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Glucose as a fetal nutrient: dynamic regulation of several glucose transporter genes by DNA methylation in the human placenta across gestation☆,☆☆

Boris Novakovic[a,b], Lavinia Gordon[c], Wendy P. Robinson[d], Gernot Desoye[e], Richard Saffery[a,b,*]

[a]Cancer and Disease Epigenetics, Murdoch Childrens Research Institute, Royal Children's Hospital, Parkville, Victoria 3052, Australia
[b]Department of Paediatrics, University of Melbourne, Parkville, Victoria 3052, Australia
[c]Bioinformatics, Murdoch Childrens Research Institute, Royal Children's Hospital, Parkville, Victoria 3052, Australia
[d]Department of Medical Genetics, University of British Columbia, Child and Family Research Institute, Vancouver, BC, Canada
[e]Department of Obstetrics and Gynaecology, Medical University of Graz, Auenbruggerplatz 14, A-8036 Graz, Austria

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Abstract

The human placenta ensures proper fetal development through the regulation of nutrient and gas transfer from the mother to the fetus and the removal of waste products from the fetal circulation. Glucose is one of the major nutrients for the growing fetus. Its transport across the placenta to the fetus is mediated by a family of facilitative transporter proteins, known as the glucose transporters (GLUTs), encoded by the *SLC2A* family of genes. There are 14 members of this gene family, and the expression of several of these has been shown in human placenta; however, aside from GLUT1 and GLUT3, little is known about the role of these proteins in placental function, fetal development and disease. In this study, we analysed previously generated genome-scale DNA methylation and gene expression data to examine the role of methylation in GLUT expression throughout gestation. We found evidence that DNA methylation regulates expression of *GLUT3* and *GLUT10*, while the constitutively expressed *GLUT1* showed no promoter methylation. We further analysed the level of DNA methylation across the promoter region of *GLUT3*, previously shown to be involved in glucose back-flux from the fetal circulation into the placenta. Using the Sequenom EpiTYPER platform, we found increasing DNA methylation of this gene in association with decreasing expression as gestation progresses, thereby highlighting the role of epigenetic modifications in regulating the GLUT family of genes in the placenta during pregnancy. These findings warrant a reexamination of the role of additional GLUT family members in the placenta in pregnancy and disease.

Keywords: Human placenta; Glucose transport; DNA methylation; Epigenetics; GLUT3

1. Introduction

The placenta is a tissue of limited lifespan that performs a diverse range of functions including the transport of maternal nutrients to the fetal circulation to ensure adequate fetal growth. Not surprisingly, therefore, the placenta has been implicated in pathologies associated with aberrant fetal growth, including gestational diabetes mellitus (GDM) and fetal growth restriction (FGR) [1–3].

The fetus has a very low capacity for glucose production, so fetal glucose levels are primarily controlled through placental transport from the maternal circulation [4,5]. This is largely a result of facilitated diffusion mediated by a family of transporter proteins, the glucose transporters (GLUTs), encoded by 14 different genes of the *SLC2A* family. To date, several GLUTs have been described in the placenta [6,7]. The ubiquitous GLUT1 is found in all cells at all gestational ages [8]. GLUT3, which is the high-affinity glucose transporter, is expressed in a cell-specific manner depending on developmental stage. Both GLUT1 and GLUT3 not only accept glucose as sole substrate but are also involved in the uptake and presumably transfer of dehydroascorbic acid (vitamin C) [9]. In the first trimester, GLUT3 expression can be detected in villous and extravillous cytotrophoblasts, but not the syncytiotrophoblast layer, suggesting that its expression is localised to rapidly proliferating and poorly differentiated cells [10], whereas in the third trimester, placenta GLUT3 is primarily localised to endothelial cells of the fetoplacental vessels and in stromal cells [10]. GLUT3 protein expression decreases over pregnancy, suggesting a greater role for this protein in glucose transport and fetal growth early in pregnancy [11]. GLUT4 is an insulin-dependent transporter localised to villous stromal cells [12] and the syncytiotrophoblast

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* Corresponding author. Murdoch Childrens Research Institute, Parkville, Victoria 3052, Australia. Tel.: +61 3 83416341.

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layer in the first trimester [13]. However, given that glucose transport across the placenta is insulin independent [14], the function of this transporter remains unclear. Generally speaking, the roles of other members of this protein family in placental function and disease, and the mechanisms regulating gene expression throughout gestation are poorly understood [15].

Epigenetic (literally 'above DNA') mechanisms play a key role in controlling both the temporal and spatial patterns of gene expression seen in all tissues, particularly during early development [16,17]. DNA methylation is the most widely studied epigenetic phenomenon, previously demonstrated to play an important role in placental function and to be dynamic in the human placenta, both across gestation and in response to cumulative environmental and stochastic factors [18]. Additionally, disruption of placental methylation profile has been implicated in several pathologies, including fetal growth restriction and gestational diabetes mellitus [19–25]. In light of these data and the potential for epigenetic mechanisms to play an important role in regulating nutrient transport in the placenta, we have investigated the relationship between *GLUT* family member expression and promoter methylation in the human placenta across gestation using publicly available genome-scale DNA methylation and gene expression array data.

2. Methods and materials

2.1. Tissue and cell collection

First- (8–12 weeks), second- (17–24 weeks) and third- (34–41 weeks) trimester placental DNA was collected as previously described [18]. Briefly, placental tissues from elective abortions or miscarriages (first and second trimester), or at delivery (third trimester) were collected from the basal plate, around the middle of the placenta. The collection of placenta from elective abortions, from miscarriages and at term was approved by the ethics committees of the University of British Columbia and the Children's & Women's Health Centre of British Columbia. Collection of term placenta in Australia was approved by the Human Research Ethics Committees at the Royal Women's Hospital (03/51), Mercy Hospital for Women (R07/15) and Monash Medical Centre (07084C). Use of DNA from additional 8- and 12-week placental villi was covered by Cambridgeshire Research Ethics committee (CREC 04/Q0108/23).

2.2. DNA extraction

DNA was extracted as previously described [18,26–28]. A cross-section was cut at random from the middle of each placenta and washed in phosphate-buffered saline prior to storage at −80°C. Tissue samples were incubated at 50°C overnight with shaking in DNA extraction buffer [100 mM NaCl, 10 mM Tris–HCl pH8, 25 mM EDTA, 0.5% (w/v) sodium dodecyl sulfate] containing 200 μg/ml proteinase K. DNA was isolated by two rounds of phenol:chloroform extraction, followed by RNAse A treatment. DNA was precipitated in absolute ethanol containing 10% (v/v) sodium acetate (3 M, pH 5.2) and resuspended in 100 μl nuclease-free water (Ambion, Austin, TX, USA). DNA was stored at −20°C until needed.

2.3. Locus-specific DNA methylation

DNA methylation levels at the *GLUT3* promoter region was quantified using the Sequenom MassARRAY EpiTYPER platform as previously described [29,30]. Primer pairs for amplification were designed using EpiDesigner Web tool (http://www.epidesigner.com/). The *GLUT3* promoter region was targeted using the following primers: forward 5′ aggaagagagTTTAAGAAAGATTTAGGGGTGATTTT 3′ and reverse 5′ cagtaatacgactcactatagggagaaggctCCTCCCTCCAAACTTTTCTAATAAT 3′. Amplification was performed after bisulfite conversion of genomic DNA with the MethylEasy Xceed bisulphite conversion kit (Human Genetic Signatures, North Ryde, Australia). Amplification conditions were 40 cycles of 95°C for 5 min, 56°C for 1 min 30 s and 72°C for 1 min 30 s, and then 72°C for 7 min.

2.4. Infinium methylation analysis

We previously performed genome-scale DNA methylation analysis of 18 first-trimester, 10 second-trimester and 14-third trimester placenta samples using the Illumina Infinium Human-Methylation27 BeadChip (HM27) [18]. Data were previously uploaded to the Gene Expression Omnibus, accession number GSE26683 [18].

2.5. Gene expression array analysis

Gene expression data in the form of CEL files were also downloaded from GEO from series GSE9984 [31] and GSE5999 [32] with both series being run on the same array platform, the Affymetrix Human Genome U133A array. Each series was normalized and summarized using the Bioconductor package 'gcrma' [33], generating log2 expression values. Gene expression data were then linked with the methylation data according to the annotated gene name, as previously described [18,34].

3. Results

3.1. GLUT genes are differentially methylated in the human placenta at birth

We extracted data related to the glucose transporter gene family (*GLUT*) from genome-scale DNA methylation data obtained from placental samples of different gestational ages [18]. The Infinium HM27 array targets about 14,000 genes and outputs the methylation levels as a beta value, with a theoretical range from 0 (completely unmethylated) to 1 (completely methylated). GLUT genes are separated into three classes: Class I (*GLUT1, 2, 3* and *4*), Class II (*GLUT5, 7, 9* and *11*) and Class III (*GLUT6, 8, 10* and *12*). Each of these genes is targeted by two probes on the HM27 methylation array, with the exception of *GLUT6* and *12*, which are only targeted by a single probe. Analysis of promoter methylation level in placental tissue collected at birth revealed highly variable methylation levels in these genes, largely independent of class of transporter. This is typified by HM27 probes associated with Class I genes *(GLUT1–4*) that showed a mean methylation level ranging from 0.03 (both probes in *GLUT4*) to 0.85 (probe cg17142134 in *GLUT2*) (Fig. 1A). Interestingly, despite a lack of previous evidence of placental expression for some GLUT family members, Class II–IV *GLUT* genes also showed evidence of minimal promoter methylation in the full-term placenta, including *GLUT6, 8, 10, 11, 12* and *13* (Fig. 1B). The level of methylation of specific genes was very consistent between different placental samples, with the highest variance of 0.009 for probe cg13323752 in *GLUT14*. This is well under the threshold of 0.02 previously used to define a variable gene in the human term placenta [18,25].

3.2. Identification of differentially methylated GLUT family genes in the placenta across gestation

Given its role as a ubiquitous glucose transporter, it was not surprising that there was little evidence of promoter methylation of the *GLUT1* gene throughout gestation in the placenta (Fig. 2Ai). A similar pattern of methylation was also apparent for *GLUT4* (Fig. 2Aiv). However, clear evidence for temporal change in DNA methylation of several other *GLUT* genes was apparent across gestation, including Class I genes *GLUT2* (*P* value<.001) (Fig. 2Aii) and *GLUT3* (*P* value<.001) (Fig. 2Aiii), Class II gene *GLUT5* (*P* value<.001) (Fig. 2Bi), and Class III genes *GLUT9* (*P* value<.001) (Fig. 2Biii) and *GLUT10* (*P* value<.001) (Fig. 2Biiii). Little evidence of change was noted in any other of the GLUT gene family (data not shown).

3.3. The high-affinity glucose transporter GLUT3 shows increasing promoter methylation across gestation

HM27 array data suggested an increase in placental methylation at two CpG sites within the *GLUT3* promoter region across gestation (Fig. 2). In order to confirm this and to examine whether this is representative of regional methylation levels, we designed a Sequenom EpiTYPER assay to interrogate several CpG sites spanning the transcription start site (Supplementary Figure 1). Of eight CpG sites in the region of interest, five were analysable using this platform and produced methylation data. These included one of the HM27 CpG sites (cg10338338) that was analysed as part of the MassARRAY

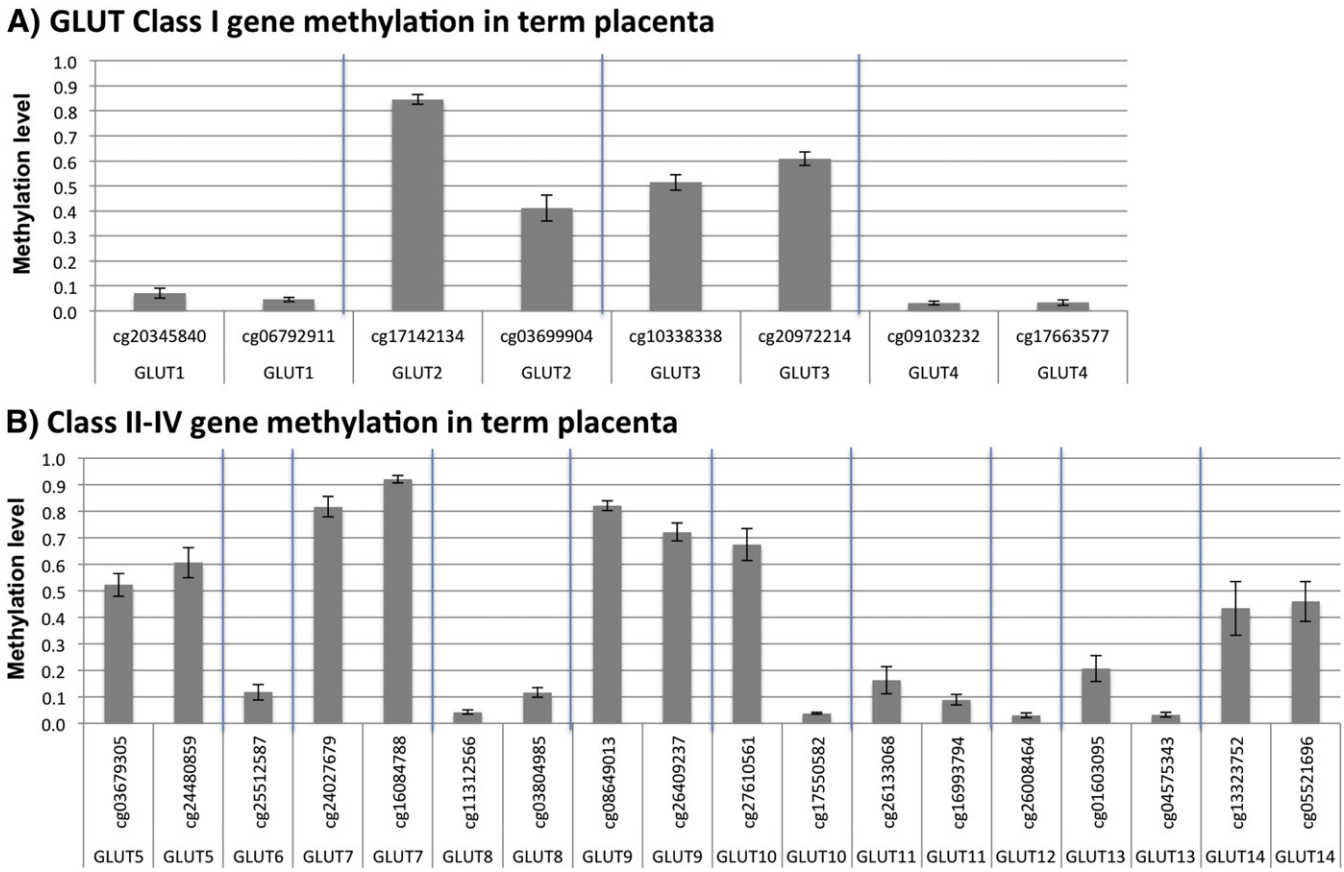

Fig. 1. Methylation of *GLUT* genes in full-term placenta. Average Infinium HM27 methylation level in third-trimester placenta (*y*-axis) was plotted for each probe linked to a *GLUT* gene (*x*-axis) (error bars=standard deviation). (A) Class I *GLUT* genes showed a range of methylation levels, with *GLUT1* and *4* showing low methylation for both probes, while *GLUT2* and *3* showed intermediate levels of methylation. (B) Class II–IV *GLUT* genes showed a range of methylation levels, with lowest levels seen for *GLUT8* and highest levels for *GLUT7*.

CpG_6.7 unit (Fig. 3, Supplementary Figure 1). This locus-specific analysis produced data that were highly correlated with the HM27 probe cg10338338 methylation levels, with $R^2=0.897$ and 0.916 for CpG_6.7 and assay mean methylation, respectively (Supplementary Figure 2). This level of correlation is comparable or higher to previously reported correlations using similar platforms [18,35] and validates the HM27 approach for the analysis of DNA methylation levels in our samples.

3.4. Complex relationship between DNA methylation and gene expression of GLUT transporters in the placenta over gestation

In order to examine the potential functional consequences of the altered DNA methylation in the placenta across gestation, we plotted the relationship between the change of methylation levels, as measured on the HM27 platform, and publically available gene expression data, measured using the HG-U133A&B Affymetrix GeneChips. Differences in methylation between first- and third-trimester placental tissue were plotted against gene expression discordance between early second-trimester and third-trimester points from unrelated individuals (Fig. 4). This revealed a clear and consistent link between increasing DNA methylation with decreasing expression of the *GLUT3* gene and a similar increase in methylation (at one of two probes) in the apparent decrease in expression of the *GLUT10* gene. No clear relationship was found between HM27 methylation levels and the apparent increasing expression of *GLUT1* and *GLUT11* with increasing gestation, or the apparent decrease in expression of *GLUT13* over the same period. Interestingly, a consistent increase in methylation of the *GLUT9* gene was noted over gestation at two independent HM27 probes, but this was not reflected in a significant change in gene expression, at least in the transcripts represented on the U133 array platform used to measure expression levels.

4. Discussion

Glucose transport across the human placenta has been extensively studied in an attempt to understand the placental contribution to fetal overnutrition, as associated with maternal diabetes, or fetal undernutrion, as found in FGR. Many studies have measured alterations in placental expression of the main glucose transporters, GLUT1 and GLUT3, in these conditions. Other members of the GLUT family have not received much attention, although their presence in the human placenta has been described. GLUT3 as a high-affinity transporter has been implicated in extraction of glucose from the fetal circulation for storage as glycogen in the placental endothelial cells [10]. GLUT3 upregulation in maternal diabetes is in line with elevated glycogen depots in the placental endothelium in this condition. Aberrant *GLUT3* expression was therefore suggested as contributing to fetal hyperglycemia in maternal diabetes [36].

The mechanisms underlying hypoglycemia, which occurs at high rates in FGR infants, are unclear [15,37]. Aberrations in placental glucose transport across the placenta have been postulated as a potential underlying mechanism; however, there are no evidence that transplacental glucose transport is unaltered in such pregnancies and no association between placental *GLUT1* expression levels and FGR pregnancies [38]. Despite this, other molecular changes in *GLUT* gene expression and FGR have been reported, including a link between

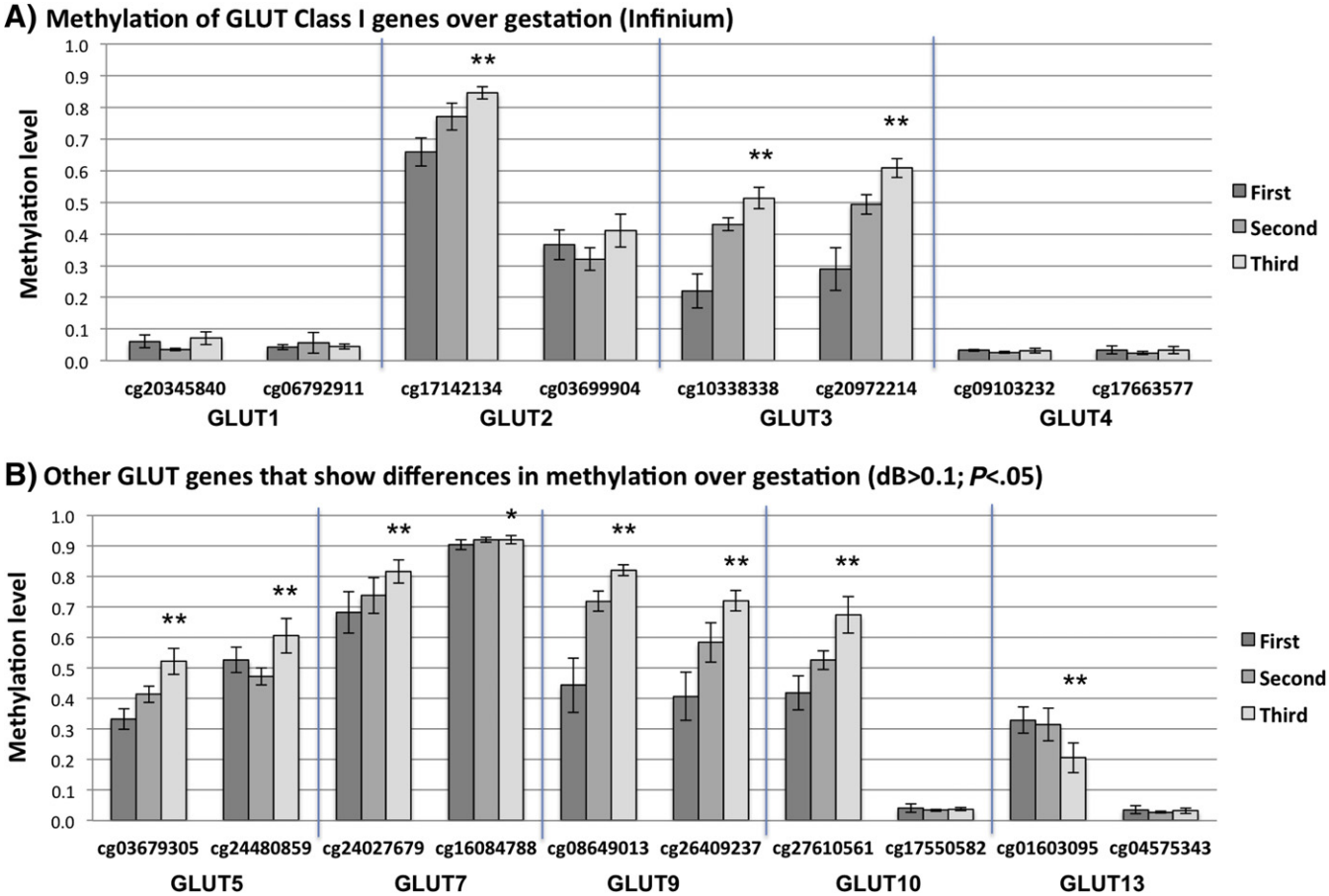

Fig. 2. Variable methylation of *GLUT* family genes across gestation. Infinium HM27 average methylation at first-, second- and third-trimester placenta is plotted on the *y*-axis for each gene (*x*-axis). Error bars=SD. (A) Class I genes *GLUT1* and *4* showed consistently low methylation across gestation, while both *GLUT2* and *GLUT3* showed increasing methylation with increasing gestation. (B) Other *GLUTs* showed a range of methylation differences between first and third trimester, with *GLUT9* showing the most consistent difference. The overall trend is an increase in methylation of *GLUT* genes over gestation. $^{*}P<.01$ and $^{**}P<.001$ between first and third trimester.

maternal undernutrition, FGR and reduced placental *GLUT3* [39]. Furthermore, lactate production has been demonstrated to be lower in FGR pregnancies, suggesting aberrations in other components of the glycolytic pathway [5]. Several studies have similarly identified altered expression of GLUT genes in GDM [40–43].

Surprisingly, little is known about the factors that regulate GLUT family gene expression in the human placenta in response to environmental or gestational factors. In particular, the role of epigenetic mechanisms, such as DNA methylation, known to be sensitive to environmental perturbation, in this process remains to be determined. DNA-methylation-mediated down-regulation of GLUT2 and 4 has previously been reported in mouse tissues [44,45]; however, human studies are lacking. As a first step to addressing this potentially important aspect of fetal growth (de)regulation by aberrant nutrition, we have explored the level of methylation of all GLUT family genes in healthy human placenta at term, the relationship between DNA methylation status and gene expression across gestation, and the level of interindividual variation in methylation at each GLUT gene.

GLUT genes show a range of promoter methylation levels in term placenta, possibly reflecting their expression level and the cell composition of the placenta at term. The low methylation of GLUT1 reflects the high expression of this gene throughout pregnancy [15]. The level of methylation for the other genes ranged from low to high methylation, with no link between the level of methylation and class of GLUT genes.

Due to its role at the interface between the maternal and fetal circulations, the human placenta is exposed to many environmental factors, which can affect its methylation patterns. A previous study identified CpG sites that show high interindividual variation in their methylation patterns in the placenta [25]. These highly variable CpG sites were defined as having a variance >0.025 and were suggested to be more susceptible to both environmental and stochastic factors during pregnancy [25]. Our group subsequently found that the number of CpG sites showing variable methylation increased over gestation. This increase in variably methylated CpG sites may be explained by the accumulation of stochastic/environmental effects and changes in placental physiology in response to fetal requirements [18]. Due to their role as placental transporters, GLUT genes may show methylation and expression differences between different individuals. In order to test this, the variance for each probe was calculated across all samples at term. Interestingly, all GLUT genes showed very low interindividual variation, with the highest variance of 0.009 for GLUT14. This suggests that GLUT promoter methylation levels may be tightly controlled in the placenta, irrespective of differing genetic background and environmental/stochastic factors. However, this will require additional testing in placental tissue from pregnancies with measured levels of these variables as such data were not available in the current study. The low level of variation is even more interesting when we consider that the samples were collected at two independent sites: Australia and Canada.

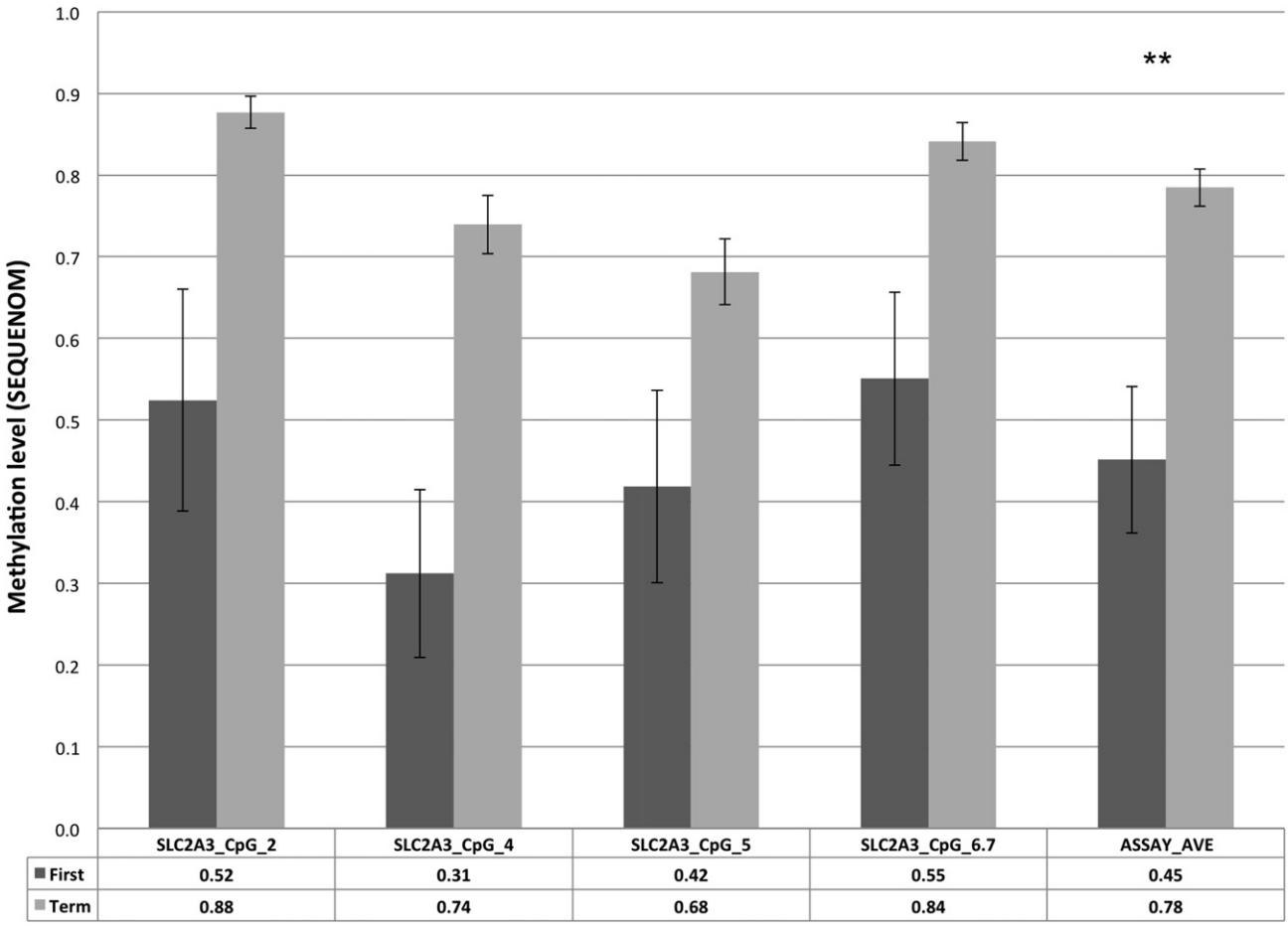

Fig. 3. Regulation of *GLUT3* high-affinity glucose transporter in placental tissue and derived cells. *GLUT3* Infinium HM27 methylation level was validated using the Sequenom EpiTYPER platform. The assay information is shown in Supplementary Figure 1. EpiTYPER results validated HM27 data, with consistently higher methylation in third trimester compared to first across all CpG sites assayed. The average increase in methylation across the assay was 0.33. $^{**}P<.001$.Regulation of *GLUT3* high-affinity glucose transporter in placental tissue and derived cells. *GLUT3* Infinium HM27 methylation level was validated using the Sequenom EpiTYPER platform. The assay information is shown in Supplementary Figure 1. EpiTYPER results validated HM27 data, with consistently higher methylation in third trimester compared to first across all CpG sites assayed. The average increase in methylation across the assay was 0.33. $^{**}P<.001$.

We next identified genes that showed differences in methylation over gestation. Of the Class I genes, GLUT1 and 4 showed very low methylation at all three gestational ages. This reflects the importance of GLUT1 as the main glucose transporter in the placenta and its ubiquitous expression across placental cell types. On the other hand, one GLUT2 and two GLUT3 probes showed significantly higher methylation at term compared to first trimester ($P<.05$). Of the other classes of GLUT genes, the largest differences between first trimester and term were observed for at least one probe in GLUT5, 7, 9 and 10. Overall, the largest and most consistent difference between first trimester and term was observed for GLUT3 and GLUT9, both of which showed an increase of $\beta>0.25$ for both probes ($P<.001$).

Given its known expression in the placenta and potential involvement in glucose back-flux from the fetal circulation into the placenta, we sought to validate the HM27 data for GLUT3 using a Sequenom EpiTYPER assay (Supplementary Figure 1). Fig. 3 shows the level of methylation of each CpG site assayed for GLUT3 and the average methylation across the assay. All CpG sites within the assay show significantly higher methylation in term placenta compared to the first trimester ($P<.001$), with an average change in methylation of 0.33 (Fig. 3). This level of methylation difference is likely to be biologically significant and agrees with the lower level of expression at term recently reported [11]. We also looked at the methylation of GLUT3 in purified first-trimester cytotrophoblasts, third-trimester endothelial cells and several trophoblast-derived cell lines (data not shown). We found low methylation in all cell types examined, which was confirmed using the Sequenom EpiTYPER platform (Supplementary Figure 2). GLUT3 expression has previously been reported in choriocarcinoma cell lines JAR, JEG-3 and BeWo cells [46,47] and endothelial cells in term placenta [10]. These data suggest that GLUT3 expression in the human placenta is mediated by promoter methylation; however, this requires direct functional testing.

In order to measure the effect of DNA methylation change over gestation to change in expression over the same period, we compared HM27 methylation data with U133 expression array data. If promoter methylation controlled the expression of a particular GLUT gene, we would expect to see an inverse relationship between promoter methylation and expression level. As expected, most of the genes that showed no difference in methylation over gestation did not show a difference in expression over this period (e.g., GLUT4, 6, 8 and 12). Several genes showed differences in methylation (GLUT9) or expression (GLUT1 and 11) over gestation, but not both. This suggests that these genes are regulated by other epigenetic mechanisms. Finally, GLUT3 showed consistent low expression at term with higher methylation, as did one probe for GLUT10. This is compelling

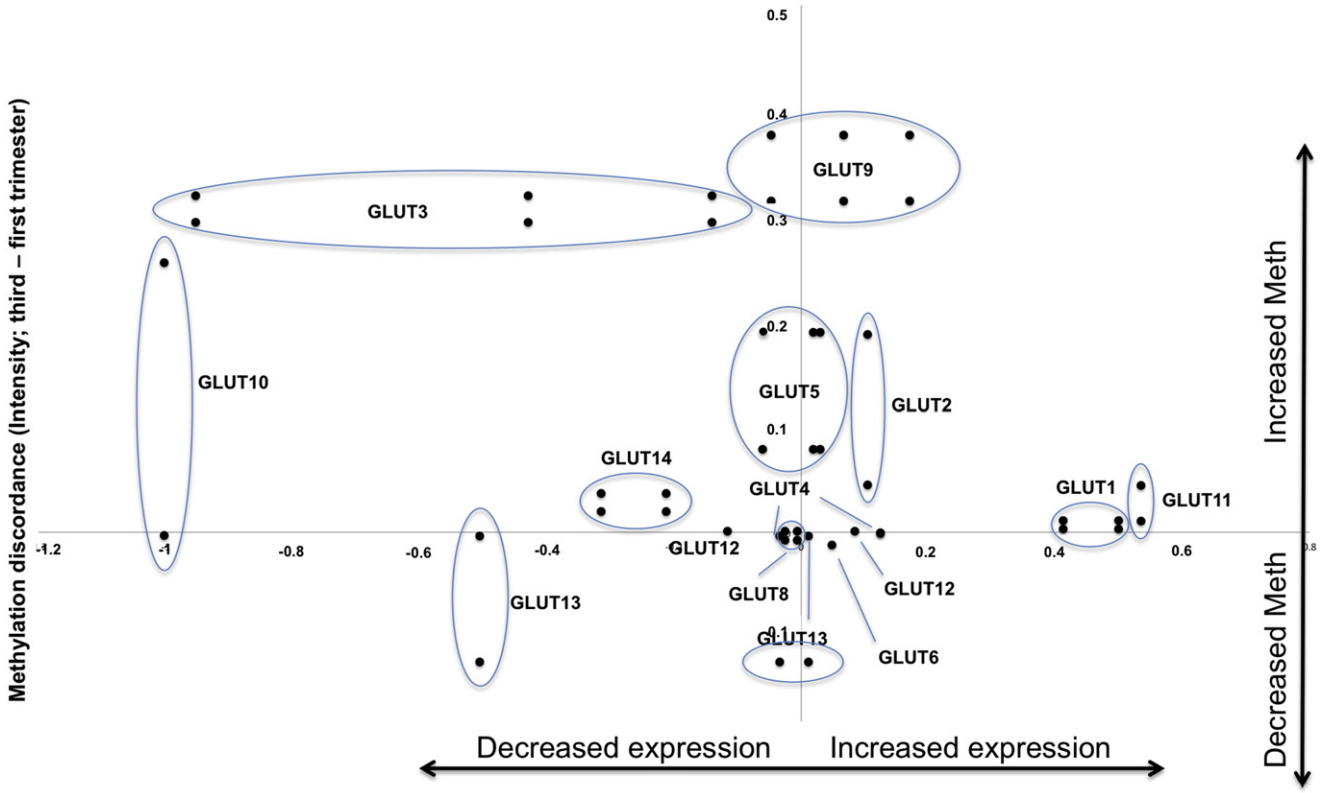

Fig. 4. Complex interplay between DNA methylation and gene expression of placental *GLUT* genes across gestation. Difference in methylation ($\Delta\beta$) for each probe across gestation [18] was plotted on the *y*-axis against difference in expression for each probe on the *x*-axis [32]. *GLUT* genes show a range of correlations between methylation and expression, with most genes showing little difference in both methylation and expression. *GLUT3* and *10* show increased methylation with decreased expression over gestation, while *GLUT9* showed high difference in methylation, with no difference in expression. Conversely, *GLUT1* and *11* showed differences in expression, with no difference in methylation. Methylation discordance vs experience discordance (every meth probe vs every expr probe [so there are multiple points per probe]).

evidence that GLUT3, and possibly GLUT10, expression is strongly influenced by promoter methylation.

The variation in both gene expression and DNA methylation levels of individual probes on the U133 expression and HM27 methylation arrays is interesting, particularly in light of the known gene variants of some of the GLUT family. For example, there are two well-characterised isoforms of the GLUT9 gene [48,49], and we identified clear evidence for increasing methylation levels in the vicinity of the GLUT9a isoform transcriptional start site across gestation, while the GLUT9b isoform transcriptional start site is not targeted by HM27. The level of GLUT9 expression does not change over this same period, which raises the potential for isoform switching or specific down-regulation of the GLUT9a isoform as gestation progresses, warranting further isoform-specific expression analysis in the future.

Despite the highly consistent data seen across individual placentas, there are several potential limitations with our study. Firstly, methylation data were acquired using the HM27 platform, which targeted two CpG sites within the promoter region of each GLUT gene. While in general surrounding CpG sites show a similar level of methylation, we cannot rule out that the CpG sites we interrogated may not represent the methylation level across the entire promoter region. However, we have shown that the Infinium probes were representative of the surrounding CpG sites within our GLUT3 Sequenom assay (Supplementary Figure 2). Secondly, the relationship between methylation and expression (Fig. 4) was based on data from unrelated individuals, raising the slim possibility that methylation does not directly alter gene expression levels. Contrary to this, we believe that combining unrelated data sets may be advantageous as it validates our data in a different population and supports the previously reported reduction in GLUT3 in third-trimester placenta [11]. Finally, we do not have information about the maternal environment, anthropometric and metabolic profile, and diet or vitamin/nutrient levels for our samples. This prevents us from linking methylation levels to a particular environmental factor (e.g., maternal serum glucose) or birth outcome. In light of recent data linking DNA methylation aberrations in placenta to gestational diabetes, preeclampsia and intrauterine growth restriction [2,20,40,50–52], it would be interesting to measure the methylation of *GLUT* genes in association with these diseases and other potential confounding factors.

In summary, our data have revealed the complex interplay between epigenetic modification and gene regulation of the *GLUT* gene family in the placenta, directly implicating increasing promoter methylation in the reduced expression of placental *GLUT3* observed in late gestation [11]. Additionally, we have confirmed variable methylation and expression of these genes in the placenta, possibly in response to both genetic and environmental factors. Further targeted studies examining this family of genes are warranted to further examine the link between glucose levels, epigenetic regulation of gene expression and fetal growth.

Supplementary data to this article can be found online at http://dx.doi.org/10.1016/j.jnutbio.2012.06.006.

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