

Maternal obesity may disrupt offspring metabolism by inducing oocyte genome hyper-methylation via increased DNMTs

Reviewed Preprint

v2 • November 7, 2024

Revised by authors

Reviewed Preprint

v1 • August 13, 2024

Shuo Chao, Jun Lu, Li-Jun Li, Hong-Yan Guo, Kui-Peng Xu, Ning Wang, Shu-Xian Zhao, Xiao-Wen Jin, Shao-Ge Wang, Shen Yin, Wei Shen, Ming-Hui Zhao, Gui-An Huang, Qing-Yuan Sun , Zhao-Jia Ge 

College of Life Sciences, Institute of Reproductive Sciences, Key Laboratory of Animal Reproduction and Germplasm Enhancement in Universities of Shandong, Qingdao Agricultural University, Qingdao, China; • College of Horticulture, Qingdao Agricultural University, Qingdao, China; • Guangzhou Key Laboratory of Metabolic Diseases and Reproductive Health, Guangdong-Hong Kong Metabolism & Reproduction Joint Laboratory, Reproductive Medicine Center, Guangdong Second Provincial General Hospital, Guangzhou, China

 https://en.wikipedia.org/wiki/Open_access

 Copyright information

eLife Assessment

This manuscript reports **important** findings on the impact of maternal obesity on offspring metabolism. It presents **solid** evidence that maternal obesity induces genomic methylation alterations in oocytes, which can be partly transmitted to F2 in females, and that melatonin is involved in regulating the hyper-methylation of high fat diet oocytes by increasing the expression of DNMTs via the cAMP/PKA/CREB pathway. This study would be of interest to biologists in the fields of epigenetics and metabolism.

<https://doi.org/10.7554/eLife.97507.2.sa2>

Abstract

Maternal obesity has deleterious effects on the process of establishing oocyte DNA methylation; yet the underlying mechanisms remain unclear. In the present study, we found that maternal obesity induced by high-fat diet (HFD) disrupted the genomic methylation of oocytes, and that at least a part of the altered DNA methylation was transmitted to the F2 oocytes and the livers via females. We further examined the metabolome of serum and found that the altered metabolites such as melatonin may play a key role in the disrupted genomic methylation in the oocytes of obese mice. We further found that exogenous melatonin treatment significantly reduced the hyper-methylation of HFD oocytes, and the increased expression of DNMT3a and DNMT1 in HFD oocytes was also decreased. To address how melatonin regulates the expression of DNMTs, the function of melatonin was inhibited or activated upon oocytes. Results revealed that melatonin may regulate the expression of DNMTs via the cAMP/PKA/CREB pathway. These results suggest that maternal obesity induces genomic methylation alterations in oocytes, which can be partly transmitted to F2 in females,

and that melatonin is involved in regulating the hyper-methylation of HFD oocytes by increasing the expression of DNMTs via the cAMP/PKA/CREB pathway.

Introduction

Obesity has become a global health problem, affecting approximately 13% of the world's adult population, and over 340 million children and adolescents (WHO). This epidemic has profound implications not only for reproductive health but also for the well-being of subsequent generations. Previous studies have demonstrated that maternal obesity reduces the function of the hypothalamic-pituitary-ovarian (HPO) axis (X. Chen, Xiao, Cai, Huang, & Chen, 2022 [\[1\]](#)), deteriorates oocyte cytoplasmic quality and nuclear maturation, and disrupts genome methylation (Broughton & Moley, 2017 [\[2\]](#)). Reduced expression of *Stella* in oocytes induced by obesity results in global hypo-methylation in zygotes, which has an important contribution to the defective embryo development (Han et al., 2018 [\[3\]](#)). Furthermore, progeny of obese females have a higher risk of non-communicable diseases, such as obesity, diabetes, and cardiovascular diseases (Godfrey et al., 2017 [\[4\]](#)). Our previous study indicated that obesity altered the methylation status of *Leptin*, which might play a role in the metabolic disorders of female offspring. However, these methylation changes are not detected in the first-generation (F1) oocytes (Ge et al., 2014 [\[5\]](#)). The influence of maternal obesity on the genomic methylation of oocytes is still obscure. Thus, more studies are necessary to explore the role of DNA methylation in mediating the transgenerational transmission of metabolic syndrome induced by female obesity.

Obesity also perturbs glucose and lipid metabolism, which has negative effects on oocyte maturation and embryo development. Elevated levels of circulating free fatty acids in obese females contribute to oocyte lipotoxicity, while concomitantly diminishing mitochondrial function within oocytes (Broughton & Moley, 2017 [\[2\]](#)). The decreased melatonin levels are also reported in animals and humans by previous studies (Overberg et al., 2022 [\[6\]](#); Virto et al., 2018 [\[7\]](#)). Melatonin has contributions to metabolism and DNA methylation in somatic cells (Davoodvandi, Nikfar, Reiter, & Asemi, 2022 [\[8\]](#)). These findings indicate that the disturbed metabolism induced by obesity is closely linked to the compromised oocyte quality and aberrant methylation patterns. This study aims to elucidate the effects of obesity-induced metabolic changes on oocyte genomic methylation and its inherited implications in a murine model.

Results

Obesity alters the genomic methylation in oocytes

The obese mouse model was induced via a high-fat diet (HFD) (Ge et al., 2014 [\[5\]](#); Han et al., 2018 [\[3\]](#)), and mice fed with standard diet were used as a control (CD). The HFD group exhibited a significantly higher average body weight compared to CD group ($n > 86$, Fig. S1A and B). Re-methylation in oocytes occurs in follicular development and is nearly complete at the germinal vesicle (GV) stage. The 5mC (5-methylcytosine) and 5hmC (5-hydroxymethylcytosine) levels in the GV oocytes of HFD mice were significantly higher than that in the CD group ($n > 30$, Fig. 1A [\[9\]](#) - C). To further explore the effects of maternal obesity on the oocyte methylation, we examined the genomic methylation of metaphase II (MII) oocytes using whole-genome bisulfite sequencing for small samples (WGBS, Novogene, Beijing, China). The information of reads count, mapping rate, conversion rate and sequencing depth was presented in Table S1. We found that global methylation in MII oocytes of HFD group was higher than that in the CD group (Fig. 1D [\[10\]](#)). Methylated cytosine (C) can be classed into three types according to the context in the genome including CG, CHG and CHH (H=A, T or C). The methylated CG have more contributions to regulate gene expression. We found that the CG methylation level in MII oocytes of HFD was significantly

higher than that in the CD group (**Fig. 1E**). Differentially methylated CG distributed across all chromosomes (Fig. S1C). To further analyze the distribution of methylation, each functional region of genes was equally divided into 20 bins, and then the average methylation levels in the functional regions were calculated, respectively. CGIs (CG islands) and CGI shores were predicted using cpGISlandExt and repeat sequences were predicted using RepeatMasker. Results showed that the hyper-methylation was distributed in the promoter, exon, upstream 2k, and downstream 2k regions (**Fig. 1F** and Fig. S1D) of genes in HFD oocytes. These findings suggest that maternal obesity results in hyper-genome methylation in oocytes.

Distribution of differentially methylated regions (DMRs)

We further analyzed the differentially methylated regions (DMRs) in oocytes, and identified 4340 DMRs between HFD and CD oocytes. These DMRs were defined by the following criteria: the number of CGs ≥ 4 and the absolute methylation difference ≥ 0.2 . Among these, 2,013 were hyper-DMRs (46.38%), and 2327 were hypo-DMRs (53.62%) (**Fig. 1G**). These DMRs were distributed across all chromosomes (**Fig. 1H**). We then annotated DMRs into different genomic regions including promoter, exon, intron, CGI, CGI shore, repeat, TSS (transcription start site), TES (transcription end site), UTR3 (3' end untranslated region), and UTR5 (5' end untranslated region) regions (Fig. S2A), and the average methylation levels of DMRs in these regions were similar between HFD and CD oocytes (Fig. S2B). However, the hypo-DMRs were enriched in the UTR3, repeat, and intron regions compared with hyper-DMRs (Fig. S3).

Methylation level at promoters strongly contributes to the regulation of gene expression. We then analyzed the enrichment of genes with DMRs at promoters in KEGG (Kyoto Encyclopedia of Genes and Genomes) pathways using KOBAS online. Results indicated that the genes with DMRs at promoters were significantly enriched in metabolic pathways including amino acid metabolism pathways, carbohydrate metabolism pathways, lipid metabolism pathways, and metabolism of cofactors and vitamins pathways (**Fig. 1I**, Table S2). A total of 35 genes with DMRs at promoters were included in metabolism pathways, and 19 of these genes were with hyper-DMRs and 16 of these genes were with hypo-DMRs (Table S3). These results suggest that the altered methylation in oocytes induced by maternal obesity may play a role in the metabolic disorders in offspring.

The disturbed methylation may be associated with the transgenerational inheritance of the metabolic disorders through females

Our recent study demonstrated that disturbed methylation in oocytes caused by uterine undernourishment can be partly transmitted to F2 oocytes via females, which may play a key role in the transgenerational inheritance of metabolic disorders (S. B. Tang et al., 2023). Here, we investigated the inheritance of altered methylation in HFD oocytes. F1 and F2 generations were produced as shown in the schedule in **Fig. 1J**: HF1 and CF1, female HFD and CD, respectively, were mated with control males; HF2 and CF2, female HF1 and CF1, respectively, were mated with control males. We examined the glucose and insulin tolerance (GTT and ITT), and found that the GTTs and ITTs of F0, F1, and F2 females were impaired (**Fig. 2A-C**). The inheritance of disrupted metabolism in females might be associated with the altered DNA methylation of oocytes. To address this question, we first examined the DNA methylation status of DMRs located at the promoters of *Bhlha15* (also known as *Mist1*, basic helix-loop-helix, a transcription factor), *Mgat1* (mannoside acetylglucosaminyltransferase 1), *Taok3* (serine/threonine-protein kinase 3), *Tkt* (transketolase), *Pik3cd* (phosphatidylinositol-4, 5-bisphosphate 3- kinase catalytic subunit delta), and *Pld1* (phospholipase D1) in the HFD and CD oocytes. We found that the methylation levels of hyper-DMRs including *Bhlha15*-DMR, *Mgat1*-DMR, and *Taok3*-DMR in HFD oocytes were significantly higher than in the CD group (**Fig. 2D**). The methylation level of hypo-DMRs, *Pik3cd*-DMR in HFD oocytes was significantly lower than that in the CD group (**Fig. 2D**). These results

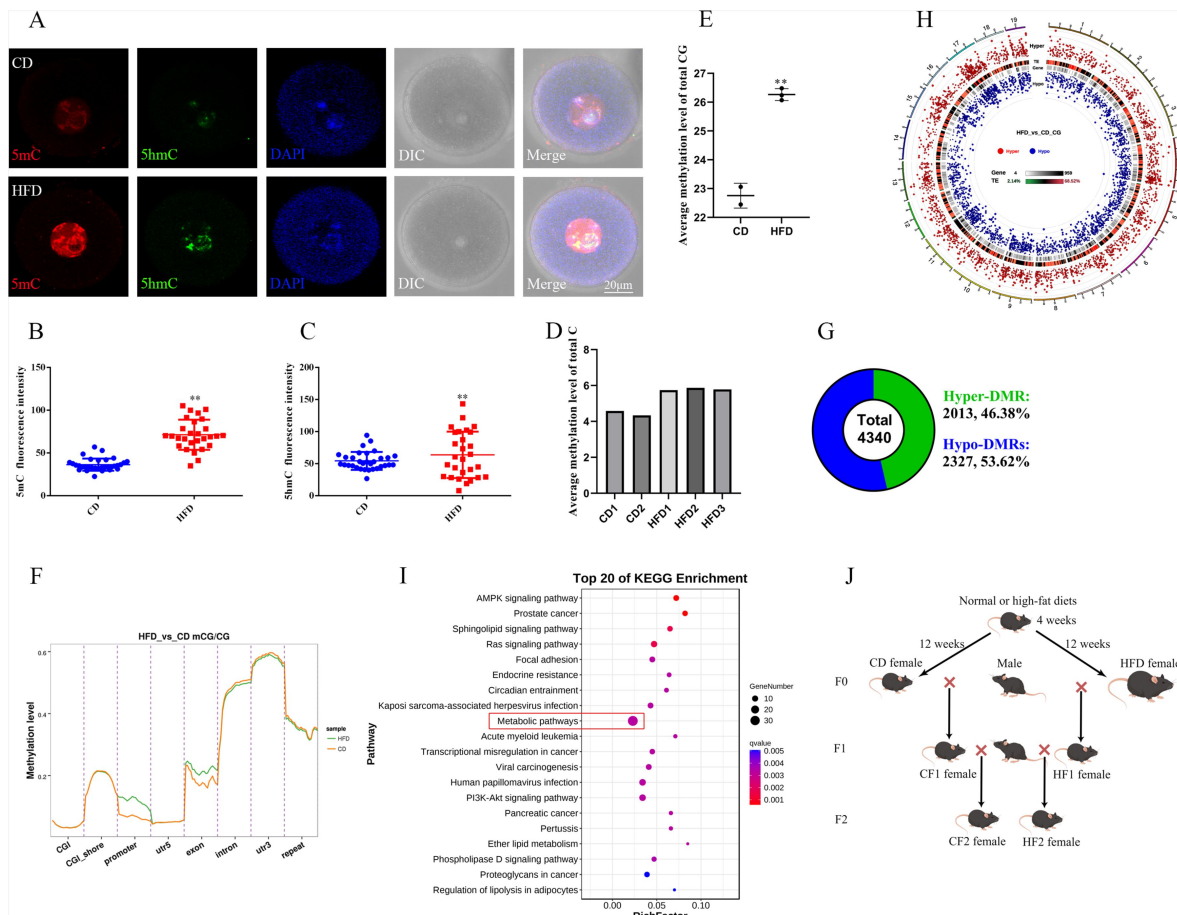


Figure 1

Maternal obesity alters the DNA methylation of oocytes.

(A) Methylation levels of 5mC and 5hmC in oocytes ($n > 30$). 5mC, 5-methylcytosine; 5hmC, 5-hydroxymethylcytosine; DAPI, chromatin.

(B, C) Relative fluorescence intensity of 5mC and 5hmC in GV oocytes.

(D) Genomic methylation level of MII oocytes examined by single-cell whole genome bisulfite sequencing. The control group (CD) has two replicates, and the obesity group (HFD) has three replicates.

(E) Average genomic CG methylation level in MII oocytes. CD, control group; HFD, obesity group; ** means p value < 0.01 .

(F) CG methylation levels at different regions in MII oocytes. CGI, CpG island; utr5, 5' untranslated region; utr3, 3' untranslated region; repeat, repeat sequence.

(G) Total differentially methylated regions (DMRs) in oocytes of control and obesity groups. Hyper-DMRs, hypermethylated DMRs; hypo-DMRs, hypomethylated DMRs.

(H) Distribution of DMRs on chromosomes in MII oocytes. Outside-to-in: chromosomes, hyper-DMRs, TEs (transcription end regions), and gene, hypo-DMRs.

(I) KEGG pathway enrichment of genes with DMRs at the promoter regions, and the top 20 pathways are presented.

(J) Schedule of breeding. Female C57BL/6 mice fed with normal (CD) or high-fat diet (HFD) for 12 weeks were marked as F0. F1 was produced by F0 mated with normal males, respectively, and marked as CF1 and HF1; F2 was produced by female F1 mated with normal males and marked as CF2 and HF2, respectively.

coincide with the genomic sequencing results. Whereas, the methylation level of *Tkt*-DMR (hypo-DMR) in HFD oocytes was higher than that in the CD group, and the methylation level of *Pld1*-DMR (hypo-DMR) was similar between the two groups, which contradicts the genomic sequencing results (Fig. S4A). These findings suggest that some regions are false positives in genomic sequencing. To exclude the effects of somatic cell contamination, we examined the methylation level of paternally imprinted gene *H19*, which was low in both HFD and CD oocytes (Fig. S4B). These findings indicate that the samples are not contaminated by somatic cells.

We then examined the methylation of DMRs in F1 livers using bisulfite sequencing (BS). Ten livers from five litters were analyzed for each group. Results revealed that the methylation levels of *Bhlha15*-DMR and *Mgat1*-DMR were higher and the methylation level of *Pik3cd*-DMR was lower in HF1 livers than that in CF1 livers (Fig. S5A-C). The methylation level of *Tkt*-DMR in HF1 livers was lower than that in CF1 (Fig. S5D), although it was higher in HFD oocytes compared with CD. This contradiction may be associated with the uterine environment of obesity and the reprogramming during early embryo development. We further examined the expression of several genes with DMRs at promoters, including hyper-methylated genes, such as *Bhlha15*, *Mgat1*, *Dgka*, *Pdpk1* and *Taok3*, and hypo-methylated genes, such as *Igf1*, *Map3k8*, *Pld1*, *Tkt*, *Pik3cd* and *Sphk2*. The expression levels of *Bhlha15*, *Mgat1* and *Pdpk1* were significantly lower and the expression levels of *Map3k8*, *Tkt*, *Pik3cd*, and *Sphk2* were significantly higher in HF1 livers compared to that in CF1 (Fig. S5E and F). The expression trends of *Bhlha15*, *Mgat1*, *Tkt*, and *Pik3cd* were consistent with the methylation status at promoters in F1 livers. The expression of *Dgka*, *Taok3*, *Igf1*, and *Pld1* was not affected in F1 livers (Fig. S4E and F). *Mgat1* is associated with lipid metabolism and obesity (Jacobsson et al., 2012; Johansson et al., 2010), *Tkt* regulates glucose metabolism (Bartakova et al., 2016; Kang et al., 2018), and *Pik3cd* is involved in lipid metabolism and diabetes (Hood, Berger, Freedman, & Law, 2019; Wojcik et al., 2014). These results suggest that the altered methylation in HFD oocytes is partly transmitted to F1 livers via oocytes, and that the abnormal methylation may be a reason for the disturbed metabolism of HF1.

If the altered methylation in HFD oocytes was inherited by HF1 oocytes, it would be transmitted to F2 generation. Therefore, we examined the methylation of DMRs in F1 oocytes, and results revealed that *Bhlha15*-DMR, *Mgat1*-DMR, and *Taok3*-DMR were significantly hyper-methylated in HF1 oocytes compared with CF1 oocytes (Fig. 2E). The methylation level of *Tkt*-DMR was significantly lower (Fig. S6), and the methylation level of *Pik3cd*-DMR was slightly lower in the HF1 oocytes than that in the CF1 oocytes (Fig. 2E). These results indicate that at least a part of the altered methylation in HFD oocytes is transmitted to F1 oocytes via females.

To confirm the transgenerational inheritance of the altered DNA methylation in HFD oocytes, we examined the methylation levels of DMRs in F2 livers. The methylation of *Bhlha15*-DMR and *Mgat1*-DMR was higher and the methylation level of *Pik3cd*-DMR was lower in HF2 livers than that in CF2 livers (Fig. S7A-C). The methylation of *Tkt*-DMR in HF2 livers was similar to that in CF2 group (Fig. S7D). In addition, the expression levels of *Bhlha15*, *Pdpk1*, and *Mgat1* were significantly lower (Fig. S7E), and the expression levels of *Pld1*, *Pik3cd*, and *Sphk2* were significantly higher in HF2 livers than that in CF2 (Fig. S7F). The expression of the other gene were similar in the livers between HF2 and CF2 (Fig. S7E and F). These results suggest that at least a part of the altered methylation in HF2 livers may be inherited from HFD oocytes, and that this alteration may be associated with the disrupted metabolism in F2 offspring.

We then examined the methylation of DMRs in F2 oocytes using BS, and found that the hyper-methylation of *Bhlha15*-DMR, *Mgat1*-DMR, and *Taok3*-DMR, and the hypo-methylation of *Pik3cd*-DMR and *Tkt*-DMR were maintained in HF2 oocytes (Fig. 2F).

To better understand the inheritance of the altered methylation, we analyzed the methylation level of DMRs among generations compared with that in HFD oocytes (Fig. 2G). For hyper-DMRs, the methylation of *Bhlha15*-DMR was maintained from HFD oocytes to HF2 oocytes. Compared to

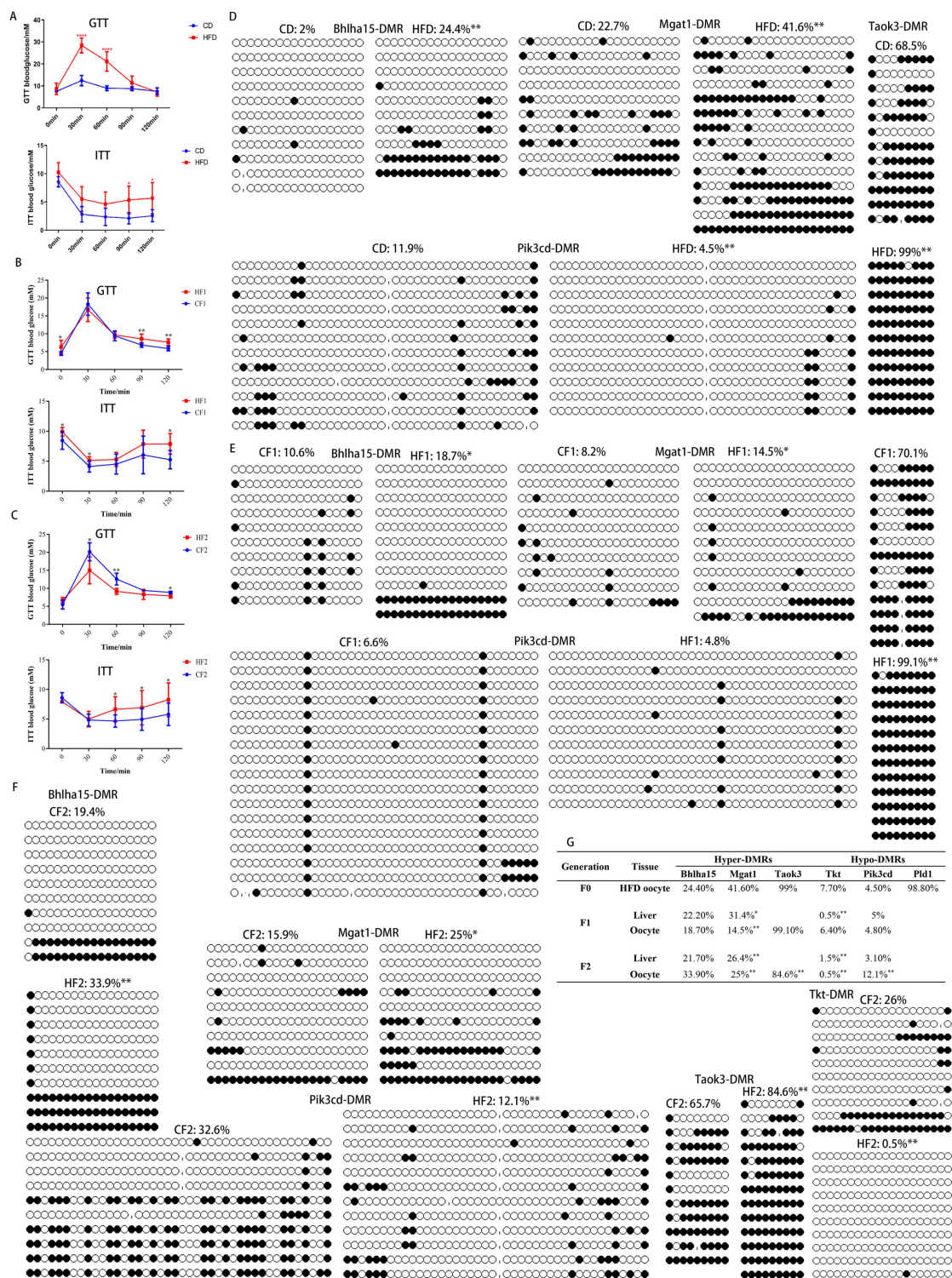


Figure 2

Transgenerational inheritance of metabolic disorders and altered DNA methylation.

(A-C) Glucose tolerance (GTT) and insulin tolerance (ITT) were tested for female F0, F1, and F2, respectively. * $p < 0.05$; ** $p < 0.01$.

(D-F) DMR methylation at the promoter regions of *Bhlha15*, *Mgat1*, *Taok3*, *Tkt*, and *Pid3cd* in F0, F1, and F2 oocytes was respectively examined using bisulfite sequencing. At least 10 available clones from 80-100 oocytes were used to calculate the methylation level. White circle, unmethylated CG; black circle, methylated CG. * $p < 0.05$; ** $p < 0.01$.

(G) Inheritance of altered methylation in different generations was analyzed. * $p < 0.05$; ** $p < 0.01$.

those in HFD oocytes, the methylation levels of *Mgat1*-DMR and *Taok3*-DMR were partly transmitted to HF2 oocytes. For hypo-DMRs, the methylation levels of *Tkt*-DMR and *Pik3cd*-DMR in HFD oocytes were inherited by HF2 (Fig. 2G). We did not examine the methylation level of *Pld1*-DMR in F1 and F2 because it was similar in oocytes between CD and HFD (Fig. 2G). These results suggest that only a part of the altered methylation in HFD oocytes can be transmitted to F2 oocytes, and that disrupted methylation may be a reason for the inheritance of the metabolic disorders in F1 and F2.

Obesity alters the metabolomics of serum

Obesity alters the metabolism of glucose, fatty acids, and amino acids, which are essential for oogenesis. Thus, we suppose the altered metabolism may play a key role in the disturbed global methylation in HFD oocytes. We examined the metabolomics of serum using non-targeted approaches (BGI, Wuhan, China). We used LC-MS/MS to identify the variation in metabolites, including amino acids, carbohydrates, lipid, and phenols. The principal component analysis (PCA) showed that the PC1 and PC2, respectively, explained 44.6% and 9.96% of the total metabolite variation, respectively (Fig. 3A). The distribution of extracts between groups was distinguishable (Fig. 3A). We identified 538 differential metabolites based on the PLS-DA (robust orthogonal partial least squares-discriminant analysis) and T-test analysis with the following criteria: VIP (variable importance in projection) ≥ 1 , fold change ≥ 1.2 or ≤ 0.83 , and p -value < 0.05 , including 288 upregulated and 250 downregulated metabolites (Fig. 3B). The enrichment of differential metabolites was analyzed using KEGG, which revealed that differential metabolites were significantly enriched in tryptophan and vitamin B6 metabolism (Fig. 3C). The top 20 differential metabolites were presented in Fig. 3D. These results suggest that obesity disturbs the metabolomics of serum.

Melatonin may play a key role in the genomic hyper-methylation of HFD oocytes

To investigate the associations between the metabolites and methylation in oocytes, we identified several differential metabolites in HFD mice, including pyridoxine (vitamin B6), L-methionine, melatonin, and L-tyrosine, which may be associated with the hyper-methylation of oocytes. The concentration of pyridoxine (vitamin B6), L-methionine, and L-tyrosine was significantly increased in HFD serum compared with CD group (Fig. 3D-G). In the methionine cycle, methionine adenosyltransferase catalyzes methionine and ATP into S-adenosyl methionine (SAM) (Shou, Pan, & Chin, 1969) which acts as a universal methyl donor (Lyon, Strippoli, Fang, & Cimmino, 2020). DNA methyltransferases (DNMTs) transfer the methyl group to cytosine and convert SAM to S-adenosyl homocysteine (SAH) which is further degraded to homocysteine (HCY) by SAH hydrolase (D. H. Chen, Wu, Hung, Hsieh, & Li, 2004). During these processes, vitamin B6 serves as a co-factor (Vaccaro & Naser, 2021). Excessive methionine and vitamin B6 intake has been reported to induce hyper-methylation (R. A. Waterland, 2006). These findings indicate that the hyper-methylation in HFD oocytes may be associated with the increased concentrations of methionine and pyridoxine. To confirm this hypothesis, we examined the concentrations of SAM, SAH, and HCY in livers and oocytes, which are crucial intermediate metabolites in the methionine cycle. Results showed that the concentrations of SAM, SAH, and HCY in HFD livers were similar to those in CD livers (Fig. 3H-J), and the concentration of SAM in HFD oocytes was lower than that in the CD group (Fig. 3K). These results suggest that the higher concentrations of methionine and pyridoxine in the serum of HFD mice may not be the main reason for the genomic hyper-methylation in oocytes.

As presented in Fig. 3D, it is curious that the abundance of genistein, daidzein and dibutylphthalate were also altered in HFD serum compared with those in CD, which might have contributed to the altered DNA methylation in the oocytes. These metabolites might be from the diets or materials used to collect samples. To confirm these results, we examined the

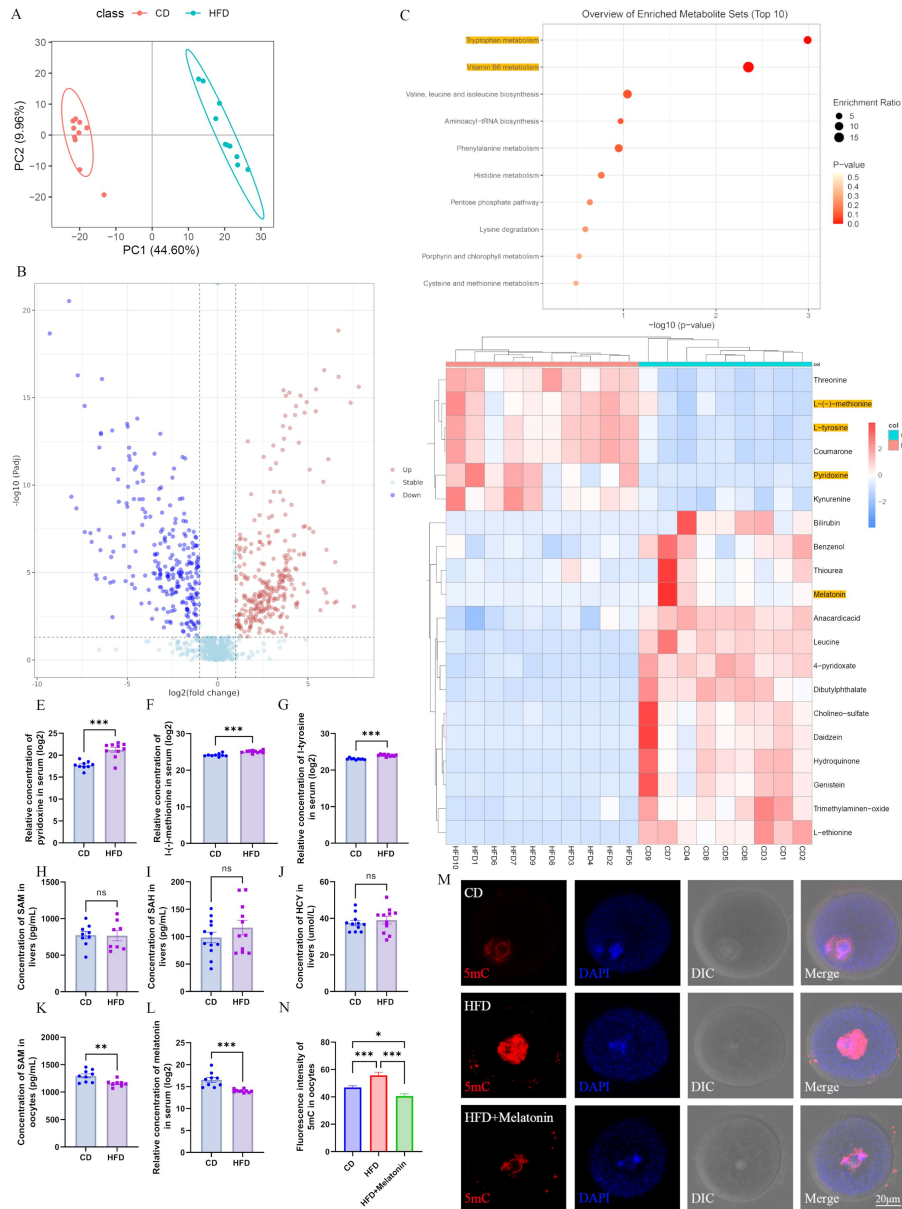


Figure 3

Maternal obesity alters metabolome of serum.

(A) Principal component analysis in CD and HFD mice.

(B) Differential metabolites in HFD serum compared with those in CD group. Red circles, upregulated metabolites; blue circles, downregulated metabolites.

(C) The enrichment of differential metabolites was analyzed using KEGG, and the top 10 enrichment terms are presented.

(D) Heat map of the top 20 differential metabolites in HFD serum.

(E-G) Comparison of the concentrations of pyridoxine, methionine, and tyrosine among the groups. * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$.

(H-J) Concentrations of SAM, SAH and HCY in the livers were examined by ELISA. Ns, there was no statistical significance between groups.

(K) The concentration of SAM in oocytes was analyzed using ELISA. ** $p < 0.01$.

(L) Relative concentration of melatonin in the serum. *** $p < 0.001$.

(M) Genomic DNA methylation in oocytes was examined using immunofluorescence. CD, control group; HFD, obesity group; HFD+melatonin, obese mice were treated with exogenous melatonin for 14 days.

(N) Relative fluorescence intensity of 5mC was examined using Image J (CD, $n = 109$; HFD, $n = 104$; HFD+melatonin, $n = 96$). * $p < 0.05$; *** $p < 0.001$.

concentrations of genistein and dibutylphthalate using ELISA. The results revealed that the concentrations of these metabolites were similar between HFD and CD serum (Fig. S8), which suggest that these metabolites may have no effect on the altered methylation in oocytes.

As presented in **Fig. 3D and L**, the concentration of melatonin in the HFD serum was significantly lower than that in the CD group. Low concentration of melatonin has also been reported in obese rats (Virto et al., 2018) and humans (Overberg et al., 2022). Exogenous melatonin supplementation reduces body weight and improves lipid and glucose metabolism in both animals and humans (Guan, Wang, Cao, Dong, & Chen, 2021). Melatonin can also inhibit cancer by regulating DNA methylation status (Davoodvandi et al., 2022), improve DNA methylation reprogramming in the development of porcine cloned embryos (Qu et al., 2020), and affect DNA re-methylation in oocytes (Lan et al., 2018; Saeedabadi, Abazari-Kia, Rajabi, Parivar, & Salehi, 2018; Xiao et al., 2019). This indicates that the reduced melatonin may contribute to the hyper-methylation of HFD oocytes. Here, we found that if HFD mice were treated with exogenous melatonin for 14 days, the genomic hyper-methylation in HFD oocytes was significantly reduced ($n \geq 96$ **Fig. 3M and N**). These results suggest that the reduced melatonin concentration may be involved in regulating the hyper-methylation of HFD oocytes.

Melatonin regulates genomic methylation of oocytes by increasing the expression of DNMTs via the cAMP/PKA/CREB pathway

Melatonin receptors (MT1 and 2), which are coupled with inhibitor G- protein (Gi), have been identified in oocytes and granulosa cells (Jin et al., 2022; Wang et al., 2021). Activated Gi inhibits the activation of adenylyl cyclases (ADCYs), resulting in a decrease of cAMP (cyclic adenosine monophosphate), which regulates the activation of protein kinase A (PKA) and CREB (cAMP response element-binding protein) (Wongprayoon & Govitrapong, 2021). Elevated cAMP level increases the expression of DNMTs resulting in hyper-methylation in HL-1 cardiomyocytes (Fang et al., 2015). Hedrich *et al.* reported that CREMa induced hyper-methylation of CD8 cluster via increasing the expression of DNMT3a (Hedrich et al., 2014). We thus supposed that melatonin may regulate genomic methylation in oocytes via increasing the expression of DNMTs through the cAMP/PKA/CREB pathway (**Fig. 4A**). To confirm this hypothesis, female C57BL/6 mice fed with normal diet were treated with luzindole, an inhibitor of melatonin receptor, and the global methylation of 5mC and 5hmC was significantly increased in oocytes ($n \geq 49$ **Fig. 4B-D**). Luzindole did not affect the concentration of melatonin in the serum (Fig. S9A). However, excessive melatonin treatment significantly increased the concentration of melatonin in the serum (Fig. S9A) and decreased the methylation levels of 5mC and 5hmC in oocytes (**Fig. 4B-D**). These findings indicate that melatonin may regulate the re-methylation process in oocytes.

To confirm whether melatonin regulates methylation in oocytes via the cAMP pathway, we examined the expression of ADCYs in oocytes using RT-PCR, and found that ADCY5, 6, and 9 were expressed in oocytes (Fig. S9B). The melatonin antagonist luzindole significantly increased the expression of ADCY6 and ADCY9 in oocytes, and melatonin reduced the expression of ADCY6 (**Fig. 4E**). However, the expression of ADCY5 was lower in the luzindole and melatonin groups compared with the control (**Fig. 4E**). In addition, the melatonin antagonist luzindole increased, while melatonin decreased the concentration of cAMP in oocytes, respectively (**Fig. 4F**). These results suggest that melatonin may regulate the synthesis of cAMP via ADCY6 in oocytes. To further confirm the role of cAMP in regulating the methylation of oocytes, we treated mice with SQ22536, an inhibitor of ADCYs, and found that this treatment significantly reduced the global methylation of 5mC and the concentration of cAMP in oocytes ($n \geq 51$ **Fig. 5A-C**). Whereas, the ADCYs activator forskolin significantly increased the cAMP concentration and global methylation of 5mC in oocytes (**Fig. 5A-C**). 8-Bromo-cAMP, a cAMP analogue, also increased the global methylation in oocytes ($n \geq 41$ **Fig. 5D, E**). cAMP functions by activating the downstream protein PKA. When we treated mice with H89 2HCL, a PKA antagonist, the global methylation of 5mC was significantly reduced in oocytes ($n \geq 24$ **Fig. 5F, G**). These results suggest that melatonin may

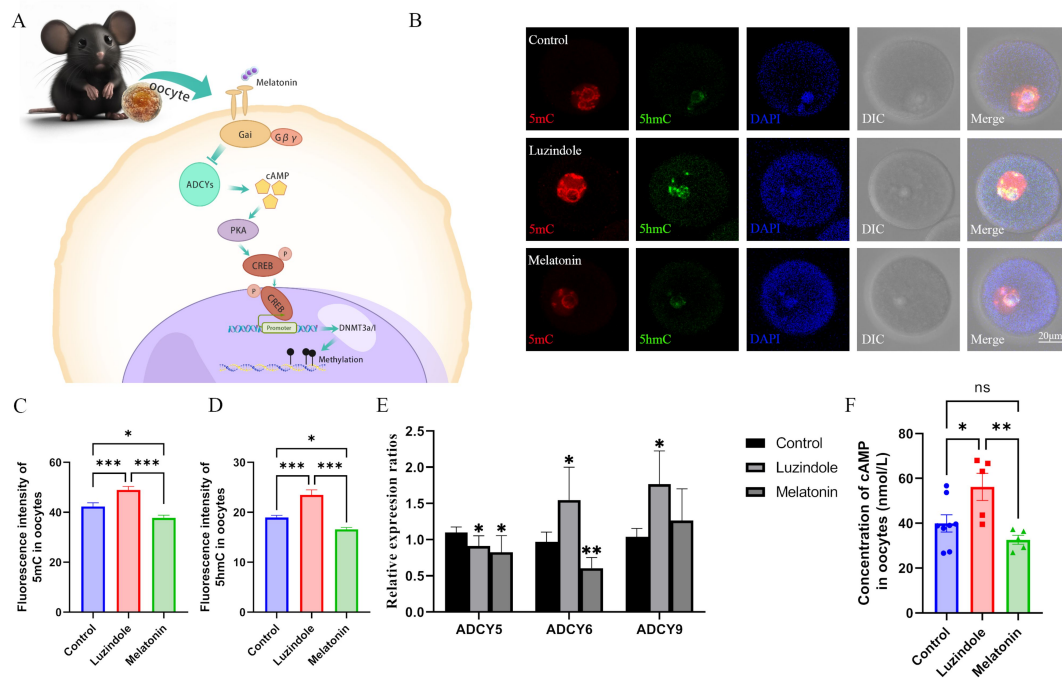


Figure 4

Melatonin regulates DNA methylation in oocytes.

(A) Schedule of the possible pathway by which melatonin regulates DNA methylation in oocytes. According to previous studies, we predicted that melatonin might regulate DNA methylation in oocytes via the cAMP/PKA/CREB pathway.

(B) Effects of melatonin and its inhibitor luzindole on oocyte methylation were examined using immunofluorescence.

(C, D) The relative fluorescence intensities of 5mC and 5hmC were analyzed using Image J (5mC: Control, n=81; Luzindole, n=83; Melatonin, n=86. 5hmC: Control, n=64; Luzindole, n=58; Melatonin, n=49). * $p < 0.05$; *** $p < 0.001$.

(E) The effects of melatonin and its inhibitor luzindole on the expression of adenylate cyclase (ADCY) in oocytes were examined by qPCR. * $p < 0.05$; ** $p < 0.01$.

(F) The concentration of cAMP in oocytes was examined by ELISA. * $p < 0.05$; ** $p < 0.01$.

mediate the methylation of oocytes via the cAMP/PKA pathway. cAMP activates PKA which further phosphorylates CREB to regulate gene expression. In oocytes, DNA re-methylation is regulated by DNMTs including DNMT3a, DNMT3l, and DNMT1. Therefore, we next investigated whether melatonin regulates DNMTs expression via the cAMP/PKA/CREB pathway in oocytes. We examined the expression of CREB1, CREM (cAMP responsive element modulator), CREB3l2 (cAMP responsive element binding protein 3 like 2), and ATF1 (activating transcription factor 1) in oocytes. Results showed that ADCYs activator forskolin treatment significantly increased the mRNA expression of CREB1 and CREM, and that the expression of CREB3l2 and ATF1 was slightly increased (**Fig. 6A**). In addition, the ADCYs inhibitor SQ22536 significantly reduced the expression of CREB1 and CREB3l2, although the expression of CREM and ATF1 was slightly decreased in oocytes (**Fig. 6A**). Furthermore, treatment with the ADCYs inhibitor SQ22536 significantly reduced the concentration of pCREB1, but it was increased by forskolin treatment in oocytes ($n \geq 36$ **Fig. 6B, C**). The pCREB1 level was also increased by 8-Bromo-cAMP and decreased by the PKA antagonist H89 2HCL in oocytes ($n \geq 28$ **Fig. 6D-G**). These results suggest that the expression and phosphorylation of CREB1 can be regulated by the cAMP/PKA pathway. Yang *et al.* demonstrated that CREB regulated DNMT3a expression in neurons of the dorsal root ganglion by binding to the promoter region (Yang *et al.*, 2021). In the present study, the binding of pCREB1 with relative regions of DNMTs was examined using CUT & Tag assay. Each sample contained 500 GV oocytes, and two replicates were involved. The sequencing result revealed that five fragments including 10 pCREB1 binding motifs (predicted using online tool JASPAR, Table S4) were associated with DNMTs, including 3 fragments at intron 1 and distill intergenic regions of DNMT3a, 1 fragment at the promoter region of DNMT1, and 1 fragment at intron 13 of DNMT3l (Table S5). These results suggest that pCREB1 may have contributions to regulate the expression of DNMTs.

Next, we investigated the expression of DNMT1, DNMT3a, and DNMT3l in oocytes. The melatonin inhibitor luzindole (slightly) and the ADCYs activator forskolin (significantly) increased the expression of DNMT1 and DNMT3a, respectively (**Fig. 7A, B**). Melatonin and the ADCYs inhibitor SQ22536 significantly and slightly reduced the expression of DNMT1 and DNMT3a in oocytes, respectively (**Fig. 7A, B**). The protein level of DNMT3a in GV oocytes was also significantly increased by 8-Bromo- cAMP and decreased by the PKA antagonist H 89 2HCL, respectively ($n \geq 48$ **Fig. 7C-F**). Although DNMT1 is well known as a maintenance methyltransferase, it also contributes to *de novo* methylation in oocytes (Li *et al.*, 2018). Therefore, we examined the localization of DNMT1 in oocytes, and found that 8-Bromo-cAMP treatment significantly increased the localization of DNMT1 in the nucleus of oocytes, but it was reduced by the PKA antagonist H89 2HCL ($n \geq 22$ **Fig. 7G-J**). When the activation of DNMTs was inhibited by 5-azacytidine, the methylation level in GV oocytes was significantly decreased ($n \geq 43$ **Fig. S10**). These results suggest that melatonin may influence the genomic methylation of oocytes via regulating the expression of DNMT1 and DNMT3a mediated via the cAMP/PKA/CREB pathway.

Increased DNMTs mediate hyper-methylation of HFD oocytes via cAMP/PKA/CREB pathway

To explore how melatonin regulates the hyper-methylation of HFD oocytes, we examined the expression of DNMTs. Results showed that maternal obesity in mice significantly increased the expression of DNMT1, DNMT3a, and DNMT3l in oocytes (**Fig. 8A**). Thus, we aimed to confirm whether the cAMP/PKA/CREB pathway mediated the increased expression of DNMTs in HFD oocytes. We examined the concentration of cAMP in oocytes, and found that maternal obesity significantly increased the concentration of cAMP in oocytes compared with that in CD group (**Fig. 8B**). The mRNA expression of CREB1, but not CREM, in the HFD oocytes was significantly increased compared with that in CD group (**Fig. 8C**), and the pCREB1 level in HFD oocytes was also significantly increased ($n \geq 49$ **Fig. 8D, E**). However, the increased level of pCREB1 was reduced by exogenous melatonin treatment (**Fig. 8D, E**). When obese females were treated with

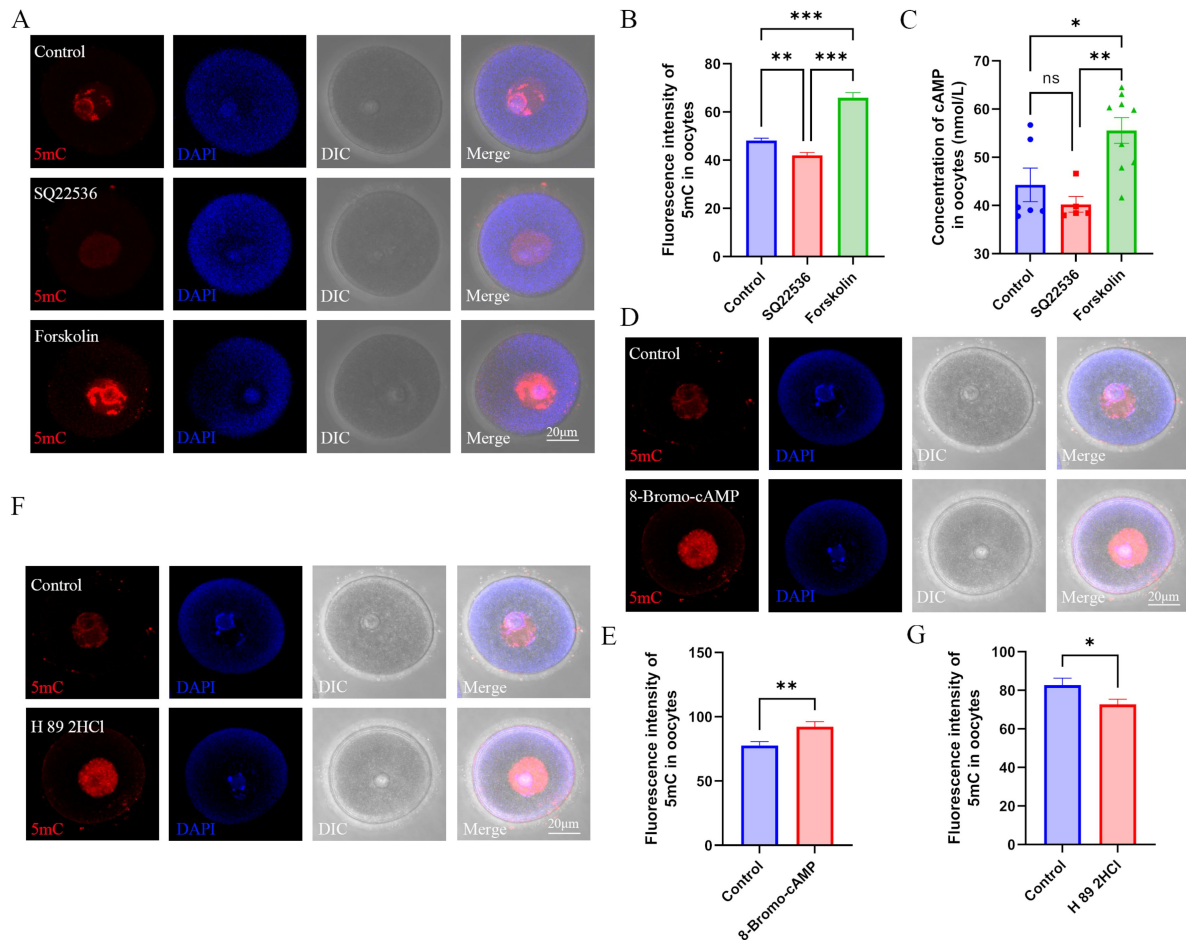


Figure 5

Role of cAMP in DNA methylation in oocytes.

(A) Female mice were respectively treated with the ADCY inhibitor SQ22536 or activator forskolin. Oocyte methylation was examined using immunofluorescence.

(B) The relative intensity of fluorescence in oocytes was analyzed using Image J (Control, n=107; SQ22536, n=51; Forskolin, n=57). ** p<0.01; *** p<0.001.

(C) cAMP concentration in oocytes was examined using ELISA. * p<0.05; ** p<0.01.

(D) Female mice were treated with the cAMP analogue 8-Bromo-cAMP, and oocyte methylation was examined using immunofluorescence. The relative fluorescence intensity of 5mC was analyzed using Image J (E) (Control, n=41; 8-Bromo-cAMP, n=42). ** p<0.01.

(F, G) Female mice were treated with the PKA (protein kinase A) antagonist H 89 2HCL, and then oocyte methylation was examined using immunofluorescence. The relative fluorescence intensity of 5mC was analyzed using Image J (G) (Control, n=24; H 89 2HCl, n=25). * p<0.05.

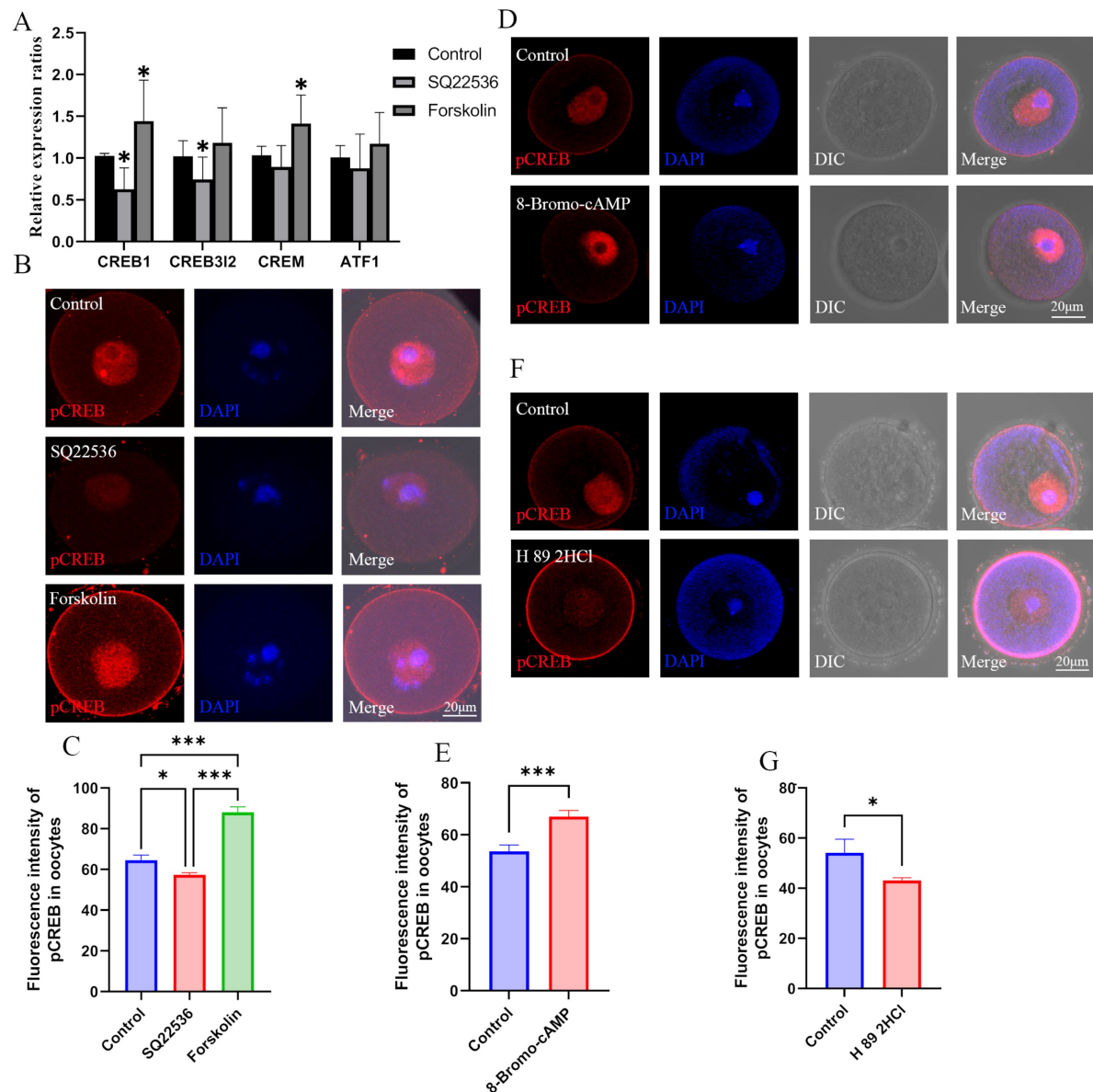


Figure 6

Effects of cAMP on CREB1.

(A) The mRNA expression of cAMP-response element binding (CREB) proteins in oocytes was examined by qPCR. * $p < 0.05$. (B and C) Phosphorylated CREB1 (pCREB1) in oocytes was examined using immunofluorescence, and the relative fluorescence intensity of pCREB1 was examined by Image J (C) (Control, $n = 36$; SQ22536, $n = 48$; Forskolin, $n = 41$). * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$. (D and E) After treatment with the cAMP analogue 8-Bromo-cAMP, pCREB1 in oocytes was examined using immunofluorescence. The relative fluorescence intensity was analyzed using Image J (E) (Control, $n = 28$; 8-Bromo-cAMP, $n = 28$). *** $p < 0.001$.

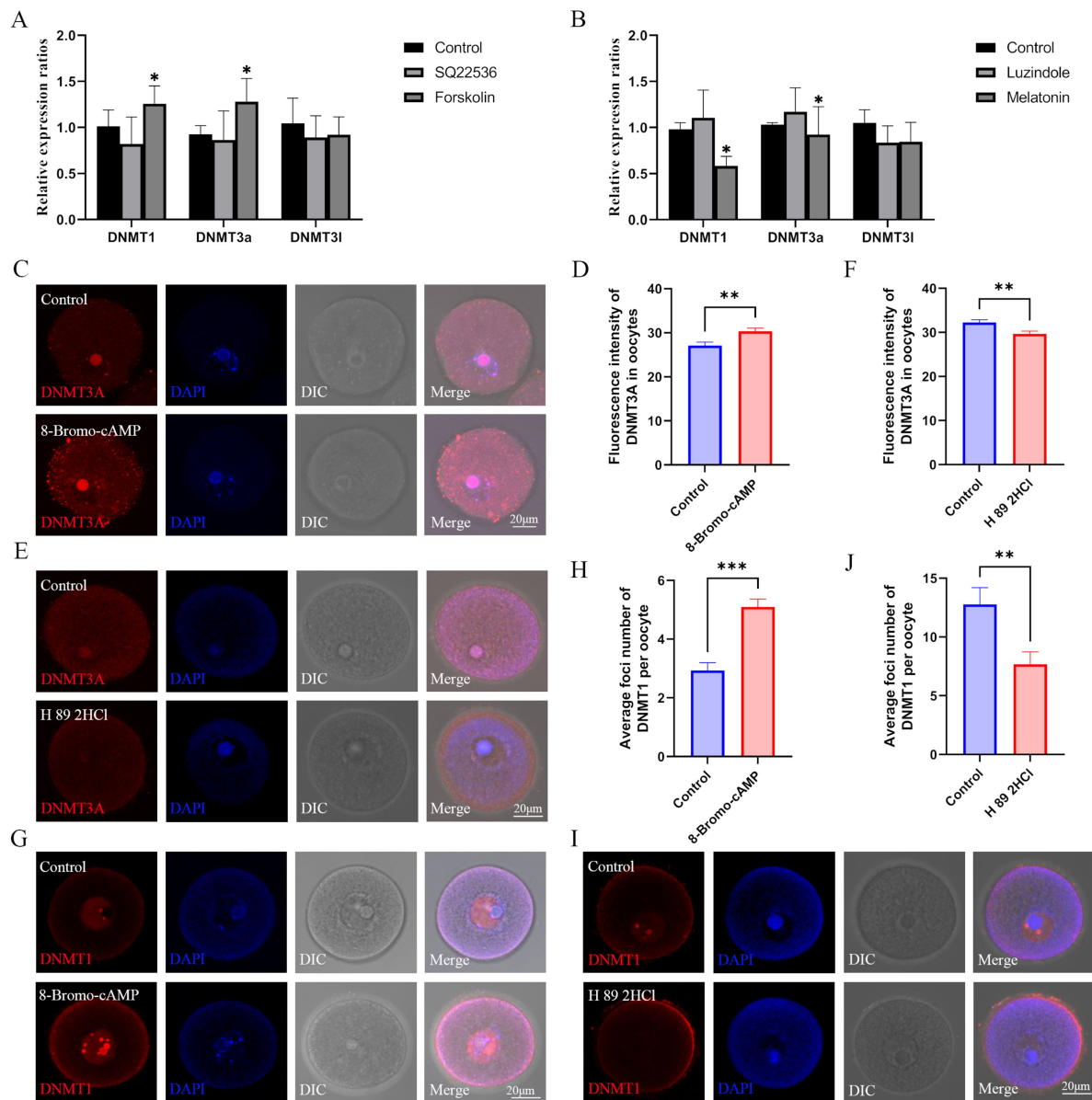


Figure 7

Role of the melatonin/cAMP/PKA pathway in the expression of DNMTs.

(A) The expression levels of DNMT1, DNMT3a and DNMT3l in oocytes were examined using qPCR after the treatment with SQ22536 and forskolin. * $p < 0.05$.

(B) The relative expressions of DNMT1, DNMT3a and DNMT3l in oocytes were examined using qPCR after the treatment with luzindole and melatonin. * $p < 0.05$.

(C) After 8-Bromo-cAMP treatment, the relative expression of DNMT3a in oocytes was examined using immunofluorescence and calculated by Image J (D) (Control, $n = 54$; 8-Bromo-cAMP, $n = 70$). ** $p < 0.01$.

(E and F) Treatment with the PKA antagonist H 89 2HCl treatment significantly reduced the level of DNMT3a in oocytes examined using immunofluorescence (Control, $n = 62$; H 89 2HCl, $n = 48$). ** $p < 0.01$.

(G and H) DNMT1 localization in the oocyte nucleus was examined using immunofluorescence after 8-Bromo-cAMP treatment (Control, $n = 30$; 8-Bromo-cAMP, $n = 31$). *** $p < 0.001$.

(I and J) The localization of DNMT1 in oocyte nucleus was reduced by the treatment with the PKA antagonist H 89 2HCl (Control, $n = 22$; H 89 2HCl, $n = 28$). ** $p < 0.01$.

the PKA antagonist H89 2HCL, both 5mC and pCREB1 levels were significantly reduced in oocytes ($n \geq 17$ **Fig. 8F-I**). These results suggest that reduced melatonin in obese mice may increase the expression of DNMTs via the cAMP/PKA/CREB pathway.

The increased expression of DNMT3a (**Fig. 8A**) may have contributed to the genomic hyper-methylation of HFD oocytes because the primary function of DNMT3a is *de novo* DNA methylation. The *de novo* methylation function of DNMT1 is usually prevented by *Stella* (also known as *Dppa3* or *PGC7*) in oocytes (Bostick et al., 2007). Nevertheless, maternal obesity significantly reduces the expression of *Stella* in oocytes (Han et al., 2018), which indicates that DNMT1 may also contribute to the hyper-methylation of the HFD oocytes. We found that the localization of DNMT1 in the nuclei of HFD oocyte was significantly increased, which could be reduced by the PKA antagonist H89 2HCL treatment ($n \geq 24$ **Fig. 8J, K**). These results suggest that the decreased melatonin induced by maternal obesity increases the expression of DNMTs via the cAMP/PKA/CREB pathway, which results in the hyper-methylation in HFD oocytes.

Discussion

Maternal obesity has negative effects on oocyte quality and offspring health, but the underlying mechanisms are not well understood. In the present study, we found that maternal obesity induced hyper-methylation in oocytes, and that the abnormal methylation, at least in part, is transmitted to F2 oocytes in females, which may be associated with the occurrence and inheritance of metabolic disorders. Maternal obesity induced metabolic changes in the serum. The decreased melatonin in serum may be involved in regulating the hyper-methylation of HFD oocytes by increasing the expression of DNMTs, which is mediated by the cAMP/PKA/CREB pathway.

Transgenerational epigenetic inheritance is common in plants, but related investigations in mammals are hindered by epigenetic reprogramming events during gametogenesis and early embryo development (Schmitz & Ecker, 2012; Xavier, Roman, Aitken, & Nixon, 2019). In mammals, isogenic agouti viable yellow (A^{vy}) and axin-fused ($Axin^{fu}$) mice, whose phenotypes are regulated by the DNA methylation level of IAP (intra-cisternal A particle long terminal repeat) respectively located at the upstream and in intron 6, are solid evidence confirming the transgenerational inheritance of epigenetic modifications (Morgan, Sutherland, Martin, & Whitelaw, 1999; Rakan, Blewitt, Druker, Preis, & Whitelaw, 2002). Nevertheless, epigenetic modifications can be affected by environmental factors such as metabolic diseases and diet. The intergenerational inheritance of phenotypes and epigenetic changes induced by maternal environmental factors have been confirmed by previous studies (B. Chen et al., 2022; Ge et al., 2014), but there are still many debates about the transgenerational inheritance of epigenetic changes induced by environment. Rats from stressed mothers are more likely to be stressed, and that can be transmitted across generations, but this transgenerational inheritance is not mediated by gametes (Weaver et al., 2004). Females cannot mediate the transgenerational inheritance of hyper-methylation induced by diet in A^{vy} mice (Robert A. Waterland, Travisano, & Tahiliani, 2007), but another study reported that if the A^{vy} allele is from the father, the hyper-methylation induced by diet during pregnancy can be retained in germ cells (Cropley, Suter, Beckman, & Martin, 2006). Anway et al. reported that the abnormal spermatogenesis induced by the exposure of vinclozolin during pregnancy can be transmitted across four generations via sperm (Anway, Cupp, Uzumcu, & Skinner, 2005). We previously reported that maternal obesity disturbed DNA methylation status of imprinted genes in oocytes (Ge et al., 2014), but the transgenerational inheritance was not observed. We also demonstrated that disturbed methylation in oocytes induced by undernourishment in utero could be inherited, at least partly, by F2 oocytes via females (S.-B. Tang et al., 2023). Recently, Takahashi et al. edited DNA methylation of promoter-associated CGIs, and found that the edited DNA methylation, associated with disrupted metabolism, was stably inherited by multiple generations (Takahashi et al., 2023). In the present study, we found that maternal obesity induced genomic hyper-methylation in oocytes, and that a

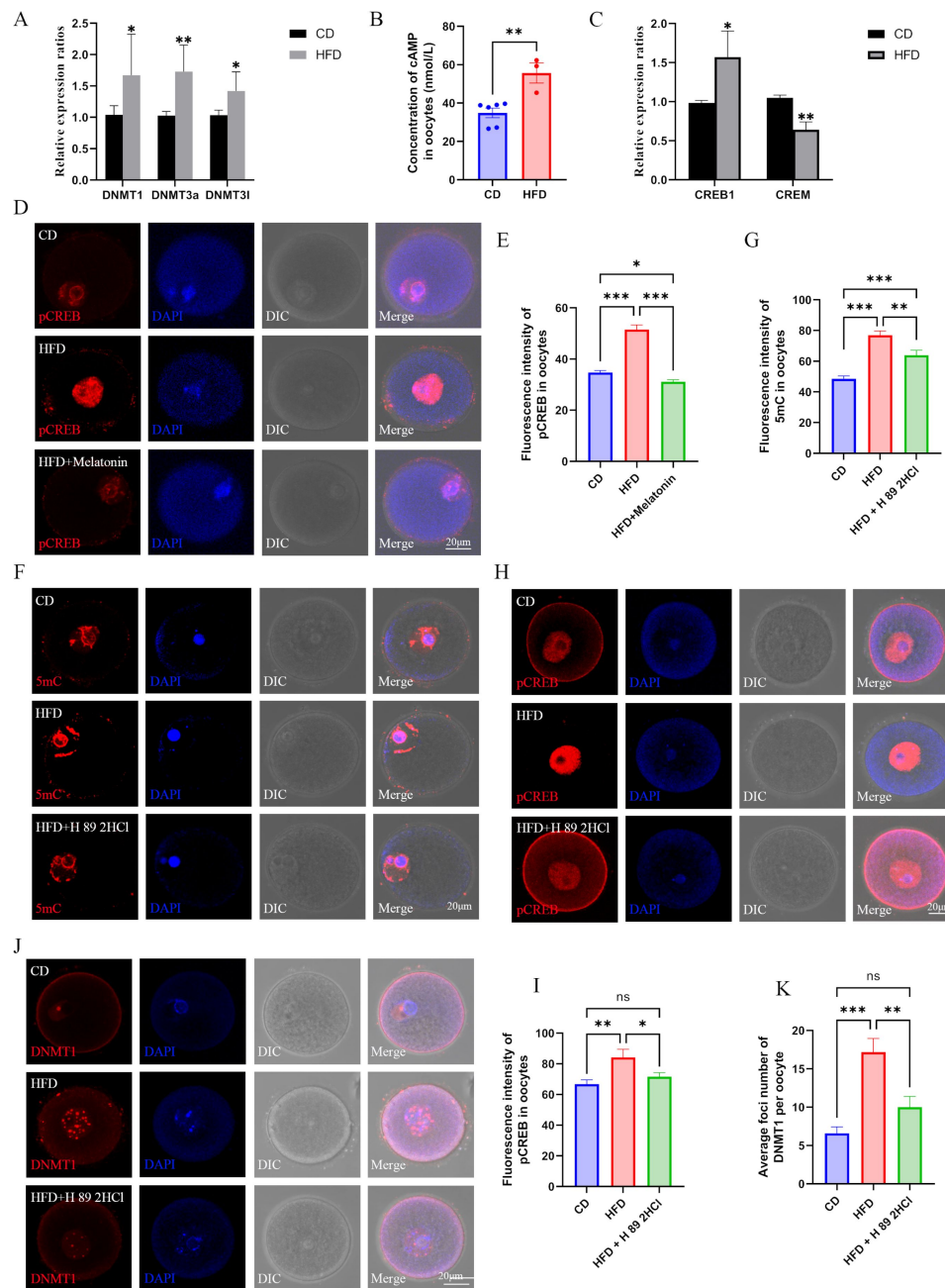


Figure 8

Melatonin regulates DNMTs expression via cAMP/PKA/CREB pathway in HFD oocytes.

(A) The relative expression of DNMT1, DNMT3a, and DNMT3l in HFD oocytes was examined using qPCR. * p<0.05; ** p<0.01.

(B) The concentration of cAMP in HFD oocytes was examined using ELISA. ** p<0.01.

(C) The relative expressions CREB1 and CREM in HFD oocytes were tested using qPCR. * p<0.05; ** p<0.01.

(D and E) The level of pCREB1 in oocytes was examined using immunofluorescence, and the relative fluorescence intensity was calculated by Image J (E) (CD, n=69; HFD, n=49; HFD+melatonin, n=61). HFD, oocytes from obese mice; CD, oocytes from control mice; HFD + melatonin, oocytes from obese mice treated with exogenous melatonin. * p<0.05; *** p<0.001.

(F and G) Treatment with the PKA antagonist H89 2HCL reduced the methylation level of HFD oocytes (CD, n=48; HFD, n=31; HFD+H 89 2HCl, n=27). ** p<0.01; *** p<0.001.

(H and I) The level of pCREB1 in HFD oocytes was also decreased by the treatment with the PKA antagonist H89 2HCL (CD, n=17; HFD, n=17; HFD+H 89 2HCl, n=22). * p<0.05; ** p<0.01; ns, no statistical significance between groups. (J and K)

Treatment with the PKA antagonist H89 2HCL reduced the localization of DNMT1 in HFD oocytes (CD, n=24; HFD, n=29; HFD+H 89 2HCl, n=25). ** p<0.01; *** p<0.001; ns, no statistical significance between groups.

part of the abnormal methylation was transmitted to F2 via female gametes. Moreover, the transmission of metabolic disorders has also been observed across two generations. These results suggest that the transgenerational inheritance of abnormal methylation induced by maternal obesity, at least in part, can be mediated by oocytes, which may be a reason for the inheritance of metabolic disorders.

During the methylation process, the methyl group is donated by SAM which is generated from homocysteine, 5-methyltetrahydrofolate (5mTHF), and methionine. 5mTHF is an intermediate of one-carbon metabolism (Mentch & Locasale, 2016 [DOI](#)). One-carbon units such as folate and vitamin B12 are crucial for the establishment of methylation (Mentch & Locasale, 2016 [DOI](#)). Disturbed glucose and lipid metabolism also has a negative influence on DNA methylation (Keller et al., 2014 [DOI](#)). These indicate that abnormal metabolism induced by maternal obesity (King, 2006 [DOI](#)) may play a key role in the genomic hyper-methylation in oocytes. In the present study, we found that the metabolomics of serum from HFD mice was distinguishable from that of serum from CD mice. Although the concentrations of vitamin B6 and methionine were higher in HFD serum than that in CD, this may not be an important reason for the genomic hyper-methylation in oocytes because the concentrations of SAM, SAH, and HCY in the livers and oocytes was similar between CD and HFD mice. In humans, obesity reduces the melatonin level in circulation (Overberg et al., 2022 [DOI](#); Virto et al., 2018 [DOI](#)). In the present study, we also found that maternal obesity induced by high-fat diet reduced the concentration of melatonin in the serum. Melatonin not only can decrease body weight, but also regulates DNA methylation of somatic cells and germ cells (Davoodvandi et al., 2022 [DOI](#); Lan et al., 2018 [DOI](#)). Nevertheless, the molecular mechanism by which melatonin regulates DNA methylation in oocytes is still unclear. Melatonin has two receptors, MT1 and MT2, both of which have been identified in oocytes (Wang et al., 2021 [DOI](#)). Melatonin receptors interact with the inhibitor G-protein and can regulate gene expression via the cAMP/PKA/CREB pathway (Wongprayoon & Govitrapong, 2021 [DOI](#)). CREM and cAMP mediate DNA methylation in somatic cells by regulating the expression of *DNMT3a* (Fang et al., 2015 [DOI](#); Hedrich et al., 2014 [DOI](#)). In neurons, CREB interacts with the promoter of *DNMT3a* to regulate its expression and DNA methylation (Yang et al., 2021 [DOI](#)). In the present study, we found that melatonin, which is mediated by the cAMP/PKA/CREB pathway, regulates methylation in oocytes by increasing the expression of DNMT1 and DNMT3a. Similar results were also observed in HFD oocytes. Hyper-methylation of HFD oocytes can be reduced by exogenous melatonin and PKA inhibitors. These suggest that decreased melatonin levels are involved in regulating the genomic hyper-methylation of HFD oocytes by increasing the expression of DNMTs, which is mediated by the cAMP/PKA/CREB pathway.

During follicular development, re-methylation in oocytes is catalyzed mainly by DNMT3a and DNMT3l (Kibe et al., 2021 [DOI](#)). DNMT1 is usually responsible for maintaining DNA methylation, but DNMT1 also contributes to CG methylation in oocytes (Shirane et al., 2013 [DOI](#)). During normal oocyte development, DNMT1 is mainly prevented from the nuclei by *Stella*. When the *Stella* level is knocked out, *Uhrf1* (Ubiquitin-like containing PHD Ring Finger 1) moves to the nucleus from the cytoplasm and recruits DNMT1 to chromatin, resulting in hyper-methylation (Li et al., 2018 [DOI](#)). Maternal obesity significantly decreases the expression of *Stella* in oocytes (Han et al., 2018 [DOI](#)). These suggest that maternal obesity may induce hyper-methylation in oocytes. In the present study, we found that maternal obesity increased the genomic DNA methylation of GV and MII oocytes, the expression of DNMT1 and its localization at chromatin in GV oocytes. These suggest that reduced *Stella* in HFD oocytes may recruit more DNMT1 into chromatin resulting in hyper-methylation. Nevertheless, Han LS *et al.* reported that maternal obesity has no significant influence on whole genome methylation of GV oocytes and Hou YJ *et al.* reported that maternal obesity reduces whole genome methylation of NSN (no Hoechst-positive rim surrounding the nucleolus) GV oocytes. This contradiction may be associated with the methods used to examine the methylation level and the sample size.

In summary, we found that maternal obesity induced genomic hyper-methylation in oocytes and that at least a part of the altered methylation can be transmitted to F2 oocytes, which may be a reason for the inheritance of metabolic disorders. Furthermore, reduced melatonin in HFD mice was involved in regulating the genomic hyper-methylation of oocytes by increasing the expression of DNMTs, and this process was mediated by the cAMP/PKA/CREB pathway. However, there are some limitations for the present study: there is not enough evidence to confirm the role of altered DNA methylation in metabolic disorders in the offspring of obese mothers. The molecular mechanisms by which DNA methylation escapes reprogramming in oogenesis have not been elucidated. There may be other mechanisms involved in regulating genomic hyper-methylation in HFD oocytes. Therefore, more studies are needed in the short future.

Materials and methods Mice

C57BL/6 mice were purchased from Jinan Pengyue Company (Jinan, China). Mice were housed in the controlled room with 12 h light and 12 h dark cycle, and at 23–25°C. The Animal Ethics Committee of Qingdao Agricultural University supported all procedures (QAU201900326).

For the obesity model, female C57BL/6 mice at the age of 4 weeks were randomly divided into two groups fed with high-fat diet (HFD, Research Diets, D12492, USA) and normal diet (CD) for 12 weeks, respectively. We examined the body weight every week. The formulation of the diets is presented in Table S6.

Offspring were produced according to the schedule in [Fig. 1J](#). For F1 offspring, female HFD and CD mice were mated with normal adult male C57BL/6 mice, respectively, and the offspring were marked as HF1 and CF1. To avoid the effects of males on methylation, the same males were used to produce F2: female HF1 and CF1 mating with normal males, marked as HF2 and CF2.

Immunofluorescence

Briefly, oocytes were fixed with 4% PFA (paraformaldehyde), permeabilized with 0.5% TritonX-100, and blocked with 1% BSA (bovine serum albumin). After that, the oocytes were incubated with primary antibodies overnight at 4°C. The secondary antibodies were stained for 1 h at room temperature. For 5mC and 5hmC staining, the oocytes were treated with 4 N HCl for 10 min after permeabilization, and then transferred into 100mM Tris-HCl for 10 min. After washed 3 times using PBS/BSA with 0.05% Tween 20, the oocytes were blocked with PBS/BSA for 1 h, and incubated with primary antibodies overnight at 4°C. Secondary antibodies were stained for 1 h at room temperature. Then, oocytes were transferred into DAPI with mounting and sealed. Fluorescence signal was examined using a laser scanning confocal microscope (Leica SP5, Germany). The relative fluorescence intensity was examined using Image J.

Antibodies

Primary antibodies used in the present study included anti-5mC antibody (Abcam, ab73938), anti-5hmC antibody (Abcam, ab214728), anti-pCREB antibody (Cell Signaling Technology, 9198S), anti-DNMT3a antibody (Active motif, 61478), and anti-DNMT1 antibody (Active motif, 39204).

Whole-genome bisulfite sequencing and analysis

Metaphase II (MII) oocytes were collected from the oviduct. For each sample, 100 oocytes from at least 10 mice were pooled together and transferred to lysis buffer. Genomic DNA was fragmented, and the end was repaired. Then, fragmentations were ligated with adapters. Bisulfite treatment was performed using EZ DNA Methylation-Direct (Zymo Research). Lambda DNA was used as a control. After that, the sequencing library was established and sequenced using Illumina HiSeq/NovaSeq (Novogene, China). The raw data quality was evaluated using FastQC, and low

quality data and adapters were trimmed using fastp. Clean data were compared to the reference genome mm10. Methylated C site calling was performed using Bismark. Differentially methylated regions (DMRs) were identified using DSS-single. The enrichment of genes in the KEGG pathway was carried out using the online tool KOBAS.

qPCR

Total RNA was extracted from oocytes or tissues using RNAprep Pure Micro Kit (Tiangen, DP420) or RNA Easy Fast Kit (Tiangen, DP451). cDNA was synthesized using Hifair III 1st Strand cDNA Synthesis Kit (Yeast, China). cDNA was used as templates to examine the relative expression of genes. Housekeeping genes *Ppia* and *Gapdh* were used as references. Relative expression was calculated as $2^{-\Delta\Delta Ct}$.

Bisulfite sequencing (BS)

Each sample included 5 oocytes and at least 20 samples were used for each DMR. The samples were treated as described in a previous study (Ge et al., 2014 [\[14\]](#)). Briefly, samples were treated with lysis buffer and 0.3 M NaOH, respectively. After that, samples were embedded in 2% low melting point agarose (Sigma), which was treated with fresh bisulfite solution (2.5 M sodium metabisulfite, Merck; 125 mM hydroquinone, Sigma; pH 5) for 4 h. Treated DNA was used as templates to amplify the target fragment using nest-PCR. PCR products were cloned to T-vector and sequenced. Methylation status was analyzed using BiqAnalyzer, which can remove the low conversion rate (<97%) and possible repeat sequences. At least 10 available clones were used for each DMR.

Glucose and insulin tolerance

Glucose and insulin tolerance (GTT and ITT) were examined as previously reported (S. B. Tang et al., 2023 [\[15\]](#)). Briefly, mice were treated with glucose at 2 g/kg body weight or insulin (Actrapid®, Novo Nordisk) at 10 IU/kg body weight after 16 h or 4 h fasting, respectively. After that, blood glucose was measured by tail blood at 0, 30, 60, 90, and 120 min, respectively.

ELISA

Concentrations of cAMP, SAM, SAH, and HCY were examined using ELISA kits (Jinma Biotechnology Co. Ltd, Shanghai, China) according to the handbook. A standard curve was produced using four-parameters logistics.

Non-target metabolomics in serum

Metabolites in the serum were examined using LC-MS/MS (BGI, Wuhan, China). Raw data were treated with Compound Discoverer 3.1 (Thermo Fisher Scientific, USA). After that, preprocessing of the exported data was performed using metaX. Metabolites were identified according to the databases of BMDB (BGI), mzCloud and ChemSpider (HMDB, KEGG, LipidMaps). Identified metabolites were annotated according to KEGG and HMDB. Differential metabolites were scanned using PCA and PLS-DA combined with fold changes and Student's t test.

Chemicals

Inhibitors used in the present study included luzindole (Sigma), SQ22536 (Selleck), forskolin (Selleck), H89 2HCL (Selleck), and azacitidine (Selleck). 8-Bromo-cAMP was purchased from Selleck. Melatonin (Sigma) was injected by tail vein for 14 days. The other chemicals were administered via intraperitoneal injection for 14 days. The control group was injected with the appropriate solutions.

CUT & Tag and sequencing

For each sample, 500 oocytes were pooled together for this experiment, and two replicates were performed. Library was established using Hyperactive® Universal CUT&Tag Assay Kit (Vazyme, China) according to the manufacturer's instructions. Briefly, oocytes were washed with washing buffer and transferred into tubes with ConA Beads Pro for 10 min at room temperature. The tubes were placed on a magnetic rack for 2 min, and then discarded the supernatant. After that, 50 µl of precooled antibody buffer and primary antibody were added and incubated overnight at 4 °C.

Then, tubes were put on a magnetic rack for 2 min, discarded supernatant, and added 50 µl Dig-wash buffer with secondary antibody (1:100) into tubes incubated at room temperature for 60 min. Samples were washed three times, and then incubated with pA/G-Tnp pro for 1 h at room temperature. After washing, 50 µl of TTBL was added to the samples and incubated at 37°C for 1 h. Then, 2 µl 10% SDS and DNA Spike-in were added to the samples, which were incubated at 55 °C for 10 min. Supernatant was transferred into a new tube after being putted on a magnetic rack for 2 min. 25 µl DNA Extract Beads Pro was added into supernatant, incubated 20 min at room temperature, and then washed two times with B&W buffer. After that, the DNA Extract Beads Pro was re-suspended in 15 µl ddH₂O, and amplified at 60°C for 20 cycles. Library quality was examined using Qubit, AATI, and QPCR, and then sequenced using NovaSeq 6000 (Novogene, China). Adaptor and low-quality reads were removed from the raw data, and clean data were used for further analysis. Reads were mapped to the mouse reference genome mm39 using Bowtie2. Peak calling was performed using MACS2 at q-value <0.05. The peak was subsequently annotated into related gene regions.

Statistical analysis

Average data are presented as mean ± SE (standard error), and the statistical difference was calculated using two-tail independent-samples t test. Methylation level is presented as a percentage, and the statistical difference was calculated using the chi-square test. If p value < 0.05, the statistical difference was considered to be significant.

Acknowledgements

This work was supported by the National Natural Science Foundation of China (31872312), National R&D Program of China (2022YFC2703500), the Breeding Plan of Shandong Provincial Qingchuang Research Team (Innovation Team of Farm Animal Cloning 012-1622001), and the Doctor Foundation of Qingdao Agricultural University (6631116008).

Conflicts of interest statement

There are no conflicts of interest to declare.

Data availability

The raw data of sequencing are submitted to the database BGI Sub with No. CRA011654.

References

1. Anway M. D., Cupp A. S., Uzumcu M., Skinner M. K (2005) **Epigenetic Transgenerational Actions of Endocrine Disruptors and Male Fertility** *Science* **308**:1466–1469 <https://doi.org/10.1126/science.1108190>
2. Bartakova V., Pleskacova A., Kuricova K., Pacal L., Dvorakova V., Belobradkova J., . . . , Kankova K. (2016) **Dysfunctional protection against advanced glycation due to thiamine metabolism abnormalities in gestational diabetes** *Glycoconj J* **33**:591–598 <https://doi.org/10.1007/s10719-016-9688-9>
3. Bostick M., Kim J. K., Esteve P. O., Clark A., Pradhan S., Jacobsen S. E (2007) **UHRF1 plays a role in maintaining DNA methylation in mammalian cells** *Science* **317**:1760–1764 <https://doi.org/10.1126/science.1147939>
4. Broughton D. E., Moley K. H (2017) **Obesity and female infertility: potential mediators of obesity's impact** *Fertil Steril* **107**:840–847 <https://doi.org/10.1016/j.fertnstert.2017.01.017>
5. Chen B., Du Y. R., Zhu H., Sun M. L., Wang C., Cheng Y., . . . , Huang H. (2022) **Maternal inheritance of glucose intolerance via oocyte TET3 insufficiency** *Nature* **605**:761–766 <https://doi.org/10.1038/s41586-022-04756-4>
6. Chen D. H., Wu K. T., Hung C. J., Hsieh M., Li C (2004) **Effects of adenosine dialdehyde treatment on in vitro and in vivo stable protein methylation in HeLa cells** *J Biochem* **136**:371–376 <https://doi.org/10.1093/jb/mvh131>
7. Chen X., Xiao Z., Cai Y., Huang L., Chen C (2022) **Hypothalamic mechanisms of obesity-associated disturbance of hypothalamic-pituitary-ovarian axis** *Trends Endocrinol Metab* **33**:206–217 <https://doi.org/10.1016/j.tem.2021.12.004>
8. Cropley J. E., Suter C. M., Beckman K. B., Martin D. I. K (2006) **Germ-line epigenetic modification of the murine Avy allele by nutritional supplementation** *Proceedings of the National Academy of Sciences* **103**:17308–17312 <https://doi.org/10.1073/pnas.0607090103>
9. Davoodvandi A., Nikfar B., Reiter R. J., Asemi Z (2022) **Melatonin and cancer suppression: insights into its effects on DNA methylation** *Cell Mol Biol Lett* **27** <https://doi.org/10.1186/s11658-022-00375-z>
10. Fang X., Robinson J., Wang-Hu J., Jiang L., Freeman D. A., Rivkees S. A., Wendler C. C (2015) **cAMP induces hypertrophy and alters DNA methylation in HL-1 cardiomyocytes** *Am J Physiol Cell Physiol* **309**:C425–436 <https://doi.org/10.1152/ajpcell.00058.2015>
11. Ge Z. J., Luo S. M., Lin F., Liang Q. X., Huang L., Wei Y. C., . . . , Sun Q. Y (2014) **DNA methylation in oocytes and liver of female mice and their offspring: effects of high-fat-diet-induced obesity** *Environ Health Perspect* **122**:159–164 <https://doi.org/10.1289/ehp.1307047>
12. Godfrey K. M., Reynolds R. M., Prescott S. L., Nyirenda M., Jaddoe V. W., Eriksson J. G., Broekman B. F (2017) **Influence of maternal obesity on the long-term health of offspring** *Lancet Diabetes Endocrinol* **5**:53–64 [https://doi.org/10.1016/S2213-8587\(16\)30107-3](https://doi.org/10.1016/S2213-8587(16)30107-3)

13. Guan Q., Wang Z., Cao J., Dong Y., Chen Y (2021) **Mechanisms of Melatonin in Obesity: A Review** *Int J Mol Sci* **23** <https://doi.org/10.3390/ijms23010218>
14. Han L., Ren C., Li L., Li X., Ge J., Wang H., . . . , Wang Q. (2018) **Embryonic defects induced by maternal obesity in mice derive from Stella insufficiency in oocytes** *Nat Genet* **50**:432–442 <https://doi.org/10.1038/s41588-018-0055-6>
15. Hedrich C. M., Crispin J. C., Rauen T., Ioannidis C., Koga T., Rodriguez Rodriguez N., . . . , Tsokos G. C (2014) **cAMP responsive element modulator (CREM) alpha mediates chromatin remodeling of CD8 during the generation of CD3+ CD4- CD8- T cells** *J Biol Chem* **289**:2361–2370 <https://doi.org/10.1074/jbc.M113.523605>
16. Hood V. L., Berger R., Freedman R., Law A. J (2019) **Transcription of PIK3CD in human brain and schizophrenia: regulation by proinflammatory cytokines** *Hum Mol Genet* **28**:3188–3198 <https://doi.org/10.1093/hmg/ddz144>
17. Jacobsson J. A., Rask-Andersen M., Riserus U., Moschonis G., Koumpitski A., Chrousos G. P., . . . , Fredriksson R. (2012) **Genetic variants near the MGAT1 gene are associated with body weight BMI and fatty acid metabolism among adults and children.** *Int J Obes (Lond)* **36**:119–129 <https://doi.org/10.1038/ijo.2011.11>
18. Jin J. X., Sun J. T., Jiang C. Q., Cui H. D., Bian Y., Lee S., . . . , Liu Z. H. (2022) **Melatonin Regulates Lipid Metabolism in Porcine Cumulus-Oocyte Complexes via the Melatonin Receptor 2** *Antioxidants (Basel)* **11** <https://doi.org/10.3390/antiox11040687>
19. Johansson A., Marroni F., Hayward C., Franklin C. S., Kirichenko A. V., Jonasson I., . . . , Consortium E. (2010) **Linkage and genome-wide association analysis of obesity-related phenotypes: association of weight with the MGAT1 gene** *Obesity (Silver Spring)* **18**:803–808 <https://doi.org/10.1038/oby.2009.359>
20. Kang H., Jo A., Kim H., Khang R., Lee J. Y., Kim H., . . . , Shin J. H (2018) **PARIS reprograms glucose metabolism by HIF-1alpha induction in dopaminergic neurodegeneration** *Biochem Biophys Res Commun* **495**:2498–2504 <https://doi.org/10.1016/j.bbrc.2017.12.147>
21. Keller M., Kralisch S., Rohde K., Schleinitz D., Dietrich A., Schon M. R., . . . , Bottcher Y. (2014) **Global DNA methylation levels in human adipose tissue are related to fat distribution and glucose homeostasis** *Diabetologia* **57**:2374–2383 <https://doi.org/10.1007/s00125-014-3356-z>
22. Kibe K., Shirane K., Ohishi H., Uemura S., Toh H., Sasaki H (2021) **The DNMT3A PWWP domain is essential for the normal DNA methylation landscape in mouse somatic cells and oocytes** *PLoS Genet* **17** <https://doi.org/10.1371/journal.pgen.1009570>
23. King J. C (2006) **Maternal obesity, metabolism, and pregnancy outcomes** *Annu Rev Nutr* **26**:271–291 <https://doi.org/10.1146/annurev.nutr.24.012003.132249>
24. Lan M., Han J., Pan M. H., Wan X., Pan Z. N., Sun S. C (2018) **Melatonin protects against defects induced by deoxynivalenol during mouse oocyte maturation** *J Pineal Res* **65** <https://doi.org/10.1111/jpi.12477>
25. Li Y., Zhang Z., Chen J., Liu W., Lai W., Liu B., . . . , Zhu B. (2018) **Stella safeguards the oocyte methylome by preventing de novo methylation mediated by DNMT1** *Nature* **564**:136–140 <https://doi.org/10.1038/s41586-018-0751-5>

26. Lyon P., Strippoli V., Fang B., Cimmino L (2020) **B Vitamins and One-Carbon Metabolism: Implications in Human Health and Disease** *Nutrients* **12** <https://doi.org/10.3390/nu12092867>
27. Mentch S. J., Locasale J. W (2016) **One-carbon metabolism and epigenetics: understanding the specificity** *Ann N Y Acad Sci* **1363**:91–98 <https://doi.org/10.1111/nyas.12956>
28. Morgan H. D., Sutherland H. G., Martin D. I., Whitelaw E (1999) **Epigenetic inheritance at the agouti locus in the mouse** *Nat Genet* **23**:314–318 <https://doi.org/10.1038/15490>
29. Overberg J., Kalveram L., Keller T., Krude H., Kuhnen P., Wiegand S (2022) **Interactions between nocturnal melatonin secretion, metabolism, and sleeping behavior in adolescents with obesity** *Int J Obes (Lond)* **46**:1051–1058 <https://doi.org/10.1038/s41366-022-01077-4>
30. Qu J., Sun M., Wang X., Song X., He H., Huan Y (2020) **Melatonin Enhances the Development of Porcine Cloned Embryos by Improving DNA Methylation Reprogramming** *Cell Reprogram* **22**:156–166 <https://doi.org/10.1089/cell.2019.0103>
31. Rakyan V. K., Blewitt M. E., Druker R., Preis J. I., Whitelaw E (2002) **Metastable epialleles in mammals** *Trends Genet* **18**:348–351 [https://doi.org/10.1016/s0168-9525\(02\)02709-9](https://doi.org/10.1016/s0168-9525(02)02709-9)
32. Saeedabadi S., Abazari-Kia A. H., Rajabi H., Parivar K., Salehi M (2018) **Melatonin Improves The Developmental Competence of Goat Oocytes** *Int J Fertil Steril* **12**:157–163 <https://doi.org/10.22074/ijfs.2018.5204>
33. Schmitz R. J., Ecker J. R (2012) **Epigenetic and epigenomic variation in Arabidopsis thaliana** *Trends Plant Sci* **17**:149–154 <https://doi.org/10.1016/j.tplants.2012.01.001>
34. Shirane K., Toh H., Kobayashi H., Miura F., Chiba H., Ito T., . . . , Sasaki H. (2013) **Mouse oocyte methylomes at base resolution reveal genome-wide accumulation of non-CpG methylation and role of DNA methyltransferases** *PLoS Genet* **9** <https://doi.org/10.1371/journal.pgen.1003439>
35. Shou L., Pan F., Chin S (1969) **Pancreatic hormones and hepatic methionine adenosyltransferase in the rat** *Proc Soc Exp Biol Med* **131**:1012–1018 <https://doi.org/10.3181/00379727-131-34029>
36. Takahashi Y., Morales Valencia M., Yu Y., Ouchi Y., Takahashi K., Shokhirev M. N., . . . , Izpisua Belmonte J. C. (2023) **Transgenerational inheritance of acquired epigenetic signatures at CpG islands in mice** *Cell* **186**:715–731 <https://doi.org/10.1016/j.cell.2022.12.047>
37. Tang S.-B., Zhang T.-T., Yin S., Shen W., Luo S.-M., Zhao Y., . . . , Ge Z.-J. (2023) **Inheritance of perturbed methylation and metabolism caused by uterine malnutrition via oocytes** *BMC Biology* **21** <https://doi.org/10.1186/s12915-023-01545-x>
38. Tang S. B., Zhang T. T., Yin S., Shen W., Luo S. M., Zhao Y., . . . , Ge Z. J. (2023) **Inheritance of perturbed methylation and metabolism caused by uterine malnutrition via oocytes** *BMC Biol* **21** <https://doi.org/10.1186/s12915-023-01545-x>
39. Vaccaro J. A., Naser S. A (2021) **The Role of Methyl Donors of the Methionine Cycle in Gastrointestinal Infection and Inflammation** *Healthcare (Basel)* **10** <https://doi.org/10.3390/healthcare10010061>

40. Virto L., Haugen H. J., Fernandez-Mateos P., Cano P., Gonzalez J., Jimenez-Ortega V., . . . , Sanz M. (2018) **Melatonin expression in periodontitis and obesity: An experimental in-vivo investigation** *J Periodontol Res* **53**:825–831 <https://doi.org/10.1111/jre.12571>
41. Wang J., Zhuo Z., Ma X., Liu Y., Xu J., He C., . . . , Liu G. (2021) **Melatonin Alleviates the Suppressive Effect of Hypoxanthine on Oocyte Nuclear Maturation and Restores Meiosis via the Melatonin Receptor 1 (MT1)-Mediated Pathway** *Front Cell Dev Biol* **9** <https://doi.org/10.3389/fcell.2021.648148>
42. Waterland R. A (2006) **Assessing the effects of high methionine intake on DNA methylation** *J Nutr* **136**:1706–1710 <https://doi.org/10.1093/jn/136.6.1706S>
43. Waterland R. A., Travisano M., Tahiliani K. G (2007) **Diet-induced hypermethylation at agouti viable yellow is not inherited transgenerationally through the female** *The FASEB Journal* **21**:3380–3385 <https://doi.org/10.1096/fj.07-8229com>
44. Weaver I. C., Cervoni N., Champagne F. A., D'Alessio A. C., Sharma S., Seckl J. R., . . . , Meaney M. J. (2004) **Epigenetic programming by maternal behavior** *Nat Neurosci* **7**:847–854 <https://doi.org/10.1038/nn1276>
45. Wojcik M., Mac-Marcjanek K., Wozniak L. A., Nadel I., Lewinski A., Cypryk K (2014) **The association of leukocyte phosphatidylinositol 3-kinase delta overexpression with gestational diabetes mellitus (GDM)** *Endokrynol Pol* **65**:17–24 <https://doi.org/10.5603/EP.2014.0003>
46. Wongprayoon P., Govitrapong P (2021) **Melatonin Receptor as a Drug Target for Neuroprotection** *Curr Mol Pharmacol* **14**:150–164 <https://doi.org/10.2174/1874467213666200421160835>
47. Xavier M. J., Roman S. D., Aitken R. J., Nixon B (2019) **Transgenerational inheritance: how impacts to the epigenetic and genetic information of parents affect offspring health** *Hum Reprod Update* **25**:518–540 <https://doi.org/10.1093/humupd/dmz017>
48. Xiao P., Nie J., Wang X., Lu K., Lu S., Liang X (2019) **Melatonin alleviates the deterioration of oocytes from mice subjected to repeated superovulation** *J Cell Physiol* **234**:13413–13422 <https://doi.org/10.1002/jcp.28018>
49. Yang Y., Wen J., Zheng B., Wu S., Mao Q., Liang L., . . . , Tao Y. X. (2021) **CREB Participates in Paclitaxel-Induced Neuropathic Pain Genesis Through Transcriptional Activation of Dnmt3a in Primary Sensory Neurons** *Neurotherapeutics* **18**:586–600 <https://doi.org/10.1007/s13311-020-00931-5>

Author information

Shuo Chao[#]

College of Life Sciences, Institute of Reproductive Sciences, Key Laboratory of Animal Reproduction and Germplasm Enhancement in Universities of Shandong, Qingdao Agricultural University, Qingdao, China;

[#]These authors contributed equally to this work.

Jun Lu[#]

College of Life Sciences, Institute of Reproductive Sciences, Key Laboratory of Animal Reproduction and Germplasm Enhancement in Universities of Shandong, Qingdao Agricultural University, Qingdao, China;

[#]These authors contributed equally to this work.

Li-Jun Li

College of Life Sciences, Institute of Reproductive Sciences, Key Laboratory of Animal Reproduction and Germplasm Enhancement in Universities of Shandong, Qingdao Agricultural University, Qingdao, China;

Hong-Yan Guo

College of Life Sciences, Institute of Reproductive Sciences, Key Laboratory of Animal Reproduction and Germplasm Enhancement in Universities of Shandong, Qingdao Agricultural University, Qingdao, China;

Kui-Peng Xu

College of Horticulture, Qingdao Agricultural University, Qingdao, China;

Ning Wang

College of Life Sciences, Institute of Reproductive Sciences, Key Laboratory of Animal Reproduction and Germplasm Enhancement in Universities of Shandong, Qingdao Agricultural University, Qingdao, China;

Shu-Xian Zhao

College of Life Sciences, Institute of Reproductive Sciences, Key Laboratory of Animal Reproduction and Germplasm Enhancement in Universities of Shandong, Qingdao Agricultural University, Qingdao, China;

Xiao-Wen Jin

College of Life Sciences, Institute of Reproductive Sciences, Key Laboratory of Animal Reproduction and Germplasm Enhancement in Universities of Shandong, Qingdao Agricultural University, Qingdao, China;

Shao-Ge Wang

College of Life Sciences, Institute of Reproductive Sciences, Key Laboratory of Animal Reproduction and Germplasm Enhancement in Universities of Shandong, Qingdao Agricultural University, Qingdao, China;

Shen Yin

College of Life Sciences, Institute of Reproductive Sciences, Key Laboratory of Animal Reproduction and Germplasm Enhancement in Universities of Shandong, Qingdao Agricultural University, Qingdao, China;

Wei Shen

College of Life Sciences, Institute of Reproductive Sciences, Key Laboratory of Animal Reproduction and Germplasm Enhancement in Universities of Shandong, Qingdao Agricultural University, Qingdao, China;

Ming-Hui Zhao

College of Life Sciences, Institute of Reproductive Sciences, Key Laboratory of Animal Reproduction and Germplasm Enhancement in Universities of Shandong, Qingdao Agricultural University, Qingdao, China;

Gui-An Huang

College of Life Sciences, Institute of Reproductive Sciences, Key Laboratory of Animal Reproduction and Germplasm Enhancement in Universities of Shandong, Qingdao Agricultural University, Qingdao, China;

Qing-Yuan Sun

Guangzhou Key Laboratory of Metabolic Diseases and Reproductive Health, Guangdong-Hong Kong Metabolism & Reproduction Joint Laboratory, Reproductive Medicine Center, Guangdong Second Provincial General Hospital, Guangzhou, China
ORCID iD: [0000-0002-0148-2414](https://orcid.org/0000-0002-0148-2414)

gejdssd313@163.com; zjge@qau.edu.cn; sunqy@gd2h.org.cn

Zhao-Jia Ge

College of Life Sciences, Institute of Reproductive Sciences, Key Laboratory of Animal Reproduction and Germplasm Enhancement in Universities of Shandong, Qingdao Agricultural University, Qingdao, China;
ORCID iD: [0000-0002-6714-6675](https://orcid.org/0000-0002-6714-6675)

gejdssd313@163.com; zjge@qau.edu.cn; sunqy@gd2h.org.cn

Editors

Reviewing Editor

Izuchukwu Okafor

Nnamdi Azikiwe University, Awka, Nigeria

Senior Editor

Wei Yan

The Lundquist Institute, Torrance, United States of America

Joint Public review:**Summary**

This manuscript offers significant insights into the impact of maternal obesity on oocyte methylation and its transgenerational effects. Chao and colleagues demonstrated the potential mechanisms behind the DNA methylation changes. The major observations of the work include transgenerational DNA methylation changes in offspring of maternal obesity and metabolites such as methionine and melatonin which correlated with the epigenetic changes. Exogenous melatonin treatment could reverse the effects of obesity. The authors further hypothesized that the linkage may be mediated by the cAMP/PKA/CREB pathway to regulate the expression of DNMTs. This work has done lots of breeding and DNA Methylation analysis across multiple generations, which provides solid data for future research. The results of this work may benefit from deeper data analysis to make more causal analyses and conclusions more concrete.

Strengths

The study employs comprehensive methodologies, including transgenerational breeding experiments, whole genome bisulfite sequencing, and metabolomics analysis, and provides the convincing data.

Weaknesses

The results of this work are correlational, which may require further analysis to establish more concrete conclusions on causal relationships.

<https://doi.org/10.7554/eLife.97507.2.sa1>

Author response:

The following is the authors' response to the original reviews.

Public Reviews:

Reviewer #1 (Public Review):

With socioeconomic development, more and more people are obese which is an important reason for sub-fertility and infertility. Maternal obesity reduces oocyte quality which may be a reason for the high risk of metabolic diseases for offspring in adulthood. Yet the underlying mechanisms are not well elucidated. Here the authors examined the effects of maternal obesity on oocyte methylation. Hyper-methylation in oocytes was reported by the authors, and the altered methylation in oocytes may be partially transmitted to F2. The authors further explored the association between the metabolome of serum and the altered methylation in oocytes. The authors identified decreased melatonin. Melatonin is involved in regulating the hyper-methylation of high-fat diet (HFD) oocytes, via increasing the expression of DNMTs which is mediated by the cAMP/PKA/CREB pathway.

Strengths:

This study is interesting and should have significant implications for the understanding of the transgenerational inheritance of GDM in humans.

Thank you for your positive comments to our manuscript.

Weaknesses:

The link between altered DNA methylation and offspring metabolic disorders is not well elucidated; how the altered DNA methylation in oocytes escapes reprogramming in transgenerational inheritance is also unclear.

Thanks. These are very good questions. There is a long way to completely elucidate the relationship between methylation and offspring metabolic disorders, and the underlying mechanisms of obtained methylation escaping the reprogramming during development. We would like to explore these in the future.

Reviewer #2 (Public Review):

This manuscript offers significant insights into the impact of maternal obesity on oocyte methylation and its transgenerational effects. The study employs comprehensive methodologies, including transgenerational breeding experiments, whole genome bisulfite sequencing, and metabolomics analysis, to explore how high-fat diet (HFD)-

induced obesity alters genomic methylation in oocytes and how these changes are inherited by subsequent generations. The findings suggest that maternal obesity induces hyper-methylation in oocytes, which is partly transmitted to F1 and F2 oocytes and livers, potentially contributing to metabolic disorders in offspring. Notably, the study identifies melatonin as a key regulator of this hyper-methylation process, mediated through the cAMP/PKA/CREB pathway.

Strengths:

The study employs comprehensive methodologies, including transgenerational breeding experiments, whole genome bisulfite sequencing, and metabolomics analysis, and provides convincing data.

Thank you for your positive comments to our manuscript.

Weaknesses:

The description in the results section is somewhat verbose. This section (lines 126~227) utilized transgenerational breeding experiments and methylation analysis to demonstrate that maternal obesity-induced alterations in oocyte methylation (including hyper-DMRs and hypo-DMRs) can be partially transmitted to F1 and F2 oocytes and livers. The authors should consider condensing and revising this section for clarity and brevity.

Thanks for your suggestions. We have re-written this parts in the revised manuscript.

There is a contradiction with Reference 3, but the discrepancy is not discussed. In this study, the authors observed an increase in global methylation in oocytes from HFD mice, whereas Reference 3 indicates Stella insufficiency in oocytes from HFD mice. This Stella insufficiency should lead to decreased methylation (Reference 33). There should be a discussion of how this discrepancy can be reconciled with the authors' findings.

Thanks for your suggestions. As reported by Reference 33, STELLA prevents hypermethylation in oocytes by sequestering UHRF1 from the nuclei which recruits DNMT1 into nuclei. Han *et al.* reported that obesity induced by high-fat diet reduces STELLA level in oocytes. These indicate that STELLA insufficiency might induce hypermethylation in oocytes, although significant hypermethylation in obese oocytes is not reported by Han *et al.* using immunofluorescence. This contradiction may be caused by the limited sample sizes (n=14) used by Han *et al.* We have added a brief discussion in the revised manuscript.

Reviewer #3 (Public Review):

Summary:

Maternal obesity is a health problem for both pregnant women and their offspring. Previous works including work from this group have shown significant DNA methylation changes for offspring of obese pregnancies in mice. In this manuscript, Chao et al digested the potential mechanisms behind the DNA methylation changes. The major observations of the work include transgenerational DNA methylation changes in offspring of maternal obesity, and metabolites such as methionine and melatonin correlated with the above epigenetic changes. Exogenous melatonin treatment could reverse the effects of obesity. The authors further hypothesized that the linkage may be mediated by the cAMP/PKA/CREB pathway to regulate the expression of DNMTs.

Strengths:

The transgenerational change of DNA methylation following HFD is of great interest for future research to follow. The metabolic treatment that could change the DNA methylation in oocytes is also interesting and has potential relevance to future clinical practice.

Thank you for your positive comments to our manuscript.

Weaknesses:

The HFD oocytes have more 5mC signal based on staining and sequencing (Fig 1A-1F). However, the authors also identified almost equal numbers of hyper- and hypo-DMRs, which raises questions regarding where these hypo-DMRs were located and how to interpret their behaviors and functions. These questions are also critical to address in the following mechanistic dissections as the metabolic treatments may also induce bi-directional changes of DNA methylation. The authors should carefully assess these conflicts to make the conclusions solid.

Thanks for the helpful comments and suggestions. As presented in Fig. 1F, there is an increase of methylation level in promoter and exon regions and there is a decrease in intron, utr3 and repeat regions. According to the suggestions, we further analyzed the distribution of DMRs, and found that hypo-DMRs were mainly distributed at utr3, intron, repeat, and tes regions compared with hyper-DMRs (Fig. S3). These suggest that the distribution of DMRs in genome is not random.

The transgenerational epigenetic modifications are controversial. Even for F0 offspring under maternal obesity, there were different observations compared to this work (Hou, YJ., et al. Sci Rep, 2016). The authors should discuss the inconsistencies with previous works.

Thanks for the suggestions. There are contradictions on the whole genome DNA methylation of oocytes in obese mice. Hou YJ *et al.* in 2016 reported that obesity reduces the whole genome DNA methylation of NSN GV oocytes using immunofluorescence. In 2018, Han LS *et al.* reported that the whole genome 5mC of oocytes is not significantly influenced by obesity using immunofluorescence, but they find the *Stella* level is reduced in oocytes by obesity. *Stella* locates in the cytoplasm and nuclei of oocytes and sequesters *Uhrf1* from the nuclei. *Stella* knockout in oocytes results in about twofold increase of global methylation in MII oocytes via recruiting more DNMT1 into nuclei. These suggest that the global methylation of oocytes in obese mice should be increased, but the similar methylation in oocytes between obese and non-obese mice is reported by Han LS *et al.* Thus, the contradiction may be induced by the different sample size in our manuscript and previous studies, and Hou YJ and colleagues just examined the methylation of NSN GV oocytes. As present in *Stella*^{+/−} oocytes, the global methylation of oocytes is normal, which suggest that the insufficiency of *Stella* may be not the main reason for the increased methylation of oocytes in obese mice. We have added a brief discussion in the revised manuscript.

In addition to the above inconsistencies, the DNA methylation analysis in this work was not carefully evaluated. Several previous works were evaluating the DNA methylation in mice oocytes, which showed global methylation levels of around 50% (Shirane K, et al. PLoS Genet, 2013; Wang L., et al, Cell, 2014). In Figure 1E, the overall methylation level is about 23% in control, which is significantly different from previous works. The authors should provide more details regarding the WGBS procedure, including but not limited to sequencing coverage, bisulfite conversion rate, etc.

Thanks for the good questions. Smallwood *et al.* reported the the CG methylation of MII oocyte is about 33.1% (Smallwood *et al.* Nature Methods, 2014) using single-cell genome-wide bisulfite sequencing. Shirane K *et al.* reported that the average methylation level of GV oocytes is 37.9%. Kobayashi H *et al.* Reported that the CG methylation in GV oocytes is about 40% (Kobayashi H *et al.* Plos Genet. 2012). CG methylation in fully grown oocytes is about 38.7% (Maenohara S *et al.* Plos Genet. 2017). The variation of methylation in oocytes is associated with sequencing methods, sequencing depth, and mapping rates. In the present study, whole genome bisulfite sequencing (WGBS) for small sample and methylation analysis were performed by NovoGene. The reads are 31613641 to 37359643, unique mapping rate is $\geq 32.88\%$, conversation rate is $> 99.44\%$, and sequencing depth is 2.45 to 2.75. Relative information is presented in Table S1. The sequencing depth might be a reason for the inconsistency. But we further confirmed our sequencing results using bisulfite sequencing (BS), and the result is similar between BS and WGBS results. These findings suggest that our results are reliable.

Recommendations for the authors:

Reviewer #1 (Recommendations For The Authors):

(1) Since the results show that melatonin may play a role in hyper-methylation, the authors need to give some basic information in the Introduction section.

Thanks. We added more information in the section of Introduction.

(2) There are many differential metabolites identified. Besides melatonin, other differential metabolites are involved in the altered methylation in oocytes

These is a good question. We firstly filtered the differential metabolites which may be involved in methylation, and then further filtered these metabolites according to the relative DNA methylation pathways and published papers. After that, we confirmed the concentrations of relative metabolites in the serum using ELISA. Certainly, we can not completely exclude all the metabolites which might involved in regulating DNA methylation.

(3) The altered methylation would be found in the F1 tissues. Did the authors examine the other parts besides the liver?

Thank you. In the present study, we didn't examined the DNA methylation in the other tissues besides the liver. We agree that the altered methylation should be observed in the other tissues.

(4) Did the authors try or guess how many generations the maternal obesity-induced genomic methylation alterations can be transmitted?

Thanks. This is a good question. Takahashi Y and colleagues reported that obtained DNA methylation at CpG island can be transmitted across multiple generations using DNA methylation-edited mouse (Takahashi Y *et al.* 2023, cell). Similar inheritance is also reported by other studies using different models.

(5) The F2 is indirectly affected by maternal obesity, so the evidence is not enough to prove the transgenerational inheritance of the altered methylation.

Thanks. We find the altered DNA methylation in F2 tissue and oocytes is similar to that in F1 oocytes. These suggest the altered DNA methylation in F2 oocytes should be at least partly transmitted to F3. Previous paper (Takahashi Y *et al.* 2023, cell) confirms that obtain DNA

methylation in CpG island can be transmitted across several generations through paternal and maternal germ lines. Certainly, it's better if it is examined in F3 tissues.

Reviewer #2 (Recommendations For The Authors):

(1) Figure Font Size: The font sizes in the figures are quite inconsistent. Please try to uniform the font size of similar types of text.

Thanks for your suggestions. We re-edited the relative figures in the revised manuscript.

(2) Figure Clarity: Ensure that all critical information in the figures is clearly visible, such as in Figure 3C.

Thank you. We revised this figure.

*(3) Figure 1B, C: The position of the asterisks ("**") is not centered in the corresponding columns, and the font size is too small. Please correct this and address similar issues in other figures.*

Thank you for your suggestions. We re-edited these in the revised figures.

(4) Line 126: The current expression is confusing. It may be revised to: "Both the oocyte quality and the uterine environment can contribute to adult diseases, which may be mediated by epigenetic modifications."

Thanks. We revised this sentence in the revised manuscript.

(5) Missing Panel in Figure 3: Figure 3 is missing panel 3N.

Thank you so much. We corrected it in the revised manuscript.

(6) Figure Panel Order: Please adjust the order of the panels in the figures to follow a logical reading sequence.

Thank you. We changed the orders in the revised manuscript.

(7) Line 493: Correct "inthe" to "in the".

Thank you. We revised it.

(8) Lines 102-106: Polish the wording and expression, an example as follows: "We analyzed the differentially methylated regions (DMRs) in oocytes from both HFD and CD groups and identified 4,340 DMRs. These DMRs were defined by the criteria: number of CG sites {greater than or equal to} 4 and absolute methylation difference {greater than or equal to} 0.2. Among these, 2,013 were hyper-DMRs (46.38%) and 2,327 were hypo-DMRs (53.62%) (Fig. 1G). These DMRs were distributed across all chromosomes (Fig. 1H). "

Thank you! We re-wrote these parts in the revised manuscript.

Reviewer #3 (Recommendations For The Authors):

The sample numbers should be annotated in the figure legend for all the bar plots using Image J. The lines in Figures 2B and 2C were without error bars. How many mice were used for these plots?

Thanks for your suggestions. We added the sample size in the revised manuscript. We made a mistake when we prepared the pictures for figure 2B and figure 2C, which resulted in missing the error bars. We have corrected these pictures. Thanks again!

The authors should revise the panel arrangement of the figures (Figure 2, Figure 5, etc) to make them more clear and readable.

Thank you! We have revised these in the revised manuscript.

The writing should be improved since there were multiple typos and unclear expressions. AI tools like Grammarly or ChatGPT may help.

Thank you! We have re-edited the language in the revised manuscript using AI tools.

Please recheck the immunofluorescence images for clear interpretability. For example, in Figure 5F (H89 treated), the GV is all the way at the edge of the oocyte, and the oocyte in the DIC image appears like it is partially lysed. The DIC images and the DAPI images are not clear enough.

Thanks for your suggestions. We have re-edited these pictures in the revised manuscript.

Another concern is that the Methods describes the immunofluorescence preparation for 5mC and 5hmC staining as a simple fixation in 4% paraformaldehyde followed by permeabilization with .5% TritonX-100, but there is no antigen exposure step described, a step that is normally required for visualizing these DNA modifications (e.g., 4N HCl).

Thanks. Sorry for that we didn't describe the methods clearly. We have added more information about the methods in the revised manuscript.

The metabolomic analysis revealed a highly significant increase in dibutylphthalate, genistein, and daidzein in the control mice. The presence of these exogenous metabolites suggests that the diets differed in many aspects, not just fat content, so it would be very difficult to interpret the results as related to a high-fat diet alone. Both daidzein and genistein are phytoestrogens and dibutylphthalate is a plasticizer, suggesting differences in the diet and/or in the materials used to collect the samples for analysis from the mice. The Methods define the high-fat diet adequately, as the formulation can be found online using the catalog number. However, the control diet is just listed as "normal diet", so one has no idea what is in it

Thank you for your good questions. The daidzein and genistein may be from the diets and the dibutylphthalate may be from the materials used to collect samples. If so, these should be similar between groups. Thus, we added the formulation of normal diet in the revised manuscript. The raw materials of normal diet include corn, bean pulp, fish meal, flour, yeast powder, plant oil, salt, vitamins, and mineral elements. According to the suggestions, we re-checked the data about these metabolites, and found that the abundance of these metabolites was low. And the result of these metabolites was at a low confidence level because the iron of these metabolites was only mapped to ChemSpider(HMDB,KEGG,LIPID MAPS). To further confirm these results, we examined these metabolites in serum using ELISA, and results revealed that the concentrations of genistein and dibutylphthalate were similar between groups. These results suggest that these metabolites may be not involved in the altered methylation of oocytes induced by obesity.

<https://doi.org/10.7554/eLife.97507.2.sa0>