Differential methylation of genes in individuals exposed to maternal diabetes in utero

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Abstract

Aims/hypothesis Individuals exposed to maternal diabetes in utero are more likely to develop metabolic and cardiovascular diseases later in life. This may be partially attributable to epigenetic regulation of gene expression. We performed an epigenome-wide association study to examine whether differential DNA methylation, a major source of epigenetic regulation, can be observed in offspring of mothers with type 2 diabetes during the pregnancy (OMD) compared with offspring of mothers with no diabetes during the pregnancy (OMND).

Methods DNA methylation was measured in peripheral blood using the Illumina HumanMethylation450K BeadChip. A total of 423,311 CpG sites were analysed in 388 Pima Indian individuals, mean age at examination was 13.0 years, 187 of whom were OMD and 201 were OMND. Differences in methylation between OMD and OMND were assessed.

Results Forty-eight differentially methylated CpG sites (with an empirical false discovery rate ≤ 0.05), mapping to 29 genes and ten intergenic regions, were identified. The gene with the strongest evidence was *LHX3*, in which six CpG sites were hypermethylated in OMD compared with OMND ($p \leq 1.1 \times 10^{-5}$). Similarly, a CpG near *PRDM16* was

hypermethylated in OMD (1.1% higher, $p = 5.6 \times 10^{-7}$), where hypermethylation also predicted future diabetes risk (HR 2.12 per SD methylation increase, $p = 9.7 \times 10^{-5}$). Hypermethylation near *AK3* and hypomethylation at *PCDHGA4* and *STC1* were associated with exposure to diabetes in utero (*AK3*: 2.5% higher, $p = 7.8 \times 10^{-6}$; *PCDHGA4*: 2.8% lower, $p = 3.0 \times 10^{-5}$; *STC1*: 2.9% lower, $p = 1.6 \times 10^{-5}$) and decreased insulin secretory function among offspring with normal glucose tolerance (*AK3*: 0.088 SD lower per SD of methylation increase, $p = 0.02$; *PCDHGA4*: 0.08 lower SD per SD of methylation decrease, $p = 0.03$; *STC1*: 0.072 SD lower per SD of methylation decrease, $p = 0.05$). Seventeen CpG sites were also associated with BMI ($p \leq 0.05$). Pathway analysis of the genes with at least one differentially methylated CpG ($p < 0.005$) showed enrichment for three relevant biological pathways.

Conclusions/interpretation Intrauterine exposure to diabetes can affect methylation at multiple genomic sites. Methylation status at some of these sites can impair insulin secretion, increase body weight and increase risk of type 2 diabetes.

Keywords Diabetes in pregnancy · Differentially methylated region · DNA methylation · Maternal diabetes exposure in utero

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Abbreviations

AIR	Acute insulin response
BAT	Brown adipose tissue
Beta-value	Ratio of methylated probe intensity and overall intensity
CIR	Corrected insulin response
FDR	False discovery rate
M-value	Log ₂ ratio of methylated probe intensity vs unmethylated probe intensity
NGT	Normal glucose tolerance

OMD	Offspring of mothers with type 2 diabetes during pregnancy
OMND	Offspring of mothers with no type 2 diabetes during pregnancy
PC	Principle component
PP1	Protein phosphatase 1
TSS	Transcription start site
UTR	Untranslated region

Introduction

Unbalanced nutrition in utero, such as hyperglycaemia, has been associated with the development of metabolic diseases in later life [1]. In particular, intrauterine exposure to maternal diabetes increases the risk that the child will subsequently develop obesity and diabetes [2]. Among Pima Indian siblings discordant for intrauterine exposure to diabetes, the risk was higher in those born after their mother developed diabetes than in those born before their mother developed diabetes, providing evidence that the increased risk is due to an effect of the diabetic intrauterine environment in addition to any effect conferred by inheritance of diabetes susceptibility alleles [3]. Studies in Pima Indians with normal glucose tolerance (NGT) have also shown that insulin secretion is lower in young adults whose mothers were diabetic during pregnancy compared with those whose mothers developed diabetes at an early age but after the birth of the child, suggesting that exposure to a diabetic intrauterine environment increases the risk of diabetes, in part because of decreased insulin secretion [4]. This reduction in insulin secretion may be mediated by epigenetic modifications during key developmental periods of the fetus [5].

One source of epigenetic modification is DNA methylation at CpG sites. In some cases, methylation at a specific site is stable; thus, assessment after birth can provide information on methylation patterns established in utero [6]. Two recent studies have reported epigenome-wide analyses that identified differentially methylated regions as a consequence of intrauterine exposure to maternal diabetes [7, 8]. One of these studies was our prior examination of promoter regions in DNA from 28 Pima Indians which did not identify any specific differentially methylated promoter that met statistical significance [7]. A third study which examined methylation of *GNAS* and *IGF2* in 168 Chinese newborns found an association between maternal diabetes and methylation of *GNAS*, in which methylation levels were also associated with obesity [9]. However, these three studies had limited statistical power due to their small sample sizes. In the current study, we sought to understand the effect of maternal diabetes on DNA methylation in 388 Pima Indian offspring, which to our knowledge is the largest epigenome-wide report to date. These individuals were, on average, 13 years old when examined, so the current study sought to determine methylation changes due to

exposure to diabetes in utero that were stable over many years. The Pima Indian population is at high risk of type 2 diabetes, but type 1 diabetes does not occur [10].

Methods

Study participants Participants in the present study were a subset of individuals from a longitudinal study among residents of an American Indian community in Arizona who are predominately Pima Indian [2]. Biennial health examinations, beginning at age ≥ 5 years, included a 75 g OGTT; in a substudy of pregnant women, a 75 g OGTT was also offered in the third trimester. In the current study, data for the OGTT during pregnancy were obtained either at a biennial examination or at a third trimester examination. Diabetes was defined by World Health Organization criteria for non-pregnant individuals, regardless of whether a woman was pregnant, i.e. 2 h post-load plasma glucose ≥ 11.1 mmol/l, fasting plasma glucose ≥ 7.0 mmol/l or a diagnosis observed during clinical care [11]. DNA analysed in the current study was isolated from peripheral blood collected at the offspring's first examination at which they were not diabetic. All available offspring of mothers with diabetes during pregnancy (OMD), defined as having a mother diagnosed with type 2 diabetes before the child's birth, were selected for methylation studies ($N=187$). Of these, 169 (90%) were offspring of a mother diagnosed more than 9 months before the child's birth. Of the remaining 18 (10%) whose mother's diabetes diagnosis was less than 9 months before the child's birth, six were born to a mother with no previous examination, four were born to a mother with a non-diabetic examination <1 year before the child's birth and eight were born to a mother whose last non-diabetic examination was ≥ 1 year before the child's birth. Thus, the current study largely examines the effect on the intrauterine environment of type 2 diabetes that predates the pregnancy. For comparison, DNA samples ($N=201$) were randomly selected from offspring of mothers with no diabetes during the pregnancy (OMND), where the mother did not have diabetes diagnosed before the child's birth and the mother had a non-diabetic OGTT (2 h post-load plasma glucose <11.1 mmol/l) in the longitudinal study ≥ 12 months after the child's birth. OMND were frequency-matched to OMD by sex, age (5 year strata) and self-reported Pima heritage (full vs partial), and all were required to have at least 50% American Indian heritage by self-report. All studies were approved by the institutional review board of the National Institute of Diabetes and Digestive and Kidney Diseases, and all participants provided written informed consent.

Characteristics of the 388 offspring analysed in the study are provided in Table 1. The mean age of the offspring when their DNA was obtained was 13.0 (range 5.2–41.6) years. The OMD group, on average, had a higher maternal age and BMI,

Table 1 Characteristics of participants

Characteristic	OMND	OMD	<i>p</i> value (ANOVA)
<i>N</i>	201	187	
Male/female (<i>n</i>)	82/119	77/110	
Siblings/unrelated (<i>n</i>)	23/178	69/118	
Diabetes prevalence at last examination (%)	7.5	23.0	
Age at DNA sample (years)	13.0 ± 5.7	12.9 ± 5.7	0.89
Age at last examination (years)	20.1 ± 7.1	18.7 ± 7.2	0.06
Age of mother at pregnancy (years)	24.7 ± 5.9	30.1 ± 5.9	1.9 × 10 ⁻⁷
Offspring's BMI (kg/m ²)	24.5 ± 7.0	29.2 ± 8.5	9.0 × 10 ⁻⁹
Mother's pre-pregnancy BMI (kg/m ²)	29.0 ± 6.4	34.8 ± 7.3	1.4 × 10 ⁻¹²
Mother's diabetes duration at child's birth (years)		5.9 ± 4.6	

Values are given as *n* or mean ± SD

and was more likely to have a sibling included in the analyses, compared with the OMND group (Table 1).

Statistical analysis to identify differential methylation

DNA methylation at CpG sites was measured using the Infinium HumanMethylation450K BeadChip (Illumina, San Diego, CA, USA; methods detailed in electronic supplementary material [ESM] [Methods](#)). To stabilise the variance for purposes of analyses, Beta-values (the ratio of methylated probe intensity and overall intensity [the sum of methylated and unmethylated probe intensities]) were converted to M-values (the log₂ ratio of methylated probe intensity vs unmethylated probe intensity) and standardised to a mean of 0 and an SD of 1 [12]. The standardised M-value was adjusted by linear regression for age, sex, a genetic estimate of American Indian ancestry in the offspring, mother's age when the child was born, array batch number, and the first four principal components (PCs) to account for cellular heterogeneity or other unmeasured confounders [13]. In these analyses, American Indian ancestry was estimated according to the method of Hanis et al [14] from 45 ancestry-informative markers [15]. The PCs were calculated using the R software package *pcaMethods* (R Foundation for Statistical Computing, Vienna, Austria), and the methylation Beta-values from 47,340 probes, with one probe selected from each gene and CpG island. The residuals of the standardised M-values obtained from this regression were tested for association with intrauterine exposure to diabetes using Procedure GLM in SAS, version 9.3 (SAS Institute, Cary, NC, USA), with M-value as the dependent variable. To estimate the effect size in biologically meaningful terms, the same model was analysed with the original Beta-value as the dependent variable. In Table 2, effect sizes are shown for the model with Beta-value as the dependent variable, while *p* values are reported for the model with M-value as the dependent variable (which generally conforms better to the assumptions of linear regression).

The empirical false discovery rate (FDR) was estimated using a nested permutation procedure to generate the null distribution. In brief, the methylation data (M-values, PCs) were permuted independently from the phenotypic data (exposure to maternal diabetes status, age, sex, maternal age and ancestry estimate). To account for familial dependence, all phenotypic data for a sibship were permuted together; thus, permutations were conducted across sibships of the same size. With 1000 permutations, the empirical FDR *p* values were calculated using the method proposed by Millstein and Volfson [16].

Differentially methylated regions were analysed to determine whether there was a general tendency for these CpGs to be hypo- or hypermethylated in OMD relative to OMND. The number of hypermethylated regions (effect >0) and hypomethylated regions (effect <0) were counted and subjected to a binomial test for the methylation proportion using the R function *binom.test* (R Foundation for Statistical Computing), in comparison with the expected percentage of hypermethylated regions of 50%.

Assessing differentially methylated sites for prediction of impaired secretory function, obesity and diabetes risk

Secretory function could be assessed in 230 of the 388 offspring who had NGT (2 h post-load plasma glucose <7.8 mmol/l) at baseline. The 2 h corrected insulin response (CIR), a surrogate measure of insulin secretion, was derived from OGTT measurements at the time of the DNA sample collection [17]. Fourteen of these NGT offspring had further been assessed for their acute insulin response (AIR) to a 25 g intravenous glucose challenge at a later time in their life [4]. The association between the insulin secretory function (defined as standardised logarithm of CIR or AIR) and standardised M-value residuals for individual CpG sites was calculated with adjustments as detailed in the Table 3 legend.

BMI could be assessed in 376 of the 388 offspring who had both height and weight measured at baseline, while risk of type 2 diabetes could be assessed in 303 offspring who had

Table 2 Forty-eight differentially methylated CpG sites in OMD vs OMND with epigenome-wide significance

CpG	CpG position	Gene/CpG island	Position in gene	Effect, %	95% CI	Association <i>p</i> value	FDR <i>p</i> value
cg21192468	chr9:139092501	<i>LHX3</i>	Body	4.5	3.1, 5.9	8.3×10^{-10}	1.0×10^{-4}
cg14381623 ^a	chr9:139091650	<i>LHX3</i>	Body	3.3	2.2, 4.5	1.5×10^{-8}	2.0×10^{-4}
cg15796459	chrX:9858910	<i>SHROOM2</i>	Body	−1.6	−2.2, −1.1	5.7×10^{-8}	6.7×10^{-4}
cg20749955	chr8:9009401	<i>PPP1R3B</i>	TSS1500	−3.7	−5.2, −2.3	1.3×10^{-7}	1.2×10^{-3}
cg12140144	chr1:2984275	<i>PRDM16</i>	TSS1500	1.1	0.5, 1.6	5.6×10^{-7}	5.4×10^{-3}
cg06717221	chr19:1790921	<i>ATP8B3</i>	Body	−3.0	−4.1, −1.8	1.3×10^{-6}	8.5×10^{-3}
cg15833797 ^a	chr9:139091875	<i>LHX3</i>	Body	2.3	1.4, 3.2	1.6×10^{-6}	9.3×10^{-3}
cg14605520 ^a	chr9:139094350	<i>LHX3</i>	Body	3.2	1.8, 4.6	1.8×10^{-6}	1.0×10^{-2}
cg10772621	chr19:54410999	chr19:54411376–54411968	-	−1.8	−2.5, −1.0	2.1×10^{-6}	1.0×10^{-2}
cg25952247 ^a	chr9:139092873	<i>LHX3</i>	Body	2.8	1.7, 4.0	2.1×10^{-6}	9.3×10^{-3}
cg20345234	chr10:65801377	chr10:65800729–65801528	-	−2.3	−3.2, −1.4	3.4×10^{-6}	1.5×10^{-2}
cg00762450	chr9:69381140	<i>ANKRD20A4</i>	TSS1500	1.4	0.8, 1.9	4.1×10^{-6}	1.7×10^{-2}
cg13700073	chr17:79495324	<i>FSCN2</i>	TSS200	−1.6	−2.3, −0.9	4.3×10^{-6}	1.7×10^{-2}
cg20769177	chr17:44928516	<i>WNT9B</i>	TSS1500	2.7	1.6, 3.8	4.5×10^{-6}	1.7×10^{-2}
cg08292290	chr14:96010497	<i>GLRX5</i>	3' UTR	−0.6	−0.8, −0.3	4.6×10^{-6}	1.6×10^{-2}
cg06268875	chr18:11147385	<i>PIEZO2</i>	Body	4.6	2.7, 6.6	5.1×10^{-6}	1.7×10^{-2}
cg04350311	chr22:37821982	<i>ELFN2</i>	5' UTR	1.6	0.9, 2.4	5.7×10^{-6}	1.8×10^{-2}
cg15183961	chr9:43134756	<i>ANKRD20A2</i>	TSS1500	1.6	0.9, 2.3	5.9×10^{-6}	1.8×10^{-2}
cg26671988	chr5:102089760	chr5:102090439–102091241	-	2.1	1.2, 3.1	6.3×10^{-6}	1.8×10^{-2}
cg17186803	chr11:118023038	<i>SCN4B</i>	Body	1.2	0.7, 1.8	7.3×10^{-6}	2.1×10^{-2}
cg24049468	chr1:65615281	<i>AK3</i>	Body	2.5	1.4, 3.7	7.8×10^{-6}	2.1×10^{-2}
cg27222147	chr12:2224755	<i>CACNA1C</i>	Body	−0.9	−1.3, −0.5	7.8×10^{-6}	2.0×10^{-2}
cg08370430	chr17:12927578	chr17:12927455–12928747	-	1.3	0.7, 1.9	8.0×10^{-6}	2.0×10^{-2}
cg15618978	chr3:160167990	<i>TRIM59</i>	TSS1500	1.4	0.8, 2.1	8.2×10^{-6}	2.0×10^{-2}
cg07464358	chr7:100952364	-	-	0.9	0.5, 1.3	8.4×10^{-6}	2.0×10^{-2}
cg24996440	chr2:3583570	chr2:3583550–3584833	-	−2.0	−2.9, −1.1	9.0×10^{-6}	2.0×10^{-2}
cg03862414	chr17:40824241	<i>PLEKHH3</i>	Body	2.2	1.2, 3.2	9.0×10^{-6}	2.0×10^{-2}
cg05772155 ^a	chr10:65801432	chr10:65800729–65801528	-	−3.4	−5.0, −1.9	9.9×10^{-6}	2.1×10^{-2}
cg21172615 ^a	chr9:139092102	<i>LHX3</i>	Body	2.6	1.5, 3.8	1.1×10^{-5}	2.4×10^{-2}
cg20941258	chr3:46618668	<i>TDGF1</i>	TSS1500	2.4	1.3, 3.4	1.1×10^{-5}	2.4×10^{-2}
cg08414676	chr15:45314943	<i>SORD</i>	TSS1500	−2.4	−3.5, −1.4	1.2×10^{-5}	2.5×10^{-2}
cg00509616	chr15:57884415	<i>GCOM1</i>	Body	1.2	0.6, 1.7	1.2×10^{-5}	2.4×10^{-2}
cg09674170	chr16:3062382	<i>CLDN9</i>	TSS200	2.9	1.6, 4.1	1.4×10^{-5}	2.8×10^{-2}
cg07993743 ^a	chr17:44928288	<i>WNT9B</i>	TSS1500	2.1	1.1, 3.0	1.5×10^{-5}	2.9×10^{-2}
cg04645534	chr8:23712550	<i>STC1</i>	TSS1500	−2.9	−4.1, −1.6	1.6×10^{-5}	2.9×10^{-2}
cg04413090	chr16:28302913	<i>SBK1</i>	TSS1500	1.0	0.5, 1.4	1.6×10^{-5}	2.9×10^{-2}
cg13427473	chr15:88406836	-	-	−0.7	−1.0, −0.4	1.7×10^{-5}	3.0×10^{-2}
cg16426215	chr16:56706854	chr16:56709677–56709953	-	−1.5	−2.1, −0.8	2.0×10^{-5}	3.6×10^{-2}
cg24503407	chr1:205819492	<i>PM20D1</i>	TSS1500	−3.9	−6.0, −1.9	2.2×10^{-5}	3.9×10^{-2}
cg12875241	chrX:9734063	<i>GPR143</i>	TSS200	4.1	2.2, 5.9	2.2×10^{-5}	3.8×10^{-2}
cg14732789	chr20:29537189	chr20:29534910–29535208	-	−1.4	−2.1, −0.8	2.2×10^{-5}	3.7×10^{-2}
cg14105781	chrX:9433597	<i>TBL1X</i>	5' UTR	1.5	0.8, 2.2	2.4×10^{-5}	4.1×10^{-2}
cg08911291	chr9:44119413	chr9:44118137–44120175	-	2.4	1.3, 3.5	2.4×10^{-5}	4.0×10^{-2}
cg05806645 ^a	chr8:9009352	<i>PPP1R3B</i>	TSS1500	−2.2	−3.3, −1.1	2.5×10^{-5}	4.0×10^{-2}
cg25629768	chr19:2428567	<i>LMNB2</i>	TSS1500	1.4	0.7, 2.1	2.8×10^{-5}	4.4×10^{-2}
cg16482344	chr10:42970987	<i>LINC00839</i>	Body	−3.6	−5.2, −1.9	2.8×10^{-5}	4.3×10^{-2}
cg25949304	chr5:140815218	<i>PCDHGA4</i>	Body	−2.8	−4.1, −1.5	3.0×10^{-5}	4.7×10^{-2}
cg27073142 ^a	chr15:45314933	<i>SORD</i>	TSS1500	4.3	2.3, 6.2	3.3×10^{-5}	5.0×10^{-2}

CpG position is given in a chromosome:base pair format

A CpG is assigned to a gene if it is located within the gene region; if it is not within a gene region, the start/stop of the CpG island is given

TSS1500, CpG is 1500 base pairs away from the TSS and located in the 5' flanking region of the TSS; TSS200, CpG is 200 base pairs away from the TSS and located in the 5' flanking region of the TSS

Effect sizes are shown for the model with Beta-value as the dependent variable, while *p* values are reported for the model with M-value as the dependent variable

The effect represents the difference in the percentage of DNA methylation in OMD compared with OMND. The 95% CI of this effect is shown in the adjacent column

^a These CpG sites are secondary signals of a differentially methylated gene/region

data from at least one outpatient follow-up examination (58 out of 303 had documented diabetes at follow-up). The association between the logarithm of BMI and standardised

M-value, as well as the association between diabetes risk and standardised M-value, was calculated with adjustments as detailed in the Table 3 legend. A Cox proportional hazards

Table 3 The association of the 48 differentially methylated CpG sites with CIR, BMI and diabetes risk in later life

CpG	Gene/CpG island	CIR			BMI			Diabetes risk		
		Effect (SD/SD)	95% CI	p value	Effect (%/SD)	95% CI	p value	HR (/SD)	95% CI	p value
cg24049468	AK3	-0.088	-0.162, -0.013	0.02*	0.9	-2.1, 4.0	5.7×10^{-1}	1.13	0.82, 1.45	0.44
cg25949304	PCDHGA4	0.080	0.010, 0.150	0.03*	-3.0	-5.7, -0.3	3.1×10^{-2} *	0.93	0.66, 1.21	0.63
cg04645534	STC1	0.072	0.002, 0.141	0.05*	-4.5	-7.2, -1.8	1.3×10^{-3} *	0.97	0.67, 1.27	0.85
cg16426215	chr16:56709677-56709953	0.123	-0.004, 0.249	0.06	-2.9	-7.6, 2.0	2.4×10^{-1}	0.95	0.40, 1.49	0.84
cg09674170	CLDN9	-0.076	-0.161, 0.008	0.08	1.0	-2.4, 4.5	5.6×10^{-1}	1.18	0.70, 1.32	0.39
cg06717221	ATP8B3	-0.065	-0.137, 0.007	0.08	-3.1	-5.9, -0.3	3.2×10^{-2} *	1.01	0.81, 1.56	0.94
cg12140144	PRDM16	0.074	-0.015, 0.163	0.10	6.0	2.4, 9.8	1.2×10^{-3} *	2.12	1.74, 2.50	9.7×10^{-5} *
cg20941258	TDGF1	0.053	-0.033, 0.139	0.23	2.6	-0.8, 6.2	1.3×10^{-1}	1.37	1.01, 1.73	0.08
cg24996440	chr2:3583550-3584833	0.049	-0.039, 0.137	0.28	-3.0	-6.3, 0.5	8.9×10^{-2}	0.85	0.49, 1.20	0.36
cg17186803	SCN4B	0.058	-0.050, 0.166	0.29	6.2	1.5, 10.7	8.9×10^{-3} *	1.25	0.77, 1.72	0.37
cg08911291	chr9:44118137-44120175	0.044	-0.041, 0.129	0.31	3.3	-0.1, 6.8	5.5×10^{-2}	1.47	1.09, 1.85	0.05*
cg07464358	-	-0.062	-0.184, 0.060	0.32	9.2	4.3, 14.4	2.3×10^{-4} *	1.21	0.71, 1.71	0.45
cg20769177	WNT9B	0.031	-0.038, 0.101	0.38	-0.7	-3.3, 2.0	6.0×10^{-1}	1.11	0.77, 1.45	0.55
cg05772155	chr10:65800729-65801528	-0.029	-0.096, 0.038	0.40	-1.6	-4.1, 1.0	2.2×10^{-1}	0.94	0.68, 1.19	0.62
cg10772621	chr19:54411376-54411968	0.029	-0.041, 0.100	0.41	-4.9	-7.4, -2.4	2.3×10^{-4} *	0.98	0.69, 1.27	0.89
cg07993743	WNT9B	0.027	-0.042, 0.097	0.44	-1.0	-3.7, 1.8	5.0×10^{-1}	1.02	0.70, 1.35	0.89
cg15618978	TRIM59	0.030	-0.055, 0.116	0.49	2.5	-1.1, 6.3	1.7×10^{-1}	0.99	0.61, 1.37	0.96
cg04350311	ELFN2	0.027	-0.052, 0.106	0.51	4.2	1.1, 7.5	9.2×10^{-3} *	0.94	0.59, 1.29	0.73
cg03862414	PLEKHH3	-0.026	-0.105, 0.053	0.52	1.5	-1.7, 4.8	3.7×10^{-1}	1.07	0.76, 1.43	0.71
cg15833797	LHX3	-0.025	-0.102, 0.052	0.52	0.1	-2.9, 3.2	9.5×10^{-1}	1.09	0.72, 1.41	0.61
cg14381623	LHX3	0.021	-0.045, 0.087	0.53	2.0	-0.7, 4.7	1.6×10^{-1}	1.07	0.78, 1.37	0.64
cg08414676	SORD	-0.023	-0.097, 0.052	0.55	-1.3	-4.1, 1.6	3.7×10^{-1}	1.01	0.71, 1.32	0.93
cg08370430	chr17:12927455-12928747	-0.021	-0.097, 0.056	0.60	-1.1	-4.1, 1.9	4.6×10^{-1}	0.98	0.67, 1.30	0.91
cg04413090	SBK1	0.018	-0.054, 0.091	0.62	5.3	2.4, 8.4	4.1×10^{-4} *	1.08	0.78, 1.38	0.62
cg20345234	chr10:65800729-65801528	-0.017	-0.086, 0.052	0.63	-2.3	-4.9, 0.3	8.7×10^{-2}	0.99	0.70, 1.28	0.96
cg16482344	LINC00839	0.017	-0.052, 0.086	0.63	-1.7	-4.3, 1.0	2.1×10^{-1}	0.96	0.69, 1.23	0.77
cg24503407	PM20D1	0.017	-0.055, 0.090	0.64	-1.0	-3.6, 1.7	4.8×10^{-1}	0.99	0.69, 1.29	0.94
cg21192468	LHX3	-0.013	-0.077, 0.051	0.69	2.3	-0.4, 5.0	9.5×10^{-2}	1.04	0.76, 1.32	0.79
cg26671988	chr5:102090439-102091241	-0.015	-0.092, 0.063	0.71	1.2	-1.9, 4.5	4.5×10^{-1}	1.17	0.84, 1.50	0.35
cg27073142	SORD	-0.012	-0.080, 0.056	0.73	-1.7	-4.2, 0.9	2.1×10^{-1}	1.10	0.81, 1.42	0.52
cg25629768	LMNB2	-0.014	-0.097, 0.068	0.73	1.3	-1.9, 4.5	4.4×10^{-1}	1.13	0.79, 1.46	0.49
cg15183961	ANKRD20A2	-0.014	-0.097, 0.068	0.73	4.4	1.4, 7.6	4.4×10^{-3} *	1.12	0.82, 1.38	0.47
cg12875241	GPR143	0.011	-0.056, 0.079	0.74	0.7	-2.0, 3.4	6.2×10^{-1}	1.03	0.74, 1.31	0.87
cg20749955	PPP1R3B	0.012	-0.060, 0.084	0.75	-4.0	-6.6, -1.2	4.7×10^{-3} *	0.96	0.64, 1.28	0.81

Table 3 (continued)

CpG	Gene/CpG island	CIR		BMI		Diabetes risk	
		Effect (SD/SD)	95% CI	p value	Effect (%/SD)	95% CI	p value
cg08292290	<i>GLRX5</i>	0.013	−0.072, 0.098	0.76	−3.5	−6.4, −0.5	2.2 × 10 ^{−2} *
cg25952247	<i>LHX3</i>	0.009	−0.056, 0.074	0.78	−0.2	−2.9, 2.5	8.6 × 10 ^{−1}
cg14105781	<i>TBLIX</i>	−0.019	−0.154, 0.117	0.79	3.9	−1.4, 9.4	1.5 × 10 ^{−1}
cg00509616	<i>GCOMI</i>	−0.012	−0.097, 0.074	0.79	3.9	0.6, 7.5	2.3 × 10 ^{−2} *
cg27222147	<i>CACNA1C</i>	0.018	−0.113, 0.149	0.79	−3.0	−7.5, 1.7	2.1 × 10 ^{−1}
cg06268875	<i>PIEZO2</i>	0.008	−0.066, 0.083	0.82	−1.2	−3.9, 1.6	3.9 × 10 ^{−1}
cg14732789	chr20:29534910–29535208	−0.009	−0.096, 0.078	0.84	−3.7	−7.0, −0.4	2.9 × 10 ^{−2} *
cg21172615	<i>LHX3</i>	−0.007	−0.082, 0.068	0.86	2.5	−0.5, 5.6	1.1 × 10 ^{−1}
cg14605520	<i>LHX3</i>	0.005	−0.071, 0.080	0.90	0.7	−2.2, 3.7	6.4 × 10 ^{−1}
cg05806645	<i>PPP1R3B</i>	0.004	−0.066, 0.073	0.91	−2.8	−5.4, 0.0	5.0 × 10 ^{−2} *
cg13700073	<i>FSCN2</i>	−0.004	−0.080, 0.073	0.93	−2.3	−5.4, 0.8	1.4 × 10 ^{−1}
cg13427473	-	0.002	−0.078, 0.081	0.97	−3.6	−6.4, −0.6	1.9 × 10 ^{−2} *
cg00762450	<i>ANKRD20A4</i>	−0.001	−0.081, 0.078	0.97	3.5	0.4, 6.8	2.8 × 10 ^{−2} *
cg15796459	<i>SHROOM2</i>	−0.001	−0.126, 0.125	0.99	−2.9	−7.4, 1.7	2.2 × 10 ^{−1}

CpG position is given in a chromosome:base pair format

A CpG is assigned to a gene if it is located within the gene region; if it is not within a gene region, the start/stop of the CpG island is given

The effect size of CIR is shown as the SD difference in CIR per SD difference in M-value. The effect size of BMI is shown as the per cent difference in BMI (expressed as a multiplier) per SD of M-value. The HR for development of diabetes is shown per SD of M-value. The 95% CI of effects/HRs are shown in the adjacent columns

All associations were adjusted for age, sex and proportion of the American Indian ancestry of the offspring, as well as for the mother's age when the child was born, array batch and four PCs (the four most significant PCs were calculated from genome-wide methylation data). CIR was additionally adjusted for BMI and HOMA-IR of the offspring. All the models were further corrected for intrauterine exposure to diabetes

* $p \leq 0.05$

model (Procedure PHREG in SAS, version 9.3; SAS Institute) was used for the analysis of diabetes risk. To assess the extent to which observed methylation differences may account for the increased diabetes risk in OMD, a formal mediation analysis was conducted as detailed in ESM Text [18, 19].

Pathway enrichment analysis Genes with differentially methylated CpG sites ($p \leq 0.005$) were analysed for pathway enrichment (KEGG pathways [20]; release date 21 March 2011; access date 4 November 2015) compared with *Homo sapiens* genetic background using the WEB-based GENE SeT Analysis Toolkit (WebGestalt) [21]. The enrichment p values were adjusted for multiple tests by the FDR method. Pathways with FDR $p \leq 0.001$ and more than three differentially methylated genes were considered enriched.

Results

Thirty-nine unique differentially methylated regions are associated with intrauterine diabetes exposure

Methylation data from 423,311 CpG sites passed our quality control metrics. When analysed in aggregate, baseline DNA methylation mean Beta-values across all 423,311 sites did not differ ($p = 0.70$) between the OMD group ($N = 187$; Beta-value 0.513 [SD 0.006]) and the OMND group ($N = 201$; Beta-value 0.513 [SD 0.012]). Analysis of each individual CpG site in OMD compared with OMND is shown by chromosome in a Manhattan plot (Fig. 1a), and the corresponding quantile–quantile plot is also shown (Fig. 1b). Forty-eight CpGs representing 39 unique regions were significantly associated with intrauterine diabetes exposure (empirical FDR ≤ 0.05 ; Table 2). At these sites, the adjusted median absolute value of the difference in DNA methylation between OMD and OMND was 2.2% (range 0.6–4.6%). Among these 48 CpGs (17 in gene bodies or 3' untranslated regions [UTRs], 20 in regions close to transcription start sites [TSSs] or 5' UTRs, 11 in intergenic regions [with two CpGs being found in the same intergenic region]), 29 were hypermethylated and 19 were

hypomethylated; the proportion of hypermethylated CpGs was not significantly greater than 0.5 (binomial $p = 0.097$). Since there was a large difference in pre-pregnancy maternal BMI between the OMD and OMND groups (Table 1), we further adjusted for pre-pregnancy BMI in the 296 individuals (154 OMD and 142 OMND) for whom maternal BMI data were available. The effect sizes were generally unchanged with the additional adjustment for maternal pre-pregnancy BMI (ESM Table 1).

Methylation at some CpG sites which associate with intra-uterine exposure also associate with impaired insulin secretory function, higher BMI or future risk of type 2 diabetes

We further assessed whether any of the 48 CpG sites that associated with exposure to intrauterine diabetes also associated with reduction in insulin secretory function, higher BMI or future type 2 diabetes. Three of the 48 sites were modestly associated with CIR (Table 3). The CpG site (cg24049468) located in the gene body of *AK3* was hypermethylated in the OMD group, and hypermethylation at this site was associated with a reduced CIR in the 230 NGT participants. Similarly, CpG sites located in the gene body of *PCDHGA4* (cg25949304) and close to the TSS of *STC1* (cg04645534) were hypomethylated in the OMD group, and hypomethylation at these sites was associated with a reduced CIR in the 230 NGT participants. Methylation at these three CpG sites was further analysed in 14 individuals who remained NGT and had participated in a subsequent inpatient examination (median follow-up was 1.8+5.8 years) in our clinical research centre to characterise insulin secretory function via a 25 g IVGTT. In this very small sample, those who were hypomethylated at *STC1* similarly tended to have a reduced AIR (effect 3.7% per SD; $p = 0.058$).

Among the 48 CpGs differentially methylated between OMD and OMND, 17 were associated with baseline BMI of the offspring ($p < 0.05$). However, only CpG sites in *SBK1* (cg04413090, $p = 4.1 \times 10^{-4}$) and *PRDM16* (cg12140144, $p = 1.2 \times 10^{-3}$) and two intergenic CpGs (cg10772621, $p = 2.3 \times 10^{-4}$; cg07464358, $p = 2.3 \times 10^{-4}$) remained significantly associated after correction for multiple tests (assuming 39 independent tests). Three of the CpG sites, cg04413090

Fig. 1 The genome-wide differential methylation signals. (a) Manhattan plot of differentially methylated CpGs in OMND vs OMD. Genome-wide significant differences are highlighted in green. (b) Quantile–quantile plot of the differential signals. The red line is the identity line

(*SBK1*), cg12140144 (*PRDM16*) and cg07464358, were hypermethylated in OMD, and hypermethylation was associated with higher BMI (5.3%, 6.0% and 9.2% higher per SD of standardised M-value, respectively). Similarly, the CpG site cg10772621 was hypomethylated in OMD, and hypomethylation was associated with higher BMI (by 4.9% per SD of standardised M-value).

Among the 303 offspring with data from a follow-up examination, 43 OMD and 15 OMND developed diabetes later in life. The adjusted HR for developing diabetes for OMD compared with OMND was 5.48 (95% CI 2.85, 10.52; $p=3.2 \times 10^{-7}$). Among these 303 individuals, methylation at cg12140144 in the TSS of *PRDM16* was associated with a higher risk of diabetes (Table 3; HR 2.12 [95% CI 1.74, 2.50] per SD of M-value; $p=9.7 \times 10^{-5}$). Another differentially methylated CpG site, cg08911291, which is located in an intergenic region, also carried diabetes risk with marginal significance (Table 3; HR 1.47 [95% CI 1.09, 1.85] per SD of M-value; $p=0.05$). Among the 303 individuals with data on development of diabetes, the adjusted standardised M-value at the cg12140144 site in *PRDM16* was 0.51 SD higher in OMD compared with OMND. After adjustment for methylation at this site, the HR for developing diabetes in OMD compared with OMND was 3.89 (95% CI 1.98, 7.65; $p=8.0 \times 10^{-5}$). In mediation analyses, this corresponded to a percentage mediation of 20% (two-sided $p=0.026$). The corresponding value for cg08911291 was 7% ($p=0.27$).

Differentially methylated genes clustered in known pathways The pathway analysis included 641 genes with a differentially methylated CpG site ($p \leq 0.005$). The differentially methylated genes were enriched for: (1) metabolic pathways (37 genes, adjusted $p=0.0002$); (2) Wnt signalling pathways (11 genes, adjusted $p=0.0004$); and (3) protein digestion and absorption (eight genes, adjusted $p=0.0005$) (ESM Table 2).

Database searches (www.ncbi.nlm.nih.gov/gene, accessed 1 September 2016) revealed that 11 of the 29 genes have known roles in embryonic development (ESM Table 3), and several genes have a physiological function consistent with a role in beta cell function/type 2 diabetes.

Discussion

We investigated genome-wide DNA methylation in peripheral blood in relation to intrauterine diabetes exposure. Compared with published studies of intrauterine exposure to maternal diabetes, our study featured a larger sample size. The Infinium (Illumina) methylation assay used in the current study covers 96% of CpG islands and 99% of reference sequence genes with an average of 17 CpG sites per gene. Forty-eight CpG sites were identified, with a statistically significant (FDR ≤ 0.05) difference in methylation among people exposed

to maternal diabetes in utero compared with those not exposed. For differentially methylated sites, the median adjusted absolute difference in methylation between OMD and OMND was 2.2% (range 0.6–4.6%). Exposure to diabetes in utero is strongly associated with higher maternal BMI, and genetically raised maternal BMI is associated with birthweight, which in turn is associated with long-term developmental outcomes and adult diseases [22, 23]. However, the additional adjustment for maternal pre-pregnancy BMI did not materially change the effect sizes of our 48 top signals.

The strongest result in our study, in terms of statistical significance, is the differential methylation of the transcriptional activator *LHX3*. Six of the 23 CpG sites assayed on the array from this region were hypermethylated in OMD ($p \leq 1.1 \times 10^{-5}$). Mutations in *LHX3* have been associated with combined pituitary hormone deficiency (CPHD), which is often characterised by reduced growth and short stature [24]. *Lhx3* expression is regulated by DNA methylation in mice, where decreased methylation has been shown to activate expression in pituitary cells that do not normally express *Lhx3* [25]. In humans, *LHX3* is broadly expressed in many tissues, including the pancreas. Although this particular transcription factor has not been previously reported to affect insulin secretory pathways, it does interact with several other transcription factors including insulin gene enhancer protein ISL1 and LIM homeobox transcription factor 1-alpha (LMX1A), which are known regulators of insulin gene expression [26].

Several of the genes identified as differentially methylated between OMD and OMND have established roles in beta cell function, including a CpG site in *STC1* that was hypomethylated in the OMD group, where hypomethylation of the same CpG was also associated with both reduced CIR and AIR, as well as increased BMI at baseline. *STC1* encodes the polypeptide hormone stanniocalcin-1 (STC-1), which co-localises with insulin in mouse pancreatic islets, and it has been proposed that STC-1 regulates beta cell mitochondria membrane potential, which is a crucial component in regulation of insulin release [27]. Another gene, *CACNA1C*, which was hypomethylated in the OMD group, has also been shown to be important for beta cell function [28]. However, hypomethylation at *CACNA1C* was not associated with reduction in insulin secretory response in the present data.

Two CpG sites that were significantly differentially methylated between OMD and OMND on an epigenome-wide basis also had nominally significant associations with diabetes risk in later life; however, only the CpG site in *PRMD16* (cg12140144) remained associated after correction for multiple testing (assuming 39 independent differentially methylated regions). This CpG was hypermethylated in OMD by 1.1% on average, and a 1 SD increase in methylation was associated with a doubled diabetes risk. Mediation analysis suggested that differential methylation at this site could potentially explain 20% of the excess risk of diabetes for OMD compared

with OMND, and this mediation effect was nominally statistically significant. This site maps within a CpG island (chr1:2983925–2987962) that spans both 5' UTRs of *LINC00982* and the promoter of *PRDM16*. In human placental tissue, methylation at two CpG sites in *PRDM16* was associated with maternal fasting glucose in the second and third gestational trimesters [29]. CpGs in *PRDM16* were also identified in another study which investigated differential DNA methylation in pancreatic islets of human donors with type 2 diabetes [30]. *PRDM16* has been predominately studied in relation to brown adipose tissue (BAT), where it regulates differentiation from myoblastic precursors to BAT [31]. A SNP (rs12409277) in its 5' flanking region was associated with lean body mass in a Japanese population, and the same SNP was demonstrated to affect the transcriptional activity of *PRDM16* [32]. An essential role for *PRDM16* in pancreatic islet development from a single fetal progenitor cell has recently been described [33], suggesting that this gene also has an important role in pancreatic development. In mice, the function of *Prdm16* is regulated by epigenetic histone modification [34]. Taken together, multiple evidence shows that *PRDM16* could be the regulatory target of hyperglycaemia and responsible in part for increased diabetes risk in OMD. The differentially methylated CpG site in *PRDM16* identified in peripheral blood might be transferable to other tissues, making it a promising biomarker for diabetes risk screening.

While our previous in vivo clinical studies in Pima Indians have shown that exposure to diabetes in utero increases the risk of type 2 diabetes primarily via impairment of beta cell function, it is possible that reduced insulin sensitivity either as a primary effect or secondary to increased adiposity may also contribute to the higher risk of type 2 diabetes. For example, *PPP1R3B*, which was differentially methylated in our study, codes for the G_L regulatory subunit of protein phosphatase 1 (PP1), which targets PP1 to activate glycogen synthase, thereby increasing insulin sensitivity of liver and skeletal muscle [35, 36]. A decreased rate of glycogen synthesis in the skeletal muscle in response to insulin is a characteristic feature of individuals with type 2 diabetes [37], and polymorphisms in another regulatory subunit (*PPP1R3A*), which also targets PP1 activation of glycogen synthase in the skeletal muscle, were associated with insulin resistance and type 2 diabetes in several different populations [38–42]. Differential methylation of a second gene, *PM20D1*, could also affect insulin sensitivity via its effect on body weight. In humans, methylation of *PM20D1* in cord blood is associated with birthweight centile [43]. Methylation of *PM20D1* has also been associated with several adverse health events during early life, indicating that *PM20D1* may be a target gene for in utero epigenetic programming in multiple organs [44, 45]. Differential methylation of *PM20D1* in obese and non-obese individuals at older ages suggests that in utero programming of *PM20D1* has sustainable effects much later in life [46].

Among the 29 genes identified in the current study which are differentially methylated in OMD but not in OMND, only *TBLIX* has been previously implicated in exposure to maternal diabetes. In our study, a CpG in the 5' UTR of *TBLIX* was hypermethylated in the OMD group, and others have reported a 27.22-fold decrease in expression following exposure to maternal diabetes [47]. *TBLIX* encodes a mediator of β -catenin proteolysis through ubiquitination in the canonical Wnt pathway, which was highlighted by our pathway analysis (ESM Table 2).

The strength of this study is its sample size and detailed clinical measures of a well-phenotyped, longitudinal cohort. There are, however, several weaknesses. First, since blood samples were not obtained at birth in our longitudinal study, the DNA analysed for methylation was from individuals with a mean age of 13 years. Thus, our study can only detect in utero differences in methylation that are stable throughout many years, and our results could also be influenced by differences in methylation that arise postnatally. Prior studies of in utero methylation have shown evidence of both transient and stable methylation sites. For example, a longitudinal analysis showed that sites that were differentially methylated at birth and associated with birthweight and gestational age were no longer different when the child reached 7 years of age [48]. By contrast, DNA methylation differences associated with in utero exposure to maternal smoking have been reported to be long-lasting [49, 50]. A second weakness of our study is that we analyse DNA from peripheral blood, although we believe that the pancreas and corresponding beta cell function are the key tissues responsible for the development of early-onset type 2 diabetes as a consequence of in utero exposure to diabetes. Therefore, our study will only detect differences in methylation that affect pancreatic development/function that are similarly differentially methylated in the blood. Fine mapping of CpG sites from DNA isolated from specific target tissues is required to further understand the detailed molecular mechanisms.

In conclusion, our study identified differentially methylated CpGs in 39 genomic regions that achieved epigenome-wide significance in their association with exposure to a diabetic intrauterine environment. Methylation at three sites also nominally associated with insulin secretion, while a fourth site associated with future risk of type 2 diabetes. However, our studies of insulin secretion and future diabetes risk were conducted in relatively small numbers of individuals and thus may be subject to considerable stochastic variation; therefore, replication in other cohorts will be very informative. In addition, further investigations on quantitative gene expression as a consequence of DNA methylation at these CpG sites, as well as causal CpG mapping, will lead to a better understanding of the mechanism of diabetes and its complications.

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Data availability These data are not publicly available for reasons of privacy. For the present study, the National Institutes of Health (NIH) has granted an exception from the expectation of broad data sharing outlined in the NIH Genomic Data Sharing Policy.

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