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Erasure of DNA methylation in rat fetal germ cells is sex-specific and sensitive to maternal high-fat diet

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Brief Report

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Abstract

In mammals, DNA methylation (DNAme) erasure and reinstatement during embryo development and germline establishment are sensitive to the intrauterine environment. Maternal intake of a high-fat diet (HFD), associated with excessive gestational weight gain, has transgenerational effects on offspring health, which may be mediated by changes in DNAme in the germline. Here, we tested the impact of a maternal HFD on embryonic germline DNAme erasure using a rat strain that expresses green fluorescent protein specifically in germ cells. DNAme was analysed by methyl-seq capture in germ cells collected from male and female F1 gonads at gestational day 16. Our data show that although HFD induced global hypomethylation in both sexes, DNAme erasure in female germ cells was more advanced compared to male germ cells. The delay in DNAme erasure in males and the greater impact of HFD suggest that male germ cells are more vulnerable to alterations by exogenous factors.

Introduction

Over 40% of women+[1] entering pregnancy experience excessive gestational weight gain (EGWG),[1] which significantly increases maternal, fetal and neonatal health complications. Indeed, EGWG is a primary risk factor for adverse childhood outcomes including obesity, with effects that can be transmitted across multiple generations.[2,3] The factors underpinning maternal-to-child risk transmittance are unclear, although it is likely to occur through heritable phenotypic changes (epigenetics). Animal models have shown that maternal EGWG produces offspring with metabolic dysfunction and reproductive deficits independent of postnatal environmental factors.[4–7] In rats, pregnant females fed a high-fat diet (HFD) produced fetuses with reduced oocyte numbers, a phenotype that correlated positively with maternal adiposity.[8] Long term, these same offspring show impaired folliculogenesis, irregular reproductive cycles, increased ovarian atretic follicle number, increased serum FSH levels (indicative of early ovarian ageing) and reduced oocyte growth factors.[8] These rodent data suggest that EGWG sets F1 embryos on a road to metabolic compromise and accelerated ovarian ageing, which impairs oocyte quality and potential fertility.[4,8] Although these reports show clear relationships between maternal diet and female germline responses in offspring, data describing impacts on male offspring germline are lacking. This is despite the fact that maternal HFD intake during pregnancy results in early puberty[9] and alters the processes involving oxidative stress balance in the testis of adult offspring,[10] which could lead to transgenerational transmittance of disease risk.

Transgenerational inheritance of disease risk has been observed in animal models after *in utero* exposure to stress, various xenobiotics or dietary interventions. Although this inheritance is likely related to the epigenome, the exact mechanisms remain largely unknown.[11,12] Because epigenetic reprogramming occurs early during germline differentiation, many have hypothesised that transgenerational impacts are embedded in the epigenetic modification of primordial germ cells (PGCs). In mammals, erasure and reinstatement of DNA methylation (DNAme) profiles occur during two main developmental windows: zygote formation and germline establishment.[13] In the germline, an epigenetic reprogramming wave occurs in fetal PGCs to create a hypomethylated epigenetic ground state. DNAme patterns are then reacquired

[1]The term women+ embraces women, transgender and non-binary individuals

in a sex-specific manner during germ cell development, with the sperm epigenome being hypermethylated compared to the oocyte.[13,14] This reprogramming wave ensures the re-establishment of parental imprints and erasure of epimutations in germ cells and ultimately establishes the epigenome of mature gametes, which will, in part, guide embryo development and the health of the progeny. DNAme has been shown to be vulnerable to dietary and environmental chemical exposures, potentially leading to abnormal cell differentiation and function, and transgenerational inheritance of disease.[15,16] Therefore, these same factors within the *in utero* environment could interfere with fundamental DNA demethylation processes in PGCs, which in turn could impede proper re-establishment of sex-specific DNAme patterns required in male and female gametes, thus having consequences on the health of future progeny.

In the present study, we hypothesised that the link between EGWG and multigenerational disease risk lies in the epigenetic information contained in PGCs. Our goal was to define whether a maternal HFD during gestation can impair the DNA demethylation processes occurring in PGCs of the developing F1 offspring and to test for sex differences.

Material and methods

Study design

Two groups of animals have been compared ($n = 5$/group): one control group fed commercial food and one group fed a HFD from the day after mating designated as GD0 until GD16. Inclusion criteria were 10 weeks old animals with expected weights and no visible health issues. Each outcome measured is described below.

Animals

All procedures were conducted in accordance with the guidelines set out by the Canadian Council of Animal Care and as reviewed and approved by the Institutional Animal Care and Use Committee of Institut National de la Recherche Scientifique (INRS) (Protocol #1802-06). Germ cell-specific green fluorescent protein (GCS-GFP) transgenic Sprague-Dawley rats (Cronkhite et al., 2005) were housed on a 12L:12D cycle and fed with commercial food (Teklad global 18% protein, Envigo, Madison, WI) and tap water *ad libitum*. Two virgin females in pro-oestrus were caged with one male overnight, and vaginal smears were done to identify sperm-positive females the following day (GD0). These females were randomly distributed alternatively into the two groups ($n = 5$/group): one group fed a control diet (18% kcal from fat; https://www.inotivco.com/rodent-natural-ingredient-2018-diets) and the other group fed a HFD (45% kcal from fat; https://www.researchdiets.com/formulas/d12451)[7] from GD0 until sacrifice at GD16. Food intake and maternal weight were recorded every 2 d until GD16, when females were sacrificed, and fetuses removed from the uterus. Fetal gonads were dissected under a binocular microscope. Testis and ovaries, determined by the morphology of the gonads, were pooled within litter and weighed separately. Per litter, one testis and one ovary were fixed for stereological analysis, while all others were pooled and used for cell sorting.

Histology

Testes and ovaries were processed and embedded in paraffin as previously described.[17,18] Briefly, 5 µm tissue sections were cut, and three representative sections/gonad were mounted on slides for immunostaining of germ cells by GFP (Life Technologies, #A21311) in ovaries and by HSP90 (BD Biosciences, #610419) in testis (Supplemental Fig. 1). Observations were performed by an experimenter blind to the treatment group.

Tissue dissociation and germ cell purification

Pooled GD16 gonads underwent a two-step enzymatic digestion used for GFP-positive cell purification by fluorescent-activated cell sorting as previously described.[17] The number of GFP-positive cells was counted before and after cell sorting using a haemocytometer under a TiS fluorescent microscope (Nikon, Mississauga, Ontario, Canada). Sorted cells were flash frozen and stored at −80°C until DNA extraction. We have shown previously the effectiveness of this method by expression microarray showing the enrichment of germline-specific RNAs in males.[17]

Genomic DNA isolation, methyl-seq capture, library preparation and sequencing

All procedures were done as previously described.[19] In brief, after genomic DNA extraction, four biological replicates ($n = 4$/group; n being one pooled litter) with the best purity, integrity and highest amount of gDNA were chosen and used for methyl capture and sequencing library preparation using the Rat SureSelectXT Methyl-Seq Target Enrichment Panels kit (Agilent) as described in the SureSelectXT Methyl-Seq Target Enrichment System for Illumina Multiplexed Sequencing protocol (Agilent, Version E0, April 2018). The resulting indexed libraries were assessed for their quantity and quality using High Sensitivity D1000 Screen Tape and 4200 TapeStation (Agilent) and pooled at the same equimolar amount of 5 ng in a single library that was sequenced in paired-end on NovaSeq6000 S4 system (Illumina, San Diego, CA, USA) at the Centre d'expertise et de services Génome Québec (Montreal, Quebec, Canada). 2 females samples with low coverage were eliminated from the final dataset. All sequencing data used in this publication have been deposited in NCBI's Gene Expression Omnibus[20] and are accessible through GEO Series accession number GSE245803 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE245803)[2]. These represent three biological replicates per condition for females and four biological replicates per condition for males.

Data analysis

Sequencing data analysis was done as previously described.[19] In brief, alignment was done against the Rattus_norvegicusRnor_6.0 assembly, and the average number of paired-end reads uniquely aligned was 22.3 million reads per sample. Extraction of methylation followed by a filtration step to obtain data with a minimum of 10X coverage was performed, and the sequencing datasets obtained included 1,820,224 CpGs in female samples and 1,911,208 CpGs in male samples. These datasets were segmented using SMART2[21] into 113,115 segments in females and 114,708 in males, which contained ≥5 CpGs each and were on average 565bp and 548bp, respectively. SMART2 was further used to determine differentially methylated regions (DMRs) with a minimum difference of 10% between control and HFD samples or between sexes (complete lists are available online in the GEO Series

[2]Theses data remain private until 29 October 2024, and reviewers can access using the password: uvaposuulzylpuj

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GSE245803). HOMER 4.11 was used for sequence annotation[22] and ClusterProfiler 4.0 for gene ontology enrichment analysis.[23]

Statistics

A total of 10 pregnant females were used. Phenotypic data are presented as mean ± standard error of the mean of 4–5 biological replicates corresponding to different litters. GraphPad Prism 6 (GraphPad Software, La Jolla, CA, USA) was used to compare HFD to control conditions. R package was used to test the difference in DNAme level between both sexes and HFD to control conditions with a Kruskal–Wallis rank sum test and an effect size measure by Cohen's *d* test.

Results

We used a validated model of maternal HFD intervention to model EGWG in rats and found that, consistent with our previous work,[7] HFD-fed pregnant rats gained more weight by GD16 compared to control (Table 1). We did not observe any HFD impacts on litter size, fetal survival rate, fetal weight or sex ratio (Table 1). A gross histological examination of the testes and ovaries revealed no major morphological changes in the gonadal structure after HFD exposure (Supplemental Fig. 1). Germ cells were isolated at GD16 with high purity (91.8% ± 0.97) using fluorescence-activated cell sorting (FACS). GD16 was chosen to test for sex-specific effects, as this is the last day that germ cells are in a similar state of proliferation in males and females and therefore comparable, despite a different somatic environment and a clonal versus a non-clonal proliferation in female and male germ cells, respectively.[24]

In control conditions, we observed that the global DNAme level in female germ cells was significantly lower than in males with a large Cohen's d effect size (value of 1.57) (Fig. 1a). Specifically, we identified 48,127 DMRs between the sexes at this stage, including 47,962 (99,62%) that were hypermethylated in males compared to females (Fig. 1b). To test whether this difference indicates that female PGCs are advanced in the timeline of methylation re-patterning, we compared datasets from GD16 female germ cell to GD18 male germ cells we obtained previously using the same experimental approach.[19] DNAme in GD16 female germ cells was significantly higher than DNAme levels in GD18 male PGCs but with a small Cohen's d effect size (value of 0.42) (Fig. 1a). Specifically, we identified 1618 DMRs between the two cell populations (Fig. 1b). Overall, this shows that the DNA landscape of female germ cells at GD16 more closely resembles the landscape we observed in male germ cells at GD18 than at GD16, suggesting that DNA demethylation is more rapid in female germ cells compared to male germ cells.

In maternal HFD conditions, we obtained a similar proportion of GFP-positive cells in gonadal cell suspensions compared to controls, suggesting no effect of HFD on germ cell development (Table 1). We did find however that maternal HFD was associated with a small yet significant decrease in DNAme levels in germ cells in both sexes (Fig. 1c). Further analysis revealed more DMRs in males than in females but similar distributions in different genomic regions between sexes, whether they were hypo- or hyper-methylated DMRs (Fig. 1d–f). Only 51 DMRs were common between both sexes, and only 23 varied in the same direction in both sexes (5 hypermethylated and 17 hypomethylated) (Supplemental Table 2), suggesting sex-specific differences due to maternal diet. The analysis of chromosomal distribution showed a broad DMR spread across chromosomes, with the majority of the DMRs being hypomethylated (Fig. 1e). GO term analysis using all DMRs but the intergenic regions in each sex failed to find any significant enrichment, suggesting a random distribution of these HFD-induced DMRs in both males and females. Yet, detailed analysis of CpG islands, imprinted genes, ncRNA and transposable elements identified in our datasets revealed some that were affected by the maternal HFD in both sexes (Supplemental Table 1). We also identified hypermethylated DMRs in both male and female PGCs after exposure to the maternal HFD (Fig. 1e,f), which could correspond to genomic hotspots escaping epigenetic reprogramming (i.e. escaping DNAme erasure). Considering only the hypermethylated DMRs with more than 50% difference in DNAme levels compared to the control group, we found 49 DMRs in males, and only 4 in females (complete lists are available online in the GEO Series GSE245803). Out of the 49 DMRs in males, 29 are in genic regions, while out of the 4 DMRs identified in females, only 2 are within genes (cut-like homeobox 2 (*Cux2*) and teneurin transmembrane protein 3 (*Tenm3*)).

Table 1. Impact of gestational HFD on maternal weight gain and pregnancy outcomes ($n = 5$ litters/group)

	Control	HFD
Maternal weight gain (g)	52.56 ± 1.4	70.41 ± 3.76*
Litter size	11.20 ± 1.11	12.40 ± 068
Live birth (%)	98.46 ± 1.54	97.14 ± 2.86
Pup sex ratio (% males)	54.05 ± 9.08	43.56 ± 8.82
GFP-positive cells/testes (%)	20.22 ± 2.51	24.24 ± 1.92
GFP-positive cells/ovary (%)	21.94 ± 1.73	22.46 ± 2.35

Maternal weight gain was determined by the difference between weight on the day of sacrifice and the first day on the diet (gestational day 0). The % GFP-positive cells were determined from the gonadal cell suspensions obtained before sorting. Data are mean ± SEM. *$p < 0.05$; HFD = high-fat diet.

Discussion

Germline epigenetic reprogramming in early embryonic development is sensitive to intrauterine environmental factors. This vulnerability is proposed as one of the underlying mechanisms of inter- and transgenerational effects. Maternal HFD has been well documented to induce impaired physiologic function over several generations.[25] The present study therefore aimed to test whether HFD gestational conditions could influence the reprogramming of DNAme in the germline of the F1 offspring and test for sex-specific effects. We show here that maternal HFD was associated with a global DNA hypomethylation in PGCs in both sexes. Importantly, we observed that the reprogramming of DNAme differs in control male and female PGCs and that this sex-specific difference likely renders males more vulnerable to factors like a HFD during early embryonic development.

In mammals, DNAme is almost completely erased in germ cells during fetal life, which occurs in both the male and female germline, despite different gonadal somatic environments.[13] This stage of germ cell development, when DNAme levels reach their lowest, corresponds to the last stage of development when male and female PGCs are comparable in their cell cycle, before pursuing different paths: quiescence for male PGCs and meiosis for female PGCs. Importantly, we observed that DNAme levels at GD16 in the rat germline are in fact different between sexes,

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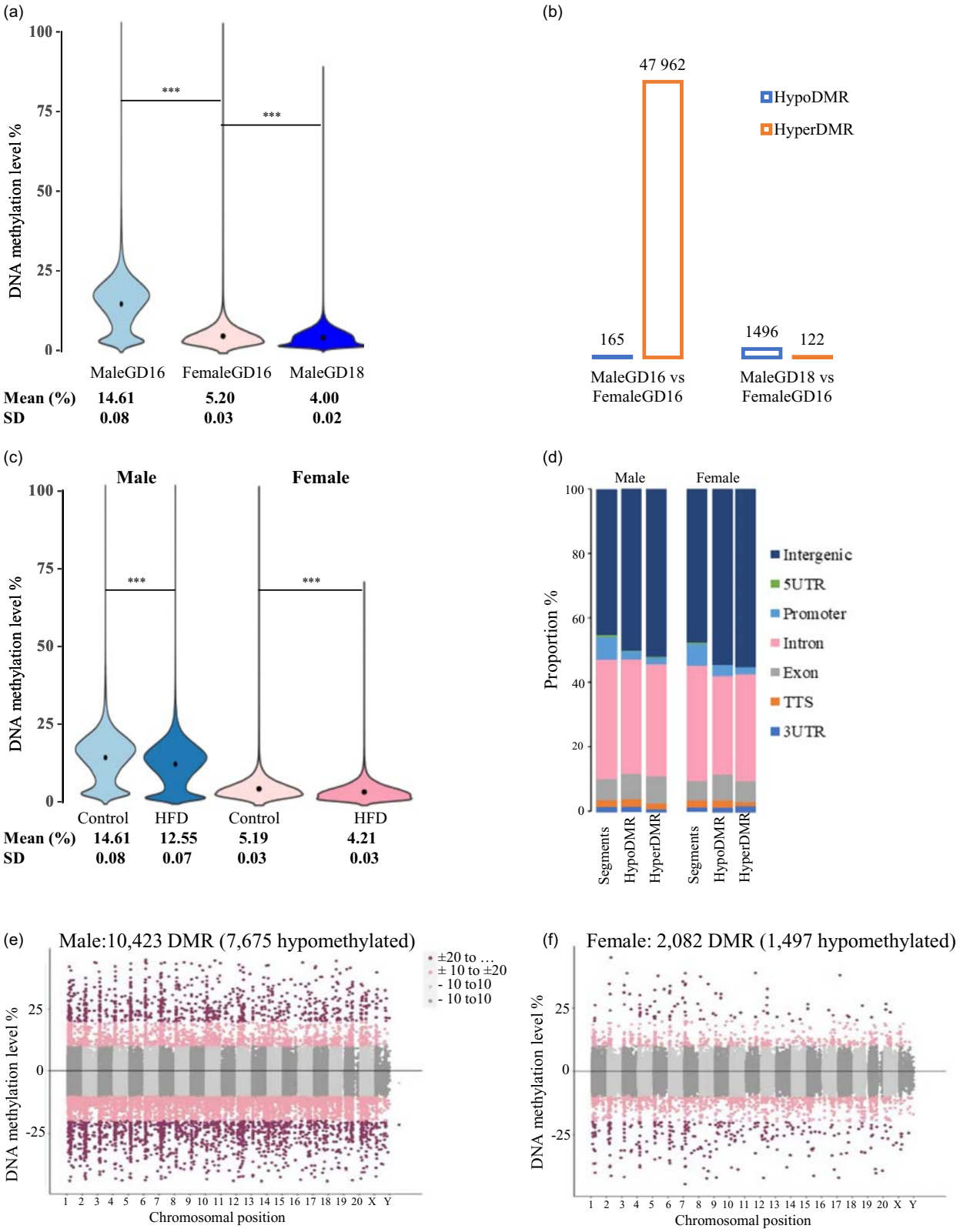

Figure 1. DNA methylation in male and female germ cells from control and maternal HFD-exposed fetuses at midgestation . (***a***) DNA methylation levels are compared between control female and male germ cells at GD16 and GD18. (***b***) The number of differentially methylated segments (DMR ≥ 10%) are represented between control female and males germ cells at GD16 and GD18. (***c***) DNA methylation levels are compared between control and HFD-exposed germ cells at GD16 in both male and female. (***d***) Proportions of DNA features are represented for male and female germ cells, respectively, in all segments analysed and in hypomethylated and hypermethylated DMRs. (***e***) Manhattan plots showing the distribution of the segments along chromosomes (*x* axis) and the differential methylation levels in control versus HFD (*y* axis) in male and female. ***$P << 2.2e{-}16$; HFD = high-fat diet.

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with levels higher in males than in females. Interestingly, we have recently demonstrated that the lowest levels in the male rat germline were reached only at GD18[19] with a pattern that resembles what we describe here for the female at GD16. The loss of DNAme in female rat germ cells therefore appears to occur earlier than in males. This difference is consistent with what was reported in mice in sorted *Oct4-Gfp*-positive germ cells at E13.5, where DNAme levels were slightly higher in males than in females.[26,27] Whether the different kinetics of DNA demethylation between males and females is due to higher activity of the active demethylation process through TET enzymes is unknown. The possibility that it is due to a faster cell cycle in females compared to males, inducing more rapid passive loss of methylation, is supported by the demonstration in mice that the proliferation index measured by incorporation of BrdU is higher in females than male germ cells.[28] However, this sex difference in DNA demethylation highlights the possibility that the windows of sensitivity to intrauterine factors differ according to fetal sex and that males, because DNAme erasure takes longer, may be sensitive for a longer period of time in terms of epigenetic disruption of the germline. This dynamic difference in DNAme could be one of the first steps that differentiates male versus female response to environmental factors, allowing downstream signalling pathways to further enhance sex-specific differences.

We observed a global hypomethylation induced by the maternal HFD in both male and female germ cells at GD16. Yet only very few DMRs were common to both sexes. In females, this global hypomethylation could mean that demethylation, which is already well advanced in controls at this stage, was even more profound in HFD-exposed germ cells. For males, HFD-induced hypomethylation could be the result of accelerated demethylation, a process that is still active in male rat germ cells at that stage, since the lowest level of DNAme is not reached until 2 d later at GD18 in rats.[19] The mechanisms by which maternal HFD may induce hypomethylation of DNA include increasing passive demethylation by accelerating the cell cycle or stimulation of active demethylation by TET enzymes. This is consistent with the fact that HFD has been shown to induce demethylation in adult male germ cells by increasing the expression of *Tet3* mRNA.[29] However, such mechanism remains to be further studied in germ cells at earlier stages. The fact that we did not observe any difference in the proportion of GFP-positive cells after FACS between groups suggests that this hypomethylation is not due to a change in cell number.

Importantly, we identified hypermethylated DMRs in both sexes that could correspond to genomic hotspots escaping epigenetic reprogramming or remethylated more rapidly. Interestingly, these were mostly sex specific. Additionally, some showed major differences in DNA methylation, higher than 50%, including a few in genic regions. Further investigation of these genes' functions could help raise the hypothesis on disruption of the F1 gametogenesis and inheritance.

In conclusion, we have shown that maternal acute HFD intake impacts fetal PGC epigenetic reprogramming, particularly at the demethylated stage in male and female germ cells. We have shown novel data demonstrating that HFD is associated with an overall hypomethylation of PGCs compared with controls, but also show that some hypermethylated sites escaped reprogramming. Future studies are needed to identify the DMRs that remain anchored in the germline and could impact gametogenesis, fertility or even heritage transmitted to the F2 generation.

Supplementary material. The supplementary material for this article can be found at https://doi.org/10.1017/S2040174424000230.

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Author contribution. DMS, SMG and GD designed the experiments. Animal work and germ cell collection was carried out by AR. DNA extractions and libraries were done by IG. Data analysis was done by REC and AL under the supervision of CR, SMG and GD. REC wrote the first draft of the manuscript that was further edited by DMS, SMG and GD and approved by all.

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Competing interests. None.

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