Folate treatment of pregnant rat dams abolishes metabolic effects in female offspring induced by a paternal pre-conception unhealthy diet

Jian Li¹ · Yong-Ping Lu^{2,3} · Oleg Tsuprykov^{2,4} · Ahmed A. Hasan^{2,5} · Christoph Reichetzeder² · Mei Tian¹ · Xiao Li Zhang¹ · Qin Zhang¹ · Guo-Ying Sun¹ · Jingli Guo^{2,6} · Mohamed M. S. Gaballa^{2,7} · Xiao-Ning Peng¹ · Ge Lin^{8,9,10} · Berthold Hochoer^{1,2}

Received: 1 June 2017 / Accepted: 16 April 2018 / Published online: 18 May 2018
© Springer-Verlag GmbH Germany, part of Springer Nature 2018

Abstract

Aims/hypothesis Paternal high-fat diet prior to mating programmes impaired glucose tolerance in female offspring. We examined whether the metabolic consequences in offspring could be abolished by folate treatment of either the male rats before mating or the corresponding female rats during pregnancy.

Methods Male F0 rats were fed either control diet or high-fat, high-sucrose and high-salt diet (HFSSD), with or without folate, before mating. Male rats were mated with control-diet-fed dams. After mating, the F0 dams were fed control diet with or without folate during pregnancy.

Results Male, but not female offspring of HFSSD-fed founders were heavier than those of control-diet-fed counterparts ($p < 0.05$ and $p = 0.066$ in males and females, respectively). Both male and female offspring of HFSSD-fed founders were longer compared with control ($p < 0.01$ for both sexes). Folate treatment of the pregnant dams abolished the effect of the paternal diet on the offspring's body length ($p < 0.05$). Female offspring of HFSSD-fed founders developed impaired glucose tolerance, which was restored by folate treatment of the dams during pregnancy. The beta cell density per pancreatic islet was decreased in offspring of HFSSD-fed rats (−20% in male and −15% in female F1 offspring, $p < 0.001$ vs controls). Folate treatment significantly increased the beta cell density (4.3% and 3.3% after folate supplementation given to dams and founders, respectively, $p < 0.05$ vs the offspring of HFSSD-fed male rats). Changes in liver connective tissue of female offspring of HFSSD-fed founders were ameliorated by treatment of dams with folate ($p < 0.01$). Hepatic *Ppara* gene expression was upregulated in female offspring only (1.51-fold, $p < 0.05$) and was restored in the female offspring by folate treatment ($p < 0.05$). We observed an increase in hepatic *Lcn2* and *Tmcc2* expression in female offspring born to male rats exposed to an unhealthy diet during spermatogenesis

Jian Li, Yong-Ping Lu and Oleg Tsuprykov contributed equally to this work.

Electronic supplementary material The online version of this article (<https://doi.org/10.1007/s00125-018-4635-x>) contains peer-reviewed but unedited supplementary material, which is available to authorised users.

✉ Berthold Hochoer
hochoer@uni-potsdam.de

¹ Key Laboratory of Study and Discovery of Small Targeted Molecules of Hunan Province, School of Medicine, Hunan Normal University, Changsha 410013, China

² Institute of Nutritional Science, University of Potsdam, Nuthetal, Germany

³ Department of Nephrology, Charité - Universitätsmedizin Berlin, Campus Mitte, Berlin, Germany

⁴ Institute for Laboratory Medicine, IFLB, Berlin, Germany

⁵ Department of Biochemistry, Faculty of Pharmacy, Zagazig University, Zagazig, Egypt

⁶ Center for Cardiovascular Research, Charité - Universitätsmedizin Berlin, Campus Mitte, Berlin, Germany

⁷ Faculty of Veterinary Medicine, Benha University, Moshthohor, Toukh, Egypt

⁸ Institute of Reproductive and Stem Cell Engineering, College of Basic of Medicine, Central South University, Changsha, China

⁹ Reproductive and Genetic Hospital of CITIC-Xiangya, Changsha, China

¹⁰ Key Laboratory of Reproductive and Stem Cell Engineering, National Health and Family Planning Commission, Changsha, China

Research in context

What is already known about this subject?

- Maternal nutrition in early life/during pregnancy may cause permanent alterations to the function and structure of the offspring organs
- Recent studies indicate that paternal nutrition during sperm maturation/before mating may also result in permanent alteration of the offspring organs
- The underlying mechanisms are parental nutrition-induced epigenetic alterations in the offspring

What is the key question?

- Can a high-fat, high-sucrose and high-salt diet given to male rats before mating (mimicking a fast-food diet often consumed by young men) induce a metabolic phenotype in the offspring and will folate treatment affect the resulting phenotype?

What are the new findings?

- Female offspring displayed impaired glucose tolerance, most likely due to alterations in gene expression of key metabolic genes in the offspring liver. Altered gene expression was accompanied by alterations in the promotor methylation pattern of these genes
- Folate treatment of pregnant dams reversed effects on female offspring's glucose metabolism induced by pre-conception unhealthy diet in male founders

How might this impact on clinical practice in the foreseeable future?

- This study demonstrates that paternal programming induced by a fast-food-like diet prior to mating is treatable with folate, a drug known to be safe in human pregnancy, and thus might stimulate translational studies in humans

before mating ($p < 0.05$ vs controls). Folate treatment of the corresponding dams during pregnancy abolished this effect ($p < 0.05$). Analysis of DNA methylation levels of CpG islands in the *Ppara*, *Lcn2* and *Tmcc2* promoter regions revealed that the paternal unhealthy diet induced alterations in the methylation pattern. These patterns were also affected by folate treatment. Total liver DNA methylation was increased by 1.52-fold in female offspring born to male rats on an unhealthy diet prior to mating ($p < 0.05$). This effect was abolished by folate treatment during pregnancy ($p < 0.05$ vs the offspring of HFSSD-fed male rats).

Conclusions/interpretation Folate treatment of pregnant dams restores effects on female offspring's glucose metabolism induced by pre-conception male founder HFSSD.

Keywords Glucose tolerance · High-fat-sucrose-salt diet · Maternal folate treatment · Paternal programming

Abbreviations

5-MC%	Per cent 5-methylcytosine
HFSSD	High-fat, high-sucrose and high-salt diet
miRNA	microRNA

Introduction

The ‘fetal programming’ hypothesis proposes that adulthood metabolic disease originates through adaptation of the fetus in early development [1]. These adaptations are tissue-specific, persist throughout life and may cause metabolic diseases in later life [2–5].

The classical events involved in fetal programming are of maternal origin [6–12] but paternal factors may also alter the

epigenome and phenotype of offspring [13–15]. Feeding male rat founders a high-fat diet before mating induces impaired glucose tolerance in female offspring [16], possibly due to epigenetic adaptations in the pancreas and liver. Treatment approaches for paternal diet-induced adverse metabolic effects in offspring include physical activity, antioxidants and improvement in pre-mating diet [17, 18]. Previously, we fed a diet resembling an unhealthy ‘western’ diet (high-fat, high-sucrose and high-salt diet [HFSSD], often consumed by men) to male rats prior to mating and analysed the effect on glycaemic control in offspring. Since folate treatment in low-protein-diet-fed pregnant rats improves glycaemic control [19, 20], here we investigated the effects of paternal folate (folic acid) treatment before mating, as well as maternal folate treatment during pregnancy, on offspring phenotype. The

unhealthy paternal diet was given before mating, during spermatogenesis, since it is known that a high-fat diet induces adverse effects on non-coding RNA in the sperm and has long-lasting adverse effects on the offspring [21–23].

Methods

Animals

The present study was performed in Sprague-Dawley rats of both sexes, including F0 generation (45 male rats, 32 female rats) and F1 generation animals. The F0 generation rats were purchased at the age of 4 weeks from Hunan SJA Laboratory Animal (Changsha, China). After acclimatisation to their new environment for 1 week, the rats were given a specific diet. The rats were housed in temperature-controlled chambers under control lighting with 12 h light–dark cycles. All rats were allowed free access to water and food. The experimental protocols were conducted in accordance with the ethical standards of the local ethics committee. See electronic supplementary material (ESM) [Methods](#) for further details.

Study design

F0 male rats were randomly divided into groups, each of which received one of the following diets: (1) control diet + tap water by oral gavage ($n = 15$); (2) HFSSD + tap water by oral gavage ($n = 15$) or (3) HFSSD + folate (HFSSD+F) at a daily dose of 3 mg/kg dissolved in tap water and given by oral gavage ($n = 15$). See ESM Table 1 for further details. The daily folate dosage of 3 mg/kg is in accordance with previous publications [24, 25].

The 14-week-old F0 founder male rats fed either the control diet or one of the two fat-rich diets were mated with F0 12-week-old, normal-weight, naturally cycling dams fed a control diet to produce F1 offspring. Depending on paternal diet before mating and on maternal diet after mating and throughout the gestational period, the F1 offspring of both sexes were allocated into one out of four study groups (Fig. 1):

PatCD/MatCD group—offspring of control-diet-fed founders and control-diet-fed dams (dams were fed a control diet from the beginning of gestation until delivery)

PatHFSSD/MatCD group—offspring of HFSSD-fed founders and control-diet-fed dams from 50% of obtained litters (dams were fed a control diet from the beginning of gestation until delivery)

PatHFSSD/MatCD+F group—offspring of HFSSD-fed founders and control-diet-fed dams from another 50% of obtained litters (dams were fed a control diet+folate [5 mg/kg daily in food] from the beginning of gestation until delivery, a folate intake comparable with that

currently recommended for women in the UK before pregnancy and during the first trimester [20, 24])

PatHFSSD+F/MatCD group—offspring of HFSSD+F-fed founders and control-diet-fed dams (dams were fed a control diet from the beginning of gestation until delivery).

The total mean (SEM) number of offspring per litter was 13.9 ± 0.3 and did not differ significantly between the groups. We sampled randomly 173 offspring (86 male offspring, 87 female offspring) out of a total of 540. Thus, the sample size was around one-third of the entire population of offspring. After delivery, all F1 offspring were fed a normal diet until adulthood. The composition of the normal diet was very similar to the control diet; the normal diet was used for reasons of economy (see ESM [Methods](#) for details). All the F0 founder male rats at the age of 18 weeks and all the F1 rats at the age of 15 weeks were killed after receiving deep anaesthesia. Blood samples were collected and the organs were harvested.

Real-time quantitative PCR

Expression levels of hepatic and pancreatic genes were assessed by real-time quantitative PCR using a Bio-Rad CFX96 cycler (Bio-Rad Laboratories, USA) and using standard protocol (see ESM [Methods](#) and ESM Table 2).

RNA sequencing in the liver

RNA sequencing was carried out in collaboration with Oebiotech (Shanghai, China). Total RNA was extracted from the livers of the PatCD/MatCD and PatHFSSD/MatCD F1 female offspring using the mirVana miRNA Isolation Kit (Ambion, Foster City, CA, USA) following the manufacturer's protocol. RNA integrity was evaluated using the Agilent 2100 Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA). See ESM [Methods](#) and ESM Table 3 for further details.

Global and gene-specific DNA methylation in the liver

DNA was extracted from the liver and the concentration and purity were assessed spectrophotometrically. Global DNA methylation was determined as per cent 5-methylcytosine (5-MC%) using MethylFlash Global DNA Methylation (5-mC) ELISA Easy Kit (Epigentek, Farmingdale, NY, USA). Gene-specific DNA methylation of CpG islands of the promoter region of *Ppara*, *Lcn2* and *Tmcc2* was analysed using MethylTarget, based on Illumina next-generation sequencing in combination with bisulfite treatment and DNA methylation mapping as previously described [26–28]. Illumina next-generation sequencing was carried out in collaboration with

Fig. 1 Study design for the F0 and F1 generations. F0 male rats were randomly divided into one of the following three study groups according to the diet type: (1) control diet + tap water by oral gavage (CD); (2) HFSSD + tap water by oral gavage; and (3) HFSSD + folate 3 mg/kg body weight daily, dissolved in tap water and given by oral gavage (HFSSD+F). F1 offspring of both sexes were allocated into one of four study groups: PatCD/MatCD—offspring of CD-fed founders and CD-fed dams (dams were fed a CD from the beginning of gestation until delivery); PatHFSSD/MatCD—offspring of HFSSD-fed founders and CD-fed

dams from 50% of obtained litters (dams were fed a CD from the beginning of gestation until delivery); PatHFSSD/MatCD+F—offspring of HFSSD-fed founders and CD-fed dams from another 50% of obtained litters (dams were fed a CD+F [5 mg/kg body weight per day folate in food] from the beginning of gestation until delivery) and PatHFSSD+F/MatCD—offspring of HFSSD+F-fed founders and CD-fed dams (dams were fed a CD from the beginning of gestation until delivery)

Genesky Biotechnologies (Shanghai, China). See ESM [Methods](#) and ESM Table 4 for further details.

Metabolic tests

When F1 offspring rats were aged 100 days, an OGTT was performed following 12 h of starvation. The offspring were administered 2 g/kg glucose (50% [wt/vol.] glucose solution) by gavage and serum glucose levels were measured (Sannuo glucometer; San Nuo, Changsha, China) at 0, 30, 60 and 120 min after glucose ingestion. Serum glucose, cholesterol, triacylglycerols, HDL-cholesterol, LDL-cholesterol, alanine aminotransferase, aspartate transaminase, blood urea nitrogen and creatinine were measured using Hitachi 7020 automatic biochemistry analyser (Hitachi High-Technologies, Tokyo, Japan). Serum insulin levels were determined by a rat insulin ELISA kit (Millipore, Billerica, MA, USA). See ESM [Methods](#) for further details.

Pancreas and liver morphology

Liver and pancreas samples were fixed, sectioned and stained with H&E for oil droplet visualisation and Sirius Red for fibrosis assessment and were immunostained for CD68 (ED-1, rabbit polyclonal anti-CD68 antibody, 1:50; Abcam, Cambridge, UK) to evaluate periportal inflammation. Pancreatic slices were H&E stained to assess islet density and number and were immunostained for insulin (guinea pig anti-insulin, 1:200; Abcam) to detect beta cell density per islet. The samples were examined with light microscopy using a BZ

9000 microscope (Keyence, Neu-Isenburg, Germany). See ESM [Methods](#) for further details.

Liver triacylglycerol measurement

Liver triacylglycerol levels were measured using an enzyme immunoassay kit (Kehua Bio-Engineering, Shanghai, China) and an automatic biochemistry analyser (Hitachi High-Technologies, Tokyo, Japan). See ESM [Methods](#) for details.

Statistics

All data are presented as means $\pm$ SEM. Two-way analysis of variance with Bonferroni post hoc test was used to analyse the body weight gain and OGTT data. For all other data, one-way analysis of variance followed by least significant difference test was applied. To account for potential bias, additional ANCOVA models considering litter size as a covariate were calculated. A p value of <0.05 was considered statistically significant. The data were analysed using SPSS version 20.0 (SPSS, Chicago, IL, USA) and GraphPad Prism version 5 (GraphPad Software, San Diego, CA, USA).

Results

F0 founders

Detailed data on body weight gain and selected metabolite serum levels in the F0 founders are presented in ESM

Table 5. The number of pups was similar in all groups; no effect of HFSSD or folate treatment on the number of pups was detectable.

F1 offspring

Body weight and length In the F1 generation, a paternal HFSSD diet resulted in body weight increase, which appeared to be more pronounced in female offspring (ESM Fig. 1). The male and female PatHFSSD/MatCD offspring were 8% and 6% heavier than their control littermates, respectively ($p < 0.05$ in male offspring, $p = 0.066$ in female offspring). Folate intervention given to pregnant dams and folate food supplementation given to founders before mating both failed to reduce F1 progeny body weight.

At the end of the study, male and female PatHFSSD/MatCD offspring were 3.7% and 3.2% longer than their control littermates, respectively ($p < 0.01$ for both sexes; Tables 1 and 2). In male and female offspring, folate given to pregnant dams decreased body length (by 3.0% and 2.7%, $p < 0.05$ and $p < 0.01$ vs PatHFSSD/MatCD, respectively). Folate given to founders before mating failed to reduce F1 progeny body length (Tables 1 and 2). The liver weight was increased in female PatHFSSD/MatCD offspring ($p < 0.01$ vs PatCD/MatCD) but not in the male offspring.

Glucose tolerance Feeding the F0 founders with HFSSD impaired the glucose homeostasis of their F1 offspring in a sex-specific manner. Female F1 progeny were more susceptible to glucose intolerance, as assessed by OGTT. When F1 female offspring of the PatHFSSD/MatCD group were 100 days old, serum glucose levels were elevated 60 min after glucose intake in the OGTT compared with levels in the PatCD/MatCD offspring ($p < 0.001$; Fig. 2b) and the AUC for glucose following the OGTT was also increased ($p < 0.01$ for PatHFSSD/MatCD vs PatCD/MatCD; Fig. 2d). In contrast to the F1 females, there were no significant alterations in glucose homeostasis in male F1 offspring except for serum glucose levels, which were elevated 30 min after glucose intake in the PatHFSSD/MatCD group compared with levels in the PatCD/MatCD offspring ($p < 0.05$; Fig. 2a, c).

In female F1 offspring, folate intervention given to pregnant dams (group) restored glucose tolerance ($p < 0.05$ for PatHFSSD/MatCD+F vs PatHFSSD/MatCD, 60 min after glucose intake, Fig. 2b) and significantly decreased glucose AUC following the OGTT ($p < 0.01$ for PatHFSSD/MatCD+F vs PatHFSSD/MatCD, Fig. 2d). Folate supplementation given to F0 founder male rats (PatHFSSD+F/MatCD) failed to restore glucose tolerance in their F1 offspring (Fig. 2a, b).

With the exception of lowered fasting glucose in PatHFSSD+F/MatCD vs PatHFSSD/MatCD male F1 offspring ($p < 0.01$), there were no significant differences in

fasting glucose and insulin levels between the groups of male and female F1 offspring (Tables 1 and 2).

Liver morphology Male and female F1 offspring of HFSSD-fed F0 founder rats revealed mild hepatic steatosis ($p < 0.01$ and $p < 0.05$ vs PatCD/MatCD, respectively; Fig. 3a, b) manifesting as microvesicular steatosis (Fig. 3c, d). Minor hepatocyte degeneration was observed in all paternally programmed groups (data not shown). In PatHFSSD/MatCD offspring, the hepatic steatosis score was increased by 57% ($p < 0.01$) and 52% ($p < 0.05$) vs control male and female counterparts, respectively (Fig. 3a, b). Neither of the folate intervention groups showed any significant beneficial effects regarding steatosis.

In female F1 offspring born to male rats exposed to the HFSSD diet prior to mating liver fibrosis was increased (65% increase in periportal fibrosis score; $p < 0.01$ PatHFSSD/MatCD vs PatCD/MatCD; Fig. 4b). The increase in liver fibrosis in the female offspring was abolished by folate treatment of the dams during pregnancy ($p < 0.01$ PatHFSSD/MatCD+F vs PatHFSSD/MatCD, Fig. 4b). None of the study groups in the F1 male progeny showed any fibrotic changes around portal vessels. There were no statistical differences in parenchymal fibrosis levels and inflammation between any of the study groups in male offspring (Fig. 4a and Table 1). Liver triacylglycerol content did not significantly differ between the groups of F1 offspring, regardless of the sex (Tables 1 and 2).

Hepatic gene expression We selected genes from studies showing that glucose tolerance in offspring was clearly differentially regulated as a result of paternal high-fat diet during roughly two full rounds of spermatogenesis before mating [16, 29, 30]. Hepatic expression levels of *Lcn2* mRNA were affected in both male and female F1 offspring of fathers fed HFSSD; folate treatment of parent rats normalised *Lcn2* expression levels (Fig. 5b). Hepatic expression of *Ppara* mRNA was elevated in female F1 offspring only (1.51-fold, $p < 0.05$) and was restored by folate treatment given to both dams and founders ($p < 0.05$), whereas *Ppara* mRNA revealed no significant regulation in male F1 progeny (Fig. 5a). The analysis of hepatic mRNA expression of other genes (selected either based on a hypothesis-driven approach or based on microarray data) revealed only minor differences between study groups in both male and female F1 progeny (Table 3).

To identify yet unknown genes that are differentially expressed in offspring of male rats given an unhealthy diet prior to mating, we performed a liver whole-genome array RNA sequencing approach. We chose the most promising candidate genes based on p values and fold change in the arrays and conducted real-time quantitative PCR (Table 3, Fig. 5a, b). The most prominent alterations were seen in the expression of *Lcn2* and *Tmcc2* genes which showed elevation in female offspring born to male rats exposed to an

Table 1 Body length, organ weights, pancreas and liver morphology and serum metabolites in male F1 offspring

Variable	PatCD/MatCD	PatHFSSD/ MatCD	PatHFSSD/ MatCD+F	PatHFSSD+F/ MatCD
Body length and organ weights				
Final body length (cm)	24.11 ± 0.22	25.01 ± 0.21**	24.27 ± 0.24 [†]	24.67 ± 0.20
Heart weight (g)	1.06 ± 0.04	1.18 ± 0.02**	1.10 ± 0.02 [†]	1.09 ± 0.02 ^{††}
Liver weight (g)	11.03 ± 0.69	11.87 ± 0.40	11.87 ± 0.30	12.03 ± 0.28
Left kidney weight (g)	1.27 ± 0.05	1.39 ± 0.03*	1.37 ± 0.02	1.36 ± 0.03
Right kidney weight (g)	1.31 ± 0.05	1.40 ± 0.03*	1.38 ± 0.02	1.38 ± 0.04
Relative heart weight (% of body weight)	0.27 ± 0.01	0.28 ± 0.01	0.26 ± 0.01 [†]	0.25 ± 0.01 ^{††}
Relative liver weight (% of body weight)	2.75 ± 0.08	2.76 ± 0.07	2.81 ± 0.05	2.79 ± 0.04
Relative left kidney weight (% of body weight)	0.32 ± 0.01	0.33 ± 0.01	0.33 ± 0.01	0.32 ± 0.01
Relative right kidney weight (% of body weight)	0.33 ± 0.01	0.33 ± 0.01	0.33 ± 0.01	0.32 ± 0.01
Glucose metabolism variables on day 105 of the study				
Fasting serum glucose (mmol/l)	8.04 ± 0.35	8.77 ± 0.25	8.03 ± 0.26	7.50 ± 0.31 ^{††}
Fasting serum insulin (pmol/l)	337.744 ± 74.10	378.99 ± 65.14	430.87 ± 65.35	336.00 ± 86.95
Pancreas morphology				
Total islet area (% of pancreas surface area)	0.61 ± 0.33	0.45 ± 0.36	0.50 ± 0.41	0.63 ± 0.40
Total no. of islets/mm ² pancreas surface area	2.36 ± 0.29	1.85 ± 0.20	1.59 ± 0.21	2.06 ± 0.28
Percentage of small islets (0–5000 μm ²) (% of total islet number)	82.38 ± 5.69	81.13 ± 5.01	81.81 ± 4.42	79.94 ± 5.86
Percentage of medium islets (5001–10,000 μm ²) (% of total islet number)	10.5 ± 3.17	12.87 ± 3.93	13.24 ± 4.37	10.94 ± 2.86
Percentage of large islets (>10,000 μm ²) (% of total islet number)	7.13 ± 5.68	2.67 ± 1.78	4.95 ± 2.27	3.24 ± 1.57 [†]
No. of small islets/mm ² pancreas surface area	1.94 ± 0.29	1.53 ± 0.18	1.34 ± 0.19	1.70 ± 0.22
No. of medium islets/mm ² pancreas surface area	0.27 ± 0.08	0.26 ± 0.09	0.17 ± 0.07	0.25 ± 0.07
No. of large islets/mm ² pancreas surface area	0.15 ± 0.13	0.06 ± 0.04	0.08 ± 0.03	0.11 ± 0.05
Liver morphology				
Periportal CD68-positive cell expression (score)	0.66 ± 0.24	0.63 ± 0.18	0.90 ± 0.23	0.94 ± 0.20
Metabolites				
Serum glucose (mmol/l)	8.04 ± 0.35	8.77 ± 0.25	8.16 ± 0.28	7.50 ± 0.31 ^{††}
Serum insulin (pmol/l)	1.97 ± 0.43	2.16 ± 0.36	2.75 ± 0.49	1.96 ± 0.51
Serum triacylglycerols (mmol/l)	0.50 ± 0.11	0.51 ± 0.06	0.85 ± 0.14 [†]	0.61 ± 0.09
Serum cholesterol (mmol/l)	1.19 ± 0.07	1.42 ± 0.07*	1.29 ± 0.06	1.48 ± 0.09*
Serum HDL-cholesterol (mmol/l)	0.50 ± 0.03	0.61 ± 0.03*	0.52 ± 0.03 [†]	0.61 ± 0.04*
Serum LDL-cholesterol (mmol/l)	0.30 ± 0.02	0.30 ± 0.02	0.28 ± 0.03	0.29 ± 0.03
Blood urea nitrogen (mmol/l)	6.42 ± 1.03	5.27 ± 0.24	6.50 ± 0.58	5.65 ± 0.31
Serum creatinine (μmol/l)	24.13 ± 3.72	24.30 ± 1.34	24.84 ± 1.49	21.87 ± 1.28
Serum aspartate aminotransferase (U/l)	90.90 ± 9.12	82.93 ± 4.64	84.88 ± 3.69	96.21 ± 6.21
Serum alanine aminotransferase (U/l)	24.75 ± 2.03	26.41 ± 1.18	25.22 ± 2.16	26.04 ± 1.66
Liver triacylglycerols (mmol l ⁻¹ mg ⁻¹)	13.53 ± 1.70	16.37 ± 0.92	18.25 ± 2.05	18.10 ± 1.75
Serum hs-CrP (pmol/l)	9.52 ± 1.90	12.38 ± 3.81	10.48 ± 2.86	12.38 ± 2.86

Data are shown as means ± SEM, *n* = 15–20 per study group

p* < 0.05 and *p* < 0.01 vs PatCD/MatCD; [†]*p* < 0.05 and ^{††}*p* < 0.01 vs PatHFSSD/MatCD

hs-CrP, high-sensitivity C-reactive protein

unhealthy diet (*p* < 0.05, Fig. 5b and Table 3). Similar alterations in *Lcn2* gene expression were seen in male offspring.

Pancreas morphology Pancreatic islets in the F1 offspring of both sexes born to male rats exposed to the HFSSD diet prior to mating had a decreased beta cell density (–20% in male and

–15% in female F1 offspring, (*p* < 0.001 PatHFSSD/MatCD vs PatCD/MatCD; Fig. 6a, b). In female F1 progeny, folate given to either pregnant dams or F0 founders resulted in elevation of beta cell density when compared with their counterparts born to parents with no folate intervention (4.3% and 3.3% after folate supplementation given to dams and founders,

Table 2 Body length, organ weights, pancreas and liver morphology and serum metabolites in F1 female offspring

Variable	PatCD/MatCD	PatHFSSD/ MatCD	PatHFSSD/ MatCD+F	PatHFSSD+F/ MatCD
Body length and organ weights				
Final body length (cm)	21.87 ± 0.16	22.56 ± 0.13**	21.94 ± 0.23 ^{††}	22.66 ± 0.13
Heart weight (g)	0.81 ± 0.03	0.86 ± 0.02*	0.83 ± 0.02	0.82 ± 0.02
Liver weight (g)	7.19 ± 0.38	8.48 ± 0.24**	8.27 ± 0.35	8.28 ± 0.21
Left kidney weight (g)	0.81 ± 0.02	0.87 ± 0.02*	0.90 ± 0.02	0.89 ± 0.02
Right kidney weight (g)	0.83 ± 0.03	0.90 ± 0.03*	0.90 ± 0.02	0.91 ± 0.012
Relative heart weight (% of body weight)	0.31 ± 0.01	0.31 ± 0.01	0.31 ± 0.01	0.29 ± 0.01 ^{††}
Relative liver weight (% of body weight)	2.83 ± 0.13	3.04 ± 0.04	3.04 ± 0.09	2.89 ± 0.05
Relative left kidney weight (% of body weight)	0.32 ± 0.01	0.31 ± 0.01	0.33 ± 0.01 ^{††}	0.31 ± 0.01
Relative weight of the right kidney (% to body weigh)	0.33 ± 0.01	0.32 ± 0.01	0.33 ± 0.01	0.32 ± 0.01
Glucose metabolism variables on day 105 of the study				
Fasting serum glucose (mmol/l)	7.04 ± 0.28	6.84 ± 0.38	7.28 ± 0.32	6.67 ± 0.32
Fasting serum insulin (pmol/l)	257.31 ± 40.00	383.85 ± 49.24	385.03 ± 53.13	316.97 ± 39.66
Pancreas morphology				
Total islet area (% of pancreas surface area)	0.46 ± 0.05	0.50 ± 0.09	0.48 ± 0.07	0.58 ± 0.10
Total no. of islets/mm ² pancreas surface area	1.41 ± 0.16	1.61 ± 0.21	1.29 ± 0.15	1.48 ± 0.15
Percentage of small islets (0–5000 μm ²) (% of total islet number)	81.53 ± 4.93	85.95 ± 4.85	78.86 ± 5.71	71.95 ± 6.19
Percentage of medium islets (5001–10,000 μm ²) (% of total islet number)	14.79 ± 4.90	13.73 ± 4.85	17.38 ± 5.45	20.53 ± 4.76
Percentage of large islets (>10,000 μm ²) (% of total islet number)	3.68 ± 2.02	1.32 ± 1.32	3.86 ± 2.52	7.47 ± 3.34
No. of small islets/mm ² pancreas surface area	1.19 ± 0.18	1.38 ± 0.19	0.97 ± 0.13	1.11 ± 0.15
No. of medium islets/mm ² pancreas surface area	0.17 ± 0.06	0.21 ± 0.05	0.28 ± 0.09	0.28 ± 0.06
No of large islets/mm ² pancreas surface area	0.05 ± 0.02	0.02 ± 0.02	0.04 ± 0.02	0.10 ± 0.04
Liver morphology				
Periportal CD68-positive cell expression (score)	1.08 ± 0.27	0.97 ± 0.16	1.45 ± 0.35	1.50 ± 0.45
Metabolites				
Serum glucose (mmol/l)	7.17 ± 0.29	6.84 ± 0.38	7.35 ± 0.30	6.67 ± 0.32
Serum insulin (pmol/l)	1.75 ± 0.33	2.21 ± 0.29	2.35 ± 0.31	1.92 ± 0.23
Serum triacylglycerols (mmol/l)	0.42 ± 0.06	0.38 ± 0.03	0.45 ± 0.04	0.47 ± 0.06
Serum cholesterol (mmol/l)	1.80 ± 0.09	1.57 ± 0.08*	1.86 ± 0.09 [†]	1.64 ± 0.09
Serum HDL-cholesterol (mmol/l)	0.67 ± 0.04	0.62 ± 0.04	0.69 ± 0.03	0.66 ± 0.04
Serum LDL-cholesterol (mmol/l)	0.14 ± 0.01	0.14 ± 0.02	0.17 ± 0.02	0.17 ± 0.02
Blood urea nitrogen (mmol/l)	6.65 ± 0.32	5.95 ± 0.34	6.70 ± 0.54	5.74 ± 0.38
Serum creatinine (μmol/l)	26.89 ± 1.03	23.89 ± 1.19	28.62 ± 1.90 [†]	24.56 ± 1.49
Serum aspartate aminotransferase (U/l)	76.92 ± 5.63	59.92 ± 3.71*	76.16 ± 7.68 [†]	69.69 ± 5.53
Serum alanine aminotransferase (U/l)	21.99 ± 1.04	19.84 ± 1.50	21.14 ± 1.83	23.12 ± 2.11
Liver triacylglycerols (mmol l ⁻¹ mg ⁻¹)	9.64 ± 0.73	11.00 ± 0.78	9.72 ± 0.73	9.79 ± 0.65
Serum hs-CrP (pmol/l)	9.52 ± 1.90	18.10 ± 1.90**	10.48 ± 2.86 [†]	14.29 ± 2.86

Data are shown as means ± SEM, $n = 15$ –20 per study group

* $p < 0.05$ and ** $p < 0.01$ vs PatCD/MatCD; [†] $p < 0.05$ and ^{††} $p < 0.01$ vs PatHFSSD/MatCD

hs-CrP, high-sensitivity C-reactive protein

respectively, $p < 0.05$ vs PatHFSSD/MatCD, Fig. 6b). However, neither of the parental folate interventions restored pancreatic beta cell density in the F1 male progeny (Fig. 6a). There were no significant differences in total number of islets, islet size distribution or islet area per mm² pancreas section between the study groups of F1 offspring (Tables 1 and 2).

Pancreatic gene expression Pancreatic candidate genes were selected as described for the liver, see above. The analysis of mRNA expression profiles of selected genes (*Pparg*, *Ikbke*, *Ppara*, *Foxo1* and *Fos*) in the pancreas revealed only minor differences between study groups in both male and female progeny of the F1 generation (Table 4).

Fig. 2 Blood glucose levels (**a, b**) and AUC (**c, d**) following an OGTT in the F1 male (**a, c**) and female (**b, d**) offspring at 100 days of age. Brown circles, PatCD/MatCD ($n = 21$ and $n = 34$ in males and females, respectively); pink squares, PatHFSSD/MatCD ($n = 35$ and $n = 23$ in males and females, respectively); green triangles, PatHFSSD/MatCD+F ($n = 15$ and $n = 18$ in males and females, respectively); blue inverted triangles, PatHFSSD+F/MatCD ($n = 18$ and $n = 15$ in males and females, respectively). Values are shown as means $\pm$ SEM. * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ vs PatCD/MatCD; † $p < 0.05$ and †† $p < 0.01$ vs PatHFSSD/MatCD

Global DNA methylation in the liver Regardless of the F1 offspring sex, the rate of global DNA methylation in the liver was 1.52-fold elevated in PatHFSSD/MatCD offspring compared with their PatCD/MatCD control counterparts ($p < 0.05$, Fig. 7a, b). In female F1 offspring, the treatment of the F0 dams with folate restored global methylation to a normal level ($p < 0.05$ PatHFSSD+F/MatCD vs PatHFSSD/MatCD, Fig. 7b). However, effects of folate treatment of either the F0 founders or dams on the reduction in global DNA methylation rate was not statistically significant in the male offspring (Fig. 7a).

DNA methylation of specific target genes in the liver The methylation rate of CpG islands in the promoter region of *Ppara*, *Lcn2* and *Tmcc2* genes was analysed. The differences among the groups were not significant, most likely due to the limited number of samples analysed ($n = 5$ per group) (ESM Table 6). We also investigated the potential correlation between the mRNA expression and the methylation rate of CpG islands in the promoter region of *Ppara*, *Lcn2* and *Tmcc2*. Interestingly, the methylation rate of some CpG islands in the promoter region of *Lcn2* showed

Fig. 3 Hepatic steatosis score in the male (**a**) and female (**b**) F1 offspring and typical photomicrographs of H&E staining for hepatic steatosis in male (**c**) and female (**d**) F1 offspring. Brown circles, PatCD/MatCD ($n = 8$ and $n = 10$ in males and females, respectively); pink squares, PatHFSSD/MatCD ($n = 12$ and $n = 10$ in males and females, respectively); green triangles, PatHFSSD/MatCD+F ($n = 12$ and $n = 11$ in males and females, respectively); blue inverted triangles, PatHFSSD+F/MatCD ($n = 8$ and $n = 5$ in males and females, respectively). Values are shown as means $\pm$ SEM. Scale bars, 100 μ m. * $p < 0.05$ and ** $p < 0.01$ vs PatCD/MatCD

Fig. 4 Hepatic periportal fibrosis score in male (**a**) and female (**b**) F1 offspring and typical photomicrographs of Sirius Red staining for hepatic periportal fibrosis in male (**c**) and female (**d**) F1 offspring. Brown circles, PatCD/MatCD ($n = 6$ and $n = 9$ in males and females, respectively); pink squares, PatHFSSD/MatCD ($n = 12$ and $n = 9$ in males and females, respectively); green triangles, PatHFSSD/MatCD+F ($n = 12$ per study group); blue inverted triangles, PatHFSSD+F/MatCD ($n = 8$ and $n = 7$ in males and females, respectively). Values are shown as means $\pm$ SEM. Scale bars, 100 μ m. ** $p < 0.01$ vs PatCD/MatCD; †† $p < 0.01$ vs PatHFSSD/MatCD

significant negative correlation with *Lcn2* mRNA expression in female offspring (ESM Table 7). Moreover, to investigate whether the methylation rate of a given CpG site is correlated with other CpG sites within the promoter region, Pearson correlation matrices were calculated and plotted as heat maps for each group (Fig. 5c, d and ESM Table 8). The resulting group-specific correlation patterns were clearly different. Regarding the correlation matrices of the methylation rate of CpG islands within the *Ppara* promoter, positive correlations, indicated by dark blue, were more predominant in the offspring born to fathers on a normal diet when compared with the offspring born to fathers on an unhealthy diet, even following folate treatment of both parents. On the other hand, regarding the correlation matrices of the methylation rate of CpG islands within the *Lcn2* promoter, positive correlations were more predominant in the offspring born to fathers on an unhealthy diet when compared with the offspring born to fathers on a normal diet or those born to fathers fed an unhealthy diet where fathers or mothers had been treated with folate.

ANCOVA models considering litter size As stated above, litter size was not significantly different between the groups. However, to investigate the influence and the potential bias of litter size, ANCOVA models, considering litter size as a covariate, were calculated for variables that were significantly different in the ANOVA analyses. Indeed, litter size was associated with several readouts, yet results overall were not affected by litter size (ESM Table 9).

Discussion

Several studies suggest that exposure of a male parent to environmental adverse factors during spermatogenesis can influence the development of traits in their offspring [14]. A model of paternal high-fat diet prior to mating is usually used. We exposed male rats to a high-fat, high-carbohydrate and high-salt diet mimicking an unhealthy fast-food diet often eaten by young men. We tested the hypothesis that the adverse effects of an unhealthy paternal diet before mating on the offspring could be ameliorated by folate treatment of either the dams or the founders before mating. Our study demonstrated that folate treatment of dams ameliorated the adverse effects on female offspring's glucose metabolism. This might be partially due to folate-induced beta cell preservation in the female offspring combined with a normalisation of hepatic connective tissue density. Furthermore, folate treatment reversed dysregulated *Ppara*, *Lcn2* and *Tmcc2* gene expression and normalised liver total DNA methylation.

Sex-dependent effects of adverse paternal diets before mating on the offspring

The effects of an adverse paternal diet on the offspring's phenotype were sex-specific. Only female offspring developed an impaired glucose tolerance. This is in agreement with the findings of published studies [16, 31, 32]. Gene expression shows sex-specific differences, which are detectable in the pre-implanted embryo, long before gonadal development

Fig. 5 (a, b) Expression levels of hepatic *Ppara* (a) and *Lcn2* mRNA (b) in male and female F1 generation offspring. mRNA expression data are given as fold change relative to PatCD/MatCD; mRNA expression levels are normalised to β -actin. Brown circles, PatCD/MatCD ($n = 10$ per study group); pink squares, PatHFSSD/MatCD (for *Ppara*, $n = 9$ and $n = 10$ in males and females, respectively; for *Lcn2*, $n = 8$ and $n = 10$ in males and females, respectively); green triangles, PatHFSSD/MatCD+F (for *Ppara*, $n = 10$ and $n = 9$ in males and females, respectively; for *Lcn2*,

$n = 10$ per study group); blue inverted triangles, PatHFSSD+F/MatCD (for *Ppara*, $n = 9$ and $n = 10$ in males and females, respectively; for *Lcn2*, $n = 10$ per study group). Values are shown as means $\pm$ SEM. * $p < 0.05$ vs PatCD/MatCD; † $p < 0.05$ and †† $p < 0.01$ vs PatHFSSD/MatCD. (c, d) Heat maps of group-specific inter-CpG site correlation coefficients of DNA methylation of *Ppara* (c) and *Lcn2* (d) in F1 offspring (both male and female)

and sex hormone production [33]. When comparing the consequences of parental nutritional insults with respect to the offspring, paternal pre-conception stimuli were shown to display a stronger effect on female offspring [16, 31, 32].

Folate treatment and fetal programming

Folate treatment during pregnancy prevents adverse developmental programming [19, 20, 25]. A recent study showed that the effects on the offspring of folate during pregnancy are sex-specific [34]. We demonstrated that folate treatment of pregnant dams prevents adverse metabolic effects of paternal programming. In other words, a **disadvantageous** diet in the male parent prior to mating can be corrected by folate treatment of the dam after mating.

Folate treatment of male rats exposed to an unhealthy diet improved pancreatic beta cell density in female offspring (Fig. 6). Since we only analysed the effect of one folate dose, the very

next task should be to establish the dose-dependency of this effect. The same is true for the supplementation of the dams.

Whereas the paternal unhealthy diet-induced fat accumulation in the liver of male and female offspring, liver fibrosis and systemic inflammation was only seen in female offspring (Tables 3 and 4). The fat accumulation in the liver of male offspring is not associated with inflammation and is thus benign, in contrast to the findings in female offspring. Beta cell density in offspring was reduced by paternal HFSSD feeding and was improved slightly by folate treatment in female offspring only. This effect in female offspring might contribute to the folate-treatment-related improvement in the OGTT measurements.

The beneficial effects of folate administration during pregnancy were associated with decreased global DNA methylation in the liver of female offspring. This is counterintuitive to previous findings suggesting that folate is a major source of methyl groups required for DNA methylation and, hence, increases DNA methylation. However, recent studies support

Table 3 Relative hepatic gene expression profile in male and female F1 offspring

Gene	PatCD/MatCD	PatHFSSD/MatCD	PatHFSSD/MatCD+F	PatHFSSD+F/MatCD
Genes chosen based on a hypothesis-driven approach				
F1 male offspring				
<i>Fasn</i>	1.00 ± 0.28	0.68 ± 0.20	1.92 ± 0.36*	1.38 ± 0.53
<i>Cpt1a</i>	1.00 ± 0.25	0.60 ± 0.20	1.02 ± 0.18	1.60 ± 0.54*
<i>Srebf1</i>	1.00 ± 0.27	0.48 ± 0.13	0.92 ± 0.21	1.03 ± 0.34
<i>Nfkb1</i>	1.00 ± 0.24	0.57 ± 0.21	1.18 ± 0.44	0.94 ± 0.41
<i>Mtl</i>	1.00 ± 0.25	0.68 ± 0.24	0.84 ± 0.32	1.31 ± 0.50
<i>G6pc</i>	1.00 ± 0.38	0.40 ± 0.14	1.03 ± 0.31	1.49 ± 0.63
<i>Acaca</i>	1.00 ± 0.20	0.46 ± 0.15	1.01 ± 0.40	1.41 ± 0.60
<i>Pck1</i>	1.00 ± 0.26	1.39 ± 0.67	1.18 ± 0.41	0.69 ± 0.23
<i>Por</i>	1.00 ± 0.37	1.21 ± 0.70	1.34 ± 0.77	0.46 ± 0.11
F1 female offspring				
<i>Fasn</i>	1.00 ± 0.18	0.62 ± 0.08*	0.47 ± 0.11	0.95 ± 0.11
<i>Cpt1a</i>	1.00 ± 0.18	1.33 ± 0.18	0.82 ± 0.13 [†]	0.95 ± 0.12
<i>Srebf1</i>	1.00 ± 0.12	1.07 ± 0.12	1.58 ± 0.80	1.37 ± 0.10
<i>Nfkb1</i>	1.00 ± 0.16	0.71 ± 0.13	0.81 ± 0.13	0.92 ± 0.26
<i>Mtl</i>	1.00 ± 0.16	0.80 ± 0.23	0.63 ± 0.10	0.82 ± 0.19
<i>G6pc</i>	1.00 ± 0.10	1.26 ± 0.24	0.80 ± 0.12	1.07 ± 0.24
<i>Acaca</i>	1.00 ± 0.18	0.77 ± 0.12	0.63 ± 0.11	0.93 ± 0.12
<i>Pck1</i>	1.00 ± 0.08	1.33 ± 0.13*	1.15 ± 0.11	0.99 ± 0.08 [†]
<i>Por</i>	1.00 ± 0.12	0.68 ± 0.06*	0.57 ± 0.07	0.84 ± 0.12
Genes chosen based on micro-array data				
F1 male offspring				
<i>Tnks2</i>	1.00 ± 0.04	0.95 ± 0.17	0.86 ± 0.17	1.52 ± 0.26 ^{††}
<i>Sult1c2</i>	1.00 ± 0.24	0.68 ± 0.16	0.48 ± 0.12	0.81 ± 0.27
<i>Rpl30</i>	1.00 ± 0.11	0.81 ± 0.21	0.71 ± 0.20	0.76 ± 0.08
<i>Slc9a9</i>	1.00 ± 0.14	0.89 ± 0.15	1.21 ± 0.24	1.24 ± 0.13
<i>Serpina10</i>	1.00 ± 0.09	0.91 ± 0.06	1.01 ± 0.10	1.04 ± 0.12
<i>Mrpl53</i>	1.00 ± 0.05	0.98 ± 0.10	1.36 ± 0.20 [†]	1.00 ± 0.08
<i>Ifit1</i>	1.00 ± 0.57	2.00 ± 0.84	0.48 ± 0.22	1.73 ± 0.83
<i>Gpat3</i>	1.00 ± 0.20	1.37 ± 0.40	0.91 ± 0.15	2.24 ± 0.64
<i>Cyp2b1</i>	1.00 ± 0.31	1.54 ± 0.68	0.64 ± 0.20	0.79 ± 0.28
<i>Il1rl1</i>	1.00 ± 0.19	1.04 ± 0.13	0.59 ± 0.14 [†]	0.52 ± 0.10 [†]
<i>LOC100362027</i>	1.00 ± 0.18	0.87 ± 0.14	1.07 ± 0.14	0.97 ± 0.14
<i>Tmcc2</i>	1.00 ± 0.14	1.66 ± 0.38	1.32 ± 0.30	1.39 ± 0.25
F1 female offspring				
<i>Tnks2</i>	1.00 ± 0.27	0.61 ± 0.10	0.43 ± 0.16	0.31 ± 0.14
<i>Sult1c2</i>	1.00 ± 0.30	0.33 ± 0.14*	0.26 ± 0.14	0.25 ± 0.09
<i>Rpl30</i>	1.00 ± 0.15	0.62 ± 0.06	1.92 ± 0.69 [†]	0.94 ± 0.11
<i>Slc9a9</i>	1.00 ± 0.09	0.89 ± 0.11	0.90 ± 0.12	0.68 ± 0.06
<i>Serpina10</i>	1.00 ± 0.05	0.85 ± 0.07	1.25 ± 0.16 [†]	1.18 ± 0.14 [†]
<i>Mrpl53</i>	1.00 ± 0.05	0.78 ± 0.10	1.05 ± 0.14	1.29 ± 0.23 [†]
<i>Ifit1</i>	1.00 ± 0.57	3.76 ± 1.12	3.04 ± 1.42	4.50 ± 1.67
<i>Gpat3</i>	1.00 ± 0.33	0.41 ± 0.12*	0.19 ± 0.05	0.37 ± 0.15
<i>Cyp2b1</i>	1.00 ± 0.37	1.03 ± 0.26	1.05 ± 0.53	0.18 ± 0.06
<i>Il1rl1</i>	1.00 ± 0.15	1.19 ± 0.26	1.22 ± 0.24 [†]	0.75 ± 0.17 [†]
<i>LOC100362027</i>	1.00 ± 0.27	0.57 ± 0.09	1.04 ± 0.17	1.51 ± 0.29 [†]
<i>Tmcc2</i>	1.00 ± 0.26	1.87 ± 0.29*	0.54 ± 0.09 [†]	0.83 ± 0.22 [†]

Values are shown as means ± SEM, *n* = 15–20 per study group**p* < 0.05 vs PatCD/MatCD; [†]*p* < 0.05 and ^{††}*p* < 0.01 vs PatHFSSD/MatCD

Fig. 6 Beta cell density per pancreatic islet (% of insulin-positive area) in the male (a) and female (b) F1 offspring and typical photomicrographs of immunostaining of the pancreatic tissue for insulin in male (c) and female (d) F1 offspring. Brown circles, PatCD/MatCD ($n = 16$ and $n = 20$ in males and females, respectively); pink squares, PatHFSSD/MatCD ($n = 30$ and $n = 21$ in males and females, respectively); green triangles, PatHFSSD/MatCD+F ($n = 21$ and $n = 19$ in males and females, respectively); blue inverted triangles, PatHFSSD+F/MatCD ($n = 16$ and $n = 17$ in males and females, respectively). Values are shown as means $\pm$ SEM. Scale bars, 100 μ m. *** $p < 0.001$ vs PatCD/MatCD; † $p < 0.05$ vs PatHFSSD/MatCD

our findings: in one study, folate supplementation was associated with genome-wide loss of methylation [35] and in another study [36] maternal plasma folate during pregnancy was associated with a decreased methylation of 416 CpGs (94%) in newborns and increased methylation of 27 CpGs (6%). Alterations of microRNAs (miRNAs) in sperm are known to affect paternal programming [21, 22]. Folate can alter miRNA status via regulation of gene expression, possibly altering synthesis/effects of DNA methyltransferases or enzymes involved in the folate-dependent one-carbon metabolism pathway, leading to decreased DNA methylation [37].

To identify underlying molecular mechanisms, we performed a candidate gene approach [29, 30, 38] as well as a whole-genome array approach. Both approaches are necessary, since open non-hypothesis-driven technologies are not yet capable of discovering all underlying alterations in gene expression [39–41]. The key finding of the whole-genome array was the identification of the dysregulated hepatic genes *Lcn2* and *Tmcc2* in female offspring (Fig. 5c, d). A high-fat, high-fructose diet was found to upregulate hepatic *Lcn2* expression in mice [42]. Importantly, lipocalin 2 levels correlate with obesity, impaired insulin sensitivity and diabetes and have been suggested

Table 4 Relative pancreatic gene expression profile in male and female F1 offspring

Gene	PatCD/MatCD	PatHFSSD/MatCD	PatHFSSD/MatCD+F	PatHFSSD+F/MatCD
F1 male offspring				
<i>Pparg</i>	1.00 $\pm$ 0.37	0.95 $\pm$ 0.21	1.20 $\pm$ 0.48	0.81 $\pm$ 0.24
<i>Ikbke</i>	1.00 $\pm$ 0.15	0.65 $\pm$ 0.13	0.96 $\pm$ 0.20	0.76 $\pm$ 0.14
<i>Ppara</i>	1.00 $\pm$ 0.18	0.95 $\pm$ 0.19	1.01 $\pm$ 0.41	1.33 $\pm$ 0.16
<i>Foxo1</i>	1.00 $\pm$ 0.16	0.88 $\pm$ 0.15	0.77 $\pm$ 0.25	0.92 $\pm$ 0.11
<i>Fos</i>	1.00 $\pm$ 0.51	0.57 $\pm$ 0.19	0.89 $\pm$ 0.36	0.38 $\pm$ 0.20
F1 female offspring				
<i>Pparg</i>	1.00 $\pm$ 0.18	1.08 $\pm$ 0.22	1.49 $\pm$ 0.65	0.80 $\pm$ 0.18
<i>Ikbke</i>	1.00 $\pm$ 0.18	0.37 $\pm$ 0.14	0.74 $\pm$ 0.09	0.55 $\pm$ 0.09 [†]
<i>Ppara</i>	1.00 $\pm$ 0.19	0.90 $\pm$ 0.15	0.78 $\pm$ 0.20	0.93 $\pm$ 0.19
<i>Foxo1</i>	1.00 $\pm$ 0.24	0.61 $\pm$ 0.10	0.50 $\pm$ 0.10*	0.48 $\pm$ 0.09*
<i>Fos</i>	1.00 $\pm$ 0.52	0.54 $\pm$ 0.08	1.07 $\pm$ 0.33	0.40 $\pm$ 0.13

Values are shown as means $\pm$ SEM, $n = 15$ –20 per study group

* $p < 0.05$ vs PatCD/MatCD; † $p < 0.05$ vs PatHFSSD/MatCD

Fig. 7 Global DNA methylation in the liver of male (**a**) and female (**b**) F1 offspring. The data are given as fold change relative to PatCD/MatCD. Brown circles, PatCD/MatCD; pink squares, PatHFSSD/MatCD; green

triangles, PatHFSSD/MatCD+F; blue inverted triangles, PatHFSSD+F/MatCD. Values are shown as means $\pm$ SEM, $n = 5$ per study group. * $p < 0.05$ vs PatCD/MatCD; † $p < 0.05$ vs PatHFSSD/MatCD

as a potential prognostic biomarker of non-alcoholic fatty liver disease [43]. Notably, a recent study showed that *Lcn2* expression is altered by maternal nutrition during the development of the fetal liver [35]. In our study, the increased hepatic expression seen in offspring born to F0 founders fed an unhealthy diet was normalised in female offspring of folate-treated F0 founders on an unhealthy diet. In contrast, treatment of pregnant dams with folate had no significant effect on hepatic *Lcn2* expression in female offspring. *Tmcc2* plays a role in Alzheimer's disease; however, its role in the pathogenesis of paternal unhealthy diet-induced liver damage is unknown so far.

The candidate gene approach revealed normalisation of liver *Ppara* expression seems to play a key role in the effects of maternal folate treatment. Notably, a recent study in mice found that a fast-food-induced increase in hepatic fibrosis was associated with an increase in hepatic *Ppara* expression [44]. Successful treatment of the liver fibrosis normalised hepatic *Ppara* expression, like in our study. Maternal high-fat diet also results in dysregulated fetal hepatic *Sirt1* expression, sirtuin 1 protein level and activity and a concomitant dysregulation of *Sirt1*-associated genes, including *Ppara* [45, 46]. However, in the current study the paternal unhealthy diet was not associated with a significant effect on *Sirt1* expression, indicating other underlying mechanisms of *Ppara* regulation.

The methylation level of certain CpG islands in the promoter region of *Lcn2* showed significant negative correlation with *Lcn2* mRNA expression exclusively in the female offspring, suggesting that liver *Lcn2* expression could be controlled through DNA methylation in a sex-dependent manner. Because it has been suggested that there may be correlations between the methylation states of neighbouring and/or functionally related CpG sites [47, 48], we analysed treatment-group-specific inter-CpG site correlation of DNA methylation and plotted the resulting correlation coefficients as heat maps. Interestingly, different patterns were observed when comparing treatment groups. Different correlation patterns between the degree of DNA methylation of one CpG site to another could result in a different net effect on gene expression [47]. However, as methylation is just one of several epigenetic modifications, other mechanisms such as histone modifications might be of importance [45]. Recent studies highlight a key role for paternal sperm-cell-derived small non-coding

RNAs [21, 49]. One study, investigating effects of a paternal high-fat diet, demonstrated sex-specific metabolic disturbances in female offspring. The authors showed that a high-fat diet alters the expression of miRNA let-7c in the sperm of F0 rats and their F1 offspring. This finding is further substantiated by studies showing that the microinjection of sperm-derived RNAs into oocytes can transmit environmentally induced paternal phenotypic changes to the resulting offspring [21, 49, 50].

In conclusion, folate treatment of pregnant dams, but not the male founders, reverses detrimental effects on female offspring's glucose metabolism induced by pre-conceptional male founder high-fat, high-carbohydrate and high-salt diet. This effect might be at least partially due to a folate-induced beta cell preservation in the female offspring combined with a partial improvement of the liver abnormalities.

Data availability The datasets collected in this study are available from the corresponding author on request.

Funding This project has been funded in whole or in part with funds from the National Natural Science Foundation of China (Grant No. 81300557), Hunan Province Science and Technology Plan (grant no. 2014SK3003) and the Programme for Excellent Talents of Hunan Normal University (grant no. ET14106).

Duality of interest The authors declare that there is no duality of interest associated with this manuscript.

Contribution statement BH designed the study. JL, MT, XLZ, QZ, OT, JG, MG, AH, CR, X-NP, G-YS, Y-PL and GL generated and analysed the data. JL, OT, BH, Y-PL, AH and CR interpreted the data and wrote the manuscript. All the authors revised the manuscript for intellectual content and approved its final version to be published. BH is the guarantor of this work.

References

1. Reichetzeder C, Dwi Putra SE, Li J, Hochoer B (2016) Developmental origins of disease - crisis precipitates change. *Cell Physiol Biochem* 39:919–938
2. Reichetzeder C, Dwi Putra SE, Pfaf T et al (2016) Increased global placental DNA methylation levels are associated with gestational diabetes. *Clin Epigenetics* 8:82

3. Novakovic B, Saffery R (2010) DNA methylation profiling highlights the unique nature of the human placental epigenome. *Epigenomics* 2:627–638
4. Hochoer B, Haumann H, Rahnenführer J et al (2016) Maternal eNOS deficiency determines a fatty liver phenotype of the offspring in a sex dependent manner. *Epigenetics* 11:539–552
5. Thompson RF, Fazzari MJ, Niu H et al (2010) Experimental intra-uterine growth restriction induces alterations in DNA methylation and gene expression in pancreatic islets of rats. *J Biol Chem* 285: 15111–15118
6. Woods LL, Weeks DA, Rasch R (2004) Programming of adult blood pressure by maternal protein restriction: role of nephrogenesis. *Kidney Int* 65:1339–1348
7. Barker DJ, Gluckman PD, Godfrey KM et al (1993) Fetal nutrition and cardiovascular disease in adult life. *Lancet* 341:938–941
8. Reichetzeder C, Chen H, Föller M et al (2014) Maternal vitamin D deficiency and fetal programming—lessons learned from humans and mice. *Kidney Blood Press Res* 39:315–329
9. Thone-Reineke C, Kalk P, Dorn M et al (2006) High-protein nutrition during pregnancy and lactation programs blood pressure, food efficiency, and body weight of the offspring in a sex-dependent manner. *Am J Phys Regul Integr Comp Phys* 291:R1025–R1030
10. Kajantie E, Dunkel L, Turpeinen U et al (2003) Placental 11 β -hydroxysteroid dehydrogenase-2 and fetal cortisol/cortisone shuttle in small preterm infants. *J Clin Endocrinol Metab* 88:493–500
11. Li J, Lu YP, Reichetzeder C et al (2016) Maternal PCaC38:6 is associated with preterm birth - a risk factor for early and late adverse outcome of the offspring. *Kidney Blood Press Res* 41:250–257
12. Hochoer B, Slowinski T, Stolze T et al (2000) Association of maternal G protein beta3 subunit 825T allele with low birthweight. *Lancet* 355:1241–1242
13. McPherson NO, Fullston T, Aitken RJ, Lane M (2014) Paternal obesity, interventions, and mechanistic pathways to impaired health in offspring. *Ann Nutr Metab* 64:231–238
14. Li J, Tsuprykov O, Yang X, Hochoer B (2016) Paternal programming of offspring cardiometabolic diseases in later life. *J Hypertens* 34:2111–2126
15. Chen Y-P, Xiao X-M, Li J et al (2012) Paternal body mass index (BMI) is associated with offspring intrauterine growth in a gender dependent manner. *PLoS One* 7:e36329
16. Ng S-F, Lin RCY, Laybutt DR et al (2010) Chronic high-fat diet in fathers programs β -cell dysfunction in female rat offspring. *Nature* 467:963–966
17. McPherson NO, Lane M, Sandeman L et al (2017) An exercise-only intervention in obese fathers restores glucose and insulin regulation in conjunction with the rescue of pancreatic islet cell morphology and microRNA expression in male offspring. *Nutrients* 9:122
18. McPherson NO, Owens JA, Fullston T, Lane M (2015) Preconception diet or exercise intervention in obese fathers normalizes sperm microRNA profile and metabolic syndrome in female offspring. *Am J Physiol Endocrinol Metab* 308:E805–E821
19. Lillycrop KA, Phillips ES, Jackson AA et al (2005) Dietary protein restriction of pregnant rats induces and folic acid supplementation prevents epigenetic modification of hepatic gene expression in the offspring. *J Nutr* 135:1382–1386
20. Torrens C, Brawley L, Anthony FW et al (2006) Folate supplementation during pregnancy improves offspring cardiovascular dysfunction induced by protein restriction. *Hypertens Dallas Tex* 1979 47:982–987
21. Chen Q, Yan M, Cao Z et al (2016) Sperm tsRNAs contribute to intergenerational inheritance of an acquired metabolic disorder. *Science* 351:397–400
22. Waldron D (2016) Non-coding RNA: inheritance of diet-induced metabolic changes via tsRNAs. *Nat Rev Genet* 17:128
23. Yan M, Zhai Q (2016) Sperm tsRNAs and acquired metabolic disorders. *J Endocrinol* 230:F13–F18
24. Shin JH, Shiota K (1999) Folic acid supplementation of pregnant mice suppresses heat-induced neural tube defects in the offspring. *J Nutr* 129:2070–2073
25. Zhao M, Chen Y-H, Chen X et al (2014) Folic acid supplementation during pregnancy protects against lipopolysaccharide-induced neural tube defects in mice. *Toxicol Lett* 224:201–208
26. Wang X, Tang D, Shen P et al (2017) Analysis of DNA methylation in chondrocytes in rats with knee osteoarthritis. *BMC Musculoskelet Disord* 18:377
27. Paul DS, Guilhamon P, Karpathakis A et al (2014) Assessment of RainDrop BS-seq as a method for large-scale, targeted bisulfite sequencing. *Epigenetics* 9:678–684
28. Feng H, Conneely KN, Wu H (2014) A Bayesian hierarchical model to detect differentially methylated loci from single nucleotide resolution sequencing data. *Nucleic Acids Res* 42:e69
29. Terashima M, Barbour S, Ren J et al (2015) Effect of high fat diet on paternal sperm histone distribution and male offspring liver gene expression. *Epigenetics* 10:861–871
30. Ormellas F, Souza-Mello V, Mandarim-de-Lacerda CA, Aguila MB (2015) Programming of obesity and comorbidities in the progeny: lessons from a model of diet-induced obese parents. *PLoS One* 10: e0124737
31. Ng S-F, Lin RCY, Maloney CA et al (2014) Paternal high-fat diet consumption induces common changes in the transcriptomes of retroperitoneal adipose and pancreatic islet tissues in female rat offspring. *FASEB J* 28:1830–1841
32. Carone BR, Fauquier L, Habib N et al (2010) Paternally induced transgenerational environmental reprogramming of metabolic gene expression in mammals. *Cell* 143:1084–1096
33. Gabory A, Roseboom TJ, Moore T et al (2013) Placental contribution to the origins of sexual dimorphism in health and diseases: sex chromosomes and epigenetics. *Biol Sex Differ* 4:5
34. Oster M, Trakooljul N, Reyer H et al (2017) Sex-specific muscular maturation responses following prenatal exposure to methylation-related micronutrients in pigs. *Nutrients* 9:74
35. Rees WD, Hay SM (2014) Lipocalin-2 (Lcn2) expression is mediated by maternal nutrition during the development of the fetal liver. *Genes Nutr* 9:380
36. Joubert BR, den Dekker HT, Felix JF et al (2016) Maternal plasma folate impacts differential DNA methylation in an epigenome-wide meta-analysis of newborns. *Nat Commun* 7:10577
37. Beckett EL, Veysey M, Lucock M (2017) Folate and microRNA: bidirectional interactions. *Clin Chim Acta Int J Clin Chem* 474:60–66
38. Skinner MK (2010) Metabolic disorders: fathers' nutritional legacy. *Nature* 467:922–923
39. Dwi Putra SE, Reichetzeder C, Meixner M et al (2017) DNA methylation of the glucocorticoid receptor gene promoter in the placenta is associated with blood pressure regulation in human pregnancy. *J Hypertens* 35:2276–2286
40. Hochoer B, Adamski J (2017) Metabolomics for clinical use and research in chronic kidney disease. *Nat Rev Nephrol* 13:269–284
41. Tsuprykov O, Ando R, Reichetzeder C et al (2016) The dipeptidyl peptidase inhibitor linagliptin and the angiotensin II receptor blocker telmisartan show renal benefit by different pathways in rats with 5/6 nephrectomy. *Kidney Int* 89:1049–1061
42. de Sousa Rodrigues ME, Bekhat M, Houser MC et al (2017) Chronic psychological stress and high-fat high-fructose diet disrupt metabolic and inflammatory gene networks in the brain, liver, and

- gut and promote behavioral deficits in mice. *Brain Behav Immun* 59:158–172
43. Asimakopoulou A, Weiskirchen S, Weiskirchen R (2016) Lipocalin 2 (LCN2) expression in hepatic malfunction and therapy. *Front Physiol* 7:430
 44. Abdelmegeed MA, Choi Y, Godlewski G et al (2017) Cytochrome P450-2E1 promotes fast food-mediated hepatic fibrosis. *Sci Rep* 7:39764
 45. Borengasser SJ, Kang P, Faske J et al (2014) High fat diet and in utero exposure to maternal obesity disrupts circadian rhythm and leads to metabolic programming of liver in rat offspring. *PLoS One* 9:e84209
 46. Williams L, Seki Y, Vuguin PM, Charron MJ (2014) Animal models of in utero exposure to a high fat diet: a review. *Biochim Biophys Acta* 1842:507–519
 47. Laubach ZM, Perng W, Dolinoy DC et al (2018) Epigenetics and the maintenance of developmental plasticity: extending the signaling theory framework. *Biol Rev Camb Philos Soc.* <https://doi.org/10.1111/brv.12396>
 48. Lövkvist C, Dodd IB, Sneppen K, Haerter JO (2016) DNA methylation in human epigenomes depends on local topology of CpG sites. *Nucleic Acids Res* 44:5123–5132
 49. de Castro BT, Ingerslev LR, Alm PS et al (2016) High-fat diet reprograms the epigenome of rat spermatozoa and transgenerationally affects metabolism of the offspring. *Mol Metab* 5:184–197
 50. Gapp K, Jawaideh A, Sarkies P et al (2014) Implication of sperm RNAs in transgenerational inheritance of the effects of early trauma in mice. *Nat Neurosci* 17:667–669