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Gestational diabetes mellitus alters DNA methylation profiles in pancreas of the offspring mice

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ABSTRACT

Gestational diabetes mellitus (GDM), which has an increasing global prevalence, contributes to the susceptibility to metabolic dysregulation and obesity in the offspring via epigenetic modifications. However, the underlying mechanism remains largely obscure. The current study established a GDM mice model to investigate the alternations in the metabolic phenotypes and genomic DNA methylation in the pancreas of the offspring. We found that in the GDM offspring, intrauterine hyperglycemia induced dyslipidemia, insulin resistance, and glucose intolerance. Meanwhile, altered DNA methylation patterns were exhibited in the pancreas and many differentially methylated regions (DMRs)-related genes were involved in glycolipids metabolism and related signaling pathways, including *Agap2*, *Plcbr*, *Hnf1b*, *Gnas*, *Fbp2*, *Cdh13*, *Wnt2*, *Kcnq1*, *Lhcgr*, *Irx3*, etc. Additionally, the overall hypermethylation of *Agap2*, verified by bisulfite sequencing PCR (BSP), was negatively correlated with its mRNA expression level. In conclusion, these findings suggest that the DNA methylation changes in the pancreatic genome of the GDM offspring may be associated with the glycolipid metabolism abnormalities, T2DM susceptibility, and obesity in the adult GDM offspring.

1. Introduction

Gestational diabetes mellitus (GDM), defined as a new-onset glucose intolerance during pregnancy especially in the second and third trimester, is one of the most common complications during pregnancy.[1] It is diagnosed in about 7% of pregnant women.[2]

Hyperglycemia during pregnancy can increase the risk of obesity and type 2 diabetes mellitus (T2DM) in the offspring[3,4]. For example, children born after the mother developed diabetes have an increased risk of obesity and T2DM when compared with their siblings born before the mother was diagnosed.[5] This demographic phenomenon is quite intriguing: a similar genetic background results in quite different outcomes. According to the theory of fetal reprogramming and the hypothesis of the Developmental Origins of Health and Diseases (DOHaD),[6] we postulate that the environmental factors during embryonic development such as intrauterine hyperglycemia may account for the pathogenesis of obesity and T2DM in later life.

Accumulating evidence suggests that maternal hyperglycemia contributes to continuous glucose transfer to the fetus, stimulating fetal islet β-cell proliferation and insulin secretion, and finally resulting in fetal hyperinsulinemia.[7,8] Epigenetically, fetal hyperinsulinemia programs a predisposition for the offspring to develop obesity and T2DM later in life[9]. In fact, it has been widely accepted that intrauterine exposure to hyperglycemia may disrupt the normal pattern of fetal development, which in turn permanently changes its body structure, physiology and metabolism, and induces chronic diseases such as obesity and T2DM in later life.[9–11] The risk transmission from the GDM mothers to their offspring appears to be partly mediated by epigenetic modifications, for instance, DNA methylation.[12,13]

Recent studies have explored the role of DNA methylation in the offspring exposed to GDM.[5,10,14–19] In these studies, DNA methylation changes are examined in tissues relevant to diabetes, such as placentas, umbilical cord blood, or maternal peripheral blood, which have no direct relevance to diabetes and therefore may implicate other confounding factors, for DNA methylation profiles vary in different tissues, even in those of the same species.[20] Therefore, tissues directly related to diabetes may serve as a better target to probe into the role of DNA methylation in the transmission. The current study utilized the pancreatic tissues from the GDM offspring and hypothesized that GDM-induced intrauterine hyperglycemia may change the DNA methylation mode in the developing pancreas, causing glucolipid

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metabolism dysfunctions, obesity, even diabetes in the adult offspring. With this hypothesis, we established a GDM model to observe the differences in metabolic phenotypes by comparing with the offspring from normal pregnancy. RRBS was applied to detect the alternation of DNA methylation profiles in pancreas from the offspring to identify the candidate genes that may be responsible for the changes in response to GDM. RRBS findings were verified by BSP, and the transcription of the candidate genes was detected by RT-PCR.

2. Material and methods

2.1. Animal treatment

All research procedures involving animals were approved by the Institutional Animal Care and Utilization Committee of Fujian Medical University. Male and virgin female (C57/BLKS/J mice (JAX stock #000662, 4 weeks of age) were purchased from the Jackson Laboratory (Bar Harbor, ME, USA). All animals were fed with normal chow diet and bred under specific pathogen-free conditions (IVC system, Tecniplast, Italy) at the laboratory animal center of Fujian Medical University. Mice were kept in a controlled environment (25 ± 1 °C, 40–70% humidity) under a 12-h light/dark shift and allowed food and water ad libitum.

Night-week-old virgin female mice (n = 40, weighted 17–22 g) were housed overnight with males of the same age to mate in a ratio of 2:1 in each cage. The presence of a vaginal plug the following morning was deemed day E0.5 of pregnancy. After pregnancy confirmation, the mice were randomly divided into two groups at random, namely the control group (CON group, n = 6) and gestational diabetes group (GDM group n = 20). After a 13-h fasting, mice in the GDM group received a single intraperitoneal injection of freshly-prepared streptozotocin (STZ, 150 mg/kg body weight; Sigma, St. Louis, MO) within 30 min to induce the GDM model as previously described.[21–23] The CON group received vehicle injections [50 mM sodium citrate (pH 4.5)]. Three days after the injection, blood glucose levels were detected from the tail venous blood with an Accu-Chek Performa (Roche Diagnostic, Germany). The mice were considered as successful GDM ones if their glucose levels were higher than 16.7 mM or 300 mg/dL. At day 7 and 8 of gestation, the gestational diabetes status was reconfirmed. Then random plasma glucose levels were monitored in both groups during gestation.

2.2. Offspring adiposity index

Fasted overnight, 12-week-old offspring from the CON group (CON-F1) and GDM group (GDM-F1) were weighted and the fasting blood glucose was detected. After the blood samples were collected for the following tests, the offspring were sacrificed to separate the inguinal fat and adiposity indices were recorded accordingly.

2.3. Lipid panel screening and glucolipid metabolism-associated hormone detection

Lipid profiles including the total cholesterol and free fatty acid were determined using a corresponding kit (Nanjing Jiancheng Biology Engineering Institute, China), according to the manufacturer's protocol. Plasma insulin, leptin and adiponectin levels were measured using enzyme-linked immunosorbent assay kits (MD6930, MD6928, MD6929, MDL, China).

2.4. Intra-peritoneal glucose tolerance test (IPGTT) and intra-peritoneal insulin tolerance test (IPITT)

To find out whether uterine hyperglycemia can influence the glycometabolism in the offspring and the dynamic changes in their different periods of their life span, we conducted the intra-peritoneal glucose tolerance test (IPGTT) and intra-peritoneal insulin tolerance test (IPITT) in the 12-week-old group, 40-week-old group, and 72-week-old group, respectively. After 16 h of food deprivation, the offspring underwent IPGTT and the blood glucose concentrations were immediately measured with a glucometer at 0, 15, 30, 60, 90, 120 min post injection (1.5 g/kg, dissolved in 0.9% NaCl, i.p.). Then after three days' recovery, the animals received IPITT (1 U/kg), following similar procedures to IPGTT.

2.5. Sample preparation and genomic DNA isolation

Six whole pancreas of the newborn mice were collected (n = 3 for each group), frozen immediately in liquid nitrogen and then stored at −80 °C until use. The DNA of pancreatic genome from the 6 samples was isolated using a tissue/cell genome DNA isolation kit (Aidlab Biotechnlogies co., Ltd.), according to the manufacturer's protocol. The quality of the genomic DNA was checked with a NANODROP UV-2100 DNA analyzer (Amersham, USA).

2.6. Library construction and RRBS

Three DNA samples from the pancreas of the GDM-F1 and three from the CON-F1 were chosen for DNA methylation analysis by RRBS. Briefly, the 3 μg genomic DNA was digested with the restriction enzyme MspI (NEB, R0106T), which can specifically recognize and cut its restriction site, and then was end-repaired and 3′-dA overhung, followed by methylated Illumina adapter ligation. Fragments with 40–220 bps were gel-selected, and bisulphite treatment was conducted with a ZYMO EZ DNA Methylation-Gold™ kit (Zymo Research, Irvine, CA, USA). The quality of library was analyzed by the Agilent 2100 Bioanalyzer (Agilent Technologies) after polymerase chain reaction (PCR) amplification and quantified by the ABI Step One Plus Real time-PCR System (Thermo Fisher, USA). The resulting fragments were sequenced with an Illumina Hiseq2000 analyzer (Illumina, USA).

2.7. Bioinformatics analysis of RRBS data

After the sequencing data were delivered, data filtering was executed, which included removing adaptor sequences, contamination and low-quality reads from raw reads. After filtering, qualified clean data were mapped to the corresponding reference genome by BSMAP.[24] Methylation level was determined by the reads which were covered in cytosine[25]. It was also equal to the mC/C ratio at each reference cytosine.[26] Putative differentially methylated regions (DMRs) were identified in comparison with the methylomes between samples using windows that contained at least 5 CpG (CHG or CHH) sites with a 2-fold change in methylation level and Fisher test was performed to determine p value.[27]

We analyzed the covered promoter and CGI number of target regions, average methylation levels of C and mC, DMRs identification in the CON-F1 and GDM-F1 samples. DMR-related genes were chosen for functional annotation, including Gene Ontology (GO) annotation, Kyoto Encyclopedia of Genes, Genomes (KEGG) pathway analysis and protein-protein interaction analysis.[28] GO enrichment analysis, which is an international standard gene functional classification system, provides all GO terms that are significantly enriched with DMR-related genes, and filters from the genome background the DMR-related genes that correspond to specific biological functions (http://www.geneontology.org/). KEGG pathway analysis is a major public pathway-related database that helps to further understand gene biological functions.[29] This analysis identified significantly-enriched metabolic pathways or signal transduction pathways in DMR-related genes when in comparison with the target regions. Rich Factor analysis was then used to determine the ratio of the DMR-related genes located in pathways to all annotated genes. A greater Rich Factor indicates a larger degree of enrichment. STRING is a widely-used biological database and web resource of known and predicted protein-protein interactions.

We used cytoscape web, basing on the information gained up to 4 levels of functional analysis: fold change of gene/protein, protein-protein interaction, KEGG pathway enrichment, and biological process enrichment to create protein-protein interaction network model.

2.8. BSP

The PCR primers were designed using the online MethPrimer software (http://www.urogene.org/cgi-bin/methprimer/methprimer.cgi). Genomic DNA extractions of GDM-F1/CON-F1 were performed using the same method as mentioned above. 1 μg of DNA samples were converted using a ZYMO EZ DNA Methylation-Gold Kit™ (ZYMO) and one-third of the elution products were used as templates. PCR amplification was performed with a thermal cycling program of 95 °C for 4 min, 40 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 40 s, and then a final incubation at 72 °C for 5 min. PCR products were purified using a QIAquick Gel Extraction Kit (QIAGEN). To clarify the methylation status of *Agap2* gene, 2 CpG sites in the CpG island of the *Agap2* gene region were amplified using BSP of the bisulfite-treated DNA. The primers used for *Agpa2*:forward,GTTTATTGAGTTYGTATTTTYG;reverse, CCCRAACCCCTCTAAACC.

2.9. RT-qPCR

The total RNAs of 6 samples from GDM-F1/CON-F1 were extracted with a RNeasy Mini kit (QIAGEN) following the manufacturer's instructions. Qualification of RNA samples were performed by agarose gel electrophoresis. RT-qPCR was designed using the Primer5 software. The primers used for *Agap2:f*orward,AACTACGCACCTCCCCTAA; reverse, ACGACTTCCCACTCCTGAC.

Real-time qPCR analysis was performed with an ABI Prism 7700 (Applied Biosystems, Tokyo, Japan) using a SYBR Green real-time PCR master mix (Toyobo Co., Japan). Relative expression levels of objective mRNAs were calculated using the $2^{-\Delta\Delta Ct}$ method and normalized to *β-actin*.

2.10. Statistics

All data were presented as the mean values ± SE. Comparisons were made using the Student's *t*-test and a two-sided *p* value <0.05 was considered statistically significant.

3. Results

3.1. Intrauterine hyperglycemia triggers dyslipidemia in the early adulthood of the offspring

We first compared the body fat percentage in the male and female separately and found that regardless of gender, GDM-F1 had a higher proportion of fat in the body composition (female: CON-F1 0.016 ± 0.001, GDM-F1 0.018 ± 0.001, $p < 0.05$; male: CON-F1 0.014 ± 0.001, GDM-F1 0.017 ± 0.001, $p < 0.05$). Furthermore, serum biomarkers showed obvious alternation in the lipid profile and glucolipid metabolism-associated hormone level. Free fatty acid (FFA) and total cholesterol (TC) both showed trends to increased levels in the GDM-F1 ($p < 0.001$), accompanied by a reduction in the levels of adiponectin. GDM-F1 also turned out to secrete more insulin and leptin (Table 1).

Table 1
Biological indicator in 12-week-old offspring (n = 6).

Biological indicator	CON-F1	GDM-F1
FFA (μmol/L)	200.17 ± 45.98	363.27 ± 49.07***
TC (mmol/L)	2.11 ± 0.50	6.33 ± 1.47***
HDL-C (mmol/L)	1.87 ± 0.76	2.45 ± 0.43
LDL-C (mmol/L)	0.71 ± 0.13	0.51 ± 0.17*
FBG (mmol/L)	4.40 ± 0.10	5.29 ± 0.46
Insulin (mU/L)	4.67 ± 0.74	5.72 ± 0.94*
Leptin (ng/mL)	5.99 ± 0.52	8.47 ± 0.23***
Adiponectin (ng/mL)	0.46 ± 0.06	0.29 ± 0.07***

FFA, Free fatty acid; TC, total cholesterol; HDL-C, high-density lipoprotein cholesterol; LDL-C, low-density lipoprotein cholesterol; FBG, free blood glucose.
* $p < 0.05$ vs. the CON-F1 group.
*** $p < 0.001$ vs. the CON-F1 group.

3.2. Intrauterine hyperglycemia induces impaired glucose tolerance and insulin resistance in GDM-F1

We assessed glucose tolerance and insulin sensitivity in the offspring. Different from the early appearance of dyslipidemia, abnormal glycometabolism did not appear until the middle adulthood. In the early adulthood, no significant difference was found in both glucose and insulin tolerance tests (GTTs and ITTs) while GDM-F1 showed impaired glucose tolerance (Fig. 1A, B, C) as well as decreased insulin sensitivity (Fig. 1D, E, F) along with advancing age. By calculating the area under the glycemic time curve (AUC), we found that the gap between CON-F1 and GDM-F1 increased along with age (Fig. 1G, H).

3.3. Intrauterine hyperglycemia affects DNA methylation patterns in the pancreatic genome

To explore the mechanisms of the glucose intolerance and insulin insensitivity observed in the offspring, we performed reduced representation bisulfite sequencing (RRBS) to characterize DNA methylation. We first calculated the ratio of three types of methylated cytosine (mCG, mCHG, mCHH, H = A, G or T) in both promoters and CpG islands (CGI) regions. We found that compared with the control group, the proportion of mCG increased while mCHG and mCHH decreased in both promoters and CGI regions in GDM-F1 group (Supplemental Table S1). We then used a sliding window approach to identify differentially methylated regions (DMRs) which contained at least five CG (CHH or CHG) sites and its methylation levels of two different groups were different ($p < 0.05$). A total of 338 DMRs were identified in 20 chromosomes (Supplemental Table S2) and differentially methylated genes (promoters- and gene bodies-) are shown in Table 2. In the promoter region, 12 differentially methylated genes were upregulated and 11 were downregulated; in the gene body region, 79 differentially methylated genes were upregulated and 97 were downregulated.

3.4. DMRs-related genes analysis

To further explore the molecular mechanism by which the offspring exposed to hyperglycemic intrauterine environment are at increased risk for developing future obesity and type 2 diabetes, DMRs-related genes were analyzed using GO functional analysis, KEGG enrichment analysis and PPI analysis. Significantly-enriched GO terms of both DMR-related genes in the gene body and promoter regions mainly participated in the single-organism process, cellular process, metabolic process, and developmental process (Supplemental Tables S3–S8). KEGG pathway analysis of differentially-methylated genes in the promoter and gene body regions were shown in Fig. 2.

Interestingly, many glucolipid metabolism-related pathways were enriched in the top 20 pathways. Aldosterone-regulated sodium reabsorption, FoxO signaling pathway, mTOR signaling pathway, calcium signaling pathway were enriched in DMR-related genes in the promoter regions (Fig. 2A). Nitrogen metabolism, methane metabolism, fructose and mannose metabolism, Wnt signaling pathway, sphingolipid metabolism, Rap1 signaling pathway, insulin secretion, pancreatic secretion were enriched in DMR-related genes in the gene body regions (Fig. 2B).

PPI analysis was used to build the DMR-related gene interaction network of DMR-related genes according to the known biological relationship among the genes, proteins, and compounds in the database. Based on this computed signaling network, many genes were involved in the glucolipid metabolic process, such as *Agap2*, *Plcbr*, *Hnf1b*, *Gnas*,

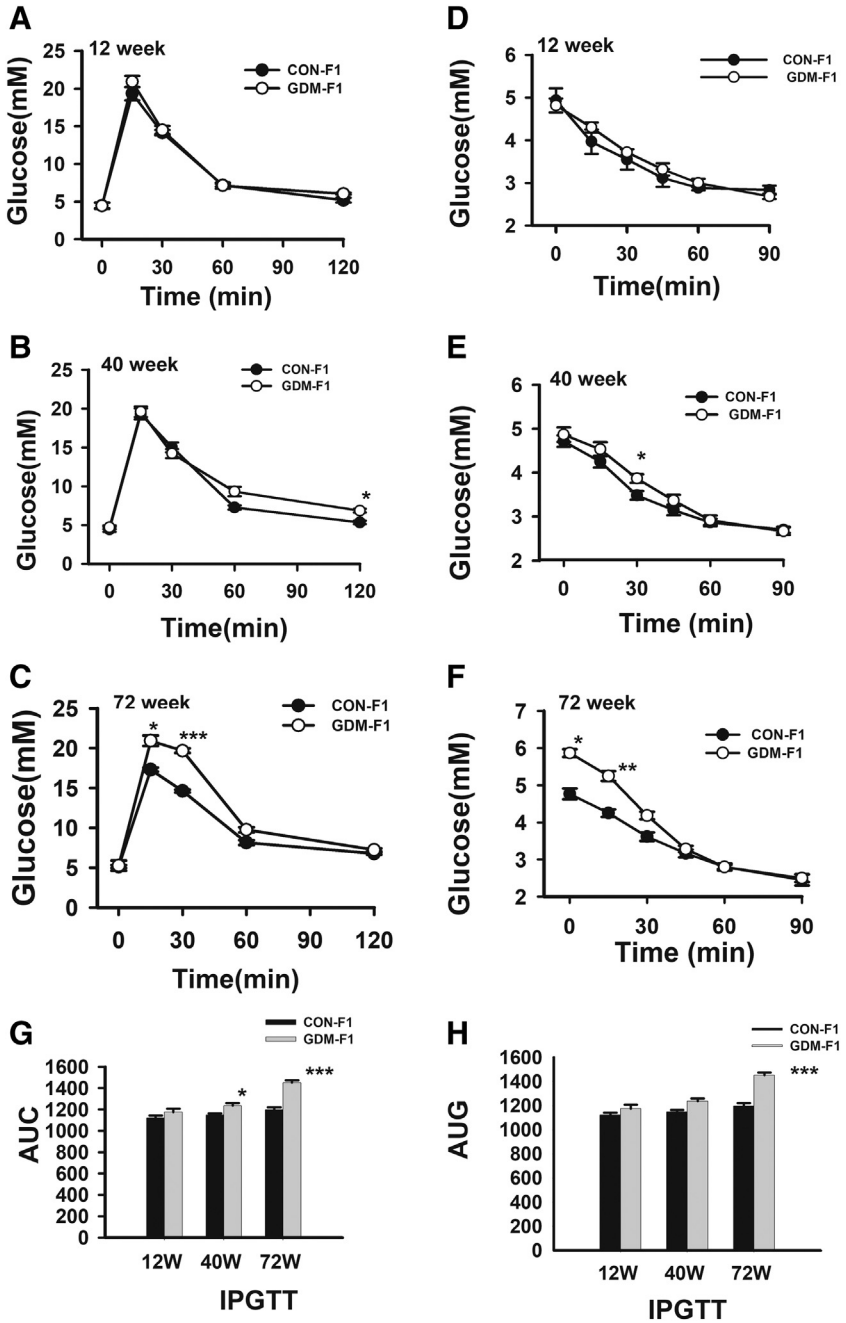

Fig. 1. Intrauterine hyperglycemia-induced impaired glucose & insulin tolerance in the GDM-F1 in the middle adulthood. A, B, C refers to the IPGTT result at 12-week, 40-week and 72-week, respectively; D, E, F for the IPITT result in the three different periods of the mice's life spans; G, H shows the area under the glycemic time curve between the two groups in both IPGTT and IPITT ($^{*}p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$).

Fbp2, *Cdh13*, *Wnt2*, *Kcnq1*, *Lhcgr*, *Irx3* and others. Moreover, these genes participated in the multiple possible signaling pathways associated with susceptibility to diabetes and obesity of the offspring, including insulin secretion, pancreatic secretion, pancreatic development, glucagon signaling pathway and regulation of lipolysis in adipocytes, etc. Intriguingly, *Gnas* and *Plcb4* were considered as the hub node, which interacts with many proteins.

3.5. Overall differential DNA methylation of AGAP2 and gene expression

Among those differentially methylated genes, the methylation level of *Agap2* in the GDM-F1 gene pool was upregulated by more than 2-fold when compared with that of the CON-F1 group. Gene functional annotation revealed that *Agap2* mainly participated in the metabolic and developmental process in gene body region and the metabolic process in the promoter region. KEGG pathway analysis showed that DMR located in *Agap2* gene promoter area was enriched in the signal transduction, especially in the FOXO signal pathway, which may be associated with insulin signal transduction. In order to verify the methylation levels of the candidate genes revealed by RRBS, we further studied one of the most significantly-upregulated genes, *Agap2*, by KEGG analysis. As shown in Fig. 4B, the corresponding cites in the *Agap2* gene promoter region underwent a series of hypermethylation after exposure to the hyperglycemia in utero, compared with the normal pregnant mice (Fig. 4A). The result demonstrated that the methylation rate of *Agap2* in the GDM offspring increased nearly 4-fold (Fig. 4D). RT-PCR was applied to confirm the abnormal expression of *Agap2* in the pancreas from the GDM offspring. As illustrated in Fig. 4E,

Table 2
Gene with differentiation methylated regions (GDM-F1 vs. CON-F1).

	Promoter	Gene body
Upregulated genes	Gbx2, Plagl1, Agap2, Il13,Krt18, Aip,Npr2, Utp3,Rps4, Leng9,Adad2, oxred1	Aff3, Angel2, Plagl1, Rab36, Bc1, Dos,Tbxa2r, Ptprb, Gns, Agap2,Mbd6, Commd1,Zrsr1, Bcl6b, Hnf1b, Acsf2, Chad, Fam171a2, Fmnl1, Aspscr1,Akap6,Mipol1, Klhdc2, Smoc1, Med10, Zswim6, Pnoc, Trappc9, Peg13, Commd5, Creld2, Krt79,Abcc5, Apod, Zbtb20, Synj2, Rn45s, Tmem151b, Mdfi, B4galt6,Arhgap26,Skor2, Tmem134, Ovol1, Macrod1, Shoc2, Gfra1, Bend7, Ttn, Ldlrad3, Plcb4, Ube2v1,Shb, Alad, Ephb2, Slc26a5, Gfi1, Myo1h, Stx1a, Wbscr22, Hoxa3, Hoxa5, Gata2, Foxp1, Tmtc1, Rps4y2, Cdc42ep5, Zfp444, Bcam, Sipa1l3, Zfp710, Dusp8,Kcnq1, Hapln4, Cbfa2t3, Srpr, Slc24a1, Mns1, Trf
Downregulated genes	Sgk1, Sox30, Eif2b2, Ltb, Lhcgr, Henmt1,NcdnSdf4,Dbp, Itgal,Cbfa2t3	Prdm14, Lrrfip1, Espnl, Kif26b, Ush2a, Sgk1, Unc5b, Lrrc20, Lingo3, Ebf1,Myo18a, Stat5b, Plekhh3, Adam11, Taco1, Sdk2, Dlk1, Ptprn2, Cd83, Rbm24,Fbp2, Irx4, Cebpe, Il17d, Spata13, Gata4, Lect1, Slitrk5, Farp1, Dock9, Wdr70,Cerk, Shank3,Tmem117, Gpr84, Galnt6, Sox8, Guca1a, Emilin2, Lhcgr, Pcdha,Pcdhb5, Sipa1, Sgms1, Cyp2c66, Sufu, Wdr96, Gm7102, Csf2ra, Gm996, Ntng2,Abl1, Pla2r1, Gnas, Car3, Lrba, Henmt1, AU040320, Bai2, Oprd1, Trim63, Emilin1, Apbb2, Ldlrap1, Ski, B3galt6, Crybb3, Fam222a, Ccdc64, Tbx3, St8sia1, Vmn2r53, Dact3, Dmwd, Gtf2ird1, Wnt2, Fbxo41, Chst13, Pou2f2, Lrrc4b, Mybpc2, Car11,Nav2, Peg12, Lrrk1, Itgal, Ctbp2, Atp11a, Ccdc124, Nfix, Irx3, Cmip, Cdh13,Kcng4,Crispld2, Cdh15, Gpd1l

the relative expression of *Agap2* mRNA in the GDM-F1 group declined remarkably in contrast to that of the CON-F1 group ($p \le 0.001$). According to the classic epigenetic mechanism, in most situations, the gene expression is repressed by methylation in the CpG site, especially in highly methylated promoter regions.[30]

4. Discussion

Previous studies have indicated that DNA methylation changes produce significant influence on the future health of mother and offspring subjected to GDM, but all these studies examined DNA methylation alterations in samples such as placentas, umbilical cord blood or maternal peripheral blood.

Different from the existing research,[10,16–19,31,32] the current study our work now first provides evidence that GDM alters the DNA methylation patterns in the pancreas of the offspring. As energy metabolism usually involves the pancreas as a whole rather than the islet alone, we accordingly apply RRBS to detect the DNA methylation alternation in the whole pancreas. On the basis of available literature, the current study for the first time screened out the DMR-related genes in the whole pancreas. Our future research efforts will explore the role of these candidate genes in the regulation of pancreatic development and even the methylation alternation in the islets.

In this study, we found that GDM offspring exhibited an altered phenotype manifested as metabolic dysfunction with advancing age. Accompanying the alternation in DNA methylation patterns in pancreas, this altered phenotype was characterized by dyslipidemia and impaired glucose tolerance. Accordingly, our research provides a comprehensive profile of the adverse epigenetic and metabolic consequences associated with intrauterine hyperglycemic during pregnancy in the offspring, and expands our understanding of the potential significance of gestational hyperglycemia in the development of embryonic pancreas.

Increasing evidence from epidemiological studies and animal models illustrates that early life experience represents a window into the phenotypic plasticity that is critically important for adulthood metabolic health.[33,34] Thus, intrauterine environments can impose a

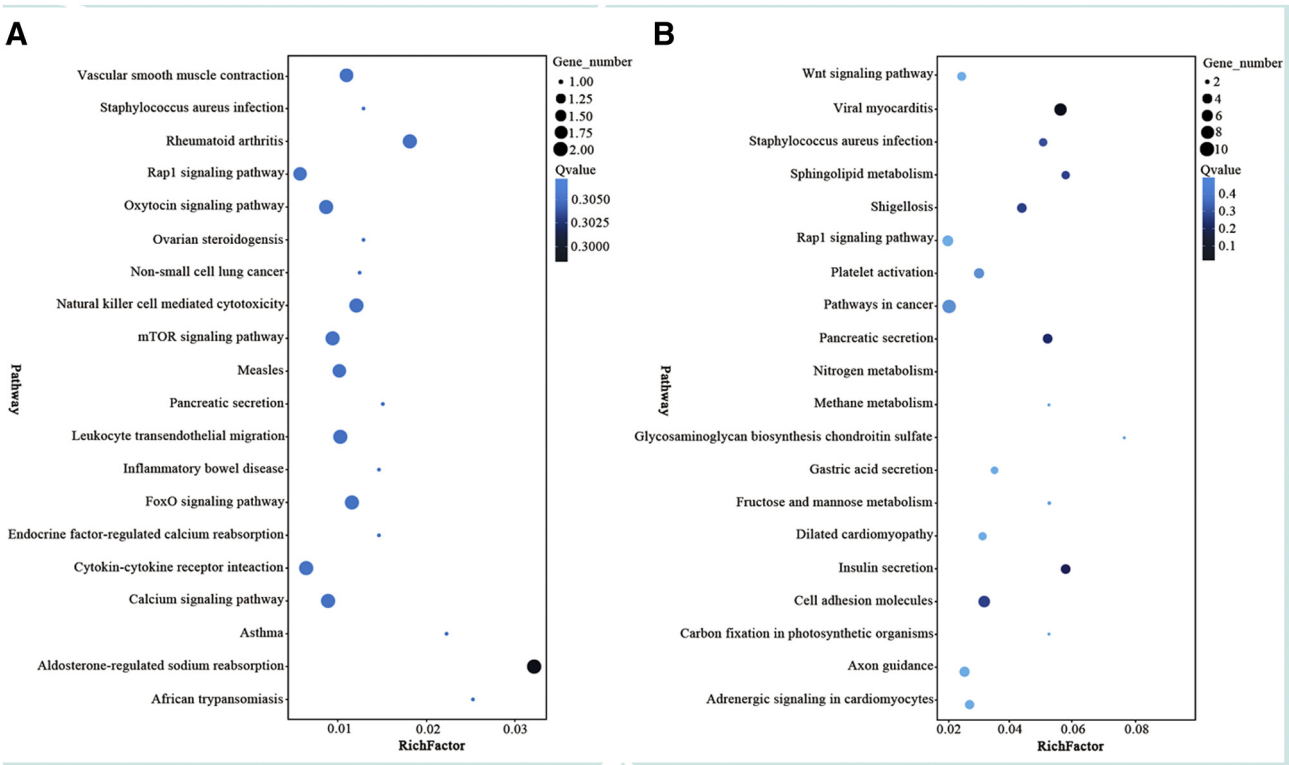

Fig. 2. Top 20 enriched pathways in the pancreatic genome of GDM offspring and CON mice by KEGG pathway analysis. (A) The promoter region and (B) the gene body region. Rich factor indicates the ratio between the number of genes enriched in the pathway and annotated in DMRs. Rich factor and enrichment are positively correlated. The Q value is the corrected *p* value after multiple hypothesis testing. Q value and enrichment are negatively correlated.

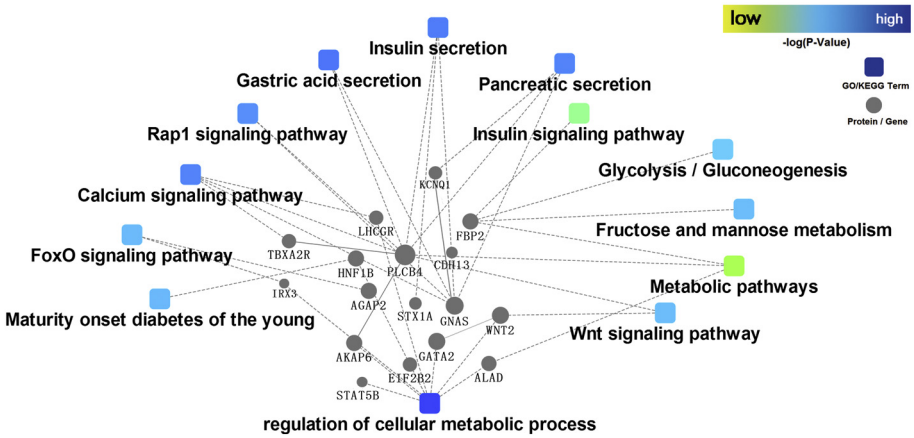

Fig. 3. The network model generated with cytoscape web application, based on STRING database (Search Tool for the Retrieval of Interacting Genes/Proteins). Pathways were indicated with gradient colors from yellow to blue, yellow for smaller *p*-value and blue for bigger *p*-value. Genes/proteins were colored in gray. Default confidence cutoff of 400 was used: interactions with bigger confident score were shown as solid lines between genes/proteins; otherwise in dashed lines.

significant long-term influence on the body weight, energy homeostasis and metabolic function in the offspring.[35,36] As observed in our experiment, different from the early dyslipidemia, glucose intolerance appeared in the middle age and deteriorated along with aging. It is considered that, in unfavorable uterine environment, some obesity-associated genes are more sensitive to DNA methylase than those diabetes candidate genes.

The present study screened out a subset of genes that were differentially methylated in the offspring pancreas, and found 23 differentially methylated genes in promoter regions and 141 differentially methylated genes in gene bodies (Table 2). Moreover, we found that many of the differentially methylated genes were involved in regulating glucose metabolism, such as *Agap2*, *Plcbr*, *Hnf1b*, *Gnas*, *Fbp2*, *Cdh13*, *Wnt2*, *Kcnq1*, *Lhcgr*, *Irx3* (Fig. 3).

GO functional analysis reported that the DMRs-related *Agap2* gene was identified in the functional groups of metabolic process and development process. Meanwhile, the molecular pathway analysis based on the KEGG database revealed that the differentially methylated

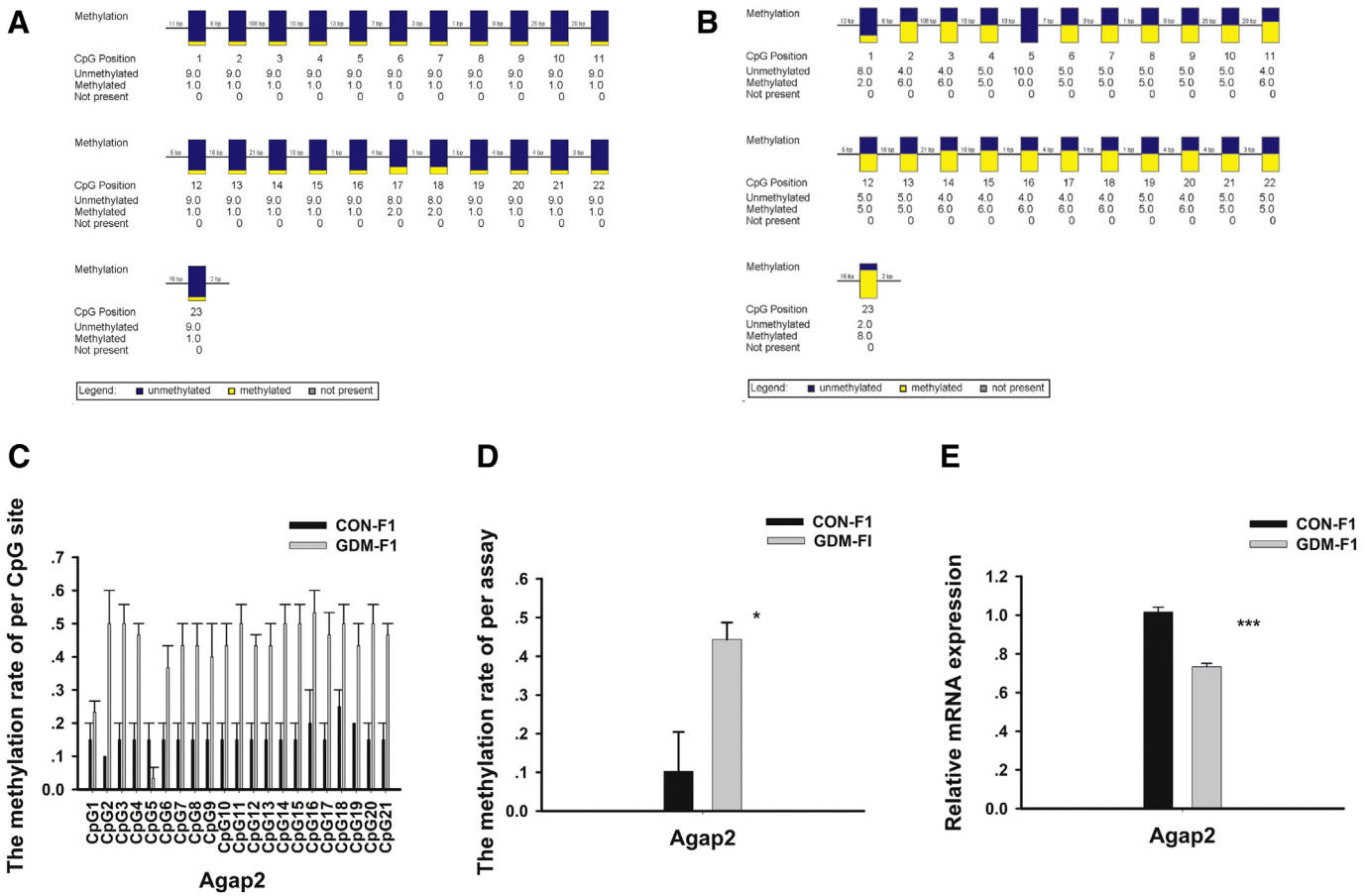

Fig. 4. DNA methylation of *Agap2* and gene expression in pancreas. (A) CON-F1 and (B) GDM-F1. (C) The methylation rate of per CpG site for *Agap2*. (D) The total methylation rate of all the CpG sites per assay for *Agap2*. (E) The relative mRNA expression of *Agap2* gene ($^{**}p < 0.01$, $^{***}p < 0.001$).

Agap2 gene was enriched in the FOXO signaling pathway, which is also involved in the insulin signal transduction. As verified by BSP, *Agap2* turned out to be hypermethylated in GDM-F1 samples.

AGAP2 belongs to the GTP protease superfamily, also named as PIKE-A. It is a kind of PI3K enhancer, directly promoting the cascade reaction of PI3K/Akt.[37] The members can be divided into three types[38], namely PIKE-L, PIKE-S and PIKE-A. The former two types are expressed specifically in the brain, while the last one is widely distributed. As we know, the role of PIKE-A protein depends on the cell type. In the central nervous system, PIKE-A can enhance the activity of Akt, thereby promoting the invasiveness of malignant gliomas.[37,39] At present, few studies focus on the role of PIKE-A in the peripheral tissues. Till now, more and more research finds that PIKE-A participates in the development of obesity and energy metabolism due to its association with glycometabolism. Recently, it is reported that Fyn, PIKE-A and STAT5a cooperate together to mediate the lipogenesis.[40] A recent study also documents that PIKE-A is involved in skeletal muscle energy metabolism mediated by TNF-α and that the interaction between PIKE-A and Akt may be one node in the regulation of glycemetabolism.[41] The interaction between PIKE-A and Akt can activate Akt as the effector of insulin, which promotes the utilization of insulin. Therefore, PIKE-A is an enhancer of insulin in the peripheral tissue, and participates in the regulation of glucose. In a similar line, the current study found that the hypermethylation of *Agap2* gene caused a lower expression of the corresponding PIKE-A protein in the peripheral tissue of GDM-F1 mice, which may reduce the effect of insulin and consequently impair the glucose tolerance.

GNAS protein encoded by *Gnas* gene is the stimulatory G-protein alpha subunit (Gs-α), a key component of many signal transduction pathways. A candidate gene study of the cord blood from 168 offspring born to GDM mothers or controls found that three sites in the *Gnas* gene were hypermethylated in the cord blood of the GDM offspring.[14] Furthermore, increased methylation of the *Gnas* gene can disrupt energy metabolism and accelerate obesity development.[42,43] As the network model displayed in Fig. 3, *Gnas*, the core gene closely related to many genes including *Kcnq1*, *Lhcgr*, and *Cdh13* may play a key role in susceptibility to T2DM, obesity disease in the adult offspring.

Interestingly, differential methylation of adiponectin-T-cadherin (*Cdh13*) was observed in our study. CDH13 is a high molecular weight receptor of adiponectin which is an important adipokine known to play a role in regulating insulin resistance.[44] Being a third receptor of adiponectin, CDH13 is not only involved in the pathophysiology of T2DM, but also directly affect insulin secretion, independent of adiponectin,[45] which suggests that methylation changes in the gene seem to be associated with GDM, in turn supporting our proposal that an exposure to GDM in utero may alter the expression of adiponectin in the offspring.

Irx3 is an important determinant of body mass and composition. Thus, we posit that the DMR-associated gene *Irx3* is functionally connected with obesity susceptibility in the adult offspring. Recent genetic and functional analyses show that the obesity-associated non-coding sequences within *FTO* gene are functionally connected with *Irx3*.[46] Specifically, the obesity-associated *FTO* region contains long-range enhancers that interact directly with the promoter of *Irx3* to regulate *Irx3* expression, suggesting that *Irx3* may be an obesity-associated susceptibility gene.[47]

Available literature reports that $Ca2^{+}$ increases can activate and enhance the activity of PLC, and in turn PLC activity enhancement stimulates insulin secretion.[48] Previous work demonstrates that *Stx1a* not only exists in islet beta cells, but also plays a negative regulatory role in the process of the synthesis and secretion of insulin.[49,50] As insulin secretion is a process dependent on the influx of Ca^{2+}, *Plcb4* and *Stx1a* genes are supposed to affect insulin release through a calcium-dependent insulin secretion.

In summary, we depict a comprehensive profile of the epigenetic alternation in the pancreas of the offspring exposed to intrauterine hyperglycemia, and finger out several candidate genes for T2DM and obesity in the adult offspring. Besides, our studies show that DMR-related genes do not act as single molecules, but rather play interrelated roles together in the context of networks. However, due to the limited sample used in the analysis, it is urgent to find more proof that focuses on the association of genome-wide epigenetic landscape in human pancreas and offspring outcome of gestational diabetic pregnancies. Furthermore, detailed studies on the candidate genes are awaited to explore their function in the regulation of pancreatic development, which could contribute to the prevention of the adverse pregnant outcome and morbidity of metabolic related diseases resulting from GDM.

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Appendix A. Supplementary data

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