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Placental and Cord Blood Methylation of Genes Involved in Energy Homeostasis: Association With Fetal Growth and Neonatal Body Composition

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Low weight at birth is associated with subsequent susceptibility to diabetes. Epigenetic modulation is among the mechanisms potentially mediating this association. We performed a genome-wide DNA methylation analysis in placentas from term infants born appropriate-for-gestational-age (AGA) or small-for-gestational-age (SGA) to identify new genes related to fetal growth and neonatal body composition. Candidate genes were validated by bisulfite pyrosequencing (30 AGA, 21 SGA) and also analyzed in cord blood. Gene expression analyses were performed by RT-PCR. Neonatal body composition was assessed by dual X-ray absorptiometry at age 2 weeks. The *ATG2B*, *NKX6.1,* and *SLC13A5* genes (respectively related to autophagy, β-cell development and function, and lipid metabolism) were hypermethylated in placenta and cord blood from SGA newborns, whereas *GPR120* (related to free fatty acid regulation) was hypomethylated in placenta and hypermethylated in cord blood. Gene expression levels were opposite to methylation status, and both correlated with birth weight, circulating IGF-I, and total and abdominal fat at age 2 weeks. In conclusion, alterations in methylation and expression of genes involved in the regulation of energy homeostasis were found to relate to fetal growth and neonatal body composition and thus may be among the early mechanisms modulating later susceptibility to diabetes.

Individuals born small-for-gestational-age (SGA) are at increased risk for developing insulin resistance, obesity, metabolic syndrome, and, subsequently, cardiovascular disease and type 2 diabetes in adulthood (1,2). Growth restraint before birth is thought to confer such risk, particularly when followed by excessive weight gain after birth (3). By the postnatal age of 4 months, SGA infants already have an altered endocrine-metabolic profile with elevated concentrations of circulating IGF-I and high–molecular weight adiponectin (4). These abnormalities are influenced by early nutrition and evolve toward a profile that includes more hepatic and visceral adiposity in childhood (5). The mechanisms underpinning these abnormalities remain poorly understood.

Epigenetic regulation of the placenta evolves during preimplantation and progresses further during gestation. Strictly regulated DNA methylation has a key role in tissue-specific gene regulation and transcription; DNA methylation in CpG islands has been the most investigated target so far (6). Although DNA methylation has traditionally been viewed as an epigenetic "silencing" mark, recent advances suggest that the impact of DNA methylation on gene expression depends more on the genomic location of the CpG site (7). An adverse intrauterine environment can modify placental epigenetic marks and gene expression profiles, resulting in fetal growth restraint (8–10). Differential patterns of placental and cord blood methylation and/or expression have been described in selected imprinted and nonimprinted genes (11,12) in heterogeneous populations, including preterm infants (11) and growth-restricted

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fetuses from pregnancies complicated by hypertension or preeclampsia (12). Genome-wide methylation abnormalities in adipose-derived stem cells from SGA individuals have also been reported (13).

We tested whether placental and cord blood methylation and/or expression of genes involved in the regulation of energy homeostasis (which may influence both early growth and later diabetes risk) differ between offspring born appropriate-for-gestational-age (AGA) or SGA after an uncomplicated, term, singleton pregnancy.

RESEARCH DESIGN AND METHODS

Study Population

The study cohort consisted of 51 mother-placenta-newborn trios, with postnatal follow-up (Supplementary Fig. 1). Thirty infants were born AGA (17 girls, 13 boys) and 21 SGA (9 girls, 12 boys). The inclusion and exclusion criteria are detailed in Supplementary Data.

Maternal age at conception, parity, and height, as well as pregestational weight and BMI [weight (kg)/height (m^2)], were retrieved from clinical records. Gestational age was calculated from last menses and confirmed by first-trimester ultrasound (~10 weeks).

The infant's weight and length were measured by the same investigator (G.S.) at birth and at the postnatal age of approximately 2 weeks. Weight was measured with a beam balance (Seca, Hamburg, Germany) and length with a length board, using the mean of three measurements.

DNA Methylation Microarray in Placenta

DNA methylation profiling was performed in eight AGA and eight SGA samples with the Agilent DNA Methylation array (ID 049738, Agilent Technologies), which examines 27,800 highly informative CpG sites located within the proximal promoter regions of 14,475 genes. Nearly 100% of these CpG sites were localized within CpG islands. The process for isolating methylated DNA from purified DNA samples, as well as labeling and hybridization to the Human DNA Methylation Array, was conducted following the manufacturer's protocol (Agilent Microarray Analysis of Methylated DNA Immunoprecipitation version 1.1, Agilent Technologies). Briefly, 5 μg of purified DNA was sonicated in PBS; DNA fragments were run in a 1.5% agarose gel and ranged in size from 200 to 1,000 base pairs. Enrichment of methylated regions was performed using an antibody against 5-methylcytosine. Enriched and unenriched DNA samples were labeled with Cy3 and Cy5, respectively, and hybridized in the microarray. The microarray chip was then washed and immediately scanned using a microarray scanner (Model G2505C, Agilent Technologies).

Gene Expression Analysis in Placenta and Cord Blood

Steady-state mRNA levels of *GPR120, ATG2B, NKX6.1*, and *SLC13A5*, as well as the housekeeping gene *GAPDH,* were measured using gene-specific primers (*GPR120*: forward 5′-CTGCCTCTCTGCGTCTTCTT-3′, reverse 5′-CTCTTGCATCCAGGAGGTGT-3′; *ATG2B*: forward 5′-ATCCCAGCCTTTTGAAGGAC-3′, reverse 5′-AGCAAAAGCAGTGGGTTGAT-3′; *NKX6.1*: forward 5′-CTTCTGGCCCGGAGTGAT-3′, reverse 5′-CCCGCCAAGTATTTTGTTTG-3′; *SLC13A5*: forward 5′- CCTGCTGGATTGGAAGGTAA-3′, reverse 5′-CACTCAGTGAACACGGCAAC-3′; GAPDH: forward 5′-GTCAGTGGTGGACCTGACCT-3′, reverse 5′-TGCTGTAGCCAAATTCGTTG-3) and SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). PCR reactions were performed using a 7500 Real-Time PCR System (Applied Biosystems) by mixing 1 μL of cDNA (20 ng) with 24 μL of reaction mixture (14 μL 2 × SYBR Green I Master Mix buffer), 2 μL forward primer (2.5 μmol/L), 2 μL reverse primer (2.5 μmol/L), and 6 μL of nuclease-free water. Relative expression was then calculated according to the $2^{-\Delta Ct}$ method (14).

The details regarding cord blood and placenta collection, DNA extraction and modification, microarray data preprocessing, validation by bisulfite pyrosequencing, RNA extraction, and retrotranscription, as well as the assays and neonatal body composition assessment procedures, are provided in the Supplementary Data.

Statistics and Ethics

Statistical analyses were performed using SPSS Statistics 23.0 (IBM Corp., Armonk, NY). Unpaired *t* test was used to study differences between AGA and SGA subgroups. Correlation and stepwise multiple regression analysis were used to study how methylation status and expression levels in placenta and cord blood were associated with auxological and body composition parameters. Covariance analysis was used to adjust for sex, gestational age, type of delivery, and pregestational BMI. The level of significance was set at $P < 0.05$.

The study was approved by the Institutional Review Board of Hospital Sant Joan de Déu at the University of Barcelona. Written informed consent was obtained before delivery.

RESULTS

Maternal and Offspring Characteristics

Table 1 summarizes the anthropometric parameters in the mothers and newborns by birth weight subgroup. The delivery rate by cesarean section was not different between the AGA and SGA subgroups (13% vs. 19%, respectively). SGA infants displayed lower circulating levels of insulin and IGF-I in cord blood and less fat and lean mass at age 2 weeks, as expected, while their mothers were shorter and had a lower pregestational weight and BMI (14).

Gene Ontology and Functional Pathway Analysis

We identified 409 genes differentially methylated in SGA newborns (false discovery rate < 0.1) (Supplementary Table 1). The gene ontology analysis disclosed that most of them were involved in the regulation of metabolic processes, DNA binding and transcription, and biosynthesis of macromolecules (Supplementary Table 2). The Kyoto Encyclopedia of Genes and Genomes (KEGG) software identified enriched numbers of epigenetic changes in 11 canonical pathways, the most significant being those related to cell

Table 1—Auxological and clinical parameters in the studied infants and their mothers

	AGA, $N = 30$	SGA, $N = 21$	P value
Mothers			
Age (years)	31.8 ± 1.1	31.8 ± 1.0	NS
Height (cm)	164 ± 0.01	159 ± 0.01	0.01
Weight (kg)	66.0 ± 2.6	53.6 ± 1.6	0.0005
BMI (kg/m^2)	24.6 ± 0.9	21.1 ± 0.6	0.003
Pregnancy weight gain (kg)	13.4 ± 0.9	11.5 ± 0.7	NS
Primiparous (%)	57	76	NS
Cesarean section (*n* [%])	4 (13)	4 (19)	NS
Singleton newborns			
Sex (% female)	57	43	NS
Gestational age (weeks)	40.2 ± 0.2	38.7 ± 0.3	0.0008
Birth weight (kg)	3.3 ± 0.1	2.3 ± 0.1	<0.0001
Birth weight *Z*-score	0.1 ± 0.1	−2.3 ± 0.1	<0.0001
Birth length (cm)	50.6 ± 0.3	45.6 ± 0.5	<0.0001
Birth length *Z*-score	0.2 ± 0.1	−1.9 ± 0.2	<0.0001
Placental weight (kg)	0.60 ± 0.02	0.51 ± 0.03	0.022
Endocrine-metabolic variables at birth			
Glucose (mmol/L)	4.4 ± 0.2	4.5 ± 0.4	NS
Insulin (pmol/L)	27.8 ± 3.5	17.4 ± 2.1	0.009
IGF-I (nmol/L)	0.09 ± 0.01	0.06 ± 0.01	0.001
HMW adiponectin (mg/L)	43 ± 3	41 ± 3	NS
Body composition at 2 weeks			
Weight gain since birth (g)	184 ± 53	310 ± 60	NS
Length gain since birth (cm)	1.7 ± 0.2	2.2 ± 0.6	NS
Bone mineral density (g/cm^2)	0.27 ± 0.01	0.24 ± 0.01	0.026
Bone mineral content (g)	94.4 ± 2.2	69.9 ± 2.8	<0.0001
Fat mass (%)	18 ± 1	16 ± 1	0.04
Fat mass (g)	696 ± 39	467 ± 38	0.0001
Abdominal fat (%)	4.1 ± 0.2	3.3 ± 0.2	0.01
Abdominal fat (g)	29 ± 2	16 ± 3	0.0007
Lean mass (%)	82 ± 1	83 ± 1	NS
Lean mass (g)	3,110 ± 55	2,279 ± 68	<0.0001

Data are mean ± SEM unless otherwise indicated. HMW, high molecular weight; NS, not significant.

development and function, lipid metabolism, autophagy, and signaling (Table 2). The top-ranked hyper- and hypomethylated genes in these pathways ($n = 39$) are listed in Supplementary Tables 3 and 4, respectively. Because validation of all 39 genes by pyrosequencing was not feasible (costly and technically complex), in accordance with the study purpose we selected those genes ($n = 8$) that were relevant to the regulation of glucose and lipid metabolism; of those, three were hypomethylated (*MAPK8IP1, CAMK4,* and *GPR120*) and five were hypermethylated (*ATG2B, KLF11, NKX6.1, NRIP1,* and *SLC13A5*) in placentas of SGA newborns (Supplementary Tables 3 and 4).

Differentially Methylated Genes in Placenta and Cord Blood

Validation of DNA methylation patterns by pyrosequencing demonstrated that *ATG2B, NKX6.1,* and *SLC13A5* were hypermethylated in SGA placenta ($P < 0.02$, $P < 0.008$, and $P < 0.0001$, respectively, vs. AGA) and SGA cord blood (all $P < 0.0001$), while *GPR120* was hypomethylated in SGA placenta ($P = 0.0006$) and hypermethylated in SGA cord blood ($P < 0.0001$) (Figs. 1 and 2). The differences in methylation were maintained after adjusting for sex, gestational age, type of delivery, and pregestational BMI. Pyrosequencing analysis failed to demonstrate differences in methylation status in *MAPK8IP1*, *CAMK4, KLF11,* and *NRIP1* genes.

Expression of Validated Genes in Placenta and Cord Blood

Expression levels of *ATG2B, NKX6.1, SLC13A5,* and *GPR120* in placenta and cord blood were opposite to their methylation status (all $P \leq 0.004$ in placenta; all $P \leq 0.01$ in cord blood) (Figs. 1 and 2). The differences in gene expression were maintained after adjusting for sex, gestational age, type of delivery, and pregestational BMI.

Correlation and multiple regression analyses between genetic and endocrine-metabolic data are provided in the Supplementary Data.

DISCUSSION

We identified differential methylation profiles in placenta and cord blood from SGA infants in 18 CpG sites along the *ATG2B, NKX6.1, SLC13A5,* and *GPR120* genes—all nonimprinted—and report for the first time associations with birth weight, endocrine-metabolic markers, and neonatal body composition. Neither the differences in methylation and expression nor the associations with growth and

Table 2—Differentially methylated genes involved in KEGG pathways

Canonical pathway	*P* value	Methylation status	Gene symbol
Cell development and function	3.7E-04	Hyper	*PHOX2B, NDEL1,* ***GFOD1****, SMAD7,* ***KCNK13****, EFNA1,* ***CHRM2****, CNTN4,* ***NKX2–5****,* ***NKX3–2****,* ***NKX6.1****,* ***LMO2****,* ***PYGO2***
Lipid metabolism	4.0E-04	Hypo	*SOAT1, LYPLA2P1,* ***GPR120****, SORL1,* ***MAPK8IP1****, ECHDC2,* ***PEX1****, LYPLA2,* ***ATG2B****, HSD17B4*
Autophagy	5.4E-04	Hyper	*DRAM1,* ***ATG2B****, AMBRA1,* ***SOGA1***
Cellular signaling	6.6E-04	Hypo	*EIF2AK1, SLK,* ***CAMK4****,* ***MAPK13****, PAK4, CHKB,* ***PRKG1****, ALK,* ***STARD3NL****, PAPSS1, AKT3*
Cation channel activity	1.3E-03	Hyper	***CALHM2****, SCN3B, SLC24A2,* ***SLC13A5****,* ***KCNK13***
Regulation of immunity	1.7E-03	Hyper	*STAT5B,* ***ICOSLG****, PTPN22, RARA*
Endoplasmic reticulum metabolism	2.4E-03	Hyper	*SOAT1, DERL2, SPTLC1,* ***CLSTN2****, LOC729316, RTN2, SYNE1, NDEL1,* ***LRP10****, LARGE, C3ORF57, FAM156A, GNAS, FAM156B,* ***EIF2AK1,*** *POM121C,* ***KPNA4****, EDA, DLG1*
DNA binding	2.4E-03	Hypo	*FLYWCH1,* ***HIST1H2B1****, HIST4H4, HIST1H4K, LOC100045887, NFKB2, SRF, GM11275, ARNT, HOXA3, LOC674678, HIST1H4A, HIST1H4B, NKX3–2, POU3F4, SRRM1, HIST1H4F, HIST1H4C, HIST1H4D, HIST1H4I, LBR, HIST1H4J, HIST1H4H, ZBTB48, CEBPD, NEUROG1, ZFP3, MECOM, HIST2H4,* ***UHRF2****,* ***SFPQ****, TOP3B*
Transcription regulation	3.2E-03	Hyper	*ZNF583, POU6F2, STAT5B, ZNF827, RHOQ, HSBP1, CNOT4, ZNF343,* ***ZGPAT****,* ***GTF2H2C****, BRPF1,* ***ZNF182****,* ***ZNF436****, GTF2H2C, ZNF680, ZFP90, PSMC3IP, RARA, SOX17, FOXB1, GATAD1, MBD3L1, PHOX2B, RCOR3*
Transcription regulation	3.2E-03	Hypo	*SCAI, JUNB, MCM5,* ***NRIP1****, GTF2H2B,* ***ZNF500****, ZNF234, TAF11, PRDM9, MED18, LIN54, EDA, HMX3, ZNF468, GPBP1L1, SCML1, NFYC, MYBL1, HOXA1, THAP1, NKX2–5,* ***MLLT3****, SMAD7, KLF11, SOX30, TFCP2,STAT1, FOXP4,* ***NKX6.1****, ZNF22, ZNF212, DR1, NARFL,* ***FMR1****, SFPQ,* ***TOP3B***
Apoptosis	3.7E-03	Hyper	*FAM176A, DOCK1, LTBR, AIMP2, STAG3L3, FAM82A2,* ***KLF11****, PAK1*
Protein interaction	8.4E-03	Hyper	*DOCK1,* ***DOCK5****, ABI1,* ***SKAP2****, EPS8L1, RASA1, DLG1*

Pathways are arranged (top to bottom) according to *P* value. The genes in boldface type (n = 39) display the lowest (top-ranked) individual *P* values.

endocrine-metabolic markers were attributable to differences in maternal BMI or infants' gestational age, sex, or delivery route. The finding that the methylation and the expression of highlighted genes mirror each other, in both placenta and cord blood, suggests that the expression of those genes is mainly under epigenetic control, presumably influenced by multiple maternal and environmental factors, most of which remain to be identified.

Hypermethylation and downregulation of *ATG2B* (autophagy-related 2B) in placenta and cord blood were associated with smaller size at birth and lower fat mass. Autophagy is critical in the maintenance of maternal-fetal material exchange during placental development, in survival immediately after birth (15), and in cell differentiation, including adipogenesis (16). In SGA infants, decreased autophagy resulting from downregulation of *ATG2B*—thus compromising the nutrient supply—could be among the mechanisms accounting for prenatal growth restraint.

NKX6.1 (*NKX6* homeodomain 1)—a transcription factor known to be involved in pancreatic differentiation and β-cell homeostasis—was hypermethylated and downregulated in placenta and cord blood from SGA infants and was associated with birth weight and neonatal fat mass. In the pancreas, *NKX6.1* is exclusively expressed in β-cells and is required for prenatal β-cell development and function and postnatal β-cell expansion (17). In placenta, it remains to be studied whether the epigenetically controlled expression of *NKX6.1* is a modulator of nutrient transfer and thus perhaps an indirect modulator of fetal β-cell development, insulin secretion, and fat mass.

Mammalian Indy homolog (*SLC13A5*, mIndy) encodes for a sodium-coupled citrate transporter that is mainly expressed in hepatocytes and serves as a link between carbohydrate catabolism and lipogenesis. Loss of *SLC13A5* augments energy expenditure and hepatic fat oxidation and attenuates hepatic lipogenesis, reducing overall growth (18). *SLC13A5* was hypermethylated and downregulated in SGA placenta and cord blood. These findings align well with the knowledge that SGA infants have less adipose tissue and are more insulin sensitive than AGA infants, not only in early infancy but, if breastfed, also in late infancy (19). Reduced expression of *SLC13A5* may conceivably be a programming mechanism attempting to protect those fetuses from excessive fat accumulation and impaired insulin action early during spontaneous catch-up.

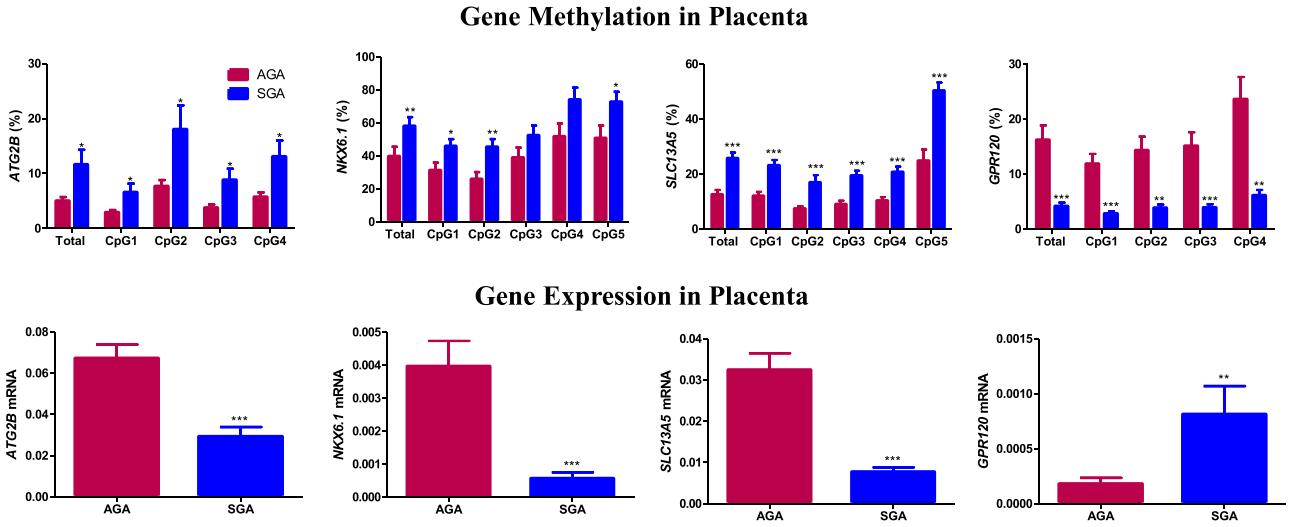

Figure 1—Methylation (top) and expression levels (bottom) of validated genes in placentas from AGA infants (n = 30) or SGA infants (n = 21). Values are mean ± SEM. *ATG2B, NKX6.1,* and *SLC13A5* were hypermethylated, whereas *GPR120* was hypomethylated, in SGA infants. Expression levels of *ATG2B, NKX6.1, SLC13A5,* and *GPR120* were opposite to their methylation status. *P < 0.05, **P < 0.01, ***P < 0.0001 vs. AGA.

GPR120, also known as free fatty acid receptor 4 (*FFAR4*), is a lipid sensor that regulates whole-body energy homeostasis in humans and rodents, mediating insulin-sensitizing effects in vivo by repressing inflammation (20). *GPR120* activation indirectly stimulates pancreatic insulin secretion by inducing the release of glucagon-like peptide 1 from the gut (21). In this study, *GPR120* was found to be hypomethylated and hyperexpressed in SGA placenta. The human placenta expresses *GPR120* mainly in the microvillous membrane of the syncytiotrophoblast (22), where it presumably transfers unsaturated long-chain fatty acids from the maternal circulation into the syncytiotrophoblast rather than into the fetal circulation. In contrast, *GPR120* was hypermethylated and hypoexpressed in cord blood of SGA infants, who have a reduced fat mass across early infancy (4). Interestingly, *GPR120* is thought to be a key contributor to adipocyte differentiation in the critical window of adipogenesis (23).

This preliminary study is the first to associate epigenetic regulation of nonimprinted genes with size at birth and markers of endocrine-metabolic homeostasis and neonatal body composition in healthy term AGA and SGA infants. Study limitations include the small sample size, the lack

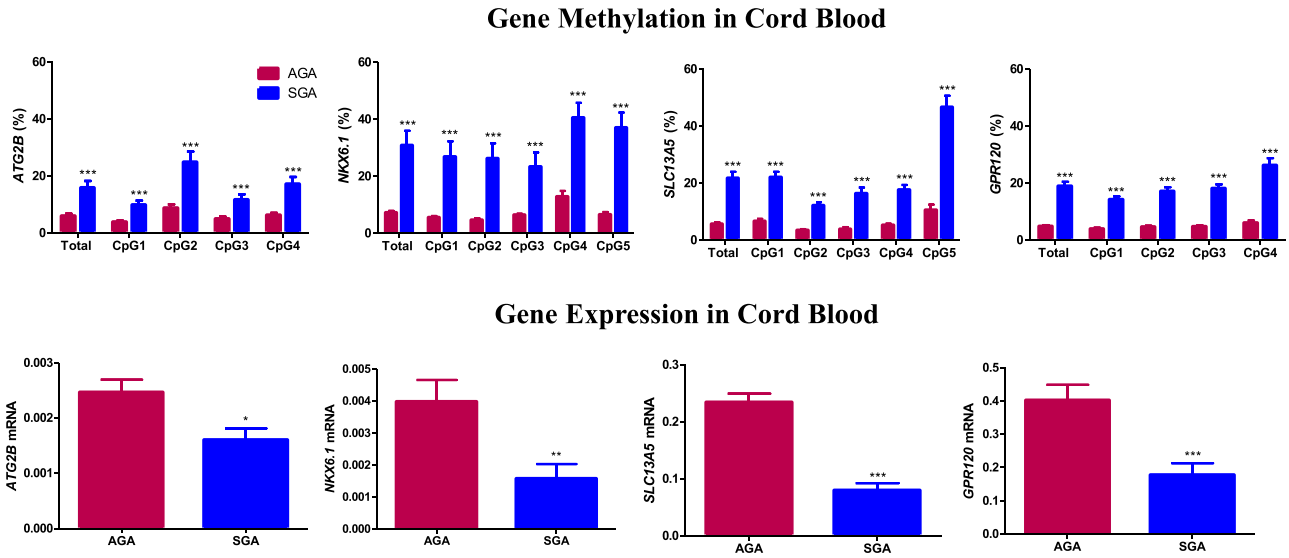

Figure 2—Methylation (top) and expression levels (bottom) of validated genes in cord blood from AGA infants (n = 30) or SGA infants (n = 21). Values are mean ± SEM. *ATG2B, NKX6.1, SLC13A5,* and *GPR120* were hypermethylated in SGA infants. Expression levels of *ATG2B, NKX6.1, SLC13A5,* and *GPR120* were opposite to their methylation status. *P < 0.05, **P < 0.01, ***P < 0.0001 vs. AGA.

of adjustment for cell composition in placenta/cord blood (24), the influence of maternal genetics on placental gene expression, the potential underrepresentation of differential methylation in CpG islands (25), and the inability to obtain—for ethical reasons—target tissues for the assessment of tissue-specific DNA methylation/expression. The strengths include the strict inclusion criteria (leading to distinct AGA and SGA populations and avoiding perinatal confounders) and the parallel assessments of placenta/cord blood pairs.

In conclusion, in SGA placenta and cord blood we identified differential methylation and expression patterns of genes involved in the regulation of glucose homeostasis and lipid metabolism. These epigenetic variations may influence fetal growth, early body composition, and lifelong diabetes risk.

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