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Paternal hyperglycemia induces transgenerational inheritance of susceptibility to hepatic steatosis in rats involving altered methylation on *Ppara* promoter

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ABSTRACT

Objective: Diabetes exerts adverse effects on the initiation or progression of diabetes and metabolic syndrome in the next generation. In past studies, limited attention has been given to the fathers' role in shaping the metabolic landscape of offspring. Our study was designed to investigate how paternal hyperglycemia exerts an intergenerational effect in mammals as well as the underlying mechanisms.

Methods: Hyperglycemia was introduced in male rats by intraperitoneally injected streptozotocin and these males were bred with healthy females to generate offspring. The metabolic profiles of the progeny were assessed; DNA methylation profiles and gene expression were investigated. Mutagenesis constructs of the Ppara promoter region, and a luciferase reporter assay were used to determine transcription factor binding sites (TFBSs) and the effects of hypermethylation on Ppara transcription.

Results: Paternal hyperglycemia induced increased liver weight, and plasma TC, TG, LDL, accumulation of triglycerides in the liver. We discovered that CpG 13 in the amplified promoter region (−852 to −601) of Ppara was hypermethylated in adult offspring liver and expression of Ppara, Acox1, Cpt-1α, and Cd36 was down regulated. Hypermethylation of CpG site 13 in the Ppara promoter inhibited the gene transcription, probably through abrogation of SP1 binding. The same epigenetic alteration was discovered in the fetus (E16.5) liver of hyperglycemic father's progeny.

Conclusions: Paternal hyperglycemia may induce epigenetic modification of Ppara in offspring's liver, probably through interaction with SP1 binding, causing impaired lipid metabolism. Our investigation may have implications for the understanding of father-offspring interactions with the potential to account for metabolic syndromes.

1. Introduction

The prevalence of diabetes is steadily increasing worldwide, and it has been revealed by epidemiology studies that family history as well as previous gestational diabetes is a known factor contributing to increased risk of developing the disease. The affected parent can predispose their offspring to youth-onset diabetes and metabolic syndrome [1–3], suggesting a risk determined by an interplay of genetic and metabolic factors. Early life represents a critical window for metabolic reprogramming by adverse environmental factors such as undernutrition [4], obesity [5–7], physical activity [8,9], stress [10,11] and toxins [12,13]. Moreover, those detrimental consequences can extend over multiple generations.

In the past, numerous studies have highlighted mother-infant interactions, whereas the fathers' legacy in shaping the metabolic landscape of offspring has drawn less attention. Notably, paternal

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nutritional status, exposure to drugs and toxins and even unpleasant social experiences could mediate transgenerational effects through epigenetic reprogramming [14,15]. Mechanistically, several types of epigenetic inheritance systems may play a role, including cytosine methylation, hydroxymethylation, and modifications of histone proteins such as lysine acetylation [16,17]. Small RNAs such as microRNAs (miRNAs) have also been shown to drive epigenetic inheritance. The methylation of DNA cytosine residues has been most widely studied, and many studies have shown that life-stage-specific changes in DNA methylation occur in certain metabolic genes during the fetal period and after birth. However, it remains poorly understood whether epigenetic remodeling induced by paternal exposure to hyperglycemia can be inherited and defined offspring metabolic status.

Nuclear receptors are ligand-activated transcription factors (TFs) that translate information about the lipid environment into specific genetic programs, facilitating adaption to fluctuations in nutrient availability. Dysregulation of these processes can have an impact on metabolic balance, leading to obesity [18,19] and related pathologies, such as type 2 diabetes [20,21], hypertriglyceridemia [22], and non-alcoholic fatty liver disease (NAFLD) [23]. New findings increasingly support the concept that nuclear receptors are involved in transgenerational adaptive changes through epigenetic mechanisms, which reflect the impact of adverse environments in early life [24]. Peroxisome proliferator-activated receptor α (*Ppara*) is a key member of the PPAR family of nuclear receptors, and is highly expressed in tissues with active fatty acid catabolism [25,26], especially in the liver. Its activation modulates the activities of fatty acid oxidation systems. It was reported that *Ppara* protects the liver from NAFLD, and its deficiency enhances hepatic steatosis and inflammation in *Ppara*-null mice when fed a high-fat diet (HFD) [27,28]. Several studies have demonstrated the consequences of epigenetic regulation on *Ppara*, specifically methylation of clusters of CpG dinucleotides in the promoter region; such methylation is induced by alterations in parental nutritional status, and perpetually reprograms metabolic hemostasis in the offspring [29,30]. As *Ppara* serves as a master transcriptional regulator of hepatic fatty acid metabolism, it is conceivable that its epigenetic landscape would be closely related to the transgenerational inheritance of metabolic dysfunction through the paternal lineage.

In previous studies, to address the transgenerational inheritance of metabolic disorders from fathers with hyperglycemia, we produced a hyperglycemic male rat model by a single injection of low-dose streptozotocin (STZ). Then, the male rats were bred with healthy female rats to generate offspring from STZ fathers (STZ-O), for comparison with offspring from citrate buffer (CB)-treated euglycemic fathers (CB-O) [31]. Metabolic derangement has been observed in STZ-O along with increased body weight and, impaired regulation of hypothalamus mediated food intake and energy expenditure. However, the detailed molecular mechanism and physiological implications of the gene- and life stage-specific changes in DNA methylation in the fathers' lineage have not been fully addressed.

In the present study, using the previous animal model, we address how paternal hyperglycemia exerts an intergenerational effect, and we further explore the underlying mechanisms. We show that the offspring of the hyperglycemic fathers develop an accumulation of fatty acid in the liver with inhibited protein expression related to fatty acid β-oxidation. Moreover, paternal hyperglycemia modifies the epigenetic signature and affects SP1-regulated transcription of *Ppara* by elevated DNA methylation of CpG sites in its promoter region.

2. Materials and methods

2.1. Animal protocols

Two-month-old male and female Sprague Dawley (SD) rats weighing 200–250 g were purchased from Chongqing Medical University Laboratory Animal Centre (Chongqing, China) and housed under a 12 h/12 h light/dark cycle with lights on from 08:00 to 20:00 in humidity-controlled rooms at 22 °C. All experimental procedures were approved by the Animal Ethics Committee, Chongqing Medical University.

The male rats randomly received two different treatments: STZ (S0130, Sigma) or CB. After a one-week acclimatization period, the hyperglycemia model was induced by intraperitoneal injection of STZ (35 mg/kg body weight) dissolved in 0.1 M CB, pH 4.3, and control rats received an equal volume of CB after 16 h of fasting. Glucose levels were measured in blood from the caudal vein by using a glucose analyzer (Roche Instruments), and rats with glucose levels that were persistently higher than 16.7 mM on the 3rd, 6th and 9th day after STZ injection and immediately before mating were considered hyperglycemic and used for subsequent experiments. STZ-treated hyperglycemic rats (n = 15) and CB rats (n = 10) were mated with age-matched healthy female rats. The males were removed from the cages immediately after the females were confirmed to be pregnant. The offspring (including fetuses) were labeled according to their fathers, forming two groups: STZ-O (STZ-offspring) and CB-O (CB-offspring). Pregnant females had ad libitum access to standard chow feed. For the experiment on early life stages, female rats were randomly chosen, and the fetal tissues were collected at E16.5 after pentobarbital anesthesia administration to the dams. The rest of the dams were fed on a normal chow diet until delivery. Subsequently, the litter size was adjusted to eight animals per dam at day 2. After weaning (21 days postpartum), the animals were housed separated by sex. All animals (both males and females) were maintained on a regular chow diet and had free access to water throughout the study. At the end of the experiment, animals aged 32 weeks were sacrificed in a fed condition by CO_2 inhalation. One rat per litter (no differentiation in gender was made) was used for each test.

2.2. Tissue dissection

For RNA and DNA extraction following dissection, tissues were snap-frozen in liquid nitrogen. Prior to extraction and to facilitate multiple extractions from the same tissue, samples were pulverized in liquid nitrogen and were never allowed to thaw.

2.3. RNA isolation and quantitative RT-PCR analysis

Total RNA from the liver was extracted with TRIzol (Invitrogen), and the RNA quantity was measured using a Nanodrop 2000 system (Thermo Scientific). cDNA was synthesized according to the manufacturer's instructions using a reverse transcription kit (TaKaRa). Quantitative real-time PCR was performed in a 10 μl final reaction volume with SYBR Green (Roche) on a C1000™ Thermal Cycler CFX96™ Real-Time System (Bio-Rad) in triplicate. Amplification was carried out at 95 °C for 4 s, 60 °C for 10 s, and 65 °C for 5 s. This sequence was repeated for 40 cycles. The mRNA expression levels were calculated using the formula $2^{-\triangle\triangle Ct}$ (where $\triangle\triangle Ct = \triangle Ct$ sample-$\triangle Ct$ reference). The primers used are listed in Table 1.

2.4. Protein extraction and western blot analysis

Total protein was extracted from frozen liver tissue using RIPA buffer (Thermo Scientific) containing protease and phosphatase inhibitors (Roche). The ultrasonic fragmentation solution was centrifuged (14,000 × *g* for 15 min) at 4 °C, and the supernatant was collected. Subsequently, the protein concentration was determined with a BCA kit (Beyotime). A total of 50 μg protein was loaded onto a 10% gel for SDS-PAGE, transferred to 0.45 μm PVDF membranes and then blocked with 5% bovine serum albumin (BSA) at room temperature for 2 h to prevent nonspecific binding. The membrane was incubated overnight at 4 °C with the following primary antibodies: anti-PPARα (1:1000, sc-398394; Santa Cruz Biotechnology, Inc., Santa Cruz, CA), anti-CD36 (1:1000, sc-7309; Santa Cruz Biotechnology), anti-acox1 (1:1000, ab184032,

Table 1
Sequences of the primer pairs used in RT-PCR.

Gene	Primers (5′ → 3′)	
β-Actin	Forward	TCACCAACTGGGACGATA
	Reverse	AGGCATACAGGGACAACA
Ppara	Forward	GGGACAAGGCCTCAGGATACCACTA
	Reverse	GACATCCCGACGGACAGGCACT
Ppara (*BSP*)	Forward	ATTTTGGGGTTTTGAAGATTAGATT
	Reverse	ATAAAAAAACTACCCAAAATCACCC
FXR	Forward	CCTCATTGTCTCCCCGACTTATCCT
	Reverse	GCCTCTAGAAAGCAGTGTTCACTTT
Pparg	Forward	CCTCCCTGATGAATAAAGATGG
	Reverse	CACAGCAAACTCAAACTTAGGC
Rxra	Forward	GCATGAAGCGGGAAGCCGTGCA
	Reverse	ACAGCGAGCTCAGCTTCCAGAA
Acox1	Forward	GAGATGGATAACGGCTACCT
	Reverse	AATTCCGTGAGCTCGGTGAC
CPT1a	Forward	CTGCTGTATCGTCGCACATTAG
	Reverse	CGGGAAGTATTGAAGAGTCGC
CD36	Forward	CTCTGACATTTGCAGGTCCA
	Reverse	AGTGGTTGTCTGGGTTCTTG
Acadsb	Forward	TGCCCTATGTTTCGCACCTC
	Reverse	TTCAATGCCCATCAT CCCTT
Adh7	Forward	ACCCGAAGCGGACATT
	Reverse	GGCATCTCCCTGAACG
Adh6	Forward	TTTGCGAAGGCCAAGACA
	Reverse	CCACGCCACCATCTATCATATC
Aldh1b1	Forward	CCAGTGTCACGAAGCCCTCT
	Reverse	GAATACAGCCGCAGCCAAGC

Abcam), anti-CPT1A (ab198494, Abcam), and anti-beta actin (1:1000, ab8227, Abcam).

2.5. Serum and liver biochemical marker assay

Serum was collected from blood after centrifugation at 3000 rpm for 10 min at 4 °C. Serum alanine aminotransferase (ALT), aspartate aminotransferase (AST), triglyceride (TG), total cholesterol (TC), high density lipoprotein (HDL), and low-density lipoprotein (LDL) were measured at the Clinical Laboratory of Children's Hospital of Chongqing Medical University, using commercial kits according to the manufacturer's instructions and a multifunctional biochemistry analyzer (AU600; Olympus, Tokyo, Japan). The absorbance of ALT and AST was read at 505 nm, and the enzyme activity was calculated as U/L. The absorbance of TG and TC was read at 510 nm, and the concentration was calculated as mM. The absorbance of LDL and HDL was read at 546 nm, and the data are expressed as mM. Free fatty acid (FFA) in liver tissue was measured by a commercial kit following the protocol provided by manufacturer (Applygen Biological Technology Co., Ltd., Beijing).

2.6. Hepatocyte morphological assay

Liver specimens were fixed overnight in 10% formaldehyde buffer, embedded in paraffin and cut into 5 μm thick sections according to a routine procedure. The sections were stained with hematoxylin and eosin (HE) for routine histopathological examination, as well as with freshly diluted Oil Red O working solution (0.5% Oil Red O in isopropanol: H2O = 3:2) for 1 h and counterstained with hematoxylin for 3 min. Sections were examined under a light microscope (BX-50; Olympus) at 200× and 400× magnification. For transmission electron microscope (TEM) observations, the hepatic samples were fixed with 0.25% glutaraldehyde in PBS (pH 7.2) for 4 h at 25 °C, washed in cacodylate buffer, postfixed with 1% osmium tetroxide solution, dehydrated in a graded series of ethanol, infiltrated with propylene oxide, and embedded in Epon. Ultrathin sections were prepared, counterstained with 4% uranyl acetate and lead citrate, and observed using an H-7650 TEM (Hitachi High-Technologies, Tokyo, Japan).

2.7. Methylation profiling by MeDIP-sequencing

Independent liver DNA samples were pooled in equimolar ratios to generate two pools for each condition. Each pool comprised three individuals from three independent litters, hence minimizing the outcomes that might be associated with inter-individual differences. Genomic DNA was sonicated to ~200–900 bp fragments, and 1 μg of fragmented sample was ligated to Illumina's genomic adapters with a Genomic DNA Sample Kit (#FC-102-1002, Illumina) following the manufacturer's instructions. Approximately 300–1000 bp ligated DNA fragments were further immunoprecipitated by the anti-5-methylcytosine antibody (Diagenode). The enriched DNA was amplified by PCR and purified by agarose gel. Sequencing was performed on an Illumina HiSeq 2000 using the TruSeq Rapid SBS Kit (#FC-402-4001, Illumina). Clean reads were aligned to the rat genome (UCSC RN5) using BOWTIE software (V2.1.0), and methylation scores were calculated for specific regions.

2.8. Microarray analysis

To identify differentially expressed mRNAs, total RNA was extracted from three rats per group (one rat was randomly selected from different STZ-O and CB-O litters). Affymetrix RTA1.0 was used in this experiment to analyze data of 6 samples. Total RNA was quantified by a NanoDrop ND-2000 (Thermo Scientific), and the RNA integrity was assessed using an Agilent Bioanalyzer 2100 (Agilent Technologies). To define the differential expression profiles and alternative splicing events within the different variants, one-way ANOVA was performed in Transcriptome Analysis Console Software (version 3.0, Affymetrix). Finally, Gene Ontology (GO) analysis and KEGG analysis were applied to determine the roles these differentially expressed mRNAs played in these GO terms or pathways.

2.9. Assessment of DNA methylation by pyrosequencing

Quantitative DNA methylation analysis was performed by pyrosequencing of bisulfite-treated DNA. One microgram of DNA was treated with bisulfite for C-T conversion using an EZ DNA Methylation-Gold kit (Zymo) according to the manufacturer's guidelines. A region of interest (252 bp) was amplified using 30 ng of bisulfite-treated genomic DNA and 5–7.5 pmol of forward and reverse primers (Table 1). Amplification was carried out at 94 °C for 30 s, 65 °C for 30 s (−1 °C/Cycle), and 72 °C for 15 s. This sequence was repeated for 10 cycles. Then, an additional 20 cycles of 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 15 s were performed. Quantification of methylation was carried out by pyrosequencing as previously described [32].

2.10. Luciferase reporter assay, plasmid construction, and transfection

Rat full-length *Ppara* and SP1 cDNAs were each subcloned into the pGL3-basic and pcDNA3.1 vectors respectively. To obtain luciferase reporter constructs containing the *Ppara* proximal promoter region with a deletion of the putative SP1 binding region ranging from −828/−808, −699/−678 and a joint deletion, relative to the transcription start site (TSS), the sequences were amplified from the genomic DNA of rat *Ppara* and subsequently inserted into a pGL3-Basic vector (designated as pGL3-P1, pGL3-P2, pGL3-P3, pGL3-P4). This site-directed mutant construct pGL3-Mut based on the pGL3-P1 structure was generated by gene synthesis. SP1 or control plasmid was cotransfected with the *Ppara* promoter constructs into 293 T cells. Firefly and Renillaluciferase activities were measured 48 h after transfection using a Dual-Luciferase reporter assay system (Promega). Relative promoter activation was represented as the ratio of firefly to Renillaluciferase activity.

2.11. Data analysis

Statistical analysis was performed using GraphPad Prism software®. All values are shown as the mean ± SD. One-way ANOVA was used to compare the differences among more than two groups. Comparisons between two groups were performed using Student's *t*-test. A P value ≤ 0.05 was considered to indicate significance.

3. Results

3.1. Paternal hyperglycemia induces lipid metabolism derangement in offspring

In our previous study, we demonstrated that paternal hyperglycemia caused impaired metabolic function in the offspring, evidenced by increased body mass and food intake, progressive impairment on the glucose tolerance test (GTT) and decreased insulin sensitivity [31]. To further determine whether the liver, the key organ closely related to metabolic function, was potentially correlated with those derangements, a series of studies was performed. Visual inspection revealed that the livers of the 32-week-old CB-O group were deep red, moist, glossy and resilient, while those of the STZ-O rats of the same age showed a gray-red color, loss of luster and tumescence correlated with a significant increase in liver weight (Fig. 1A and B). Moreover, a liver histological examination of the STZ-O group also revealed full fat vacuoles in lobule cells, infiltration of inflammatory cells, and cell swelling as well as triglyceride accumulation evidenced by the Oil Red O staining (Fig. 1C). As was expected, the liver TG level was significantly increased in the STZ-O liver (Fig. 1D), suggesting impaired lipid clearance. To further investigate systemic changes in lipid metabolism, plasma lipid profiles were tested. It was revealed that the TC, TG, and LDL levels were significantly increased in the STZ-O group, and a lower plasma HDL level was observed (Fig. 1F). The liver histology after Oil Red O staining of the STZ-O group (aged 21 days) was also examined, and lipid accumulation was present. These results suggest systemically impaired lipid metabolism in the offspring of hyperglycemic fathers, which may be present as early as the day after weaning.

To further investigate the detrimental consequences of paternal hyperglycemia on hepatocytes, hepatocellular ultrastructure was examined by transmission electron microscopy (Fig. 1G). It was found that hepatocytes in CB-O rats had a normal ultrastructure with abundant mitochondria and rough endoplasmic reticulum (RER) in the cytoplasm, whereas vacuolated mitochondria and RER dilation were present in the hepatocytes of the STZ-O group. These hepatocellular ultrastructural alterations could have been associated with endoplasmic reticulum (ER) stress and the generation of reactive oxygen species (ROS), which are well-known factors that represent a complex reaction causing hepatocyte apoptosis and lipid accumulation [33]. Notably, serum levels of AST and ALT, known biomarkers for the evaluation of hepatic injury, were not significantly changed although incremental alterations were observed in the STZ-O group (Fig. 1E).

Taken together, these results indicate paternal hyperglycemia can provoke lipid metabolism derangement in offspring liver characterized by the build up of FFA with an impaired systemic lipid clearance capacity, which further impairs hepatocyte ultrastructure.

3.2. Paternal hyperglycemia alters the global methylation levels in offspring liver

As CpG islands are highly conserved between humans and chimpanzees, their changes in methylation status are strongly correlated with gene expression [34]. To investigate the consequences of paternal hyperglycemia on the epigenetic profiles of offspring, we examined the DNA methylation alterations of CGIs in the livers of 32-week-old offspring. The liver DNA CGIs were susceptible to changes in methylation status as a result of paternal hyperglycemia (Fig. 2A). CGIs are grouped into three classes on the basis of their distance to RefSeq genes: promoter islands, intragenic islands and intergenic islands. We further examined the distribution of differentially methylated CGIs in those three regions. It was found that both hypomethylated and hypermethylated CGIs were predominantly located in the promoter region (Fig. 2B). Additionally, CGIs in the promoter region with a methylation change > 2-fold in both of the independent comparisons were clustered into 11456 differentially methylated regions (DMRs), of which 6124 were hypomethylated and 5332 were hypermethylated in STZ-O rats compared with CB-O rats. The promoter Medip signal profiles of 261 genes related to lipid metabolism are illustrated in Fig. 2C and Table S1. In normal somatic cells, the promoter CGIs are typically unmethylated and corresponding genes are frequently expressed [35]. Thus, our findings raised the intriguing possibility that paternal hyperglycemia may potentially interfere with gene expression through DNA methylation alterations of CGIs in the gene promoter regions. Noticeably the discrepancy in CGI methylation levels was also evidenced by the MeDIP analysis in four nuclear receptor family members, *Pparg*, *Ppara*, *Fxr2* and *Rxra* (Table 2). Because of their well-known role as transcriptional regulators of metabolic pathways, the methylation changes in their promoter CGIs and subsequent gene expression are worthy of further research.

We next performed the GO and KEGG analyses of differentially methylated genes. For the genes with increased methylation levels in CGIs, lipid metabolic process was among the top three GO terms with the smallest P value (which denotes the significance of the GO term enrichment in the differentially methylated genes) (Fig. 2D, Table S2), and lipid digestion was among the top 10 GO terms with the highest fold enrichment in biological process (Fig. 2D, Table S3). In addition, KEGG analysis revealed that bile secretion and fatty acid elongation, two pathways closely related to lipid metabolism were also highly enriched (Fig. 2F). The genes with lost DNA methylation did not produce highly enriched GO terms or KEGG pathways related to lipid metabolism (Fig. 2E and F). Overall, these findings highlighted the correlation of differentially methylated genes in STZ-O liver with lipid metabolic processes, and were consistent with the alterations in liver morphology and systemic impairment of lipid metabolism in the progenies of hyperglycemic fathers.

3.3. Paternal hyperglycemia decreases Ppara expression in offspring liver

To obtain molecular insight into the impact of paternal hyperglycemia on liver lipid accumulation in offspring, liver tissues from the 32-week-old CB-O and STZ-O offspring were subjected to microarray gene expression profiling. Statistical analysis of the data from CB-O and STZ-O rats revealed significant fold changes between the two groups (Fig. 3A). Under paternal hyperglycemic conditions, 3381 genes were differentially expressed ($P < 0.05$), some of which are key players for regulating fatty acid metabolism, including acyl-CoA dehydrogenase (*Acadsb*), carnitine palmitoyltransferase2 (*Cpt2*), alcohol dehydrogenase1b1, 6 and 7 (*Adh1b1*, *Adh6* and *Adh7*) and *Ppara* (Fig. 3B). KEGG pathway analysis indicated that fatty acid metabolism was highly enriched with significantly differentially expressed genes (Fig. 3C and Tables S4, S5). Notably, there was an inverse correlation between *Ppara* gene expression and its CGI methylation level in the promoter region determined by MeDIP sequencing (Table 2).

Some of the results were then confirmed by quantitative real-time PCR analysis. Gene expression of nuclear receptor *Ppara* in offspring liver was reduced by paternal hyperglycemia (Fig. 3D). The other nuclear receptors *FXR*, *Pparg*, and *Rxra*, of which the promoter region CGIs were also differentially methylated, did not show any significant changes in gene expression (Fig. 3D). *Acox1*, the first and rate-limiting enzyme of the beta oxidation pathway, and *Cpt1a* which is the key enzyme in the carnitine-dependent transport of fatty acid across the mitochondrial membrane were also significantly downregulated (Fig. 3D). Although no significant change was identified for expression

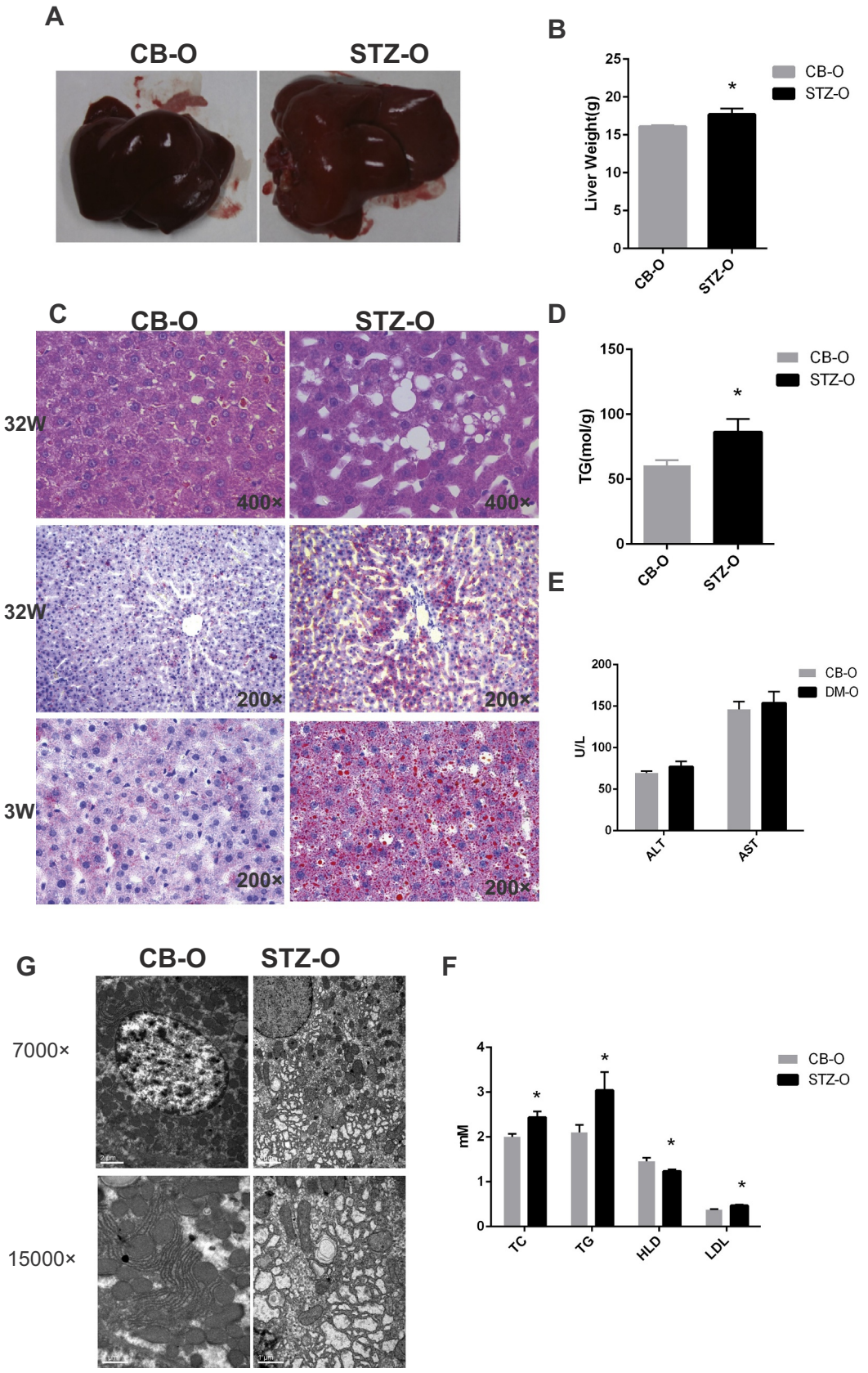
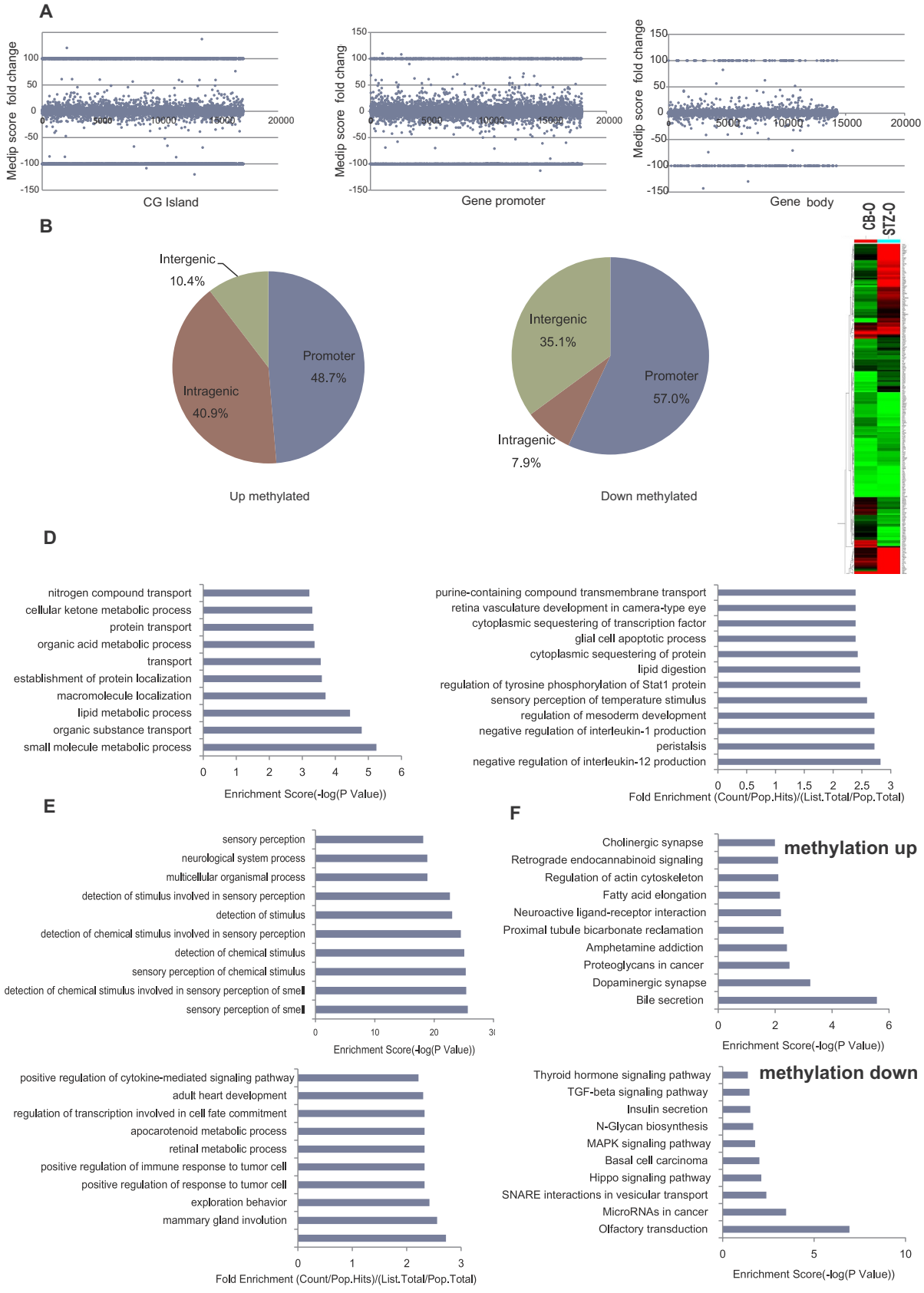


Fig. 1. Paternal hyperglycemia induces impaired systemic lipids clearance capacity and accumulated lipid in the liver.
(A) Visual inspection of liver from 32-week-old STZ-O and CB-O. Left: CB-O. Right: STZ-O. (B) Liver weight of the offspring aged 32 weeks. n = 6 per group. (C) Top: HE staining of livers excised from two groups of offspring. Bottom: Oil Red staining of livers excised from two groups of offspring. (D) Triglyceride levels in the livers of offspring aged 32 weeks. n = 6 per group (E) Plasma ALT, and AST level of two groups of offspring aged 32 weeks. n = 6 per group (F) Plasma lipid profiles including TG, TC, HDL, and LDL levels of two groups of 32-week-old offspring. n = 6 per group. (G) Hepatocellular ultrastructure examination by TEM.



(caption on next page)

Fig. 2. Paternal hyperglycemia alters global DNA methylation status of genes in the offspring liver.
(A) Methylation level fold change in differentially methylated regions including gene promoter, CGIs and gene body, in livers from 32-week-old STZ-O and CB-O. (B) Differentially methylated CGIs are predominantly enriched in the promoter region. Fold change cut off = 2.0 (C) Heat map including increased expressed genes (Fold change cut off-2.0) that belong to the lipid metabolic process Gene Ontology (D) GO analysis result with of the top ten terms with highest enrichment score and fold enrichment for upmethylated genes. Left: top ten terms with highest enrichment score. Right: top ten terms with highest fold enrichment. (E) GO analysis result of top ten terms with highest enrichment score and fold enrichment for down-methylated genes. Top: top ten terms with highest enrichment score. Bottom: top ten terms with highest fold enrichment. (F) KEGG analysis result of top ten terms with highest enrichment score. Top: up-methylated genes. Bottom: down-methylated genes.

of *Cd36*, the fatty acid transporter, there was a tendency toward a decrease (Fig. 3D). Two other genes closely related to fatty acid synthesis Fasn-1 and ACC-a, showed no significant changes in expression. The reduced expression of PPARα, ACOX1, CPT-1α and CD36 was then further confirmed by western blot (Fig. 3E and F). *Acox1* and *Cpt1a* are known to be components in the regulatory network of fatty acid β-oxidation by *Ppara* [36], and their deficiency together with reduced *Ppara* expression indicates a downregulation of the *Ppara* network, which would probably result in decreased fatty acid beta oxidation. Collectively, these data supports the derangement of lipid metabolism and liver fatty acid accumulation induced by paternal hyperglycemia, which could be explained, at least partially, by altered expression of *Ppara*.

3.4. Paternal hyperglycemia increases CpG site methylation in the Ppara promoter region in adult offspring liver that could be traced back to early life stages

We hypothesized that the deregulated expression of *Ppara* might, at least in part, be explained by altered epigenetic modifications. As visualized with the UCSC Genome Browser, the methylation status in different regions of *Ppara* was altered in the liver of 32-week-old STZ-O rats compared with that in CB-O rats of the same age (Fig. 4A). Because DNA methylation modifications in promoter regions are highly sensitive to gene expression, the *Ppara* promoter was then amplified to show more detailed information about DNA methylation alterations (Fig. 4B). Given that DNA methylation of CGIs in the promoter region is unambiguously linked with transcriptional repression [37], we further explored the methylation patterns of CGIs in the *Pparα* promoter, and two CGIs were identified (Fig. 4C) (MethPrimer software; http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi). The first one (CGI-A) spans from −953 to −607 based on the TSS, and the second one (CGI-B) spans from −536 to −55. As visualized with the UCSC Genome Browser, CGI-A was in the range of the hypermethylated region (Chr7:126329851-126330401) previously identified by MeDIP sequencing, but the other one was not; therefore we next chose CGI-A as the target for further research, and region −852 to −601 was amplified.

DNA methylation analysis by pyrosequencing showed that the methylation profile of CGI-A in the *Ppara* promoter of STZ-O rat (32 week-old) liver was significantly changed. Of the 23 CpG sites identified, the methylation level of CpG sites 8, 12, 13, and 15 were significantly increased in liver samples from the STZ-O rats compared with those in samples from the CB-O rats (Fig. 4D), and CpG sites 2, 11, 16, 17, 18, and 21 showed decreased methylation levels, suggesting epigenetic reprogramming in the *Ppara* promoter CGI-A induced by paternal high blood sugar levels.

The key question was to determine whether the altered methylation patterns at the *Ppara* locus were pathological consequences of paternal hyperglycemia, or emerged later as the STZ-O rats developed metabolic dysfunction. To address this, we determined the CGI-A methylation in the fetal liver (E16.5) from STZ-O rats. Variations in epigenetic signature were detected, but interestingly, CpG site 13 was significantly hypermethylated, as observed in adult offspring liver, and CpG site 21 was significantly hypomethylated (Fig. 4E). These data suggest that there is an epigenetic reprogramming of the transmitted marks induced by compromised paternal health status, some of which could be traced back to the early stages in life and affect the development of the next generation.

3.5. Up methylated CpG site 13 in the amplified promoter region may be a potential binding site for transcriptional factor SP1

We investigated transcriptional factor binding motifs in the amplified 252 bp sequence from the promoter region of *Ppara* as shown in Fig. 5A using Genomatix MatInspector (Genomatix Software, GmbH). Five motifs were significantly enriched in the CpG sites that gained DNA methylation in adult liver (V$5F5, V$ZF08, V$ZF04, V$SP1F, V$CTCF), and eight motifs were significantly enriched in the CpG sites that lost DNA methylation. Interestingly, CpG site 13, of which the methylation pattern was extended from gestation to adulthood, was deduced to have several TF binding sites including SP1. This TF binds to GC-rich motifs of many promoters, and regulates the expression of a large number of genes involved in a variety of processes, including the regulatory network of lipid metabolism by *Ppara* [38]. Thus we speculated that the DNA methylation of CpG site 13 in the promoter region of *Ppara* may lead to the abrogation of SP1 binding, contributing to the suppression of gene expression.

3.6. Methylation of CpG site 13 in the Ppara promoter inhibits transcription in vitro

To investigate whether DNA methylation of CpG site 13 in the proximal promoter region affects the transcriptional regulation of *Ppara*, we first needed to confirm that this binding site is functionally required for SP1-regulated *Ppara* promoter activation. As the sequence (−828/−808) containing CpG sites 1, 2, and 3 was also the putative binding site of SP1 predicted by MatInspector, we therefore generated sequential deletions of the two binding sites (−828/−808, −699/−678, Fig. 4C) individually and in combination (Fig. S1), and those sequences were inserted into a luciferase reporter construct. The luciferase reporter assay was conducted in the presence of plasmids subcloned with the rats SP1 cDNAs. pGL3-P1, which contained the two putative SP1-binding sites, showed maximum promoter activity, and exhibited a prominent increase in activity in cells transfected with SP1 (Fig. 5B), suggesting SP1-induced upregulation of *Ppara* promoter activity. Deletion of the region containing only the (−699/−678) site (pGL3-P2) caused a significant decrease in *Ppara* promoter activity but could be rescued by cotransfection with SP1, which could be explained if other SP1 binding sites were also present in the cloned sequence. Indeed, the deletion constructs pGL3-P3 (−828/−808) in transfected cells showed a pattern of alterations in promoter activity similar to that of pGL3-P2, as the recovery of promoter activity was also observed with the overexpression of SP1. A significant reduction in *Ppara* promoter activity was observed when the (−828/−808) and (−699/−678) sites were jointly deleted. Such a reduction in activity could not be rescued with cotransfection with SP1. Similarly, when the substitution mutation was generated with CpG site 13, by substituting “CCGG” with “CAGG” (Fig. 5C), the luciferase activity was largely reduced, and this reduction could be partially rescued by the over-expression of SP1 (Fig. 5D). Taken together, these results suggest that the two speculative SP1-binding sites are essential for transcriptional activation of *Ppara*. DNA methylation contributes to the constrained TF binding [39], and thus, it can be speculated that DNA methylation in CpG site 13, located within

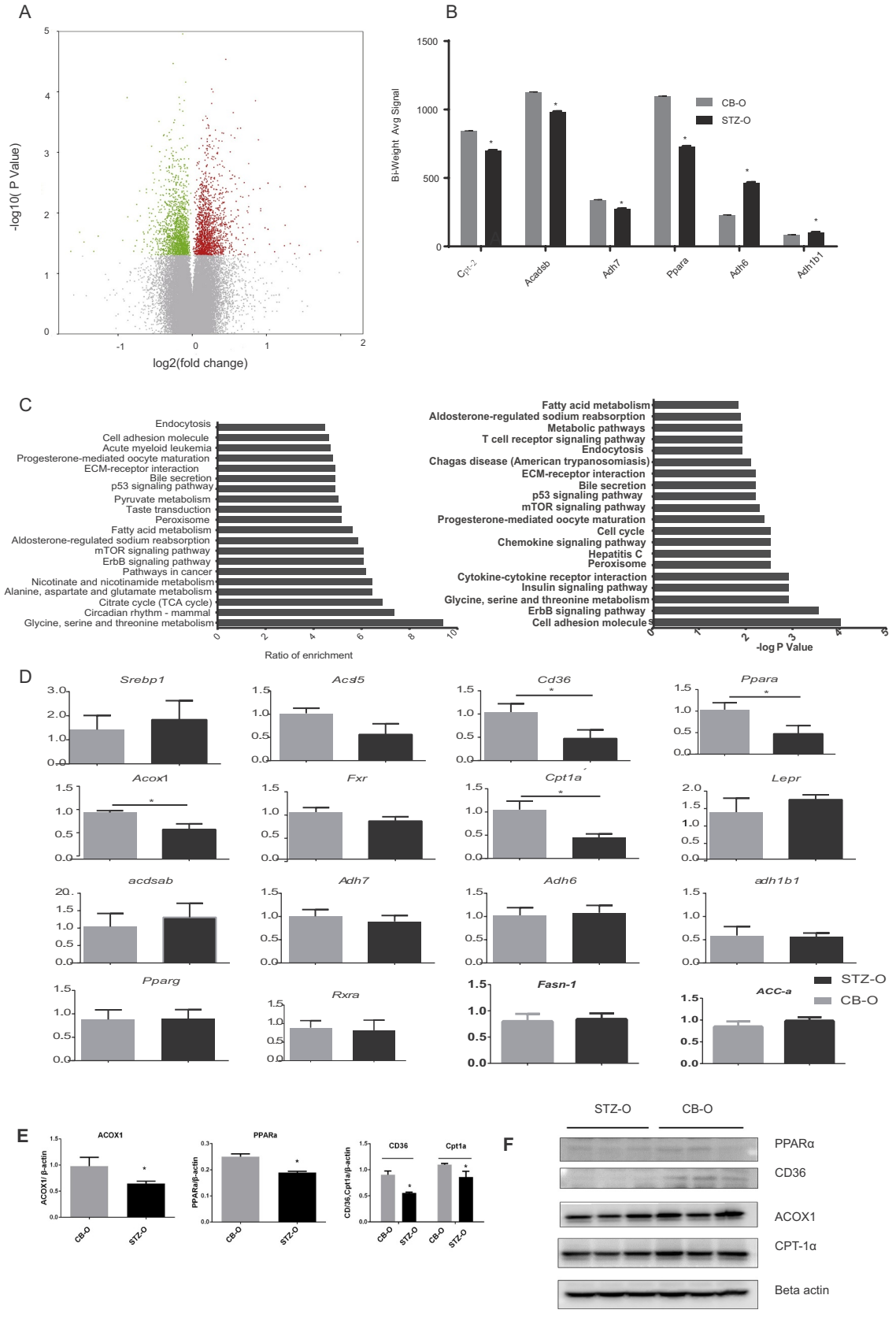


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Fig. 3. Paternal hyperglycemia causes changes in expression of genes related to lipid metabolism.
(A) Volcano plots displaying P value and fold change (CB-O vs STZ-O rats) of gene expression in livers from 32-week-old STZ-O and CB-O. Points labeled red have significantly increased ($P < 0.05$) gene expression between the groups. Points labeled green have significantly decreased ($P < 0.05$) gene expression. Points in gray have no significant difference in gene expression between CB-O and STZ-O rats ($P > 0.05$). n = 3 per group. (B) mRNA levels of genes enriched in fatty acid metabolism by microarray analysis, *$P < 0.05$. Data are expressed as the mean ± SD, n = 3 per group. (C) KEGG analysis of differentially expressed genes. Left: top 20 mRNAs with the highest ratio of enrichment. Right: top 20 mRNAs with the lowest P value. Differentially expressed: Fold change (STZ-O vs. CB-O) > 2, $P < 0.05$. n = 3 per group. (D) PCR verification of genes related to fatty acid metabolism and differentially methylated nuclear receptors by Medip analysis. Data are expressed as mean ± SD. n = 6 per group. (E) Quantification of PPARα, CD36, ACOX1, CPT-1α protein levels. The data were expressed as mean ± SD, n = 6. per group and the differences between the two groups were analyzed with Student's *t*-test, *$P < 0.05$. (F) Western blots of PPARα, CD36, ACOX1, and CPT-1α. β-actin was used as a loading control. n = 6 per group.

the SP1 binding site, may strongly affect *Ppara* expression.

To test the above possibility, methylase M.HPII was used to generate methylated CpG site 13. As M.HPII methylates the CpG within the sequence 5′-CCGG-3′, CpG sites 5 and 11 can also be methylated if the *Ppara* promoter sequence was treated with M.HPII. We therefore generated substitution mutations for 5′-CCGG-3′ (C > A, Fig. 6A) at CpG sites 5 and 11 in combination (pGL3-Mut), so CpG sites 5 and 11 could not be methylated, and subsequently, the mutated sequence was treated with M.HPII. Proper methylation of the inserts was confirmed by digestion with the restriction enzyme *Hpa*II. The luciferase reporter assay showed a significant increase in *Ppara* promoter activity when CpG sites 5 and 11 were jointly mutated (Fig. 6B); this could be explained by the possibility that these two sites are potential binding sites for TFs functioning as repressors for *Ppara* expression. *Ppara* promoter activity was significantly repressed after methylation with M.HPII methylase of both PGL3-P1 and the mutated sequence (Fig. 6B). Taken together, these in vitro studies revealed that DNA methylation of CpG site 13 in CGI-A of the *Ppara* promoter region significantly repressed the transcriptional activity of *Ppara*, possibly through restricting accessibility of SP1 to its binding sites thus attenuating the SP1-regulated *Ppara* promoter activation.

4. Discussion

Here, we described a mouse model in which paternal exposure to hyperglycemia leads to metabolic derangement and accumulation of fatty acid in the adult offspring liver. Deregulation of genes related to hepatic lipid metabolism can be explained, in part, by reduced expression of the key factor *Ppara*. In turn, altered *Ppara* expression can be partially attributed to changes in DNA methylation in the promoter region mediated by abrogation of SP1 binding. This epigenetic signature was already present in E16.5 fetuses. Hence, our data strongly suggest paternal hyperglycemia alters patterns of liver DNA methylation in early life stages that are subsequently maintained in adult livers, thereby influencing liver lipid metabolism and increasing the risk of metabolic derangement in the offspring.

The rapid global rise in the incidence of chronic metabolic diseases such as diabetes, obesity, and cardiovascular disease suggest nongenetic environmental factors are contributors to the disease risk. Disruptions in mother-infant interactions during both prenatal and perinatal periods can have profound consequences for offspring development [40]. Remodeling by environmental factors, which is referred to as “early life reprogramming”, is associated with increased risk of disease, contributing to the transmission of pathologies such as cardiovascular disease, metabolic syndrome, and cognitive impairments through generations [41]. Increasing evidence has confirmed that paternal lifestyle and particular environmental factors, such as stress [17], nutrition [42], and obesity [43] can influence offspring development either through direct care of offspring or even in the absence of direct contact with offspring. Although it has been proven that intrauterine exposure to maternal diabetes conveys high risk for obesity and type 2 diabetes in the offspring [44], the detrimental consequences of paternal hyperglycemia on the offspring's metabolic profile remains obscure. Together with our previous studies, the present investigations have demonstrated hyperglycemia in male rats influences the systemic and liver lipid metabolism in the next generation, increasing their risk for metabolic syndrome, and thus transmits environmentally induced effects to the offspring. This amplification of pathological disaster through generations can, at least in part, explain the rising prevalence of diabetes that mirrors the global increase in the number of people who are overweight or obese.

It has been noted that paternal effects on offspring can emerge even in species that are not biparental and where direct contact between fathers and their progeny is absent. Moreover, this phenomenon is unlikely to be attributed to inherited genetic variation as it can also occur in isogenic species [45]. Emerging evidence has proved the role of epigenetic mechanisms in shaping the phenotype of the offspring. Indeed, our findings confirmed that changes in genome-wide epigenetic reprogramming still occurred in the offspring of hyperglycemic fathers, even when the fathers were removed from the cages after fertilization, and parenting provided by fathers was absent, and thus influences evoked by paternal care seemed unlikely. Additionally, MeDIP analysis revealed that the lipid metabolism pathway was highly influenced by epigenetic effects, accompanied by dramatic changes in mRNA and protein expression of genes regulating fatty acid metabolism. All those alterations at the epigenetic and transcriptional levels coincided with a phenotype characterized by liver function that was compromised in terms of lipid metabolism, massive fat deposition in liver and higher hepatic triglyceride levels, thus indicating transgenerational consequences of adverse environmental factors through the paternal lineage.

One of the major findings of this study is that paternal

Table 2
Differentially methylated CGIs in promoter regions of nuclear receptors (STZ-O liver vs CB-O liver). Methylation level calculated by Medip sequencing in STZ-O and CB-O liver.

CGI information				Fold change, regulation		MeDIP-score (read-counts/kb)	
CGI_name	CGI_length	CpG number	Gene information	Fold change (STZ-O vs CB-O)	Regulation	CB-O	STZ-O
chr4:210562738-210564002	1264	104	*Pparg*: NM_001145366: −822: −125212;|| Pparg: NM_001145367: 405: −125212	100	Up	0	1.2504
chr7:126329797-126331099	1302	133	*Ppara*: NM_013196: −384: −62300	18.2978	Up	0.7961	14.5669
chr10:55983442-55983719	277	25	*Fxr2*: NM_001100647: 129: −19935	5.1843	Up	18.71	96.9984
chr3:11569674-11571212	1538	151	*Rxra*: NM_012805: −193: −83516	−100	Down	2.6958	0

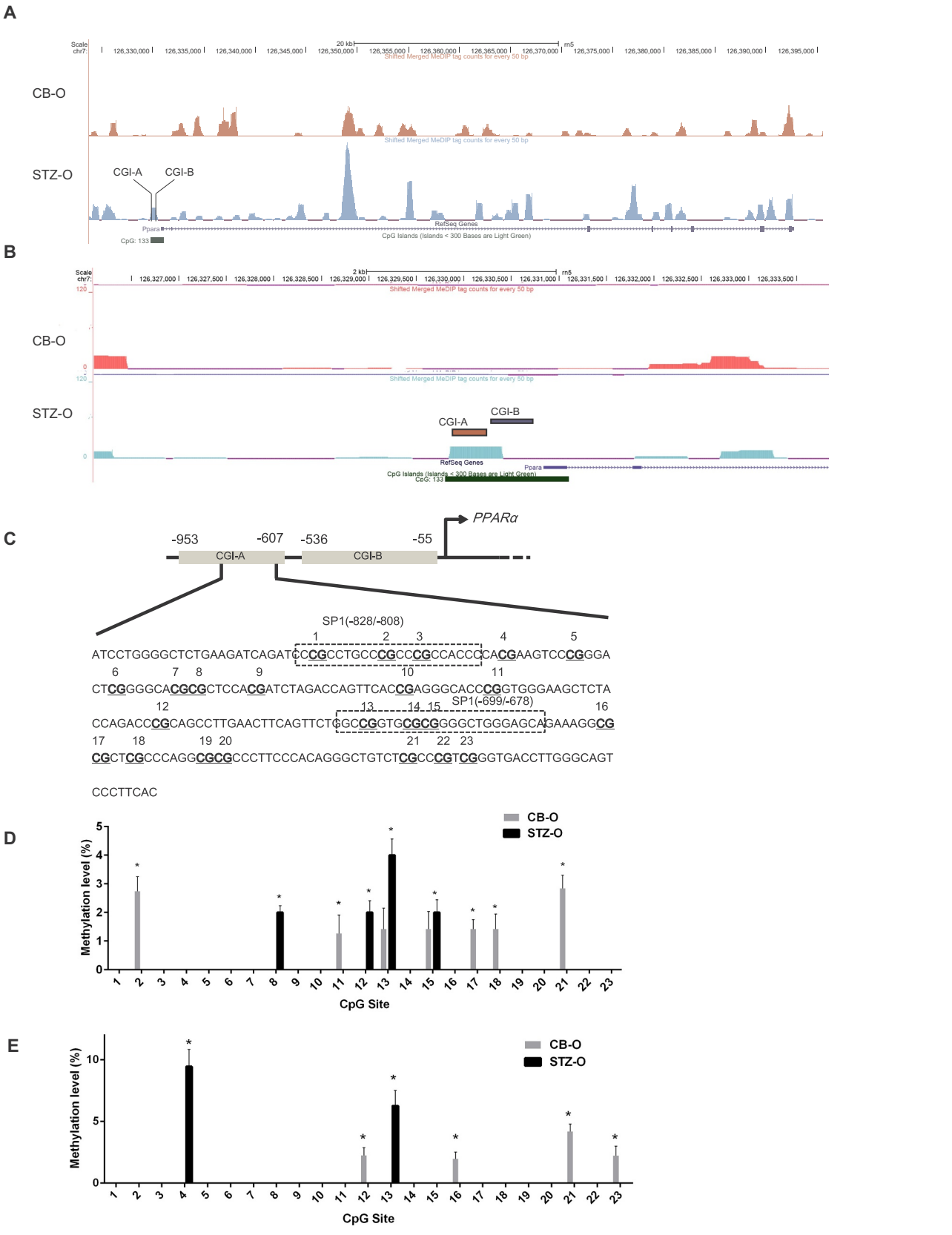

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Fig. 4. Paternal hyperglycemia increases specific CpG site 13 methylation in the *Ppara* promoter region in adult offspring liver that could be traced back to early life stage.
(A) Visualization of methylation status in different regions of *Ppara* by the UCSC Genome Browser in liver sample from 32-week-old STZ-O and CB-O. (B) Amplification of methylation status in the promoter region of *Ppara* by the UCSC Genome Browser. (C) Structure of CGI-A (−852 to −601) in *Ppara* promoter. The location of the CpG islands was identified using MethPrimer (www.urogenen.org/methprimer). Putative SP1 (Specificity Protein1) binding sites are predicted by Genomatix MatInspector (Genomatix Software GmbH). (D) Bisulfate sequencing analysis of CpG site methylation status for CGI-A (−852 to −601) in *Ppara* promoter region in 32-week old and E16.5 fetus STZ-O and CB-O liver. (E) Bisulfate sequencing analysis of CpG site methylation status for CGI-A (−852 to −601) in *Ppara* promoter region in embryos (E16.5) liver. *P < 0.05, n = 5 per group. Student's *t*-test was used for statistical analysis.

hyperglycemia induced changes in DNA methylation patterns of *Ppara* in both adult and fetal liver of the offspring. *Ppara* is a key factor in controlling systemic energy balance, including adipocyte differentiation, energy hemostasis, and lipoprotein and glucose metabolism. Many previous findings have confirmed epigenetic modifications of *Ppara* can mediate the impact of the nutritional environment of parents on offspring development, such as high-fat-diet induced obesity [46], or a protein restricted diet [47], and that ligand-activated *Ppara* -dependent DNA methylation can regulate the fatty acid β-oxidation genes in the postnatal liver [29]. However, these findings revealing epigenetic alterations as an important link between environment and genes were all based on transmission of deleterious conditions from mothers to their children. Our findings, for the first time, reveal the significance of the adverse impact of fathers, namely, hyperglycemia, on the DNA methylation patterns of *Ppara* in the offspring liver, exposing them to high risk of devastating metabolic diseases.

The present findings also show that paternal hyperglycemia induced hypermethylation of specific CpG dinucleotides in the *Ppara* promoter region of the offspring liver, rather than altering the methylation of all CpGs in the *Ppara* promoter, and the magnitude of variation in CpG methylation was sufficient to alter transcription. The CpG dinucleotides in the *Ppara* promoter coincided with the putative binding sites of a number of TFs that have an important regulatory role in a wide range of metabolic processes. Compared to imprinted genes, which usually have a high methylation level causing silencing of the genes inherited from one parent [48,49], epigenetic variations of *Ppara* expressed in somatic cells with relatively low methylation level allow fine control of transcription by changing the balance of TF regulation. This finding is consistent with the work of other researchers, where adverse environmental factors caused a small change in the relatively low methylation level of the promoter region of genes that have a crucial role in controlling mammalian metabolic function [29,46,47]. Imprinted genes are monoallelically expressed with one of the copies of the gene silenced in a parent of origin-dependent manner, and only one copy is functional; thus, any epigenetic alterations on one allele may lead to detrimental consequences, causing fetal growth disruption, lower birth weight, and cancer. However, DNA methylation alterations on biallelically expressed genes such as the one we discussed here seem to be less lethal and are more often linked to “soft” consequences such as metabolic disorders, e.g., gluconeogenesis [17] and lipogenesis [50] abnormalities, and impaired glucose tolerance and insulin secretion [42].

We confirmed that the methylation signature of the *Ppara* locus was also present in the liver samples of STZ-O fetuses well before they developed metabolic alterations that may secondarily lead to epigenetic de novo modifications. In accordance with this finding, we found lipid accumulation was present in the STZ-O group as early as the day after weaning, long before they gained more weight than the CB-O group [31]. Therefore, the presence of the same signature in the liver of STZ-O fetuses and STZ-O adults, and the lipid derangement in the liver of STZ-O fetuses as early as the day after weaning, strongly supports epigenetic marks caused by paternal lifestyle and the persistence of environmental factors from early life into adulthood. It would help to further elaborate epigenetic inheritance through the paternal lineage if the DNA methylation status in the sperm of STZ rats was studied. Genomes undergo a massive epigenetic reprograming during gametogenesis and post-zygotic divisions, and consequently epigenetic modifications in the germ cells could be erased and then be re-established, except for in some regions, primarily IAPs, which remain substantially methylated in all stages of germ cell development [15,51]. Therefore investigating the methylation changes in the *Ppara* promoter region in sperm, which undergo the dynamic process of erasing and re-establishing their epigenetic profile, would be practically difficult, and proving the consistency of DNA methylation marks between sperm and mature somatic cells could even be obscure.

DNA methylation is known to play a critical role in the expression of genes, and one of the major mechanisms is through methylation-dependent TF-DNA interaction. TFs usually bind to nonmethylated DNA motifs and promote or repress gene transcription. However, such interactions can be directly disrupted by methylation of the CpG sites in the motifs [52]. To further investigate the correlation of paternal hyperglycemia-induced changes in the *Ppara* promoter methylation status and lipid dysregulation in the offspring, we tested the effect of potential TFs on *Ppara* promoter activity. By using bioinformatics, SP1 has been identified as a potential TF binding to the hypermethylated CpG site 13 on the *Ppara* promoter. Following the deletion of this possible binding site of SP1, a luciferase reporter assay showed remarkably down-regulated *Ppara* gene expression, which demonstrated that CpG site 13 is critically involved in *Ppara* gene activation and that an elevated methylation level at this site can negatively affect gene expression. Indeed, through in vitro studies, we demonstrated *Ppara* expression was effectively inhibited with methylation of CpG site 13. However, it should be noted that after M.HPII treatment, the methylation level of CpG site 13 changed from 0% to near 100%, but this dramatic change in vitro could be different from in vivo conditions. Our research showed that methylation of this specific CpG is potentially relevant to the phenotype we observed in the animal studies, but further investigations are needed to confirm its actual physiologic and pathogenic contribution.

In support of our findings, in several population-based studies investigating the relationship between family diabetic history and the metabolic function of children and adolescents, paternal hyperglycemia was found to have a long-term effect on metabolic regulation of the next generation. Children whose fathers are diabetic are at a higher risk for developing circulatory system diseases [53], lower birth weight, higher BMI and higher plasma leptin concentrations in childhood [54,55]. Similarly, in a comparative survey of children of parents with diabetes mellitus and metabolic syndrome, the results revealed that BMI, SBP, and total cholesterol were higher in the group of children with diabetic fathers with metabolic syndrome but healthy mothers than in the group with both healthy parents [56]. Although controversies exist regarding how much paternal glycaemia can define the offspring metabolic state [3,57], most of the studies on human population have shown the intergenerational effects of father's diabetes status on the future offspring.

It should be noted that the visualization of the PPARa MeDIP analysis showed that the intragenic regions were different between the groups. DNA methylation of the promoter region is a well-studied repressive modification for gene expression. However, emerging evidence shows the correlation between gene expression and DNA methylation status in other regions of the genome besides the promoter [58–61]. Therefore, the possible contribution of DNA methylation to Ppara expression cannot be ruled out by our present study, and tis worthy of further investigations.

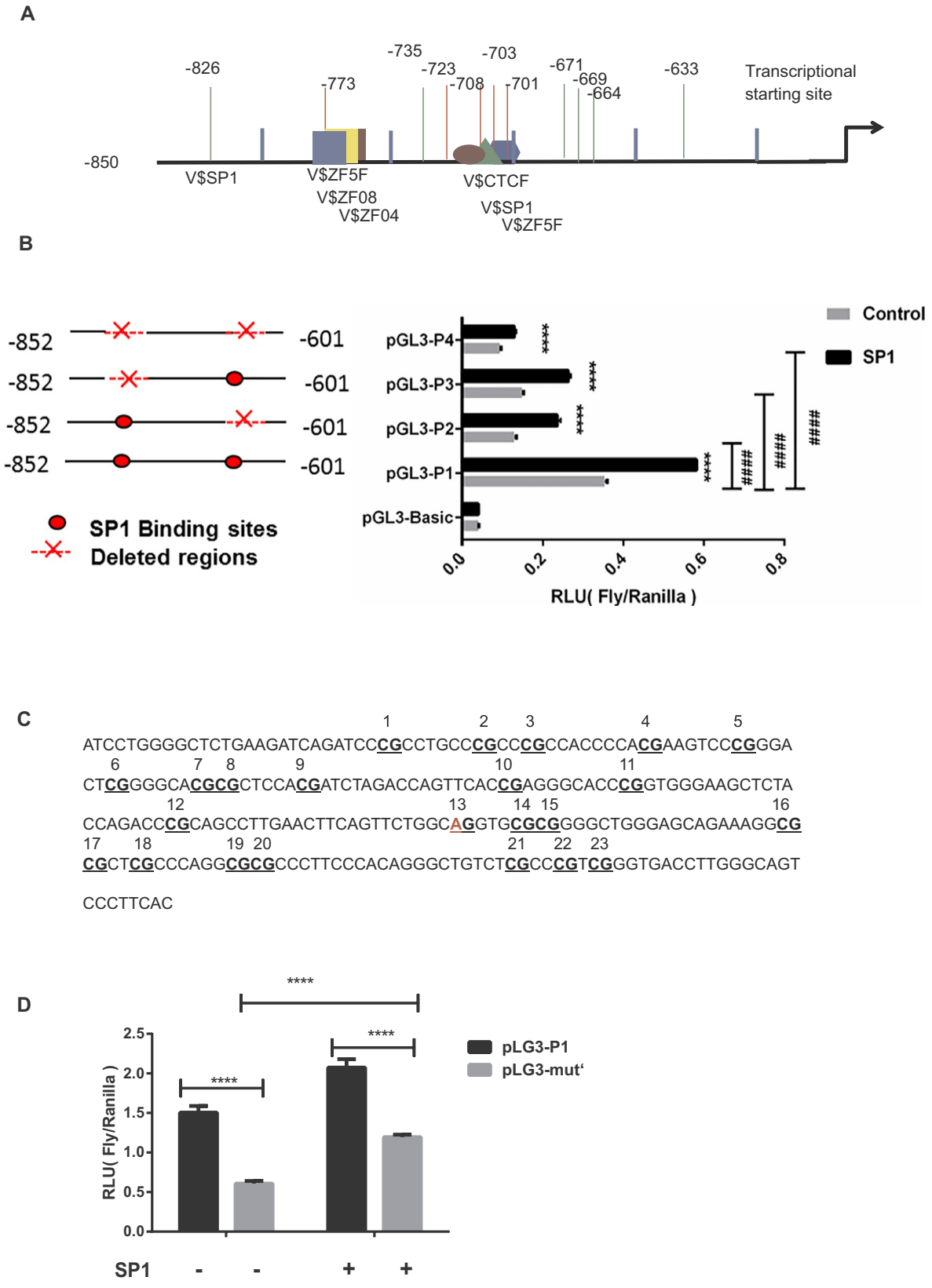

Fig. 5. CpG site 13 is functionally required for SP1-regulated *Ppara* promoter activation.
(A) Schematic diagram of methylation alterations in CpG sites in the amplified *Ppara* promoter region in STZ-O liver and potential transcription factor binding motifs. Green vertical line represents down methylated CpG sites. Red vertical line represents up methylated CpG sites. Squares, triangles, ovals and hexagons represent potential transcription factor binding motifs near the differentially methylated CpG sites. (B) Sequential deletion analyses identified SP1-responsive regions in the amplified *Ppara* promoter region. Complete sequence pGL3-P1 (−852 to −601) and Serially truncated *Ppara* promoter constructs pGL3-P2, pGL3-P3, pGL3-P4 were cotransfected with SP1 plasmids and vector, and the relative luciferase activities were determined. Data are presented as the mean ± SD, n = 5 per group. ****,$^{\#\#\#\#}$P < 0.0001 (C) PLG3-Mut': Substitution mutation of "CCGG" to "CAGG" in CpG site 13. (D) Mean luciferase intensity in 293 T cells transfected with pGL3-P1 and submission mutation sequence (PLG3-Mut'). n = 5 per group, ****P < 0.0001. Student's *t*-test was used for statistical analysis.

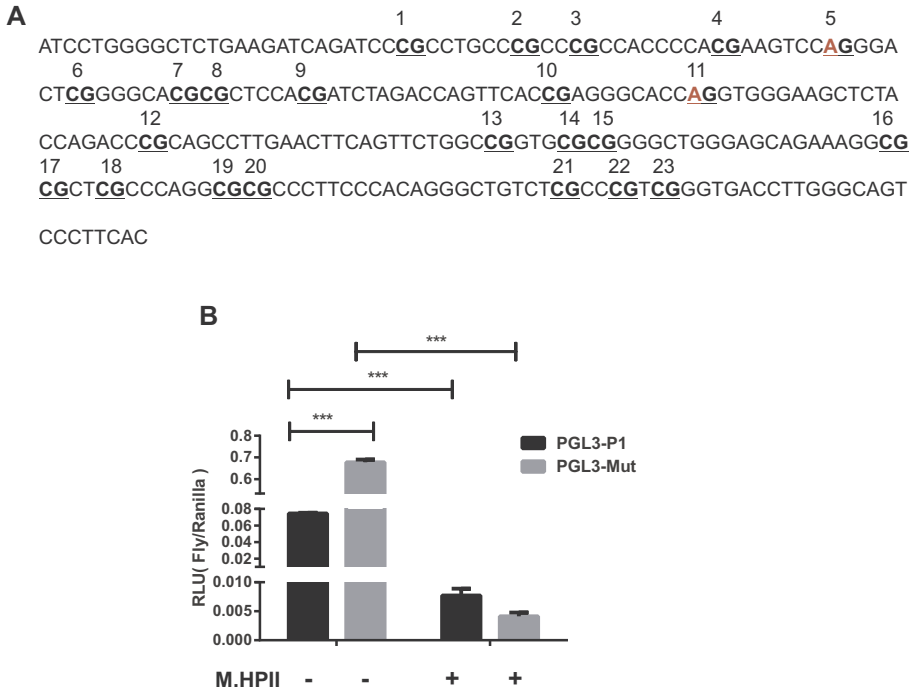

Fig. 6. Methylation of specific CpG site 13 in the *Ppara* promoter inhibits transcription activity. (A) Substitution mutation of "CCGG" to "CCAG" in CpG site 5 and 11 by methylase M.HpaII. (B) Mean luciferase intensity in 293 T cells transfected with pLG3-P1 and submission mutation sequence (pLG3-Mut) being treated with or without M.HPII methylase. n = 5 per group, ***P < 0.001. * P < 0.05. Student's *t*-test was used for statistical analysis.

5. Conclusions

In conclusion, the present study reveals that paternal hyperglycemia induces epigenetic modifications in the liver cells of offspring, which are maintained from early life to adulthood, and contribute partly to the development of lipid dysregulation in second-generation offspring. Our data suggest transmission of environmentally acquired epigenetic modifications through the patriline may play a more critical role in shaping the metabolic profile of the progeny than previously expected. This new finding offers novel insights into a more integrated understanding of parent-offspring interactions with the potential to account for metabolic syndromes in offspring of diabetic parents.

Supplementary data to this article can be found online at https://doi.org/10.1016/j.bbadis.2018.10.040.

Transparency document

The Transparency document associated with this article can be found, in online version.

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Declaration of interests

None.

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