. $n = 8$ /experimental group. * $P < 0.01$.

dysregulation of miRNA expression is associated with obesity and imposes profound effects on the onset and progression of associated metabolic disorders (51). Among the large number of candidate miRNAs, increased miR-103/107 expression has been associated with obesity-induced insulin resistance (33). For example, miR-103/107 expression

levels were upregulated in obese mice, and silencing of these two specific miRNAs improved glucose homeostasis and insulin sensitivity (33). The increased expression of miR-103/107 in SF-exposed offspring is therefore compatible with their assigned metabolic functions. Similarly, it has been shown that *Cav1*, a critical regulator of the insulin receptor, is a direct target gene of miR-103/107 and is downregulated upon miR-103/107 activation in adipocytes (33). Caveolins are 21- to 24-kDa integral membrane proteins that serve as scaffolds to recruit numerous signaling molecules and have been implicated in membrane traffic, signal transduction, substrate transport, and endocytosis (52). *Cav1* gene expression was significantly decreased in visceral adipose tissue of obese subjects (32). Thus, the increased expression of miR-103/107 (Fig. 6) and the reduced expression of *Cav1* are aligned with the insulin resistance of offspring mice born to SF-exposed dams.

This study has several limitations. First, we explored only the last one-third of the gestation period because this period of human pregnancy is the most likely to present with sleep perturbations, such as fragmented sleep. Of note, the magnitude of SF imposed here is clearly in the moderate to severe range compared with the

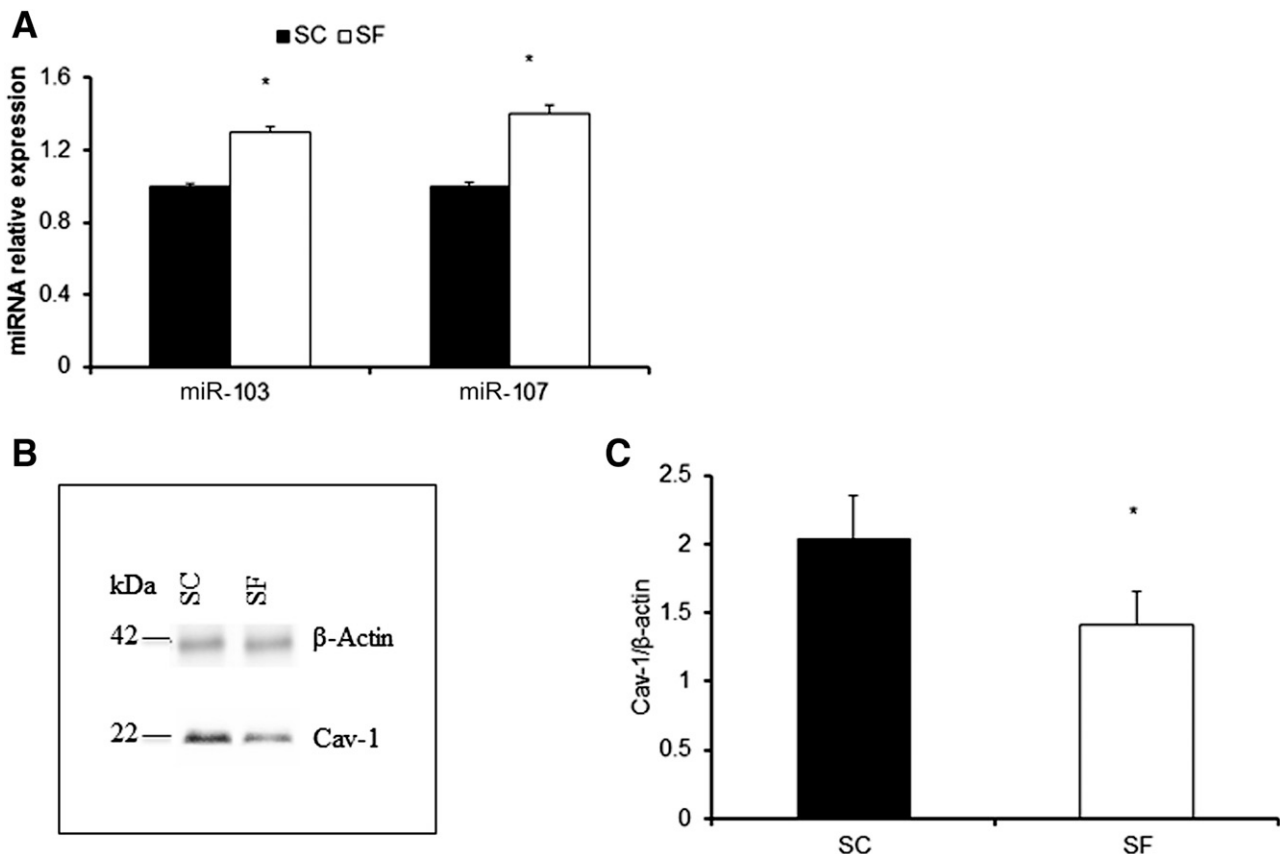


Figure 6—Late gestational SF exposures alter miR-103/107 expression profiles and their target protein *Cav1* in VWAT of offspring mice. **A:** miR-103/107 expression in VWAT. Data were normalized against miR-RNU6 ($n = 8$ /experimental group). * $P < 0.01$. **B:** Western blot for *CAV1* protein expression in VWAT. β -actin was used as control ($n = 6$ /experimental group). **C:** Histogram for signal intensity. * $P < 0.01$.

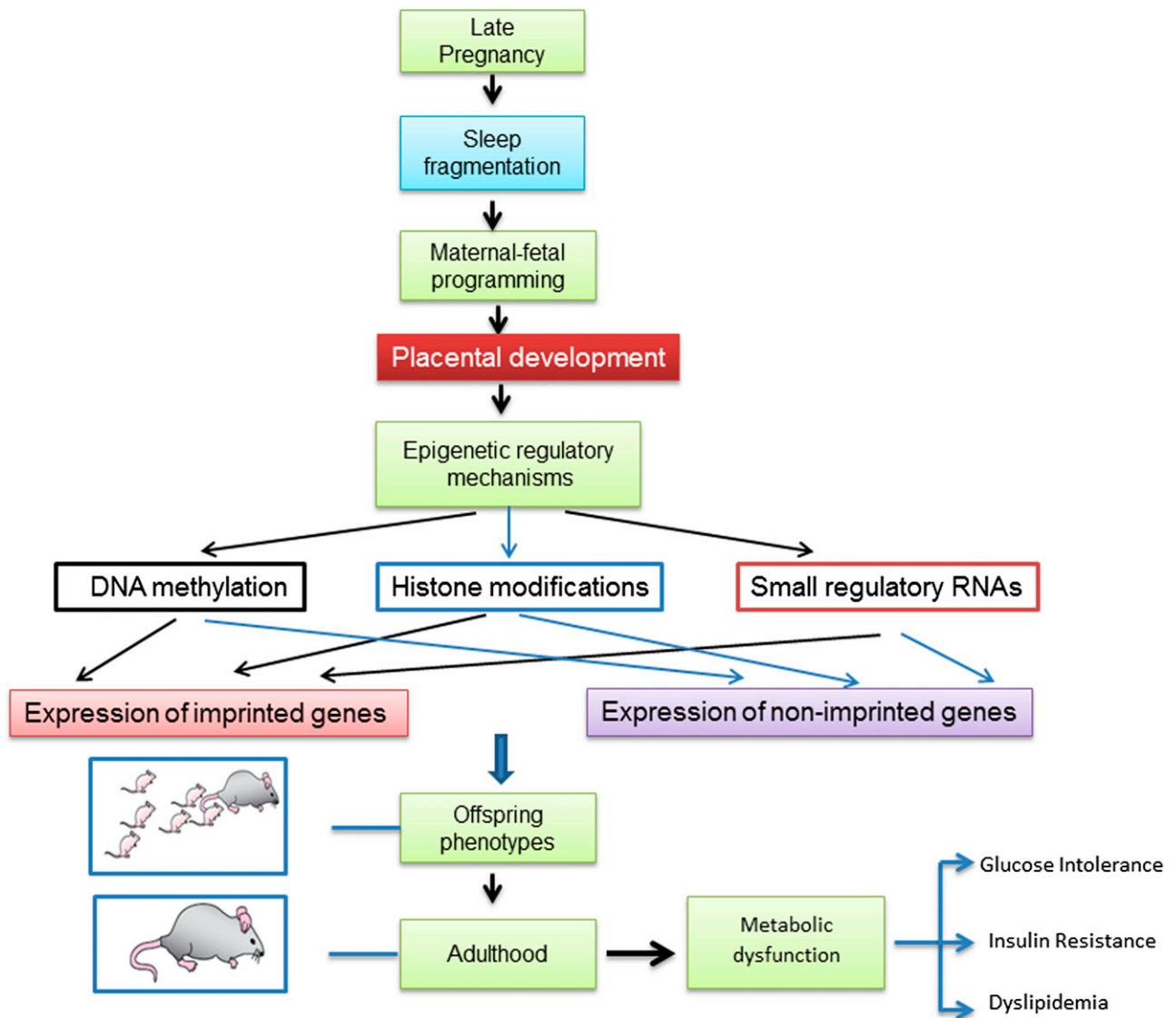


Figure 7—Schematic of the putative interactions between late gestational SF and downstream effects on fetal metabolic reprogramming in offspring.

milder sleep disruption more frequently observed. Therefore, extrapolation from current experiments to humans would be premature. Notwithstanding, it will be important to examine the impact of SF severity and the effect of SF when imposed during other delimited periods of gestation or throughout the duration of gestation. Although body weights were not assessed in pregnant mice during the late gestational period, it is highly unlikely that divergent body weight changes would have occurred during the 5-day SF procedures because accelerated weight accrual was only apparent after 3–4 weeks of SF (53). Second, we restricted our assessments to male offspring to avoid potential hormonally related differences that may obscure the overall phenotypic and epigenetic picture in the experiments, particularly when considering the sex dimorphism that has been reported for some of the epigenetically reported phenotypes (54).

Third, except for somatic weight and food consumption, we did not explore the temporal trajectory of the metabolic alterations in the experiments and examined only their presence during adulthood (i.e., 24 weeks). Therefore, whether all the metabolic and epigenetic changes coincide and become manifest around the same time frame when body weight increases in SF offspring or whether each of the phenotypic characteristics has a different timeline is unclear. In addition, body composition and more accurate adipose tissue distribution patterns were not specifically assessed. Fourth, we explored only one of the visceral adipose tissue depots, namely epididymal fat, and more extensive assessment of such depots and other metabolic organs, such as muscle and liver, for epigenetic marks and metabolic function (e.g., hepatic glucose production) is warranted to enable more reliable extrapolation of the findings to humans. Fifth, it would

have been interesting to see whether interactions between gestational SF and both pre- and postnatal diets or other food supplements (e.g., folic acid) or physical activity either alleviate or exacerbate the SF-induced phenotype in the offspring. Finally, we did not explore whether the epigenetic changes in *AdipoQ* would be transmitted to subsequent generations. Notwithstanding, we believe that the changes in metabolic phenotype in the offspring of SF pregnant dams reflect the occurrence of coordinated epigenetic modifications in gene pathways in a tissue-dependent manner and that this exploration of *AdipoQ* in visceral adipose tissue represents an illustrative and compelling proof of concept that sleep is an important modulator of offspring biological traits through epigenetic mechanisms.

In summary, SF exposures in utero during late gestation lead to a metabolically dysfunctional phenotype in the male offspring mice that includes epigenetic modifications of the *AdipoQ* gene. Given the increasing prevalence of obesity and metabolic disease in the past few decades, it is of crucial importance to understand the mechanisms underlying this process. We propose that fragmented sleep during gestation is an additional and important risk factor for the emergence of metabolic syndrome in the subsequent generation through epigenetic misregulation of tissue-specific target genes such as *AdipoQ* in VWAT.

Funding. This study was supported in part by a Herbert T. Abelson Endowed Chair in Pediatrics and Comer Children's Hospital Research grant to A.K. D.G. is the recipient of National Institutes of Health grants HL-065270 and HL-086662.

Duality of Interest. No potential conflicts of interest relevant to this article were reported.

Author Contributions. A.K. contributed to the performance of the experiments, data analysis, and drafting of the initial version of the manuscript. V.M. contributed to the study design, performance of the experiments, and editing of the manuscript. A.C., A.A.K., and F.H. contributed to the performance of the experiments. D.G. contributed to the study concept and design, data analysis, and writing of the manuscript. D.G. is the guarantor of this work and, as such, had full access to all the data in the study and takes responsibility for the integrity of the data and the accuracy of the data analysis.

References

- Kelsey MM, Zaepfel A, Bjornstad P, Nadeau KJ. Age-related consequences of childhood obesity. *Gerontology* 2014;60:222–228
- Borrell LN, Samuel L. Body mass index categories and mortality risk in US adults: the effect of overweight and obesity on advancing death. *Am J Public Health* 2014;104:512–519
- Barker DJ. The developmental origins of adult disease. *J Am Coll Nutr* 2004; 23(Suppl.):588S–595S
- Barker DJ. The origins of the developmental origins theory. *J Intern Med* 2007;261:412–417
- McMillen IC, Robinson JS. Developmental origins of the metabolic syndrome: prediction, plasticity, and programming. *Physiol Rev* 2005;85:571–633
- Barker DJ, Osmond C, Forsen TJ, Kajantie E, Eriksson JG. Maternal and social origins of hypertension. *Hypertension* 2007;50:565–571
- Armitage JA, Poston L, Taylor PD. Developmental origins of obesity and the metabolic syndrome: the role of maternal obesity. *Front Horm Res* 2008;36: 73–84
- Murabayashi N, Sugiyama T, Zhang L, et al. Maternal high-fat diets cause insulin resistance through inflammatory changes in fetal adipose tissue. *Eur J Obstet Gynecol Reprod Biol* 2013;169:39–44
- Ananth CV, Vintzileos AM. Distinguishing pathological from constitutional small for gestational age births in population-based studies. *Early Hum Dev* 2009;85:653–658
- Grün F, Blumberg B. Endocrine disruptors as obesogens. *Mol Cell Endocrinol* 2009;304:19–29
- Harrison M, Langley-Evans SC. Intergenerational programming of impaired nephrogenesis and hypertension in rats following maternal protein restriction during pregnancy. *Br J Nutr* 2009;101:1020–1030
- O'Keeffe M, St-Onge MP. Sleep duration and disorders in pregnancy: implications for glucose metabolism and pregnancy outcomes. *Int J Obes (Lond)* 2013;37:765–770
- Bourjeily G, Ankner G, Mohsenin V. Sleep-disordered breathing in pregnancy. *Clin Chest Med* 2011;32:175–189
- O'Brien LM, Bullough AS, Owusu JT, et al. Snoring during pregnancy and delivery outcomes: a cohort study. *Sleep* 2013;36:1625–1632
- Pien GW, Schwab RJ. Sleep disorders during pregnancy. *Sleep* 2004;27: 1405–1417
- Izci-Balserak B, Pien GW. The relationship and potential mechanistic pathways between sleep disturbances and maternal hyperglycemia. *Curr Diab Rep* 2014;14:459
- Fox CS, Massaro JM, Hoffmann U, et al. Abdominal visceral and subcutaneous adipose tissue compartments: association with metabolic risk factors in the Framingham Heart Study. *Circulation* 2007;116:39–48
- Gnacińska M, Małgorzewicz S, Stojek M, Łysiak-Szydłowska W, Sworczak K. Role of adipokines in complications related to obesity: a review. *Adv Med Sci* 2009;54:150–157
- Zhou H, Song X, Briggs M, et al. Adiponectin represses gluconeogenesis independent of insulin in hepatocytes. *Biochem Biophys Res Commun* 2005;338: 793–799
- Nawrocki AR, Rajala MW, Tomas E, et al. Mice lacking adiponectin show decreased hepatic insulin sensitivity and reduced responsiveness to peroxisome proliferator-activated receptor gamma agonists. *J Biol Chem* 2006;281:2654–2660
- Kadowaki T, Yamauchi T. Adiponectin and adiponectin receptors. *Endocr Rev* 2005;26:439–451
- Nair D, Zhang SX, Ramesh V, et al. Sleep fragmentation induces cognitive deficits via nicotinamide adenine dinucleotide phosphate oxidase-dependent pathways in mouse. *Am J Respir Crit Care Med* 2011;184:1305–1312
- Ramesh V, Nair D, Zhang SX, et al. Disrupted sleep without sleep curtailment induces sleepiness and cognitive dysfunction via the tumor necrosis factor- α pathway. *J Neuroinflammation* 2012;9:91
- Carreras A, Kayali F, Zhang J, Hirotsu C, Wang Y, Gozal D. Metabolic effects of intermittent hypoxia in mice: steady versus high-frequency applied hypoxia daily during the rest period. *Am J Physiol Regul Integr Comp Physiol* 2012;303: R700–R709
- Mutskov V, Felsenfeld G. The human insulin gene is part of a large open chromatin domain specific for human islets. *Proc Natl Acad Sci U S A* 2009;106: 17419–17424
- Mutskov VJ, Farrell CM, Wade PA, Wolffe AP, Felsenfeld G. The barrier function of an insulator couples high histone acetylation levels with specific protection of promoter DNA from methylation. *Genes Dev* 2002;16:1540–1554
- Mutskov V, Felsenfeld G. Silencing of transgene transcription precedes methylation of promoter DNA and histone H3 lysine 9. *EMBO J* 2004;23:138–149
- Koshiishi C, Park HM, Uchiyama H, Tanaka Y. Regulation of expression of the mouse adiponectin gene by the C/EBP family via a novel enhancer region. *Gene* 2008;424:141–146
- Jaenisch R, Bird A. Epigenetic regulation of gene expression: how the genome integrates intrinsic and environmental signals. *Nat Genet* 2003;33 (Suppl.):245–254

30. Wu H, Zhang Y. Mechanisms and functions of Tet protein-mediated 5-methylcytosine oxidation. *Genes Dev* 2011;25:2436–2452
31. Williams K, Christensen J, Pedersen MT, et al. TET1 and hydroxymethylcytosine in transcription and DNA methylation fidelity. *Nature* 2011;473:343–348
32. Fernández-Real JM, Catalán V, Moreno-Navarrete JM, et al. Study of caveolin-1 gene expression in whole adipose tissue and its subfractions and during differentiation of human adipocytes. *Nutr Metab (Lond)* 2010;7:20
33. Trajkovski M, Hausser J, Soutschek J, et al. MicroRNAs 103 and 107 regulate insulin sensitivity. *Nature* 2011;474:649–653
34. Gluckman PD, Hanson MA, Beedle AS. Non-genomic transgenerational inheritance of disease risk. *Bioessays* 2007;29:145–154
35. Khalyfa A, Carreras A, Hakim F, Cunningham JM, Wang Y, Gozal D. Effects of late gestational high-fat diet on body weight, metabolic regulation and adipokine expression in offspring. *Int J Obes (Lond)* 2013;37:1481–1489
36. Tamashiro KL, Moran TH. Perinatal environment and its influences on metabolic programming of offspring. *Physiol Behav* 2010;100:560–566
37. Gavrilu A, Chan JL, Yiannakouris N, et al. Serum adiponectin levels are inversely associated with overall and central fat distribution but are not directly regulated by acute fasting or leptin administration in humans: cross-sectional and interventional studies. *J Clin Endocrinol Metab* 2003;88:4823–4831
38. Barth N, Langmann T, Schölmerich J, Schmitz G, Schäffler A. Identification of regulatory elements in the human adipose most abundant gene transcript-1 (apM-1) promoter: role of SP1/SP3 and TNF-alpha as regulatory pathways. *Diabetologia* 2002;45:1425–1433
39. Masuyama H, Hiramatsu Y. Effects of a high-fat diet exposure in utero on the metabolic syndrome-like phenomenon in mouse offspring through epigenetic changes in adipocytokine gene expression. *Endocrinology* 2012;153:2823–2830
40. Inagaki T, Tachibana M, Magoori K, et al. Obesity and metabolic syndrome in histone demethylase JHDM2a-deficient mice. *Genes Cells* 2009;14:991–1001
41. Tateishi K, Okada Y, Kallin EM, Zhang Y. Role of Jhdm2a in regulating metabolic gene expression and obesity resistance. *Nature* 2009;458:757–761
42. Musri MM, Corominola H, Casamitjana R, Gomis R, Párrizas M. Histone H3 lysine 4 dimethylation signals the transcriptional competence of the adiponectin promoter in preadipocytes. *J Biol Chem* 2006;281:17180–17188
43. Sakurai N, Mochizuki K, Goda T. Modifications of histone H3 at lysine 9 on the adiponectin gene in 3T3-L1 adipocytes. *J Nutr Sci Vitaminol (Tokyo)* 2009;55:131–138
44. Attig L, Gabory A, Junien C. Early nutrition and epigenetic programming: chasing shadows. *Curr Opin Clin Nutr Metab Care* 2010;13:284–293
45. Griffiths EA, Gore SD. MicroRNA: miR-ly regulators of DNMT? *Blood* 2009;113:6269–6270
46. Okano M, Bell DW, Haber DA, Li E. DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. *Cell* 1999;99:247–257
47. Kamei Y, Suganami T, Ehara T, et al. Increased expression of DNA methyltransferase 3a in obese adipose tissue: studies with transgenic mice. *Obesity (Silver Spring)* 2010;18:314–321
48. Ito S, D'Alessio AC, Taranova OV, Hong K, Sowers LC, Zhang Y. Role of Tet proteins in 5mC to 5hmC conversion, ES-cell self-renewal and inner cell mass specification. *Nature* 2010;466:1129–1133
49. Tahiliani M, Koh KP, Shen Y, et al. Conversion of 5-methylcytosine to 5-hydroxymethylcytosine in mammalian DNA by MLL partner TET1. *Science* 2009;324:930–935
50. Ito S, Shen L, Dai Q, et al. Tet proteins can convert 5-methylcytosine to 5-formylcytosine and 5-carboxylcytosine. *Science* 2011;333:1300–1303
51. Hulsmans M, De Keyzer D, Holvoet P. MicroRNAs regulating oxidative stress and inflammation in relation to obesity and atherosclerosis. *FASEB J* 2011;25:2515–2527
52. Frühbeck G, López M, Diéguez C. Role of caveolins in body weight and insulin resistance regulation. *Trends Endocrinol Metab* 2007;18:177–182
53. Wang Y, Carreras A, Lee SH, et al. Chronic sleep fragmentation promotes obesity in young adult mice. *Obesity (Silver Spring)* 2014;22:758–762
54. Gabory A, Attig L, Junien C. Sexual dimorphism in environmental epigenetic programming. *Mol Cell Endocrinol* 2009;304:8–18