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# Differences of DNA methylation patterns in the placenta of large for gestational age infant

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**Abstract**

To investigate the molecular mechanisms of later metabolic health changes in large for gestational age (LGA) newborns by analyzing deoxyribonucleic acid (DNA) methylation patterns in the placenta of LGA and appropriate for gestational age (AGA) newborns.

A total of 6 placentas of LGA and 6 placentas of AGA newborns were enrolled as LGA group and AGA group. DNA methylation was analyzed using the Illumina Infinium Human MethylationEPIC BeadChip microarrays and verified via pyrosequencing and reverse transcription-quantitative real-time polymerase chain reaction. Functional enrichment analysis were constructed by gene ontology and Kyoto Encyclopedia of Genes and Genomes pathway analysis based on the differentially methylated regions between LGA and AGA groups.

Clinical investigation showed that LGA newborns had significantly lower hemoglobin and blood glucose compared to AGA newborns. Birth weight was negatively correlated to hemoglobin and blood glucose. Genome-wide DNA methylation analysis identified 17 244 methylation variable positions achieving genome-wide significance (adjusted $P < .05$). 34% methylation variable positions were located in the gene promoter region. A total of 117 differentially methylated regions were revealed by bump hunting analysis, which mapped to 107 genes. Function analysis showed 13 genes enriched in "adhesion and infection process, endocrine and other factor-regulated calcium reabsorption, calcium signaling pathway and transmembrane transport". Four genes linked to type II diabetes mellitus. Among the 13 genes, we selected *GNAS* and calcium voltage-gated channel subunit alpha1 G for independent verification of pyrosequencing, and the messenger ribonucleic acid levels of guanine nucleotide binding protein, calcium voltage-gated channel subunit alpha1 G, *DECR1*, and FK506 binding protein 11 were verified by reverse transcription-quantitative real-time polymerase chain reaction.

DNA methylation variation and gene expression differences in placental samples were associated with LGA newborns, which linking the effect of intrauterine environment to regulation of the offspring's gene expression. Furthermore, pathway analysis suggested that intrauterine environment affecting fetal growth might had a functional impact on multiple signaling pathways involved in fetal growth, metabolism, and inflammation. Further studies were required to understand the differences of methylation patterns.

**Abbreviations:** AGA = appropriate for gestational age, *CACNA1G* = calcium voltage-gated channel subunit alpha1 G, *CDKN1B* = cyclin dependent kinase inhibitor 1B, cDNA = complementary DNA, *DECR1* = 2,4-dienoyl-CoA reductase 1, DMRs = differentially methylated regions, DNA = deoxyribonucleic acid, *DNM2* = dynamin 2, *DOCK1* = dedicator of cytokinesis 1, *FKBP11* = FK506 binding protein 11, *GNAS* = guanine nucleotide binding protein, alpha stimulating, GO = gene ontology, *KCNQ1* = potassium

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*In this study, we confirmed that all methods were carried out in accordance with relevant guidelines and regulations, all experimental protocols were approved by the Medical Ethical Committee of the Children Hospital of Zhejiang University School of Medicine (Approval Number: 2015-HP-025) and informed consent was obtained from all subjects or, if subjects are under 18, from a parent and/or legal guardian.*

*I would like to declare on behalf of my co-authors that the work described was original research that has not been published previously, and not under consideration for publication elsewhere, in whole or in part. All the authors listed have approved the manuscript that is enclosed.*

*All data generated or analysed during this study are included in this published article.*

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*The datasets generated during and/or analyzed during the current study are available from the corresponding author on reasonable request.*

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voltage-gated channel subfamily Q member 1, KEGG = Kyoto Encyclopedia of Genes and Genomes, LGA = large for gestational age, MVPs = methylation variable positions, *PDX1* = pancreatic and duodenal homeobox 1, RNA = ribonucleic acid, RT-qPCR = reverse transcription-quantitative real-time polymerase chain reaction, SGA = small for gestational age.

**Keywords:** methylation, deoxyribonucleic acid, large for gestational age, placenta

## 1. Introduction

With the development of socioeconomic levels in China, the prevalence of metabolic syndrome increased dramatically. Although some genetic factors have been associated with the risks of metabolic syndrome, which cannot be explained entirely by the changes of Mendelian inheritance. Sustained changes in metabolism following an adverse fetal or early neonatal environment have been connected to the mechanisms involving environmental factors regulation of gene expressions.[1] Environmental factors can trigger long-lasting changes through epigenetic processes, which regulate gene expressions without affecting the genetic sequences.[2–4] Notably, specific epigenetic changes have been linked to growth restriction.[5] Recently, impaired fetal growth decreased expressions of molecular regulators of β-cell mass and function, shown in some cases to be due to epigenetic changes initiated by an adverse fetal environment.[6,7] However, with the development of socioeconomic levels, the prevalence of small for gestational age (SGA) infant was dramatically declined while large for gestational age (LGA) was dramatically increased in China.

There is widespread knowledge that maternal obesity, diabetes and hyperinsulinemia are main causes of an infant being LGA. But now, women without apparent pregnancy complications also common have LGA newborns. LGA is regarded to be a well-established risk factor for metabolic syndrome as well.[8–10] Nevertheless, there is a mismatch between the given attentions versus the increasing number of LGA babies.

Epigenetic modifications, an important mechanism for diseases, can alter the expression patterns of genes, followed with the influence of glucose metabolism, fetal growth and even later life. Recently, methylation status of both coding and non-coding regions of the genome from cord blood was reported to be linked with birth weight.[11,12] Factors which contribute to reduced birth weight, such as maternal stress and depression, have also been associated with gene-specific promoter methylation patterns.[13] Epigenetic changes in placenta may have a great impact on fetal development and fetal adulthood, but less related research. Gene specific changes in methylation have also been reported in human placenta from SGA infants,[14] but not found in LGA.

Deoxyribonucleic acid (DNA) methylation is arguably the major epigenetic modification,[15] which controls a variety of cellular and developmental processes. During the last decade, more and more evidences have demonstrates that this process was involved in the occurrence and development of the human diseases.[16] Until now, the effect of LGA on DNA methylation and metabolic syndrome is poorly understood. Therefore, we analyzed the differences of DNA methylation in placenta to investigate the epigenetic mechanism of later metabolic changes in LGA newborns.

## 2. Methods

### 2.1. Subjects

In this study, we confirmed that all methods were carried out in accordance with relevant guidelines and regulations, all experimental protocols were approved by the Medical Ethical Committee of the Children Hospital of Zhejiang University School of Medicine (Approval Number: 2015-HP-025) and informed consent was obtained from all subjects or, if subjects are under 18, from a parent and/or legal guardian.

A case-control study was designed. A total of 150 newborns and their parents were recruited in the Women and Children's Hospital of Shaoxing (Zhejiang Province of China) from January to April 2015. Data of newborns and their parents were recorded before and after the newborn delivery.

According to the gender, gestational age, birth weight, and delivery, 6 LGA and 6 appropriate for gestational age (AGA) newborns were enrolled for further study. All the newborns were natural labor full-term. Each group included 3 boys and 3 girls. The diagnosis of LGA and AGA was according to the 9th edition of Chinese pediatrics textbook. Mother with hyperglycemia, infection, chronic diseases of the heart, lung, liver and kidney, immunologic diseases or other diseases and smoking, or children with asphyxia, meconium-stained amniotic fluid, premature rupture of membranes, production process extension, precipitates, analgesics or anesthetics administration was excluded. The physical parameters of the newborns and their parents were listed in Table 1. The differences of gender distribution and gestational age were not significant between these 2 groups ($t=0.91$, $P=.39$, respectively).

### 2.2. Placental samples collection

Two $1\times2$ cm placenta samples near the umbilical cord were collected immediately after delivery. After decidua and blood removal, then saline wash and frozen in liquid nitrogen for 1-5 hourr. Then, these samples were stored in -80°C for further measurement.

### 2.3. Biochemical parameters

Baseline blood samples were obtained from the heel of newborns and immediately measured. White blood cells and hemoglobin of whole blood were determined with a blood cell automated analyzer (Sysmex XS-500i). Blood glucose was measured by the glucose oxidase method.

### 2.4. DNA extraction and DNA methylation data collection

Genomic DNA was purified with QIAamp DNA Mini Kit (Qiagen, Catalog No: 51304) and bisulfite treated with EZ DNA Methylation Kit (Zymo Research, Catalog No: D5001&D5002) according to the manufacturer's instructions. DNA methylation was then analyzed using the Illumina Infinium Human Methylation EPIC BeadChips (850K) (Illumina, San Diego, CA, USA) following the protocol guide (infinium-hd-methylation-experienced-user-card-manual-15019522-01) by Genergy Boi Technology Inc. (Shanghai, China). All samples were run on the microarray together within the same batch and quality

**Table 1**

**Characteristics of the newborns and their parents.**

| | | AGA group | LGA group | t/$\chi^2$ | *P* value |
|---|---|---|---|---|---|
| Mother | Age (yr) | 28.33 ± 6.44 | 25.67 ± 1.86 | 0.974 | .353 |
| | Height (m) | 162.67 ± 2.88 | 162.67 ± 3.78 | 0.000 | 1.000 |
| | Weight 1 (kg)† | 51.17 ± 3.87 | 55.83 ± 6.56 | 1.502 | .164 |
| | Weight 2 (kg)‡ | 68.67 ± 7.06 | 72.75 ± 7.55 | 0.968 | .356 |
| | Weight increment (kg) | 17.50 ± 5.68 | 16.92 ± 4.10 | 0.204 | .843 |
| | BMI1 (kg/m$^2$)† | 19.35 ± 1.64 | 21.08 ± 2.11 | 1.583 | .145 |
| | BMI2 (kg/m$^2$)‡ | 25.94 ± 2.52 | 27.44 ± 1.91 | 1.154 | .275 |
| | BMI increment (kg/m$^2$) | 6.59 ± 1.99 | 6.36 ± 1.39 | 0.234 | .820 |
| Father | Age (yr) | 29.83 ± 6.24 | 26.50 ± 1.64 | 1.265 | .235 |
| | Height (m) | 173.33 ± 2.34 | 174.17 ± 2.40 | 0.609 | .556 |
| | Weight (kg) | 67.17 ± 7.57 | 73.92 ± 2.53 | 2.070 | .065 |
| | BMI (kg/m$^2$) | 22.37 ± 2.57 | 24.38 ± 1.21 | 1.737 | .113 |
| Newborn | Gender (male/female) | 3:3 | 3:3 | | |
| | Gestational age (week) | 39.25 ± 1.09 | 39.82 ± 1.09 | 0.903 | .388 |
| | Birth weight (kg) | 3.20 ± 0.40 | 4.08 ± 0.07 | 5.242 | <.003 |
| | White blood cells (×10$^9$/L) | 16.90 ± 3.64 | 16.92 ± 2.68 | 0.063 | .951 |
| | Hemoglobin (g/L) | 158.50 ± 6.72 | 146.67 ± 9.25 | 2.537 | .030 |
| | Blood glucose (mmol/L) | 5.23 ± 0.81 | 4.15 ± 0.34 | 2.966 | .018 |

AGA = appropriate for gestational age, LGA = large for gestational age, BMI = body mass index.

† weight 1 and BMI 1 are at begin of pregnancy.

‡ weight 2 and BMI 2 are the last time measured during pregnancy.

control (QC) for obtained DNA methylation data was performed with the Minfi R package tools.

### 2.5. DNA methylation data analysis

In order to obtain good quality probes on the microarray, the 853 307 methylation probes were filtered on the following 4 conditions as previous report[17]: first were probes in 65 internal control SNPs; second were probes with SNP and probes that align to multiple locations; third were probe methylated loci with a *P*-value >.01; fourth were probes with low bead count (<3 beads). Additionally, a sample will be excluded in the further analysis if the ratio of filter out probes more than 5% or zero signal. The ratio of fluorescent signals was then computed from the two alleles using the β value. Beta Mixture Quantile dilation Beta method was used to standardize of β value.[18] Differentially methylated CpG sites were found by Limma R package tools with a *P*-values <0.05. Differentially methylated regions (DMRs) were found based on methylation variable positions (MVPs) by Probe Lasso method.[19]

### 2.6. Validation of DNA methylation status by pyrosequencing

In the validations of HM850K results, based on the analysis results and methylation levels observed, we chose to verify the methylation level of specific CpG sites with bisulfite pyrosequencing in the placenta of another 8 LGA and 7 AGA in the following genes guanine nucleotide binding protein (GNAS) and calcium voltage-gated channel subunit alpha1 G (CACNA1G). The forward, biotinylated-reverse and sequencing primers were designed using Pyro-Mark Assay Design 2.0 and were listed in Supplemental Table S1, http://links.lww.com/MD/E915. All procedures were performed according to the manufacturer's protocols. Pyrosequencing was performed on a PyroMark Q24 system (Qiagen) and cytosine methylation was quantified using PyroMark Q241.010 software.

### 2.7. Validation of gene expression level by reverse transcription-quantitative real-time polymerase chain reaction (RT-qPCR)

To confirm the DNA methylation microarray analysis results, RT-qPCR was performed to investigate messenger ribonucleic acid (RNA) levels of FK506 binding protein 11 (*FKBP11)*, 2,4-dienoyl-CoA reductase 1(*DECR1)*, *GNAS* and *CACNA1G* in the placenta of another 8 LGA and 8 AGA. Total RNA was purified with RNeasy Protect Mini Kit (Qiagen, Catalog No: 74124). Reverse transcription of total RNA into complementary DNA (cDNA) use PrimeScript 1st Strand cDNA Synthesis Kit (TaKaRa, Catalog No: 6110A). The gene-specific primers were designed based on the cDNA sequences (Supplemental Table S2, http://links.lww.com/MD/E915). RT-qPCR was performed using GoTaq qPCR SYBR Green Master Mix (Promega, Catalog No: A6001) and ABI StepOne Plus™ Sequence Detection System (Applied Biosystems; Thermo Fisher Scientific Inc.). The relative expression of each gene was determined using the $2^{-\Delta CT}$ method.

### 2.8. Gene ontology analysis and functional enrichment

To identify prominent clinical mechanisms affecting and/or affected in LGA, genes with evidence of differential methylation were investigated with respect to their functions and major biological roles. Gene ontology (GO) and Kyoto Encyclopedia of Genes and Genomes (KEGG) (http://www.kegg.jp/kegg/pathway.html) pathway analysis were constructed based on the DMRs between LGA and AGA. The *P* value threshold was limited to $10^{-3}$.

### 2.9. Statistics analyses

Anthropometric and clinical characteristics of individuals are expressed as mean and standard deviation. Differences in these data between AGA and LGA placenta were tested by unpaired Student *t* test. The methylation level of each cytosine was expressed as a β value calculated as the fluorescence intensity

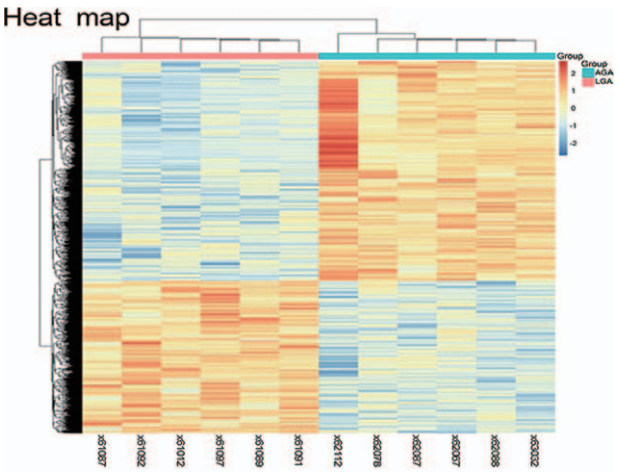


**Figure 1.** Heat map of methylation variable regions across LGA and AGA groups. Each probe set is represented by a single row of colored bars. Red color denotes higher methylation level. Blue color denotes low methylation levels. While yellow color denotes no change of methylation levels. Each line represents a placentas sample from AGA ($n=6$) and LGA ($n=6$) groups. AGA = appropriate for gestational age, AGA = appropriate for gestational age, LGA = large for gestational age.

ratio of the methylated to the unmethylated versions of the probes. β values with detection *P*-values >.01 were considered to fall below the minimum intensity and threshold, and these values were consequently removed from further analysis.

Differences in DNA methylation levels detected by pyrosequencing and expression of selected genes between AGA and LGA placenta were assessed by the nonparametric Mann–Whitney T test using the SPSS version 16.0 software (SPSS Inc, Chicago). *P*-values ≤.05 were considered statistically significant.

## 3. Results

Among 853 307 methylation probes, the ratios of filter out probes of 12 samples were all less than 0.001%. Hence, all samples were enrolled in the further analysis. Following quality filtering and normalization 803 146 individual probes were included in downstream data analyses. Density plots showed a typical pattern for each of the samples and highly comparable distributions indicating success with the Mixture Quantile dilation Beta normalization procedure (Supplemental Fig S1, http://links.lww.com/MD/E915).

### 3.1. Genome-wide DNA methylation patterns

Analysis the methylation levels with 803 146 probes, methylation levels in 17 244 MVPs were significantly different ($P<.05$, respectively) between LGA and AGA, which accounted for 2.02% probes (Fig. 1). Among these 17 244 MVPs, 705 were hypermethylated (> 1.7-fold) and 351 were hypomethylated (< 0.5-fold). Among 705 hypermethylated loci, the average β values of MVPs were 0.17 ± 0.13 (LGA) and 0.08 ± 0.06 (AGA) with significant difference (t = 18.09, $P<.001$), as shown in Figure 2A. Among 351 hypomethylated loci, the average β values of MVPs were 0.04 ± 0.03 (LGA) versus 0.96 ± 0.08 (AGA) with significant difference (t = 12.58, $P<.001$), as shown in Fig. 2B.

The identified MVPs between AGA and LGA were mainly located in CpG islands. Moreover, these MVPs were also enriched within the promoter regions. Among these 17 244 MVPs, 2 357 overlapped the transcription start site 1500 promoter regions (909 in GpG island, 44 in shelf and 1014 in shore), 1 897 overlapped the transcription start site 200 promoter regions (1 374 in GpG island, 15 in shelf and 282 in shore), 1 598 overlapped the 5'UTR (890 in GpG island, 104 in shelf and 271 in shore), 1 452 overlapped the exon one (1 233 in GpG island, 9 in shelf and 78 in shore), 561 overlapped the 3'UTR (136 in GpG island, 50 in shelf and 104 in shore) and 5 416 in the body (1 867 in GpG island, 525 in shelf and 1 064 in shore), as shown in Table 2.

To reduce data dimensionality and to demonstrate differential methylation integrated over several gene areas, we analyzed our methylation data for differentially methylated regions (DMR). We identified a total of 117 statistically significant DMRs between LGA and AGA groups, including 54 hypermethylated and 63 hypomethylated. Among these, the greatest mean Δ was 0.2121, and 25 of the DMRs had a mean Δ≥0.10. The top DMRs resided in genes such as *NTN4, FKBP11, PDX1, DECR1, ACTC1* and *SMOC2*, with many of them related with metabolism. Most of the above mentioned DMRs resided within gene promoters.

After analyzing the methylation levels of 22 pair chromosomes, we noted that the chromosome 6 had lots of DMRs (9 hypermethylated and 9 hypomethylated), accounting for 15% of DMRs. In contrast, no DMR was found in the chromosome 9. Meanwhile, highest rate of hypermethylated/hypomethylated (6 hypermethylated and 1 hypomethylated) was noticed in chromosome 12 while lowest rate of hypermethylated/hypomethylated (1 hypermethylated and 6 hypomethylated) was in chromosome 17 (Table 3).

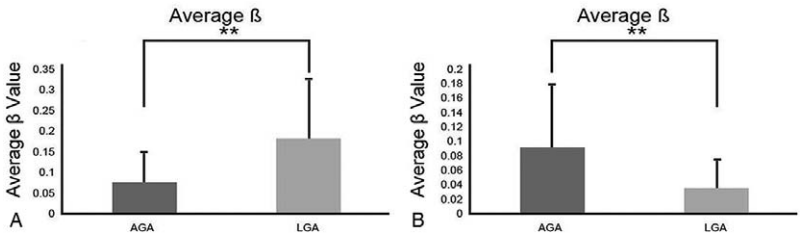


**Figure 2.** MVPs between the LGA and AGA groups. (A). The average β value of 705 hypermethylated locis between the LGA and AGA groups; (B). The average β value of 351 hypomethylated locis between the LGA and AGA groups (Independent *t* test; Data: mean ± SD; **: $P<.01$). AGA = appropriate for gestational age, LGA = large for gestational age.

**Table 2**

**The location distribution of MVPs on the gene.**

| Location | CpG island | Shelf | Shore | Others | Total |
|---|---|---|---|---|---|
| TSS1500 | 909 | 44 | 1014 | 390 | 2 357 (13.7%) |
| TSS200 | 1 374 | 15 | 282 | 226 | 1 897 (11%) |
| 5'UTR | 890 | 104 | 271 | 333 | 1 598 (9.3%) |
| Exon 1 | 1 233 | 9 | 78 | 132 | 1 452 (8.4%) |
| Body | 1 867 | 525 | 1064 | 1960 | 5 416 (31.4%) |
| 3'UTR | 136 | 50 | 104 | 271 | 561 (3.3%) |
| Others | 932 | 587 | 661 | 1783 | 3 963 (22.9%) |
| Total | 7 341 (42.6%) | 1 334 (7.7%) | 3 474 (20.1%) | 5 095 (29.6%) | 1 7244 |

AGA = appropriate for gestational age, MVPs = methylation variable positions, TSS200 = transcription start site 200, TSS1500 = transcription start site 1500.

### 3.2. GO and KEGG pathway analysis of DMRs

These hypermethylated DMRs matched 54 genes while hypomethylated DMRs matched 53 genes (Supplemental Table S3, http://links.lww.com/MD/E915). A GO analysis showed that most DMRs were related to adhesion and fetal developmental process (Biological Processes), plasma membrane part (Cellular Components), calcium, metal ion, other ion, cation, protein, microtubule binding, and transmembrane transporter (molecular functions) (Fig. 3A). A KEGG pathway analysis showed that DMRs might mainly involved in 9 pathways, such as sulfur relay system, type 2 diabetes mellitus, endocrine and other factor-regulated calcium reabsorption and so on (Fig. 3B). These DMRs matched the promoters of 13 genes, including dynamin 2 (*DNM2), GNAS*, pancreatic and duodenal homeobox 1 (*PDX1), CACNA1G*, potassium voltage-gated channel subfamily Q member 1, adenomatous polyposis coli 2, RAN binding protein 1, solute carrier family 25 member 4, major histocompatibility complex, class I, G, dedicator of cytokinesis 1, cytosolic thiouridylase subunit 2, *DECR1* and cyclin dependent kinase inhibitor 1B (Table 4).

**Table 3**

**The location distribution of DMR on chromosome.**

| Chromosome | DMR Numbers (%) | Hypermethylation | Hypomethylation | Hyper/Hypo |
|---|---|---|---|---|
| 1 | 9 (7.7%) | 2 | 7 | 0.29 |
| 2 | 5 (4.3%) | 2 | 3 | 0.67 |
| 3 | 2 (1.7%) | 1 | 1 | 1 |
| 4 | 6 (5.1%) | 2 | 4 | 0.5 |
| 5 | 8 (6.8%) | 4 | 4 | 1 |
| 6 | 18 (15.4%) | 9 | 9 | 1 |
| 7 | 6 (5.1%) | 2 | 4 | 0.5 |
| 8 | 2 (1.7%) | 1 | | 1 |
| 9 | 0 (0%) | 0 | 0 | 0 |
| 10 | 4 (3.4%) | 3 | 1 | 3 |
| 11 | 6 (5.1%) | 4 | 2 | 2 |
| 12 | 7 (6.0%) | 6 | 1 | 6 |
| 13 | 7 (6.0%) | 3 | 4 | 0.75 |
| 14 | 5 (4.3%) | 1 | 4 | 0.25 |
| 15 | 4 (3.4%) | 2 | 2 | 1 |
| 16 | 3 (2.7%) | 1 | 2 | 0.5 |
| 17 | 7 (6.0%) | 1 | 6 | 0.17 |
| 18 | 2 (1.7%) | 1 | 1 | 1 |
| 19 | 9 (7.7%) | 6 | 3 | 2 |
| 20 | 2 (1.7%) | 1 | 1 | 1 |
| 21 | 1 (0.9%) | 0 | 1 | / |
| 22 | 4 (3.4%) | 2 | 2 | 1 |
| Total | 117 | 54 | 63 | |

### 3.3. FKBP11, DECR1, GNAS and CACNA1G validation

A further analysis was performed by searching for these DMRs located in promoter regions with the ratio of β values $\geq 1.0$ or $\leq 0.70$ and *P*-values $\leq .01$. Under these conditions, 58 DMRs sites were detected. Among those, *GNAS* and *CACNA1G* were chosen for validation by targeted bisulfite pyrosequencing and RNA detection by RT-PCR because they were involved in type 2 diabetes mellitus pathways and *GNAS* paternal mutations could led to severe intrauterine growth retardation.[20] Another 2 genes, *FKBP11* and *DECR1*, were chosen for validation by RT-PCR because they exhibited the largest methylation differences in microarray analysis.

We used pyrosequencing to evaluate further DNA methylation levels for GNAS and CACNA1G in an independent cohort (validation cohort, n = 15). The results for 3 CpG sites and total CpGs within the *GNAS* DMRs and 5 CpG sites and total CpGs within the *CACNA1G* DMRs were showed in Figure 4. These results suggested that the changes in methylation levels of *GNAS* and *CACNA1G* were consistent with the microarray results.

DNA methylation has previously been associated with transcription repression. Accordingly, LGA samples showed significantly ($P = .0074$) higher expression levels of the gene *CACNA1G* ($11.5679 \pm 4.6735$) and ($P = .0059$; $P = .0019$; $P = .0043$) lower expression levels of the genes *FKBP11* ($13.59 \pm 7.55$), *DECR1* ($10.99 \pm 6.90$) and *GNAS* ($2.77 \pm 1.65$) than AGA ($4.06 \pm 3.28$; $61.06 \pm 34.93$; $50.58 \pm 10.65$; $8.06 \pm 3.23$), and these expressions were associated with the DNA methylation levels of *CACNA1G, FKBP11, DECR1* and *GNAS* (Fig. 5). These results suggested that the epigenetic regulation of these genes could have a relevant biologic function.

### 3.4. Functional enrichment

We also analyzed the enrichment of the 107 DMRs associated genes. Enrichment analysis showed a significant over-representation of functional groups highly relevant to the LGA phenotype. The functional categories detected were related to transmembrane transporter, developmental processes (eg, anatomical structure development, nervous system development) and cell adhesion (eg, homophilic cell adhesion, biological adhesion). Among 107 genes, 41 were involved in "anatomical structure development" and 23 genes were associated with "nervous system development." As many as 79 genes, or 73.8% of our list, were annotated as "binding." Other enriched functional groups were related to cell adhesion, developmental processes, and regulation of transcription.

## 4. Discussion

Environmental and lifestyle factors are recognized as influence in an individual's DNA methylation patterns. So we hypothesized that LGA might correlated with changes in placental DNA methylation and expressions of genes conferring risk for metabolic disease. As far as we are aware, our study was the first time to compare the DNA methylation level between AGA and LGA neonates of women without medical or obstetric complications at term, using the Illumina Infinium Human

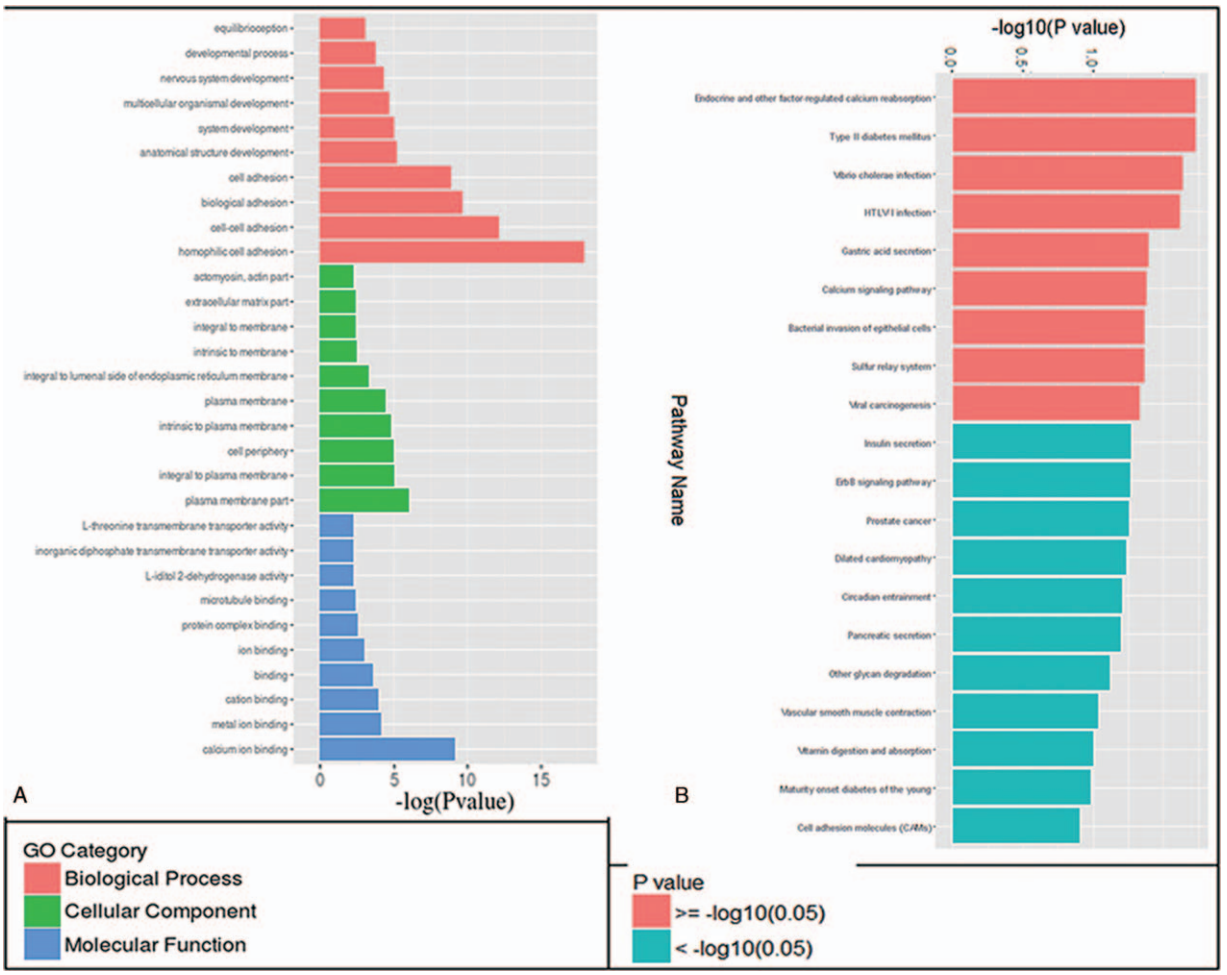


**Figure 3.** GO and KEGG pathway analysis for the differentially Methylated Regions (DMRs) between LGA and AGA groups. (A) GO analysis showed that LGA associated with biological process (red), cellular component (green) and molecular function (blue); (B) KEGG pathway analysis showed 9 differentiate pathways (red). GO = gene ontology. KEGG = Kyoto Encyclopedia of Genes and Genomes. AGA = appropriate for gestational age, LGA = large for gestational age.

Methylation EPIC BeadChips (850K), and identify potential DNA methylation signatures.

Although, epigenetics changes have been associated with the intrauterine growth and later health, previous studies were mostly focus on the epigenetics changes of newborn genome, but not the placenta. To our knowledge, whether epigenetics play a role in the placenta of LGA neonates has remained unknown, and this is the first report. Our study revealed DNA methylation changes in LGA group, which have 17 244 significantly different MVPs, including hypermethylated 705 MVPs and 351 hypo-

**Table 4**

**KEGG pathway analysis showed 9 differentiate pathway between AGA and LGA group.**

| Pathway | Genes | KEGG ID | Fold | *P* value |
|---|---|---|---|---|
| Endocrine and other factor-regulated calcium reabsorption | *GNAS, DNM2* | hsa04961 | 9.38 | .019 |
| Type 2 diabetes mellitus | *PDX1, CACNA1G, DECR1, GNAS* | hsa04930 | 9.38 | .019 |
| Vibrio cholerae infection | *KCNQ1, GNAS* | hsa05110 | 8.34 | .023 |
| HTLV-I infection | *APC2, RANBP1, HLA-G, SLC25A4* | hsa05166 | 3.42 | .024 |
| Gastric acid secretion | *KCNQ1, GNAS* | hsa04971 | 6.17 | .040 |
| Calcium signaling pathway | *GNAS, CACNA1G, SLC25A4* | hsa04020 | 3.73 | .042 |
| Bacterial invasion of epithelial cells | *DOCK1, DNM2* | hsa05100 | 5.92 | .043 |
| Sulfur relay system | *CTU2* | hsa04122 | 22.51 | .043 |
| Viral carcinogenesis | *CDKN1B, RANBP1, HLA-G* | hsa05203 | 3.57 | .047 |

AGA = appropriate for gestational age, *APC2* = adenomatous polyposis coli 2, *CACNA1G* = calcium voltage-gated channel subunit alpha1 G, *CDKN1B* = cyclin dependent kinase inhibitor 1B, *CTU2* = cytosolic thiouridylase subunit 2, *DECR1* = 2,4-dienoyl-CoA reductase 1, *DNM2* = dynamin 2, *DOCK1* = dedicator of cytokinesis 1, *GNAS* = guanine nucleotide binding protein, alpha stimulating, *HLA-G* = major histocompatibility complex, class I, G, KEGG = Kyoto Encyclopedia of Genes and Genomes, *KCNQ1* = potassium voltage-gated channel subfamily Q member 1, LGA = large for gestational age, *PDX1* = pancreatic and duodenal homeobox 1, *RANBP1* = RAN binding protein, *SLC25A4* = solute carrier family 25 member 4.

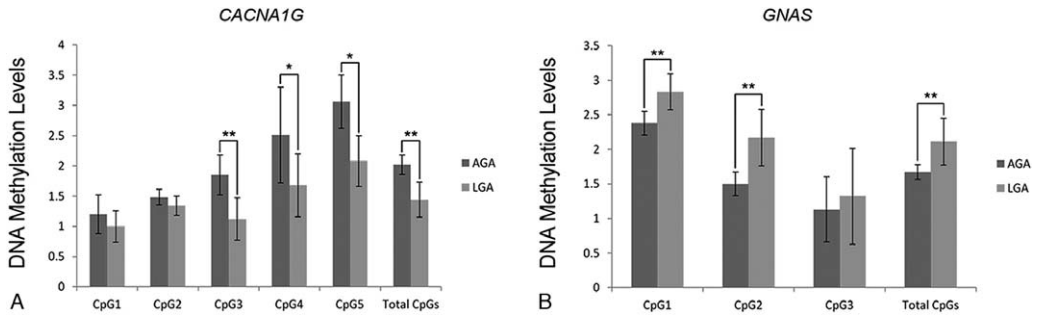


**Figure 4.** Validation of candidate LGA regions by bisulfite pyrosequencing. Bar charts representing the DNA methylation levels at five CpG sites and total CpGs overlapping *CACNA1G* (A), and three CpG sites and total CpGs overlapping *GNAS* (B) in LGA (red color) and controls (blue color) (Nonparametric Mann–Whitney *T* test; Data: mean ± SD; *: $P < .05$; **: $P < .01$). LGA = large for gestational age, DNA = deoxyribonucleic acid, *GNAS* = guanine nucleotide binding protein, alpha stimulating.

methylated MVPs, between the LGA and AGA groups. These significantly different MVPs were mainly located in the promoter region (42.4%) and GpG island (42.6%). It is well established that DNA methylation patterns are associated with gene expression levels, especially in regions defining enhancers and promoters. In fact, the differentially MVPs identified are preferentially located within regulatory regions of the genome, the CpG islands, as these are known to have important implications on transcriptional regulation. Based on these results, we proposed that some intrauterine environment resulting in LGA might alter the genomic methylation, thereby affecting the expression levels, or even later health.

DMR analyses showed that a total of 117 DMRs, including 54 hypermethylated and 63 hypomethylated DMRs, were found in LGA groups. An analysis of DMRs of LGA compared to AGA also identified many hypomethylated/hypermethylated DMRs in LGA in metabolism-related genes, such as *PDX1, CACNA1G, DECR1, GNAS* and so on. It was interesting that the methylated changes were different among different chromosomes. Chromosome 6 had the most number of DMRs and the chromosome 9 had no change of DMRs. Moreover, the chromosome 12 has the highest rate of hypermethylated to hypomethylated (6:1) while the chromosome 17 has the lowest rate (1:6). These might be associated with the gene distributions and indicated that methylated changes caused by LGA were imbalance among different chromosomes. Certainly, further studies about these imbalances will need to be conducted to understand the accurate mechanisms of epigenetic regulation.

The functions of 107 genes associated with 117 DMRs were analyzed. They tend to be associated with developmental and adhesion biological processes. These biological processes could promote the survival and adaptation of LGA fetuses because the relevant genetic networks identified important pathways in fetal development. Major networks identified by multiple pathway

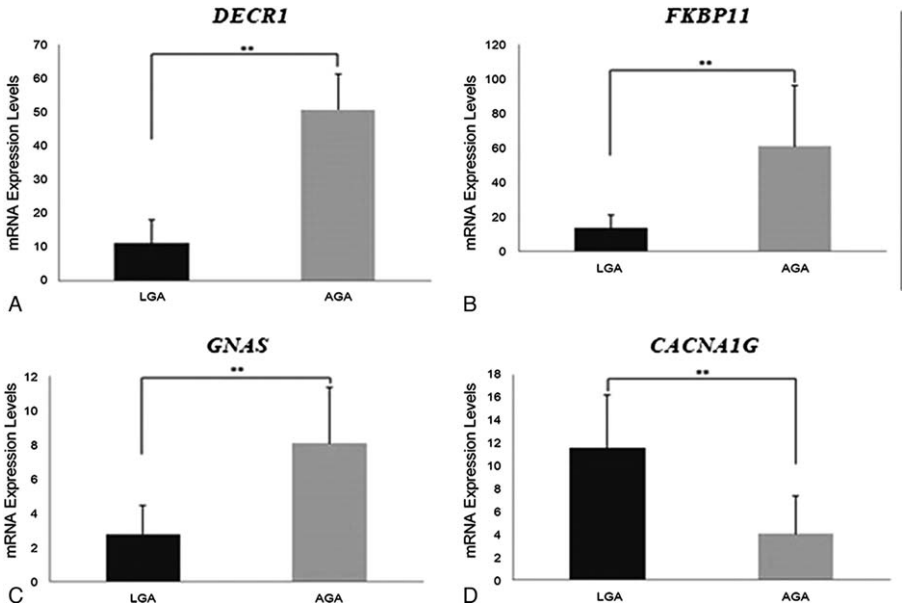


**Figure 5.** Validation the microarray results of LGA candidate genes by qRT-PCR. Bar charts representing the mRNA expression levels at *DECR1*(A), *FKBP11*(B), *GNAS* (C), and *CACNA1G* (D) in LGA (black color) and controls (yellow color) (Independent t test; Data: mean ± SD; **: $P < .01$). LGA = large for gestational age, *DECR1* = 2,4-dienoyl-CoA reductase 1. *GNAS* = guanine nucleotide binding protein, alpha stimulating, mRNA = messenger RNA.

and functional analyses highlighted the importance of ion binding, protein binding and cell-to-cell signaling, adhesion, transport, as well as system development and function. However, network analysis also interestingly identified methylation differences in genes that were important in the eventual risk of metabolic disease, such as endocrine and other factor-regulated calcium reabsorption, calcium signaling pathway and type 2 diabetes mellitus genes (e.g *GNAS*, *DNM2*, *PDX1*, *CACNA1G*, *DECR1* and solute carrier family 25 member 4. In addition, several interesting genes that might played role in immunity were identified, including potassium voltage-gated channel subfamily Q member 1, adenomatous polyposis coli 2, RAN binding protein 1, major histocompatibility complex, class I, G, dedicator of cytokinesis 1, and *DNM2*. Although these findings implied that this epigenomic data set might reflect true pathophysiological processes, further mechanistic studies were needed to be carried out. Indeed, it was important to consider using pathway analysis to explore biological mechanisms affected by fetal growth-associated epigenetic variation.

We found that 3 differentially methylated genes (*PDX1*, *DECR1* and *GNAS*) had been previously associated with SGA,[21,22] suggesting (at least in part) the changes of some genes in SGA and LGA were similar in some pathways and regulatory effects. In this study, the largest methylation difference was demonstrated in the promoter region of the *FKBP11* gene. This gene encodes FKBP11, which belongs to the FKBP family of peptidyl-prolyl cis/trans isomerases, and catalyzes the folding of proline-containing polypeptides. The peptidyl-prolyl isomerase activity of FKBP proteins is inhibited by the immunosuppressant compounds FK506 and rapamycin. The C-terminal of this protein contains a putative transmembrane domain followed by a motif found in endoplasmic reticulum membrane proteins. Thus, FKBP11 has been conjectured to be involved in protein folding and secretion.[23] The different expression of *FKBP11* gene in type 2 diabetes mellitus has been reported.[24] The next largest methylation difference identified was hypermethylation in the promoter of the *DECR1* gene in the LGA group. This gene encodes DECR protein, the rate-limiting mitochondrial enzyme in unsaturated fatty acid beta oxidation, which is required for growth and neurodevelopment.[25] An association between unsaturated fatty acids and intrauterine growth has been previously reported in that prenatal supplementation of the unsaturated fatty acid, omega-3, which has been shown to increase birth weight.[26] Prior studies of *Decr1*$^{-/-}$ mice suggested that the enzyme was responsible for the induction of gluconeogenesis during fasting.[27] Whether this gene is related to metabolic syndrome requires further research and exploration. Another highly ranked candidate gene is the *GNAS* gene, which encodes, through the use of alternative first exons, the signaling proteins Gαs and XLαs and the NESP55. The *GNAS* locus furthermore gives rise to an AS transcript as well as the A/B transcript that contributes to the regulation of Gαs expression and may be translated into an amino-terminally truncated Gαs variant that appears to antagonize the actions of the full-length signaling protein.[28]*GNAS* is a complex imprinted locus involved in metabolic regulation. A recent study had further demonstrated that a single nucleotide polymorphism in *GNAS* in the maternal genotype of African American women (SNP rs6026576) was significantly associated with birth weight.[29] In other studies, it was also found that the expression level of *GNAS* in the placenta of SGA individuals was decreased,[20,30] but the importance of its reduced expression in SGA was not immediately clear. To date, our study was the first to link changes of *FKBP11*, *DECR1* and *GNAS* and so on methylation with metabolic syndrome caused by LGA in humans. These could provide assistance in understand the development of potential epigenetic biomarkers and signals in altering fetal growth and arousing the risk for adult metabolic disease. However, the role and mechanism of FKBP11 DECR1 and GNAS changes in LGA-induced metabolic syndrome was still unclear and the further studies were required.

There were several limitations of our study, although we obtained several significant results. First, the sample sizes were relatively small. Second, due to the placental tissue samples limitation, the samples used for microarray detection, pyrosequencing verification and messenger RNA verification did not come from the same placental tissue. Third, the consistency of placental samples might be biased because of the inability to fully control the state of the pregnant woman and the fetus. Further study with larger sample is required to investigate the associations between DNA methylation patterns and the mechanisms of LGA and adult metabolic syndrome. Certainly, this study had its strengths, which might be helpful in the development of potential epigenetic biomarkers and novel therapeutic targets in the prevention and treatment of abnormal fetal growth and adult metabolic disease.

## 5. Conclusion

In conclusion, our genome-wide DNA methylation study, carried out using paired AGA and LGA placenta tissues, demonstrated specific differential DNA methylation profiles in LGA cases. Furthermore, our results of epigenetic variation and gene expression differences in placenta of LGA infants suggested that intrauterine environment might be resulted in epigenetic and gene expression changes of placental genome, then the abnormal intrauterine growth and even later health. Based on the results obtained from this study might be helpful in the development of potential epigenetic biomarkers and novel therapeutic targets in the prevention and treatment of abnormal fetal growth and adult metabolic disease. Future studies including larger sample sizes to improve our findings and investigate the mechanisms are necessary.

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## Author contributions

Professor Zou response for the design of the study, validation analysis, and drafted the manuscript. Mr Shen take participated in the design of the study, performed the bioinformatic analyses. Mrs Tang and Mrs Song take part in the recruited of subjects, data and sample collection. Mrs Shen performed the sample selection and methylation array interpretation. All authors read and approved the final manuscript.

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