Metabolic Programming of *MEST* DNA Methylation by Intrauterine Exposure to Gestational Diabetes Mellitus

Nady El Hajj,[1] Galyna Pliushch,[1] Eberhard Schneider,[1] Marcus Dittrich,[2] Tobias Müller,[2] Michael Korenkov,[3] Melanie Aretz,[4] Ulrich Zechner,[5] Harald Lehnen,[4] and Thomas Haaf[1]

Epigenetic processes are primary candidates when searching for mechanisms that can stably modulate gene expression and metabolic pathways according to early life conditions. To test the effects of gestational diabetes mellitus (GDM) on the epigenome of the next generation, cord blood and placenta tissue were obtained from 88 newborns of mothers with dietetically treated GDM, 98 with insulin-dependent GDM, and 65 without GDM. Bisulfite pyrosequencing was used to compare the methylation levels of seven imprinted genes involved in prenatal and postnatal growth, four genes involved in energy metabolism, one anti-inflammatory gene, one tumor suppressor gene, one pluripotency gene, and two repetitive DNA families. The maternally imprinted *MEST* gene, the nonimprinted glucocorticoid receptor *NR3C1* gene, and interspersed ALU repeats showed significantly decreased methylation levels (4–7 percentage points for *MEST*, 1–2 for *NR3C1*, and one for ALUs) in both GDM groups, compared with controls, in both analyzed tissues. Significantly decreased blood *MEST* methylation (3 percentage points) also was observed in adults with morbid obesity compared with normal-weight controls. Our results support the idea that intrauterine exposure to GDM has long-lasting effects on the epigenome of the offspring. Specifically, epigenetic malprogramming of *MEST* may contribute to obesity predisposition throughout life. *Diabetes* 62:1320–1328, 2013

E pidemiological studies in humans and experimental work in various animal models suggest that adverse intrauterine conditions during fetal development can be associated with negative health outcomes later in life, particularly increased rates for many metabolic diseases (1,2). According to the developmental origins hypothesis (3), different prenatal environmental experiences have long-lasting effects on the setting of metabolic pathways and, thus, influence disease susceptibility. This requires a molecular mechanism for gene–environment interactions that can permanently alter gene expression regulation in exposed individuals. Accumulating evidence suggests that epigenetic changes induced by environmental factors can stably modulate gene expression and provide a link between early life conditions and susceptibility to complex disease (4,5).

The prevalence of type 2 diabetes mellitus and obesity is increasing in most populations worldwide. In addition to overnutrition and physical inactivity, prenatal factors appear to make a significant contribution to the metabolic disease epidemic. It is well-known that the offspring of diabetic and/or obese mothers who are exposed to high concentrations of glucose, free fatty acids, and amino acids in utero are at increased life-long risk for development of metabolic disorders (6,7). Gestational diabetes mellitus (GDM) presents during pregnancy in women without pregestational diabetes and typically disappears again after delivery. It affects 3–10% of all pregnancies in developed countries and is associated with adverse consequences not only during fetal development (i.e., hypoxemia, visceromegaly, and macrosomia) but also later in life (8,9). Studies of discordant siblings born before and after the development of diabetes in the mother demonstrated a significantly higher risk for metabolic disorders in the offspring who were exposed to a hyperglycemic intrauterine environment (6).

Studies of the epigenetic effects of an adverse prenatal environment focused on maternal undernutrition. Famine at the approximate time of conception has been associated with persistent blood methylation changes of multiple genes, i.e., the anti-inflammatory cytokine *IL10* and the peptide hormone leptin (*LEP*), which plays an essential role in regulation of the body weight (10). Later-life phenotypic consequences of prenatal famine exposure include an increased risk for obesity, coronary artery disease, accelerated cognitive aging, and schizophrenia (11–13). To explore the epigenetic effects of fetal overnutrition, which is a more frequent problem in Western societies, we have analyzed the methylation levels of 14 candidate genes for metabolic reprogramming and two repetitive DNA families in newborns of mothers with and without GDM. Recently, decreased methylation levels of the adiponectin gene in fetal placenta have been associated with mothers' blood glucose levels (14).

Genomic imprinting results in the preferential expression of one of the two parental alleles. Imprinted genes, including the paternally methylated *H19* and *MEG3* genes and the maternally methylated *LIT1*, *MEST*, *NESPAS*, *PEG3*, and *SNRPN* genes, are essential for the regulation of human fetal and placental growth, somatic differentiation, as well as neurologic and behavioral functions (15,16). The nonimprinted glucocorticoid receptor NR3C1 is a transcription factor that is involved in many cellular processes. Epigenetic programming of *NR3C1* (i.e., by diet and early maternal care) has been associated with long-term effects on metabolism, stress response, and behavior in both rats (17,18) and humans (19,20). Feeding pregnant rats a protein-restricted diet altered methylation and expression of the peroxisome proliferator–activated receptor α (*PPARA*) gene, an essential transcription factor for lipid

metabolism (21). NDUFB6 is a nuclear-encoded subunit of the mitochondrial NADH ubiquinone oxidoreductase. Epigenetic factors are important for *NDUFB6* expression in human skeletal muscle and insulin sensitivity (22). Promoter methylation can inactivate tumor suppressor genes such as *APC* that prevent neoplastic transformation in normal cells (23). The transcription factor *OCT4* is the key gene for maintaining pluripotency in mammalian cells (24). Interspersed ALU and LINE1 repeats comprise 10 and 17% of the human genome (25), respectively, and can be used as surrogate markers for genome-wide methylation changes (26). In somatic tissues, most retrotransposon-derived elements are densely methylated to prevent retrotransposition activity (27).

RESEARCH DESIGN AND METHODS

Umbilical cord blood and chorionic villi samples (CVS) from newborns of 88 mothers with dietetically treated gestational diabetes (D-GDM group), 98 with insulin-dependent GDM (I-GDM group), and 65 without GDM (non-GDM group) were collected by obstetricians at the Municipal Clinics, Moenchengladbach, Germany. Tissue samples were immediately frozen at −80°C until further use. Informed written consent was obtained from all mothers participating in the study, which was approved by the ethics committee of the Medical Faculty at Würzburg University. None of the mothers with GDM had type 1 or 2 diabetes before pregnancy.

GDM was diagnosed between gestational weeks 24 and 27 by an elevated fasting (for 8–12 h) plasma glucose (>95 mg/dL) and a pathological oral glucose tolerance test (>180 mg/dL at 1 h and/or >155 mg/dL at 2 h after drinking a solution with 75 g glucose). Glucose levels were determined with the hexokinase method on a Cobas 8000 modular analyzer (Roche Diagnostics, Mannheim, Germany). HbA$_{1c}$ was measured by high-performance liquid chromatography (TOSOH Bioscience, Stuttgart, Germany). After diagnosis, women received dietary counseling by a diabetologist. Following the recommendations of the German Society of Gynecology and Obstetrics and the American Diabetes Association, they were advised to consume a diet consisting of approximately 45% carbohydrate, 30–35% fat, and up to 20% protein. Protein intake was limited to approximately 0.8 g per kilogram of body weight. The patients were not allowed to fast. If dietary treatment did not decrease glucose (<92 mg/dL after fasting, <140 mg/dL at 1 h, and <120 mg/dL at 2 h after meals) and HbA$_{1c}$ levels (<6%), then patients were treated with the basis bolus insulin and, rarely, insulin pump therapy. In addition, insulin was recommended in cases of polyhydramnion or fetal macrosomia.

Blood samples of 37 obese adults (10 males and 27 females) with a BMI >35 kg/m^2 and 37 sex-matched and age-matched controls with BMI <25 kg/m^2 were obtained with approval of the ethics committee of the Medical Council of Rhineland-Palatinate. All subjects were Caucasians from Southwestern Germany. Mean age (and SD) of the obese patients who were examined before bariatric surgery at Univeristy Medical Centre, Mainz, Germany, was 36 ± 10 years. The BMI was 48.3 ± 8.1 kg/m^2 and waist-to-hip ratio was 0.96 ± 0.11. Systolic blood pressure was 147 ± 22 mmHg and diastolic blood pressure was 92 ± 11 mmHg. Fasting plasma glucose was 108 ± 42 mg/dL and HbA$_{1c}$ was 6.5 ± 1.3%. The triglyceride level was 224 ± 135 mg/dL, HDL cholesterin was 45.5 ± 12.3 mg/dL, and LDL cholesterin was 126 ± 33 mg/dL. Approximately 30% of the patients had manifest type 2 diabetes, and 70% used drugs for hypertonia, hyperlipidemia, and/or hyperglycemia. White blood cell types of one adult control were separated by magnetic activated cell sorting using the Whole Blood Column Kit and a QuadroMACS Separator (Miltenyi Biotec, Bergisch Gladbach, Germany).

Bisulfite pyrosequencing. Genomic DNA was isolated with the DNeasy blood and tissue kit (Qiagen, Hilden, Germany) and bisulfite conversion was performed with the Epitect 96 bisulfite kit (Qiagen). Bisulfite pyrosequencing was performed on a PyroMark Q96 MD pyrosequencing system with the PyroMark Gold Q96 CDT reagent kit (Qiagen). Gene-specific PCR and sequencing primers (Table 1) were designed using the PyroMark Assay Design 2.0 software (Qiagen). We estimate that at least 10,000 mainly evolutionarily young ALU and the majority of approximately 4,000 full-length LINE1 repeats are amplified by the consensus primers used (25,26). The PCR reaction mixture consisted of 2.5 μL 10× buffer, 20 mmol/L MgCl$_2$, 0.5 μL 10 mmol/L dNTP mix, 1 μL (10 pmol) of each primer, 0.2 μL (1 unit) FastStart Taq DNA Polymerase (Roche Diagnostics), 18.8 μL PCR-grade water, and 1 μL (~100 ng) of bisulfite-converted DNA. Amplifications were performed with an initial denaturation step at 95°C for 5 min, 35 cycles of 95°C for 30 s, primer-specific annealing temperature for 30 s, and 72°C for 45 s, and a final extension step at 72°C for 5 min. The Pyro Q-CpG software (Qiagen) was used for data analysis.

In our experience, the average methylation difference between technical replicates (including bisulfite conversion, PCR, and pyrosequencing) is approximately 1 percentage point.

Bisulfite plasmid sequencing. Classical bisulfite plasmid sequencing was performed to determine the methylation patterns of individual *MEST* DNA molecules. The 161-bp fragment (chromosome 7: 130,132,756–130,132,917 bp; Ensembl release 61) amplified by forward primer 5′-TTTTGGTGY-GATTTAAAGGATAGGTTTTAG-3′ and reverse primer 5′-AATACCTAAATCT-TAAAATCCTAAACTACACC-3′ contains 10 CpG (cytosine-phosphatidyl-guanine) sites and a cytosine/guanine-SNP (rs2301335) to distinguish the two parental alleles. It is approximately 500 bp downstream of the pyrosequencing target region in the same CpG island. PCR products were cloned into pCR2.1-TOPO vector using T4-DNA ligase, the TA cloning kit, and One Shot TOP10 chemically competent *Escherichia coli* (Invitrogen, Karlsruhe, Germany). Plasmid DNA of individual clones was isolated with the ZR Plasmid Miniprep Classic Kit (Zymo Research, Irvine, CA). Clones were sequenced using dye terminator cycle sequencing with M13 primers on an ABI 3730 automated sequencer.

Statistical testing. Statistical analyses were performed with the software package R version 2.14.0. For all blood and placenta samples, a gene-wise Kruskal-Wallis nonparametric ANOVA was calculated. Pair-wise (Spearman) correlations were calculated for all single CpG methylation measurements in a given *cis*-regulatory region. Correlations of the methylation profiles with the maternal HbA$_{1c}$ and gestational age were performed using the Spearman correlation coefficient (ρ); a two-sided statistical test has been used to compute P values based on the algorithm AS 89 as implemented in R (28). Analogously, the correlation between maternal BMI, birth weight, and gene methylation was tested. For normally distributed data, multivariate parametric ANOVA models including the covariates maternal age, BMI, and birth weight were calculated to account for potential confounding effects. Subsequent post hoc tests between the three different groups (D-GDM, I-GDM, and non-GDM) were performed using Tukey honest significant difference method. For percentages in the very low or high ranges, where normality assumption might be violated, nonparametric tests (Kruskal-Wallis ANOVA and Wilcoxon rank-sum test) were performed. All P values were multiple testing–corrected using the Holm method (29). Only $P < 0.05$ was considered to be significant.

RESULTS

Table 2 shows anthropometric and metabolic parameters of the studied groups. Mothers with GDM had a significantly ($P < 0.001$) higher prepregnancy BMI (27.1 ± 6.6 kg/m^2 in D-GDM and 30.2 ± 7.4 kg/m^2 in I-GDM) than the controls (23.4 ± 3.7 kg/m^2). Average weight gain during pregnancy and gestational age were similar in all groups. HbA$_{1c}$ was significantly ($P = 0.012$) higher in mothers with I-GDM (5.7 ± 0.5) than in mothers with D-GDM (5.4 ± 0.4). Birth weight was significantly ($P = 0.012$) increased in the GDM groups (3,346 ± 457 g in D-GDM and 3,438 ± 607 g in I-GDM) compared with controls (3,285 ± 457 g). There was a trend toward higher placenta weight in I-GDM (565 ± 141) than in D-GDM (516 ± 104 g) and controls (513 ± 100 g).

Gene-specific methylation changes associated with GDM. Quantitative bisulfite pyrosequencing was used to determine the methylation levels of 14 candidate genes for metabolic programming (Table 1). Because individual CpGs usually do not maintain methylation states that differ from those of the neighboring CpGs (30), the average methylation of all analyzed sites was used as a gene-specific epigenetic marker. Table 3 presents the methylation values of all studied genes in blood samples. Variable numbers of individuals with D-GDM, with I-GDM, and without GDM were analyzed per gene. Some measurements did not pass the quality criteria for bisulfite pyrograms (implemented in the Pyro Q-CpG software). Because of limited amounts of DNA, methylation data were first obtained in a subset of samples and the most promising candidate genes were then tested in the remaining samples. In an exploratory analysis of all genes to identify candidates susceptible to epigenetic programming, we used the Kruskal-Wallis nonparametric ANOVA, which

TABLE 1
Genes and primers for bisulfite pyrosequencing

Gene	Primer	Sequence (5'-3')	Amplicon length (bp)	Gene region and chromosomal localization (bp)*	Number of CpGs
Imprinted genes (paternally methylated)					
H19	Outer forward	TTTTTGGTAGGTATAGAGTT		Intronic ICR 1 on chromosome 11p15.5; CTCF binding site	
	Outer reverse	AAACCATAACACTAAAACCC	343	2,020,989–2,021,332	
	Nested forward†	TGGGTATTTTTGGGAGGTTTTTT			
	Nested reverse	ATAAATATCCTATTCCCAAATAA	216	2,021,075–2,021,291	
	Sequencing	ATATATAAACCTACACTACC			4
MEG3 promoter	Forward	GATTTTTTTATATATGTGTTTG		Secondary DMR on chromosome 14q32; 5' (upstream) promoter; CpG island	
	Reverse†	CTCATTTCTCTAAAAATAATTAACC	220	101,292,154–101,292,374	
	Sequencing	GTGTTTGAATTTATTTGTTTGG			3
MEG3 intergenic	Forward	AGGGTTAGGAAGTTTAGTAGGTTA		Intergenic ICR (between *DLK1* and *MEG3*) on chromosome 14q32	
	Reverse†	ACTACTCCTTAAACAAAAAAACACATAAT	150	101,275,645–101,275,795	
	Sequencing	GTAGTAAATTAAAGTGTATTAGAGA			5
Imprinted genes (maternally methylated)					
LIT1	Forward	AATTAGTAGGTGGGGGG		Intronic ICR 2 on chromosome 11p15.5; CpG island	
	Reverse†	CTAAAAAACTCCCTAAAAATC	122	2,721,175–2,721,297	
	Sequencing	GGGGGTAGTYGGAG			2
MEST	Forward†	TYGTTGTTGGTTAGTTTTGTAYGGTT		Intron/exon-spanning DMR on chromosome 7q32; CpG island	
	Reverse	CCCAAAAAACAACCCCAACTC	219	130,132,066–130,132,285	
	Sequencing	AAACAACCCCAACTC			5
NESPAS	Forward†	GATGAAGGGGTGGTTAGTA		Exonic ICR on chromosome 20q13.3; CpG island	
	Reverse	CCAAAAATACCTTCTTAACCTTAA	229	57,430,015–57,430,244	
	Sequencing	TAAACTAAAAACTCTCAAAT			4
PEG3	Forward	GGTGTAGAAGTTTGGGTAGTTG		ICR on chromosome 19q13.4; 5' UTR	
	Reverse†	CTCACCTCACCTCAATACTAC	153	57,351,944–57,352,097	
	Sequencing	TGTTTATTTTGGGTTGGT			3
SNRPN	Forward†	AGGGAGTTGGGATTTTTGTATT		ICR on chromosome 15q11.2; 5' UTR; CpG island	
	Reverse	CCCAAACTATCTCTTAAAAAAAAC	237	25,200,012–25,200,249	
	Sequencing 1	ACACAACTAACCTTACCC			3+
	Sequencing 2	CCAACCTACCTCTAC			3
Metabolic genes					
LEP	Forward	GGAGTTTTTGGAGGGATATTAAGGATT		5' (upstream) promoter on chromosome 7q31.3; CpG island	
	Reverse†	CTACATCCCTCCTAACTCAATT	417	127,881,050–127,881,467	
	Sequencing	GGGAGGTATTTAAGGGTG			6

TABLE 1
Continued

Gene	Primer	Sequence (5'-3')	Amplicon length (bp)	Gene region and chromosomal localization (bp)*	Number of CpGs
NDUFB6	Forward	AGGAATTTGGGGGTAATTTTTAAATAT		5' (upstream) promoter on chromosome 9p21.1 32,573,545–32,573,775	
	Reverse†	ATTCAAATCCAACTATACCAATAAC	230		3
	Sequencing	TTTTAATATTTTGGTATTATGA			
NR3C1	Forward†	AATTTTTTAGGAAAAAGGGTGG		Exon 1F promoter on chromosome 5q31.3; intronic CpG island 142,783,505–142,783,909	
	Reverse	CCTTCCCTAAAACCTCCC	405		8
	Sequencing	AAATAACACACTTCA			
PPARA	Forward	TGTAGTTAAGGGAAATTGAGG		5' (upstream) promoter on chromosome 22q13.31 46,545,813–46,546,164	
	Reverse†	CCCCTTCTCCTTATTTTACAT	351		4
	Sequencing	GTAGTTAAGGGAAATTGAG			
Anti-inflammatory gene					
IL10	Forward†	GTATTTTGGAATGGGTAATTTG		5' (upstream) promoter on chromosome 1q31-q32 206,945,876–206,946,214	
	Reverse	CTCAAAAAAACCTCTTCATTCATT	338		3
	Sequencing	CCTCAACTATAAATTCTCAT			
Tumor suppressor gene					
APC	Forward†	GGTTAGGGTTAGGTAGGTTGT		Intronic promoter 1A on chromosome 5q21-q22 112,073,375–112,073,568	
	Reverse	ACTACACAATACAACCACACATATC	193		7
	Sequencing	CCACACCCAACCAA			
Pluripotency gene					
OCT4	Forward	AAGTTTTTGTGGGGGATTTGTAT		5' (upstream) promoter on chromosome 6p21.31 31,138,482–31,138,667	
	Reverse†	CCACCCACTAACCTTAACCTCTA	185		2+
	Sequencing 1	TGAGGTTTTGGAGGG			1
	Sequencing 2	GTTATTATTATTAGGTAAATATTTT			
Repeats					
ALU	Forward	GGGACACCGCTGATCGTATA‡ TTTTTATTAAAAATATAAAAATTAGT	~150	Interspersed repeats All chromosomes	
	Reverse	CCAAACTAAAATACAATAA			
	Universal†	GGGACACCGCTGATCGTATA			~3
	Sequencing	AATAACTAAAATTACAAAC			
LINE1	Forward	TTTTGAGTTAGGTGTGGGATA		Interspersed repeats All chromosomes	
	Reverse†	CTCACTAAAAAATACCAAACAA	~245		~4
	Sequencing	GTTAGGTGTGGGATATAGTT			

DMR, differentially methylated region; CTCF, CCCTC-binding factor; ICR, imprinting control region; UTR, untranslated region. *According to Ensembl version 61. †Biotinylated primer. ‡Linker sequence that is recognized by the biotinylated universal primer.

TABLE 2
Anthropometric and metabolic parameters of mothers and newborns

	Non-GDM	D-GDM	I-GDM	$P*$
Maternal age (years)	30.2 ± 5.1 ($n = 57$)	31.1 ± 5.5 ($n = 80$)	32.6 ± 5.6 ($n = 88$)	0.392
Prepregnancy BMI (kg/m^2)	23.4 ± 3.7 ($n = 56$)	27.1 ± 6.6 ($n = 80$)	30.2 ± 7.4 ($n = 87$)	<0.001
BMI before birth (kg/m^2)	28.0 ± 3.9 ($n = 52$)	31.8 ± 6.6 ($n = 75$)	34.7 ± 7.0 ($n = 82$)	<0.001
Height (cm)	167.0 ± 6.8 ($n = 55$)	165.9 ± 5.5 ($n = 80$)	166.6 ± 7.0 ($n = 87$)	1.000
Weight before pregnancy (kg)	65.0 ± 10.4 ($n = 56$)	74.5 ± 18.0 ($n = 80$)	84.2 ± 21.5 ($n = 87$)	<0.001
Weight before birth (kg)	78.3 ± 11.7 ($n = 53$)	87.5 ± 17.7 ($n = 75$)	96.4 ± 20.2 ($n = 83$)	<0.001
Smokers (% of women)	0 ($n = 15$)	14.0 ($n = 71$)	12.8 ($n = 86$)	1.000
HbA$_{1c}$ (%)	NA	5.4 ± 0.4 ($n = 64$)	5.7 ± 0.5 ($n = 85$)	0.012
Gravida (average n)	1.9 ($n = 54$)	2.3 ($n = 80$)	2.0 ($n = 87$)	1.000
Parity (average n)	1.8 ($n = 54$)	1.8 ($n = 80$)	2.0 ($n = 87$)	1.000
Cesarean section (% of deliveries)	34 ($n = 54$)	22 ($n = 80$)	39 ($n = 87$)	0.238
Placenta weight (g)	513 ± 100 ($n = 30$)	516 ± 104 ($n = 73$)	565 ± 141 ($n = 83$)	0.225
Birth weight (g) of the baby	$3,285 \pm 457$ ($n = 57$)	$3,346 \pm 457$ ($n = 80$)	$3,438 \pm 607$ ($n = 87$)	0.012
Gestational weeks	38.6 ± 1.1 ($n = 65$)	38.6 ± 1.4 ($n = 85$)	38.2 ± 1.7 ($n = 97$)	1.000
Sex	27 males, 40 females	73 males, 62 females	61 males, 71 females	0.100
Base excess	-0.77 ± 2.3 ($n = 56$)	-1.9 ± 3.2 ($n = 79$)	-1.0 ± 3.0 ($n = 87$)	0.744

Data are mean $\pm$ SD. NA, not applicable. *With the exception of gravida, parity (Pearson χ^2 test), smoking, and cesarean section rate (Fisher exact test), the Kruskal-Wallis test was used for calculating the P value for a between-group difference. Corrected for multiple testing using the Holm method.

cannot include covariates. A parametric ANOVA could not be applied because genes with very high (i.e., *OCT4*) or low (i.e., *NR3C1*) methylation values would violate the normality assumption. Between-group comparisons revealed a significant difference in 5 of the 14 studied loci, namely *MEST* ($P = 5.58E-12$), *IL10* ($P = 8.25E-03$), *NR3C1* ($P = 0.012$), *OCT4* ($P = 0.031$), and *NDUFB6* ($P = 0.049$). All P values (throughout the manuscript) have been corrected for multiple testing. Mean methylation of the imprinted *MEST* gene was 39.9 and 39.1% in D-GDM and I-GDM, respectively, and 43.7% in the control group. Mean methylation of the glucocorticoid receptor *NR3C1* was 3.3 and 3.2% in the two GDM groups, compared with 4% in controls. Thus, GDM was associated with decreased blood *MEST* and *NR3C1* methylation. Mean methylation of *IL10* was 32.4% in D-GDM, 28.5% in I-GDM, and 27.2% in controls, and that of *NDUFB6* was 86.9% in D-GDM, 89% in I-GDM, and 88.4% in controls, suggesting epigenetic changes in the D-GDM group, compared with I-GDM and controls. Pair-wise Spearman correlations were calculated to test whether individual CpG sites in a given region were correlated with each other. The average correlation between the five CpGs in the *MEST* differentially methylated region was 0.82, and that of the three sites in the *IL10* promoter was 0.88. Therefore, the average methylation value of all region-specific sites can be used for ANOVA. For *NR3C1*, *OCT4*, and *NDUFB6*, the average pairwise correlations were 0.37, 0.39, and 0.25, respectively. When the eight sites in the *NR3C1* promoter were tested separately, CpG 1 ($P = 6.80E-06$), CpG 2 ($P = 6.41E-04$), CpG 4 ($P = 4.70E-07$), CpG 5 ($P = 0.006$), and CpG 7 ($P = 0.017$) showed highly significant between-group differences.

Similar methylation analyses were performed in fetal placenta (Table 4). *MEST* ($P = 1.06E-10$), *PPARA* ($P = 4.15E-04$), *NR3C1* ($P = 2.23E-03$), and *NESPAS* ($P = 0.032$) showed significant between-group differences. GDM was associated with decreased placenta methylation values in all four genes. The most dramatic difference was observed for *MEST*, which showed a mean methylation of 39.3 and 38.3% in D-GDM and I-GDM, respectively, and showed a mean methylation of 45.5% in controls. Mean *PPARA* methylation was 2.6% in D-GDM and 2.4% in I-GDM, compared with 4.8% in controls. Mean *NR3C1* methylation was 3.5 and 3.3% in D-GDM and I-GDM but 5.6% in controls. Mean *NESPAS* methylation was 34.9 and 35.7% in the two GDM groups and 38.3% in controls. The average pair-wise correlations between the five analyzed CpGs in *MEST*, the three CpGs in *PPARA*, the eight CpGs in *NR3C1*, and the four CpGs in *NESPAS* were 0.82, 0.67, 0.60, and 0.78, respectively. For *NR3C1*, single-site ANOVAs revealed a highly significant between-group difference for CpG 2 ($P = 0.004$), CpG 3 ($P = 0.001$), CpG 5 ($P = 2.81E-04$), CpG 6 ($P = 0.001$), CpG 7 ($P = 0.002$), and CpG 8 ($P = 0.002$).

Pair-wise comparisons revealed a significant *MEST* methylation difference between D-GDM and controls ($P = 3.71E-08$ for blood and $P = 3.60E-09$ for CVS), as well as between I-GDM and controls ($P = 2.00E-10$ and $P = 1.58E-08$, respectively), but not between the two GDM groups. Similarly, pair-wise comparisons of *NR3C1* methylation values revealed a significant difference between D-GDM and controls ($P = 0.008$ for blood and $P = 0.001$ for CVS) and between I-GDM and controls ($P = 0.001$ for both tissues), but not between D-GDM and I-GDM.

Relaxation of *MEST* imprinting. *MEST* is expected to be methylated on the maternal but not on the paternal allele. Bisulfite plasmid sequencing of a SNP-containing segment in the studied *MEST* promoter revealed 51% (20/39) methylated alleles in two informative GDM blood samples and 53% (21/39) in two controls. Approximately 90% (70/78) of alleles in both groups showed the correct parent-specific methylation imprints. Four maternal alleles were aberrantly demethylated and four paternal alleles were aberrantly methylated. The rate of single CpG methylation errors (excluding abnormal alleles) was comparable in GDM (4.5%; 17/375) and control samples (5.9%; 21/355). Similar results were obtained in one GDM and one control CVS sample (data not shown).

Repeat methylation. To assess genome-wide changes in DNA methylation, we analyzed interspersed ALU and LINE1 elements (Table 5). In both analyzed tissues, ALU repeats showed ~1 percentage point lower methylation in the GDM groups than in controls ($P < 0.001$ for blood and

TABLE 3
Descriptive statistics of gene-specific methylation in cord blood

Gene	Group	n	Mean ± SE	Median (range)	P^*
H19	Non-GDM	37	50.3 ± 0.5	50 (45–62)	1.00E+00
	D-GDM	8	48.5 ± 1.0	48 (45–54)	
	I-GDM	13	49.8 ± 0.4	50 (47–52)	
MEG3 promoter	Non-GDM	52	46.7 ± 0.5	47 (38–55)	1.92E-01
	D-GDM	49	44.8 ± 0.5	45 (35–52)	
	I-GDM	56	45.2 ± 0.5	45 (38–53)	
MEG3 intergenic	Non-GDM	48	54.8 ± 0.4	55 (46–60)	6.93E-01
	D-GDM	28	53.9 ± 0.5	53 (50–62)	
	I-GDM	33	54.2 ± 0.5	54 (47–59)	
LIT1	Non-GDM	56	47.4 ± 0.5	47 (41–63)	6.93E-01
	D-GDM	49	46.1 ± 0.5	46 (37–54)	
	I-GDM	58	46.2 ± 0.5	46 (34–54)	
MEST	Non-GDM	57	43.7 ± 0.6	43 (27–59)	5.58E-12
	D-GDM	80	39.9 ± 0.4	39 (33–47)	
	I-GDM	83	39.1 ± 0.4	39 (27–45)	
NESPAS	Non-GDM	55	37.6 ± 0.5	37 (32–49)	1.00E+00
	D-GDM	46	37.0 ± 0.3	37 (33–40)	
	I-GDM	55	36.9 ± 0.3	37 (29–42)	
PEG3	Non-GDM	46	43.8 ± 0.3	44 (41–48)	6.93E-01
	D-GDM	28	43.3 ± 0.4	43.5 (40–48)	
	I-GDM	32	42.9 ± 0.3	42.5 (40–48)	
SNRPN	Non-GDM	41	37.3 ± 0.3	37 (34–44)	1.00E+00
	D-GDM	23	37.0 ± 0.9	37 (30–49)	
	I-GDM	29	36.1 ± 0.6	37 (26–43)	
LEP	Non-GDM	43	17.3 ± 0.4	16 (12–24)	7.92E-01
	D-GDM	26	16.3 ± 0.4	17 (13–20)	
	I-GDM	29	15.9 ± 0.5	16 (10–23)	
NDUFB6	Non-GDM	45	88.4 ± 0.3	88 (81–92)	4.85E-02
	D-GDM	25	86.9 ± 0.5	87 (81–92)	
	I-GDM	23	89.0 ± 0.5	89 (83–93)	
NR3C1	Non-GDM	43	4.0 ± 0.2	4 (2–8)	1.20E-02
	D-GDM	60	3.3 ± 0.1	3 (2–7)	
	I-GDM	62	3.2 ± 0.1	3 (2–5)	
PPARA	Non-GDM	18	3.3 ± 0.3	3 (1–6)	1.00E+00
	D-GDM	8	4.0 ± 0.4	3.5 (3–6)	
	I-GDM	14	3.8 ± 0.2	4 (3–5)	
IL10	Non-GDM	45	27.2 ± 0.8	27 (17–39)	8.25E-03
	D-GDM	28	32.4 ± 1.0	31.5 (24–48)	
	I-GDM	32	28.5 ± 0.9	27 (21–42)	
APC	Non-GDM	35	4.3 ± 0.2	4 (3–6)	1.00E+00
	D-GDM	8	4.0 ± 0.4	4 (3–6)	
	I-GDM	11	3.8 ± 0.3	4 (3–6)	
OCT4	Non-GDM	47	94.4 ± 0.3	94 (90–100)	3.09E-02
	D-GDM	26	92.0 ± 0.7	92.5 (79–96)	
	I-GDM	32	93.0 ± 0.6	94 (81–97)	

*Nonparametric ANOVA (Kruskal-Wallis test) for an association between gene-specific methylation and GDM. Corrected for multiple testing using the Holm method.

$P = 0.015$ for CVS). LINE1 methylation was ~3 percentage points higher in blood and 3 percentage points lower in CVS of newborns of mothers with GDM ($P < 0.001$ and $P = 0.001$, respectively).

Between-tissue differences. Most studied gene loci showed significant methylation differences between tissues. In the three patient groups, mean methylation of the imprinted *MEG3* promoter ranged from 44.8 to 46.7% in cord blood and from 34.8 to 38.6% in CVS (Wilcoxon test; $P = 4.15E-11$). In contrast, the intergenic differentially

methylated region showed a higher methylation in CVS than in blood (59.7–61.0% vs. 53.9–54.8%; $P = 3.58E-07$). Mean *SNRPN* methylation was 36.1–37.3% in blood and 42.9–44.4% in CVS ($P = 1.55E-10$). Tissue differences were even larger for the nonimprinted genes *LEP* (15.9–17.3% in blood and 26.6–29.3% in CVS; $P = 5.51E-14$), *NDUFB6* (86.9–89.0% in blood and 62.1–64.3% in CVS; $P = 6.00E-12$), *IL10* (27.2–32.4% in blood and 85.1–86.4% in CVS; $P = 3.15E-12$), *APC* (3.8–4.3% in blood and 38.4–40.1% in CVS; $P = 4.34E-07$), and *OCT4* (92.0–94.4% in blood and 62.7–63.8% in CVS; $P = 1.22E-15$). Both interspersed repeats showed significantly ($P < 0.001$) lower methylation in CVS (22.2–23.0 for ALU and 37.7–41.0 for LINE1) than in blood (24.5–25.4% and 68.1–71.5%, respectively).

Effects of confounding factors. BMI-related parameters and birth weight significantly differed between GDM and controls; all other anthropometric and metabolic parameters were comparable between groups (Table 2). To exclude that the observed *MEST* methylation differences were mainly associated with BMI (or other possible confounding factors) instead of GDM, we used multivariate parametric ANOVA models including maternal age, pre-pregnancy BMI, and birth weight. For fetal *MEST* methylation, BMI was the only significant ($P = 0.037$ in blood and $P = 1.04E-07$ in CVS) confounding factor. However, even after correction, the *MEST* methylation difference between GDM and non-GDM samples remained highly significant ($P = 3.93E-12$ for blood and $P = 9.72E-13$ for CVS). We used a nonparametric ANOVA model to confirm the significant association between GDM and *NR3C1* methylation in cord blood ($P = 0.0001$) and CVS ($P = 0.001$). Gene-wise correlations of HbA_{1c}, an indicator of the maternal blood glucose level, with the CVS and blood methylation levels did not show a significant effect on any of our studied genes. Similarly, there was no significant correlation between gestational age and *MEST* or *NR3C1* methylation levels. Most genes, including *MEST* and *NR3C1*, did not show significant associations of DNA methylation with fetal sex. Only *MEG3* and *LEP* methylation differed significantly ($P < 0.01$ and $P = 0.04$) between male and female cord blood samples; *NDUFB6* showed a trend difference in CVS.

Adult BMI and *MEST* methylation. To support the hypothesis that fetal programming of *MEST* contributes to adult obesity, we compared the methylation levels in peripheral blood of 37 obese adults and 37 sex-matched and age-matched controls. Consistent with the observed hypomethylation in newborns of mothers with GDM, Wilcoxon rank-sum test showed a significantly ($P = 1.46E-06$) lower *MEST* methylation in obese individuals (38.7 ± 3.7%; range, 34–47) than in normal-weight controls (42 ± 2.1%; range, 39–50). When tested separately, all five CpG sites showed a highly significant between-group difference. *NR3C1* methylation was similar (range, 0–4%) in both groups. In one adult control, *MEST* and *NR3C1* methylation were determined in specific white blood cell types. *MEST* showed 40% methylation in CD3-positive T cells, 41.5% in CD19-positive B cells, 39% in CD15-positive granulocytes, and 39% in CD14-positive monocytes. *NR3C1* methylation was 2.5, 2, 3.5, and 3%, respectively.

DISCUSSION

The main goal of our study was to identify gene-specific epigenetic changes in the offspring of mothers with GDM. *MEST* and *NR3C1* exhibited highly significant methylation

TABLE 4
Descriptive statistics of gene-specific methylation in CVS

Gene	Group	n	Mean ± SE	Median (range)	P*
H19	Non-GDM	50	49.3 ± 0.9	49 (30–80)	4.12E-01
	D-GDM	29	50.2 ± 1.7	50 (12–62)	
	I-GDM	34	53.0 ± 1.1	52.5 (45–65)	
MEG3 promoter	Non-GDM	34	36.5 ± 1.4	39 (20–49)	1.00E+00
	D-GDM	29	34.8 ± 1.6	35 (20–51)	
	I-GDM	32	38.6 ± 1.8	36.5 (22–61)	
MEG3 intergenic	Non-GDM	42	61.0 ± 1.3	60 (46–82)	1.00E+00
	D-GDM	29	59.7 ± 1.8	60 (37–83)	
	I-GDM	33	59.9 ± 1.7	59 (32–75)	
LIT1	Non-GDM	50	46.8 ± 0.6	47 (36–58)	1.00E+00
	D-GDM	29	47.9 ± 0.5	48 (43–53)	
	I-GDM	34	47.9 ± 0.6	49 (39–57)	
MEST	Non-GDM	44	45.5 ± 0.5	46 (38–51)	1.06E-10
	D-GDM	27	39.3 ± 0.7	39 (32–49)	
	I-GDM	29	38.3 ± 0.6	38 (31–46)	
NESPAS	Non-GDM	36	38.3 ± 1.1	38 (22–57)	3.23E-02
	D-GDM	29	34.9 ± 0.5	35 (27–42)	
	I-GDM	34	35.7 ± 0.7	36 (27–50)	
PEG3	Non-GDM	48	45.8 ± 0.5	45 (39–56)	1.13E-01
	D-GDM	29	43.7 ± 0.4	44 (40–49)	
	I-GDM	34	43.6 ± 0.6	44 (34–53)	
SNRPN	Non-GDM	41	43.4 ± 0.9	44 (32–54)	1.00E+00
	D-GDM	24	44.4 ± 1.1	43.5 (33–56)	
	I-GDM	31	42.9 ± 0.8	42 (36–54)	
LEP	Non-GDM	41	29.3 ± 1.5	30 (15–54)	1.00E+00
	D-GDM	27	28.6 ± 1.5	29 (13–43)	
	I-GDM	32	26.6 ± 1.6	27 (12–45)	
NDUFB6	Non-GDM	35	64.3 ± 0.5	65 (57–71)	3.50E-01
	D-GDM	24	62.4 ± 0.9	61 (54–71)	
	I-GDM	27	62.1 ± 0.7	62 (53–68)	
NR3C1	Non-GDM	36	5.6 ± 0.5	4.5 (2–16)	2.23E-03
	D-GDM	28	3.5 ± 0.4	3 (1–11)	
	I-GDM	28	3.3 ± 0.3	3 (1–10)	
PPARA	Non-GDM	46	4.8 ± 0.4	3.5 (2–12)	4.15E-04
	D-GDM	24	2.6 ± 0.4	2 (0–7)	
	I-GDM	26	2.4 ± 0.4	3 (0–7)	
IL10	Non-GDM	24	85.1 ± 0.6	85 (80–89)	1.00E+00
	D-GDM	23	85.2 ± 1.4	86 (56–90)	
	I-GDM	30	86.4 ± 0.4	86 (82–90)	
APC	Non-GDM	33	38.6 ± 2.0	36 (14–67)	1.00E+00
	D-GDM	16	40.1 ± 2.2	38.5 (25–54)	
	I-GDM	23	38.4 ± 2.0	35 (27–66)	
OCT4	Non-GDM	40	62.7 ± 1.6	63 (49–86)	1.00E+00
	D-GDM	26	63.8 ± 1.7	64.5 (45–76)	
	I-GDM	34	63.8 ± 2.1	65 (39–90)	

*Nonparametric ANOVA (Kruskal-Wallis test) for an association between gene-specific methylation and GDM. Corrected for multiple testing using the Holm method.

TABLE 5
Descriptive statistics of interspersed repeat methylation

Repeat	Tissue analyzed	Group	n	Mean ± SD	Median (range)	P*
ALU	Blood	Non-GDM	32	25.4 ± 0.8	25 (24–27)	<0.001
		D-GDM	22	24.5 ± 0.8	24.5 (23–26)	
		I-GDM	25	24.6 ± 1.3	25 (21–28)	
	CVS	Non-GDM	24	23.0 ± 1.3	23 (18–25)	0.015
		D-GDM	19	22.2 ± 0.8	22 (21–23)	
		I-GDM	18	22.4 ± 0.9	22 (21–24)	
LINE1	Blood	Non-GDM	31	68.1 ± 2.9	69 (62–74)	<0.001
		D-GDM	21	71.5 ± 2.3	70 (68–76)	
		I-GDM	23	70.9 ± 2.8	71 (62–76)	
	CVS	Non-GDM	21	41.0 ± 2.4	41 (38–48)	0.001
		D-GDM	19	37.7 ± 3.0	38 (31–42)	
		I-GDM	30	38.6 ± 2.0	38 (35–42)	

*Nonparametric ANOVA (Kruskal-Wallis test) for an association between repeat methylation and GDM. Corrected for multiple testing.

different epidemiological risk factors and health outcomes (31). The majority of genes contain ALU repeats in promoters and/or first exons (25), which may play a role in the regulation of transcription (32). Overall, GDM appears to interfere with the mechanisms controlling methylation patterns both at specific loci and at interspersed repeats.

Because epigenomes can differ between tissues (33), methylation changes in blood and CVS may not necessarily reflect events in the target tissues (i.e., fat, hypothalamus, liver, skeletal muscle, and/or pancreatic islets) for fetal metabolic programming. When analyzing whole blood or fetal placenta, it is also difficult to exclude minor differences in cell composition between groups. Although whole and differential blood cell counts varied to some extent among individuals, all parameters were within the normal range for newborns. A previous study did not find an association between ALU methylation and blood cell count (34), suggesting that other factors are responsible for ALU hypomethylation in GDM blood. We found very similar *MEST* and *NR3C1* methylation levels in different white blood cells, making it unlikely that variations in the differential cell count distort the results. Blood of newborns of mothers with GDM may contain a higher amount of nucleated erythrocytes than that of controls (35). However, because of the 50-fold to 100-fold excess of white blood cells, this also is not a likely explanation for the observed between-group methylation differences. ALU repeats as well as *MEST* and *NR3C1* exhibited methylation changes acting in the same direction in tissues from different (embryonal and extraembryonal) layers. These epigenetic signatures in blood and CVS may eventually reveal information on genes susceptible to metabolic reprogramming in the target tissues.

Human *MEST* is endowed with two promoters that use alternative first exons. The *MEST* promoter studied here is differentially methylated on the maternal chromosome in cord blood, CVS, and other tissues. It controls monoallelic (paternal) expression of isoform 1, which is specifically upregulated in fat tissue of obese individuals (36). The isoforms controlled by the other promoters may show biallelic expression in adult lymphocytes (37). *MEST* methylation was 4–7 percentage points lower in offspring of mothers with GDM. This could be attributable to single CpG errors (i.e., individual methylated CpGs in overall

differences between GDM and controls in two different tissues, making them primary candidates for metabolic reprogramming. Other genes (i.e., *OCT4*, *PPARA*, and *NESPAS*) showed GDM-associated changes in only one analyzed tissue. Although false-positive results are extremely unlikely, the limited number of analyzed samples for some genes does not allow us to exclude false-negative results, particularly when the expected effect size is small. ALU repeats were significantly hypomethylated in both GDM tissues. ALU methylation has been used as a biomarker to associate genome-wide epigenetic changes with

demethylated paternal and/or single unmethylated CpGs in overall methylated maternal alleles) or to the presence of entirely methylated paternal and/or hypomethylated maternal alleles in addition to alleles with normal methylation imprints. Although our sequence analysis of a limited number of plasmids did not allow any group comparisons, it clearly demonstrated relaxation of imprinting in both newborn tissues. The rate of single CpG errors that may be attributable to stochastic methylation errors (biological variation) or bisulfite conversion errors (technical variation) was similar to that in other studies (38,39). We propose that aberrantly (de)methylated alleles in a percentage of cells are at least partly responsible for the observed *MEST* methylation variation. Our finding that obese adults displayed lower blood methylation levels of the imprinted *MEST* gene than normal-weight controls supports the hypothesis that alterations in *MEST* regulation contribute to the life-long risk of development of obesity and other metabolic disorders. Knockout mice revealed that *Mest* not only acts as a fetal and placental growth factor but also plays a role in adult behavior, particularly maternal care (40). Loss of *Mest* imprinting was associated with increased organ and body weight (41). *Mest* belongs to a group of genes that were upregulated by early postnatal overnutritional environment of mice (42). Variations of *Mest* expression in genetically identical mice (before overnutrition) correlated with the development of diet-induced obesity (43). *Mest* overexpression (particularly of isoform 1) in transgenic mice resulted in the enlargement of adipocytes and fat mass expansion (44).

The 5′ untranslated region of exon 1 of the glucocorticoid receptor *NR3C1* encodes different splice variants that are fused to exon 2 in a tissue-specific manner. We studied the human exon 1_F promoter (orthologous to rat exon 1_7), which is highly expressed in the hippocampus. This neuron-specific *NR3C1* 1_F promoter was hypomethylated (mean, 3.3–5.6%) in all analyzed patient samples. First, it has been shown in the rat model that maternal diet (17) and behavior (18) can reprogram the *NR3C1* 1_7 promoter in the offspring. In humans, increased *NR3C1* 1_F methylation in blood and brain, respectively, has been associated with prenatal exposure to maternal depression (19) and childhood abuse (20). Although with small effect size (~1–2 percentage points), our results revealed highly significant methylation differences between newborns of mothers with GDM and newborns of mothers without GDM. Fetal *NR3C1* programming could be attributable to an increased maternal–fetal exchange of circulating glucocorticoids. At the individual level, it may be difficult to phenotypically distinguish between 3 and 5% *NR3C1* methylation. However, a recent study of monozygotic twins who were discordant for recurrent tumors demonstrated that relatively small differences (<10 percentage points) in the constitutive methylation of a tumor suppressor gene may have dramatic phenotypic consequences (39). We propose that similar to genome-wide association studies with genetic markers, even a minor methylation difference between newborns of mothers with GDM and newborns of mothers without GDM, can uncover genes that may play a major role in the etiopathogenesis of the studied disease.

In fetal cord blood, two genes, *IL10* and *NDUFB6*, showed significant methylation differences between D-GDM and I-GDM, with the I-GDM group resembling controls. The anti-inflammatory cytokine IL10 can be induced by exercise and may protect against chronic low-grade systemic inflammation in type 2 diabetes (45). Similar to

famine exposure in utero (10), specific dietetic measures in D-GDM may be associated with increased *IL10* methylation in the offspring. Reduced methylation of *NDUFB6* also could reflect lifestyle factors in D-GDM. However, we cannot exclude that these epigenetic signatures are specific for blood cells.

Our bisulfite pyrosequencing assays revealed GDM-associated methylation changes in 2 of 14 genes for metabolic programming. Hypomethylation of ALU repeats suggests that the epigenetic effects of GDM affect multiple loci. Therefore, an important next step is genome-wide studies of DNA methylation profiles by microarrays or next-generation sequencing. Although the number of analyzed samples meets the current standards in epigenetic studies, it is desirable to increase sample size in the next few years to reach genetic study standards. To detect small between-group differences in DNA methylation, which may well be biologically relevant, we need more statistical power. Epidemiological data link GDM to an increased risk for metabolic disorders in later stages of life. Although the observed alterations in DNA methylation patterns at birth may contribute to the different health outcomes by permanently changing the regulation of essential genes, so far this evidence is circumstantial. Rodent studies and the observed *MEST* hypomethylation in obese adults argue in favor of the notion that decreased *MEST* methylation in newborns of mothers with GDM is a risk factor for development of obesity later in life. However, large prospective studies will be needed to prove that such epigenetic changes are associated with changes in disease end points.

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