Gestational diabetic transcriptomic profiling of microdissected human trophoblast

Muhammad Furqan Bari1,*, Sherry Ngo2,*, Claire C Bastie3, Allan M Sheppard2 and Manu Vatish4

1Department of Pathology, Dow International Medical College, Karachi, Pakistan
2Liggins Institute, University of Auckland, Auckland, New Zealand
3Division of Biomedical Sciences, Warwick Medical School, Coventry, UK
4Nuffield Department of Obstetrics and Gynaecology, University of Oxford, Oxford, UK
(*M F Bari and S Ngo contributed equally to this work)

Abstract

Gestational diabetes mellitus (GDM), the most common metabolic complication of pregnancy, is influenced by the placenta, and its prevalence directly increases with obesity. Therefore, to define the aetiology of GDM requires that the confounding influence of obesity and the heterogeneous nature of the placenta impairing accurate quantitative studies be accounted for. Using laser capture microdissection (LCM), we optimized RNA extraction from human placental trophoblast, the metabolic cellular interface between mother and foetus. This allowed specific transcriptomic profiling of trophoblast isolated from GDM, and obese and normal human placentae. Genome-wide gene expression analysis was performed on the RNA extracted from the trophoblast of GDM and obese and normal placentae. Forty-five differentially expressed genes (DEGs) specifically discriminated GDM from matched obese subjects. Two genes previously linked with GDM, pregnancy specific beta-1 glycoprotein 6 (PSG6) and placental system A sodium-dependent transporter system (SLC38A1), were significantly increased in GDM. A number of these DEGs (8 ubiquitin-conjugating enzymes (UBE) splice variants (UBE2D3 variants 1, 3, 4, 5, 6, 7, and 9) and UBE2V1 variant 4)) were involved in RNA processing and splicing, and a significant number of the DEGs, including the UBE variants, were associated with increased maternal fasting plasma glucose. It is concluded that DEGs discriminating GDM from obese subjects were pinpointed. Our data indicate a biological link between genes involved in RNA processing and splicing, ubiquitination, and fasting plasma glucose in GDM taking into account obesity as the confounder.

Key Words
- placenta
- obesity
- gestational diabetes
- laser capture microdissection
- RNA processing
- ubiquitination

Introduction

Gestational diabetes mellitus (GDM), defined as glucose intolerance resulting in hyperglycaemia of variable severity during pregnancy, is the commonest metabolic complication of pregnancy (Virjee et al. 2001), with increasing prevalence directly linked to increasing obesity in women of reproductive age (Dabelea et al. 2005). Women with GDM and obesity (and their offspring) have a higher prevalence of metabolic syndrome than those
without a history of GDM or obesity (Catalano et al. 2012; Vohr & Boney 2008) as well increased GDM recurrence in subsequent pregnancies (Bottalico 2007). Approximately 50% of women with GDM are expected to develop type 2 diabetes within 5–10 years of delivery (Bellamy et al. 2009). Other risks of GDM include an increased likelihood of pregnancy complications, such as pre-eclampsia and offspring metabolic syndrome (Boney et al. 2005; Bryson et al. 2003).

GDM is linked with aberrant placental function and a number of placenta-derived molecules implicated in the manifestation of insulin resistance associated with GDM (Desoye & Hauguel-de Mouzon 2007). The key functioning unit of the placenta is the chorionic villus, in which a multi-nucleated trophoblast (comprising of syncytiotrophoblast and cytotrophoblast) lies in direct contact with maternal blood and remains the primary site of metabolic exchange at the maternal–foetus interface (Huppertz 2008). In addition to the trophoblast, however, there is an underlying stromal tissue and foetal capillaries as well as placental macrophages (known as Hofbauer cells) (Huppertz 2008). The heterogeneous nature of this tissue has rendered quantitative studies of the trophoblast difficult. While flow cytometry and cell culture of cellular fractions can be used to examine other cell types within the placenta, the trophoblast (part of which is a true syncytiun) is not amenable to these techniques without contamination. This issue can be avoided by using LCM, a technique that allows isolation of distinct cell types (in our case, trophoblast) from frozen section or formalin-fixed samples of tissue. Studies have been hampered both by the difficulties of separating the effects of obesity from those of GDM and by extracting RNA of sufficient quality to perform meaningful downstream analysis. Thus our data allows the effects of obesity to be subtracted from GDM at the level of the trophoblast with high quality RNA subjected to microarray.

Our findings indicate that GDM placental trophoblast is fundamentally, biologically distinct from the states of normality and obesity at gene expression levels. Taking into account obesity as the confounder, genes that showed increased expression in GDM were largely involved in RNA processing and splicing. We also found increased expression of multiple splice variants of the ubiquitin-conjugating enzymes UBE2D3, and UBE2V1 variant 4, in GDM. The expression of these genes was significantly associated with maternal fasting plasma glucose, suggesting an association of these genes with glucose intolerance, a hallmark of GDM.

Materials and methods

Samples

All study participants were pregnant women scheduled for elective Caesarean section at 39–40 weeks of gestation. The indication for Caesarean section was either breech presentation or repeat Caesarean section. Oral glucose (75 g) tolerance tests (OGTTs) were carried out at 26–28 weeks of gestation in all participants to diagnose or exclude GDM. All GDM patients were well controlled on insulin and only those with male offspring were recruited. The Coventry Local Research Ethics committee approved the study, and all patients gave written informed consent (Research Ethics Committees 07/H1210/118). Women with multiple pregnancies, as well as patients with cardiovascular disease, pre-eclampsia, or other relevant diseases were excluded. Placental biopsies were taken in the operating theatre and immediately snap-frozen in liquid nitrogen and transferred to a −80°C freezer until needed. Placental biopsies (by a random sampling method (Burton et al. 2014)) from four lean (normal; Norm), four obese (Ob), and four gestational diabetic (GDM) subjects were used.

Laser microdissection (LCM): efficacy of staining and duration of LCM procedure on RNA Integrity Number (RIN)

The sections of 8 µm thickness were cut using a cryostat (Leica Microsystems, Milton Keynes, UK) at −25°C and adhered onto MembraneSlides (Molecular Machines & Industries AG, Glattbrugg, Switzerland) pre-cooled inside the cryostat and maintained dehydrated in −80°C freezer until needed. All slides, LCM caps, and solutions were obtained from Molecular Machines & Industries. The slides were dehydrated in graded alcohols and xylene and placed in a desiccator until laser capture. The sections were stained with (a) Acridine orange (Life Technologies), (b) Alcoholic cresyl violet (Life Technologies), (c) Alcoholic cresyl violet and alcoholic eosin Y (Sigma-Aldrich), (d) Arcturus Paradise stain (Life Technologies) in combination with RNAse inhibitor, (e) Arcturus Paradise stain, (f) haematoxylin (Leica Microsystems, Milton Keynes, UK) and alcoholic eosin Y in combination with RNAse inhibitor, and (g) haematoxylin and alcoholic eosin Y together with alcoholic eosin-Y. RIN values were assessed using Nano/Pico Chips on an Agilent 2100 Bioanalyzer (Agilent Technologies Inc.) (Clement-Ziza et al. 2008). Having identified the optimal stain, LCM was performed in 0, 30 and 50 min to assess the effect of duration of LCM on RIN values.

et al. 2005; Bryson et al. 2003).
RNA extraction and gene expression profiling

The RNA was extracted using RNeasy Micro Kit (Qiagen Inc.) for the analysing RIN on the Bioanalyzer, while for gene expression profiling, microdissected samples were exposed to Prelude Direct Lysis Module (NuGEN, CA, USA) and hybridized to Affymetrix HuGene ST 1.0 arrays using manufacturer's protocols (Affymetrix Inc.). One chip was used for each clinical sample. Raw intensity data files (.CEL) were read using the publicly available oligo package in R (Carvalho et al. 2007). Background subtraction, quantile normalization and summarization via median-polish, and finally log2 transformed were performed on the raw data using the R package RNA (Robust Multichip Average preprocessing methodology) (Irizarry et al. 2003). Analysis of differential gene expression using a linear models approach was performed using the R package Limma (Smyth 2004). Adjusted P-values <0.05 using FDR correction were considered statistically significant. Results were filtered using ranked two-fold change in gene expression from pairwise comparisons between the three subject groups (Norm, Ob and GDM). Heatmap illustration of sample hierarchical clustering was generated using the R package gplots (http://www.cran.r-project.org/).

Validation of gene expression microarray data using qRT-PCR

The four genes that were upregulated by two-fold in GDM group compared with Ob and Norm groups were selected for validating the gene expression microarray data in the same four samples for each group as used for gene expression microarrays. All experiments were carried out in triplicate. The genes and primers sequences used in this study are UBE2V1 (Forward: CAAGACGACGCAAGATG; Reverse: TCTGGGTATTAGTGTCACATT); UBE2D3 transcript variant 6 (Forward: GCAGAAAGGATACCTGTG; Reverse: CAGATGTCTCTGCCTCTCA); UBE2D3 transcript variant 5 (Forward: CATTGGAGGGCGGACC; Reverse: AAATCGTGGCTGCTGTA), and PSG6 Forward: CCTATACATCACTACATACAA; Reverse: TCCGA CTCCTAGGCAC). All the primers were designed as described previously (Bari et al. 2013). RNA from laser microdissected samples was extracted using the Arcturus PicoPure RNA Isolation Kit (Life Technologies) with on-column DNase digestion (Qiagen) and converted to cDNA using High-Capacity cDNA Reverse Transcription Kit (Life Technologies) as recommended by the manufacturer. Genomic DNA was digested using recombinant shrimp DNase (Affymetrix). The qRT-PCR was performed using SYBR green-based chemistry on an Applied Biosystems 7500 Fast PCR system using Fast SYBR green master mix (Life Technologies), following the manufacturer’s recommendations. The qRT-PCR data were analysed using DataAssist software ver 3.01 (Life Technologies), 18S was taken as endogenous control, and P-values were adjusted using Benjamini-Hochberg false discovery rate.

Statistical analysis

All statistical analysis was performed in R (http://www.bioconductor.org/). Assessment of statistically significant difference in clinical measures between subject groups was performed using Tukey method of multiple comparisons test. Linear regression analysis was performed to determine association of gene expression with maternal fasting plasma glucose using deltaGT. ‘deltaGT’ was calculated as [Glucose]2h − [Glucose]baseline measured using oral glucose tolerance test. KEGG pathway data base is available at http://www.genome.jp/kegg/pathway.html

Results

Maternal and foetal clinical characteristics

The subjects used in this study averaged 39 weeks of gestation at delivery (Table 1). The BMI for Norm group (24.1 ± 1.0 kg/m²) was significantly lower than those in GDM (32.3 ± 0.8 kg; P = 0.0001) or Ob (30.8 ± 0.3; P = 0.0003) groups. Baseline glucose levels were similar between all subjects (GDM: 5.3 ± 0.2 mM; Norm: 5 ± 0.2 mM; Ob: 5.3 ± 0.1 mM). As expected, fasting plasma glucose following an oral glucose tolerance test (OGTT) (8.6 ± 0.3 mM) was significantly increased in GDM subjects compared with Norm (5.5 mM; P = 3.2 × 10⁻⁶) or Ob subjects (5.6 ± 0.1 mM; P = 3.2 × 10⁻⁶). Maternal insulin resistance has been recently reported to correlate with foetal insulin resistance (Wang et al. 2013). In our study, maternal insulin level was mildly elevated in GDM (25 ± 12.3 μIU/mL) and Ob (22 ± 3.5 μIU/mL) groups compared with Norm (14 ± 8.1 μIU/mL) group, albeit not significant. Maternal glucose was mildly elevated in the GDM (4.8 ± 0.3 mM) compared with Ob (4.3 ± 0.2 mM) or Norm (4.4 ± 0.1 mM) group. Foetal insulin level was lower in GDM (6.1 ± 0.7 μIU/mL) group compared with Norm (12.4 ± 1.2 μIU/mL; P = 0.11) or Ob (15.8 ± 4.4 μIU/mL; P = 0.07) group. Foetal glucose was mildly elevated in GDM (4.6 ± 0.3 mM) group compared with Norm
Table 1  Subjects characteristics.

<table>
<thead>
<tr>
<th>Variable</th>
<th>GDM Mean</th>
<th>S.E.M.</th>
<th>Norm Mean</th>
<th>S.E.M.</th>
<th>Ob Mean</th>
<th>S.E.M.</th>
<th>P-value</th>
<th>GDM min</th>
<th>GDM max</th>
<th>Norm min</th>
<th>Norm max</th>
<th>Ob min</th>
<th>Ob max</th>
</tr>
</thead>
<tbody>
<tr>
<td>Maternal characteristics</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Age (years)</td>
<td>32.8</td>
<td>3.0</td>
<td>34.5</td>
<td>1.3</td>
<td>34.3</td>
<td>5.1</td>
<td>n.s.</td>
<td>27.0</td>
<td>41.0</td>
<td>32.0</td>
<td>38.0</td>
<td>22.0</td>
<td>47.0</td>
</tr>
<tr>
<td>Weeks of gestation</td>
<td>38.6</td>
<td>0.3</td>
<td>39.4</td>
<td>0.1</td>
<td>39.1</td>
<td>0.3</td>
<td>n.s.</td>
<td>38.1</td>
<td>39.4</td>
<td>39.3</td>
<td>39.6</td>
<td>38.3</td>
<td>39.6</td>
</tr>
<tr>
<td>Body mass index (kg/m²)</td>
<td>32.3</td>
<td>0.8</td>
<td>24.1</td>
<td>1.0</td>
<td>30.8</td>
<td>0.3</td>
<td>0.0001 (N vs GDM)</td>
<td>0.0003 (N vs Ob)</td>
<td>1.5</td>
<td>1.6</td>
<td>1.6</td>
<td>1.7</td>
<td></td>
</tr>
<tr>
<td>OGTT at baseline (mM)</td>
<td>5.3</td>
<td>0.2</td>
<td>5.0</td>
<td>0.2</td>
<td>5.3</td>
<td>0.1</td>
<td>n.s.</td>
<td>4.9</td>
<td>5.8</td>
<td>4.6</td>
<td>5.3</td>
<td>5.0</td>
<td>5.6</td>
</tr>
<tr>
<td>OGTT post glucose load (mM)</td>
<td>8.6</td>
<td>0.3</td>
<td>5.5</td>
<td>0.0</td>
<td>5.6</td>
<td>0.1</td>
<td>3.2x10⁻⁴ (N vs GDM)</td>
<td>3.9x10⁻⁶ (GDM vs Ob)</td>
<td>8.0</td>
<td>9.2</td>
<td>5.4</td>
<td>5.6</td>
<td></td>
</tr>
<tr>
<td>Maternal haemoglobin (g/dL)</td>
<td>11.3</td>
<td>0.3</td>
<td>12.5</td>
<td>0.3</td>
<td>10.8</td>
<td>0.9</td>
<td>n.s.</td>
<td>10.5</td>
<td>11.8</td>
<td>11.9</td>
<td>13.3</td>
<td>8.4</td>
<td>12.9</td>
</tr>
<tr>
<td>Maternal insulin (μIU/mL)</td>
<td>25.6</td>
<td>12.3</td>
<td>14.5</td>
<td>3.5</td>
<td>22.4</td>
<td>8.1</td>
<td>n.s.</td>
<td>5.6</td>
<td>61.2</td>
<td>8.8</td>
<td>24.7</td>
<td>7.7</td>
<td>42.3</td>
</tr>
<tr>
<td>Maternal glucose (mM)</td>
<td>4.8</td>
<td>0.3</td>
<td>4.4</td>
<td>0.1</td>
<td>4.3</td>
<td>0.2</td>
<td>n.s.</td>
<td>4.0</td>
<td>5.6</td>
<td>4.2</td>
<td>4.6</td>
<td>3.9</td>
<td>4.9</td>
</tr>
<tr>
<td>Maternal HOMA-IR</td>
<td>5.8</td>
<td>3.2</td>
<td>2.9</td>
<td>0.7</td>
<td>4.5</td>
<td>1.8</td>
<td>n.s.</td>
<td>1.1</td>
<td>15.2</td>
<td>1.7</td>
<td>4.9</td>
<td>1.4</td>
<td>9.2</td>
</tr>
<tr>
<td>Maternal leptin (pg/mL)</td>
<td>44,782.1</td>
<td>5407.3</td>
<td>18,554.2</td>
<td>1289.3</td>
<td>38,175.3</td>
<td>2647.7</td>
<td>0.01 (N vs Ob)</td>
<td>0.001 (N vs GDM)</td>
<td>33,287.2</td>
<td>59,049.3</td>
<td>15,427.0</td>
<td>21012.9</td>
<td>31,500.7</td>
</tr>
<tr>
<td>Foetal characteristicsᵃ</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Foetal insulin (μIU/mL)</td>
<td>6.1</td>
<td>0.7</td>
<td>12.4</td>
<td>1.2</td>
<td>15.8</td>
<td>4.4</td>
<td>0.069 (GDM vs Ob)</td>
<td>0.007 (GDM vs Ob)</td>
<td>4.8</td>
<td>8.1</td>
<td>10.3</td>
<td>15.8</td>
<td>3.0</td>
</tr>
<tr>
<td>Foetal glucose (mM)</td>
<td>4.6</td>
<td>0.3</td>
<td>3.9</td>
<td>0.3</td>
<td>4.0</td>
<td>0.1</td>
<td>n.s.</td>
<td>3.9</td>
<td>5.4</td>
<td>3.2</td>
<td>4.5</td>
<td>3.7</td>
<td>4.2</td>
</tr>
<tr>
<td>Foetal HOMA-IR</td>
<td>1.3</td>
<td>0.2</td>
<td>2.2</td>
<td>0.3</td>
<td>2.8</td>
<td>0.8</td>
<td>n.s.</td>
<td>0.9</td>
<td>1.9</td>
<td>1.5</td>
<td>2.8</td>
<td>0.6</td>
<td>4.3</td>
</tr>
<tr>
<td>Foetal leptin (pg/mL)</td>
<td>4352.7</td>
<td>400.5</td>
<td>1702.3</td>
<td>136.8</td>
<td>7354.7</td>
<td>1371.0</td>
<td>0.071 (GDM vs Ob)</td>
<td>0.002 (N vs Ob)</td>
<td>3765.8</td>
<td>5522.6</td>
<td>1429.3</td>
<td>2062.9</td>
<td>3954.3</td>
</tr>
<tr>
<td>Foetal birthweight (kg)</td>
<td>3.70</td>
<td>0.05</td>
<td>3.42</td>
<td>0.09</td>
<td>3.81</td>
<td>0.06</td>
<td>0.005 (N vs Ob)</td>
<td>0.01 (GDM vs Ob)</td>
<td>3.59</td>
<td>3.82</td>
<td>3.1</td>
<td>3.55</td>
<td>3.7</td>
</tr>
<tr>
<td>Foetal length</td>
<td>48.0</td>
<td>1.47</td>
<td>47.8</td>
<td>0.62</td>
<td>49.8</td>
<td>1.7</td>
<td>n.s.</td>
<td>45.0</td>
<td>52.0</td>
<td>46.0</td>
<td>49.0</td>
<td>45.0</td>
<td>53.0</td>
</tr>
</tbody>
</table>

Maternal and foetal clinical measures of the Norm, GDM, and Ob subjects expressed as mean ± standard error or the mean (s.e.m.), and minimum (min) and maximum (max) value. Statistical significance was performed using the Tukey’s method of multiple comparisons test. OGTT, oral glucose tolerance test. n.s., not significant (P > 0.05).
ᵃFrom cord blood collected at delivery.
(3.9 ± 0.3 mM) or Ob (4.0 ± 0.1 mM) group, but was not statistically significant. We found maternal leptin was significantly elevated in GDM (44,782.1 ± 5407.3 pg/mL; P = 0.001) and Ob (38,175.3 ± 2647.7 pg/mL; P = 0.01) compared groups with Norm (18,554.2 ± 1289.3 pg/mL) group. Similarly, foetal leptin was significantly elevated in GDM (45,782.1 ± 5407.3 pg/mL; P = 0.001) and Ob (38,175.3 ± 2647.7 pg/mL; P = 0.01) compared with Norm (17,554.2 ± 1289.3 pg/mL) group.

**Acridine orange and alcoholic cresyl violet stains are most effective in preserving human placental trophoblast RNA**

LCM was performed as described above (Fig. 1). The tissues processed for RNA with all steps of hydration and dehydration without any stain yielded a RNA Integrity Number (RIN) of 8.6 (Supplementary Figure 1, see section on supplementary data given at the end of this article). Staining with Acridine orange gave the highest RIN value of 8.5 (a drop of only 0.1; Supplementary Figure 1). However as the filter for this fluorescent dye is not available in all instruments, we used other non-fluorescent dyes as well. Among the non-fluorescent dyes, the alcoholic cresyl violet was the most effective dye in preserving RIN and yielded a RIN of 8.0 (drop of 0.6; Supplementary Figure 1). As cytoplasmic staining was also needed in dissecting the trophoblast, it was combined with alcoholic eosin Y, which dropped the RIN to 7.8 (drop of 0.8; Supplementary Figure 1). The Arcturus Paradise stain with the addition of RNase inhibitor at a concentration of 1 unit/µL of stain yielded a RIN of 6.7 (drop of 1.9; Supplementary Figure 1), whereas the use of Arcturus Paradise stain alone dropped the RIN to 6.0 (drop of 2.6; Supplementary Figure 1). In contrast, the use of routine haematoxylin in combination with alcoholic eosin Y together with RNase inhibitor yielded a RIN of 5.4 (drop of 3.2; Supplementary Figure 1) and finally haematoxylin with alcoholic eosin Y without the RNase inhibitor dropped the RIN to 3.0 (drop of 5.6; Supplementary Figure 1). Having identified alcoholic cresyl violet and alcoholic eosin-Y as the optimal non-fluorescent stain, we next assessed the effect of duration of LCM on RIN values.

**LCM within 30 min maintains RIN**

Time points at 0, 30, and 50 min were selected and resulting RIN values 7.5, 6.9, and 6.2 respectively (Supplementary Figure 2) indicated that the degradation is proportional to the duration of dissection; with 30 min being optimal to allow laser-capture to be performed while maintaining RNA integrity. Tissue extraction for microarray experiments was thus performed under 30 min in tissues stained with alcoholic cresyl violet and alcoholic eosin-Y. The tissues destined for microarray were placed directly in the
NuGEN Direct Lysis Module before hybridisation on Affymetrix HuGene ST 1.0 array.

**Differential gene expression profiles of Norm, Ob, and GDM subjects**

Among the 28,869 transcript-level probes comprising the main design of the microarray, 329 probes exhibited ranked differential gene expression of at least two-fold change (up/down) between the subject groups. Of these, 130 transcripts were annotated with Entrez Gene ID (corresponding to 106 genes) and these were used for downstream functional enrichment analysis. We assessed the gene expression profile differences between Norm, Ob, and GDM subjects. We found 46 DEGs from Norm versus GDM comparison (Contrast 1); 23 genes from Norm versus Ob (Contrast 2); 70 genes from GDM vs Ob comparison (Contrast 3) (Fig. 2A). Of the DEGs from Contrast 1, 19 DEGs were unique to this pairwise comparison; these genes characterise GDM subjects who are also obese. We found that nine DEGs were unique to Contrast 2 i.e., Ob individuals compared with normal individuals. In contrast, 45 DEGs were unique to Contrast 3 wherein GDM individuals were subject-matched to obese individuals. As such, genes of interest from Contrast 3 characterise a state of GDM taking into account obesity as a confounder (Fig. 2A). We also found that 8 DEGs were common between Contrast 1 and Contrast 2. In addition, 6 DEGs were common between Contrast 2 and Contrast 3, whereas 19 genes were common between Contrast 1 and 3 (Fig. 2A). The list of DEGs identified from each pairwise comparison (Contrast 1–3) is found in Supplementary Table 1 (see section on supplementary data given at the end of this article). Probe level information including annotated GenID and fold change in gene expression between subject groups are listed in Supplementary Table 2.

Array intensities (after preprocessing and data normalization) of all probes for genes with at least two-fold change (i.e., 391 probes) were visualized using a heatmap (Fig. 2B). As illustrated in the dendrogram on the horizontal axis of the heatmap, hierarchical sample clustering generated two main spot clusters. The

**Figure 2**

Gene expression profile characterising Norm, Ob, and GDM subjects. (A) The number of differentially expressed genes that are unique or common between Norm, Ob, and GDM pairwise comparisons. Contrast 1: Norm vs GDM; Contrast 2: Norm vs Ob; Contrast 3: GDM vs Ob. Criterion: >2 ranked fold change (up/down) between any pairwise comparison. (B) Hierarchical clustering of subjects showing GDM as distinctly different from Norm and Ob individuals. Array intensities for probes with >2-fold change (up/down) between any pairwise comparisons were centred and scaled (row z-score), and then used to generate the heatmap. Agglomeration method: complete linkage. Distance measure: Euclidean. Red-Black-Green color key denotes low to high gene expression. "_Integer" on the horizontal axis denotes individual subjects in each group.
GDM subjects comprise one spot cluster, whereas the Norm and Ob subjects comprise the second larger spot cluster. Overall, the heatmap illustrates GDM subjects as a distinct group of individuals relative to Norm and Ob subjects based on their gene expression profiles. In support of this result, functional Gene Ontology term enrichment analysis showed that the significantly enriched GO terms for Contrast 1 and Contrast 2 were virtually identical and related to a wide range of processes including protein metabolic process, RNA processing, gene expression, and translational processes with the exception of GO:0022904 (respiratory electron...
transport chain) which was associated with Contrast 1 but not Contrast 2. However, the significantly enriched GO terms for Contrast 3 are distinct from those of both Contrasts 1 and 2: enriched GO terms annotated to RNA splicing processes, intracellular transport of proteins to Golgi (Supplementary Table 3). Similarly, several KEGG pathways including oxidative phosphorylation (Kegg:00190) and ribosome (Kegg:03010) pathways were predicted as being significantly enriched in both Contrasts 1 and 2. However, only the spliceosome pathway (Kegg:03040) was significantly over-represented in Contrast 3 (Supplementary Table 4). Three miRNAs (hsa-miR-876-5p, hsa-miR-151-3p, hsa-miR-641) were predicted as being significantly over-represented in Contrast 3 (GDM), whilst no miRNA enrichment was found for Contrasts 1 and 2 (Supplementary Table 5), suggesting that these miRNAs may be the useful indicators of GDM taking into account obesity as the confounder.

**Genes uniquely differentially expressed between GDM and Ob subjects correlate with maternal fasting plasma glucose**

Given that elevated fasting plasma glucose following an OGTT characterises a state of GDM, regression analyses were performed to determine correlations between the DGEs unique to Contrast 3 (GDM vs Ob) and maternal OGTT response. As illustrated in Fig. 3, among the DEGs, 17 genes were significantly associated with maternal fasting plasma glucose (P<0.05; Fig. 3). The expression of these genes (EIF1AXP1, FMR1, HNRNPA3, HNRNPC, IL6ST, OSER1, PSG6, RASA1, RGPD5, RGPD8, SCARNA7, SETDS, SLC38A1, SMA4, SNORD13, SNORD13P3, and ZNF91) was increased in GDM by two- to five-fold compared with Ob group (Fig. 4). Of these, several are known to be involved in RNA processing and splicing (FMR1, HNRNPA3, HNRNPC, SCARNA7, SNORD13); full gene names are found in Fig. 4 legend.

![Figure 4](http://joe.endocrinology-journals.org)
We also assessed the presence of differentially expressed splice variants between Norm, Ob, and GDM groups. We applied the two-fold change criterion to assess ‘lmmRNA’ probes (which are assigned to mRNA transcripts based on the Affymetrix annotation metafile). We found several differentially expressed transcript variants in Contrast 1 (Norm vs GDM), Contrast 2 (Norm vs Ob), and Contrast 3 (GDM vs Ob) (Table 2). Interestingly, transcript variants of ubiquitin-conjugating enzymes (UBE2D3) and (UBE2V1) were heavily represented in Contrasts 1 and 3. The levels of UBE2D3 transcript variants were increased in GDM group by 2.5- to 3.0-fold relative to Norm, and 2.8- to 3.3-fold relative to Ob groups. Similarly, UBE2V1 transcript variant 4 level was increased in the GDM group by three-fold compared with Norm, and by four-fold compared with Ob group (Table 2). Distribution of the log2 intensities of these eight mRNA variants across the Norm, Ob, and GDM groups is shown in Fig. 5A. Microarray data were further confirmed by separate qRT-PCR (Supplementary Table 6). Furthermore, our regression analysis showed that increased maternal fasting plasma glucose (post OGTT) is significantly associated with increased levels of these eight UBE variants (Fig. 5B; P < 0.05). These results suggest that increased UBE2D3 and UBE2V1 expression is associated with maternal glucose intolerance, which characterizes a state of GDM.

Table 2  Differentially expressed mRNA transcripts in GDM compared with Norm or Ob subjects.

<table>
<thead>
<tr>
<th>Group</th>
<th>FC</th>
<th>mRNA_AccNo</th>
<th>mRNA_Name</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 (Norm-v-GDM)</td>
<td>−2.68</td>
<td>NM_003340.4</td>
<td>Homo sapiens ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)</td>
</tr>
<tr>
<td></td>
<td>−2.78</td>
<td>NM_181887.1</td>
<td>Homo sapiens ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)</td>
</tr>
<tr>
<td></td>
<td>−2.84</td>
<td>NM_181888.1</td>
<td>Homo sapiens ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)</td>
</tr>
<tr>
<td></td>
<td>−2.93</td>
<td>NM_181889.1</td>
<td>Homo sapiens ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)</td>
</tr>
<tr>
<td></td>
<td>−2.63</td>
<td>NM_181890.1</td>
<td>Homo sapiens ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)</td>
</tr>
<tr>
<td></td>
<td>−3.01</td>
<td>NM_181891.1</td>
<td>Homo sapiens ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)</td>
</tr>
<tr>
<td></td>
<td>−2.47</td>
<td>NM_181893.1</td>
<td>Homo sapiens ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)</td>
</tr>
<tr>
<td></td>
<td>−2.21</td>
<td>NM_001187.1</td>
<td>Homo sapiens B melanoma antigen (BAGE), mRNA</td>
</tr>
<tr>
<td></td>
<td>2.07</td>
<td>NM_001029945.1</td>
<td>Homo sapiens chromosome 1 open reading frame 178 (C1orf178), transcript</td>
</tr>
<tr>
<td></td>
<td>−3.26</td>
<td>NM_001032288.1</td>
<td>Homo sapiens ubiquitin-conjugating enzyme E2 variant 1 (UBE2V1), transcript</td>
</tr>
<tr>
<td>2 (Norm-v-Ob)</td>
<td>2.10</td>
<td>NM_145326.1</td>
<td>Homo sapiens similar to hypothetical protein FLJ13659 (LOC115648), mRNA</td>
</tr>
<tr>
<td></td>
<td>2.09</td>
<td>NM_001014450.1</td>
<td>Homo sapiens small proline-rich protein 2F (SPRR2F), mRNA</td>
</tr>
<tr>
<td>3 (GDM-v-Ob)</td>
<td>3.01</td>
<td>NM_145326.1</td>
<td>Homo sapiens similar to hypothetical protein FLJ13659 (LOC115648), mRNA</td>
</tr>
<tr>
<td></td>
<td>3.08</td>
<td>NM_003340.4</td>
<td>Homo sapiens ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)</td>
</tr>
<tr>
<td></td>
<td>2.94</td>
<td>NM_181887.1</td>
<td>Homo sapiens ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)</td>
</tr>
<tr>
<td></td>
<td>3.15</td>
<td>NM_181888.1</td>
<td>Homo sapiens ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)</td>
</tr>
<tr>
<td></td>
<td>3.23</td>
<td>NM_181889.1</td>
<td>Homo sapiens ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)</td>
</tr>
<tr>
<td></td>
<td>2.84</td>
<td>NM_181890.1</td>
<td>Homo sapiens ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)</td>
</tr>
<tr>
<td></td>
<td>3.30</td>
<td>NM_181891.1</td>
<td>Homo sapiens ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)</td>
</tr>
<tr>
<td></td>
<td>2.77</td>
<td>NM_181893.1</td>
<td>Homo sapiens ubiquitin-conjugating enzyme E2D 3 (UBC4/5 homolog, yeast)</td>
</tr>
<tr>
<td></td>
<td>4.16</td>
<td>NM_001032288.1</td>
<td>Homo sapiens ubiquitin-conjugating enzyme E2 variant 1 (UBE2V1), transcript</td>
</tr>
</tbody>
</table>

FC denotes fold change in expression. Positive FC indicates increased expression in Norm for Contrasts 1 and 2, and increased expression in GDM for Contrast 3. mRNA_AccNo denotes GenBank Accession Number.
Discussion

The impact of GDM on the progeny is made difficult to assess because of the confounding influence of maternal obesity in the manifestation of foetal phenotypes. The strength of the current study is the inclusion of obese subjects for comparison with GDM subjects with the aim of more accurately defining the molecular basis for disturbed placental function in GDM pregnancies. Indeed, our findings suggest that genes involved in RNA splicing processes and the intracellular transport of proteins to the Golgi apparatus may be differentially expressed in GDM subjects compared with normal or obese subjects, implying that GDM may be fundamentally distinct from states of normality or obesity at the molecular level.

Additional strengths include careful selection of patient to exclude other pathologies that might have impacted data analysis, optimization of RNA integrity using a variety of commonly used stains, and finally using patients who had undergone elective Caesarean section, which excluded potential stresses placed on the placenta by labour (Reddy et al. 2008).

A further strength is our approach of using LCM to investigate the changes in placental trophoblast gene expression. As the placenta is a heterogeneous organ (Huppertz 2008), our approach of using LCM facilitates molecular analysis of trophoblast without contamination from other cell types. In this study, we present an optimized protocol for RNA extraction using LCM, which demonstrates that RNA integrity (RIN) is sustained when staining with alcoholic cresyl violet and alcoholic eosin-Y (Clement-Ziza et al. 2008). Moreover, we demonstrate that LCM can be performed with this stain for up to 30 min per slide with no significant degradation of RNA. We note that other publications using laser capture of placenta (Kim et al. 2013b) have used haematoxylin and eosin (which we have shown does not stabilize RNA) without demonstrating any formal assessment of RIN before hybridisation.

GDM is linked to placental function, and a number of placenta-derived molecules in particular from the trophoblast have been implicated in causing insulin resistance in GDM (Desoye and Hauguel-de Mouzon 2007). The expression of pregnancy-specific 1 glycoprotein (PSG), a common placental factor produced by trophoblast to allow for a viable pregnancy, has been reported to increase in preeclampsia (also known to be associated with increased insulin resistance (Catalano...
et al. 2012) and GDM (Okazaki et al. 2007, Tamsen et al. 1983). Increased PSG levels are associated with elevated carbohydrate levels in humans with GDM (Neufeld et al. 1984). Consistent with this, we found increased PSG6 expression in GDM group compared with Ob group. In addition, increased PSG6 expression in our GDM subjects significantly associated with increased maternal fasting plasma glucose as a result of impaired glucose tolerance. Moreover, PSG6 and PSG11 genotypes have recently been found to be located with a genomic region enriched in segmental duplication in preeclampsia patients (Zhao et al. 2012) (a disease, perhaps suggesting a linkage between PSG6 and GDM and preeclampsia). Further studies into how glucose modulates placental PSG6 function may improve our understanding of the pathogenesis of GDM.

The trophoblast cell layer, in direct contact with maternal blood, is the transporting epithelium of the human placenta integrating foetal and maternal signals to modulate placental growth in response to alterations in maternal nutrient supply (Jansson & Powell 2013). The placental system A sodium-dependent transporter system, comprising of the members SNAT1 (SLC38A1), SNAT2 (SLC38A2), and SNAT4 (SLC38A4), is important for supplying neutral amino acids necessary for foetal growth (Jansson & Powell 2013). In addition, SLC38A1 is a key contributor to System A activity in term placenta (Desforges et al. 2010). The molecular mechanisms by which diabetes in pregnancy affects placental nutrient transport is still unclear; however Jansson and coworkers reported that System A-mediated amino acid transport activity is significantly increased in the trophoblast microvillous plasma membrane (MVM) in GDM, and the authors suggested the increase to be related to increased amino acid catabolism in the placenta (Jansson et al. 2002). In another study, SLC38A1 mRNA levels were found to be unchanged in placentae from preeclampsia, intrauterine growth-restricted, and small-for-gestational-age pregnancies i.e., models of intrauterine undernutrition. The authors concluded that regulation might occur at the transporter expression level or activity (Malina et al. 2005). Indeed, other studies found decreased System A transport activity in the MVM of intrauterine growth compared with the MVM of normal foetal growth (Glazier et al. 1997, Jansson et al. 2002). Our findings of increased SLC38A1 expression in trophoblast (Fig. 3) are in agreement with Jansson and coworkers study who reported increased transport activity with this transporter in the MVM from GDM pregnancies.

The novel finding of increased expression of ubiquitin-conjugating enzyme UBE2D3 and UBE2V1 transcript gene profiling in GDM is intriguing. The ubiquitin–proteasome system is multi-functional (Lecker et al. 2006); it is involved in rapid removal of unwanted or misfolded proteins (Lecker et al. 2006) and tightly regulates gene transcription (Dhananjayan et al. 2005, Lecker et al. 2006) and immunological processes (Lecker et al. 2006). Ubiquitin-conjugating enzymes (UBE2 variants facilitate the binding of ubiquitin molecules to ubiquitin ligases (UBE3 variants) to target a protein for proteasome-dependent degradation (David et al. 2010). A recent study has reported the potential association of UBE2E2 polymorphisms with fasting plasma glucose and GDM (Kim et al. 2013a). Furthermore, UBE2 variants D1 and V2 have been recently found to be significantly differentially methylated in 8 to 12-year-old children exposed to maternal GDM in utero (West et al. 2013). Our finding and the emerging literature collectively suggest a link between UBE function and fasting plasma glucose in GDM.

The pattern of the DEGs we observed suggests that the ubiquitin-conjugating system is functionally more active in GDM compared with Ob, implying greater turnover rates of translation products. Interestingly, a recent proteomics study has reported accumulation of misfolded proteins in the urine of preeclampsia subjects, thus implying compromised proteasome function (Buhimschi et al. 2014). Given that ubiquitination and the proteasomal system are tightly coupled, it is conceivable that perturbed regulation of protein turnover may underlie the pathogenesis of common pregnancy disorders such as GDM and preeclampsia. That these phenotypes may share the elements of common aetiology are perhaps not entirely unexpected. A considerable literature describes a propensity for maternal GDM to be associated with postnatal occurrence of offspring insulin resistance (reviewed in (Borgono et al. 2012)). Similarly, mid-trimester maternal insulin resistance has been identified as a possible potentiating factor in the incidence of preeclampsia (Hauth et al. 2011). Furthermore, the incidence of preeclampsia has also been identified as an harbinger of infant type 2 diabetes (Libby et al. 2007). Collectively, these observations suggest that maternal insulin resistance in pregnancy increases the likelihood of preeclampsia complications at parturition, and that both clinical features are strongly associated with the subsequent emergence of metabolic syndrome in the infant. Our findings warrant expansion into larger studies using populations representing wide multi-cohort comparisons, and encourage the development of appropriate in vitro models to functionally validate the relevance of the emerging candidate genes in a GDM context.
Supplementary data

This is linked to the online version of the paper at http://dx.doi.org/10.1530/JOE-15-0424.

Declaration of Interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

Funding

This study was supported by the National Institutes of Health (NIH) DK81412 (B.C.C.).

Author contribution statement

M V and A M S originated the concept and designed the study. M B F and C C B optimized the laser capture methodology, and carried out the experiments. M B F, C C B and S N performed the microarray data analysis and participated in the drafting of the manuscript. All authors participated in the critical interpretation of the results, reviewed, and approved the final version of the manuscript.

References


Received in final form 4 February 2016
Accepted 11 February 2016
Accepted Preprint published online 11 February 2016


West NA, Kechris K & Dabelea D 2013 Exposure to maternal diabetes in utero and DNA methylation patterns in the offspring. *Immunometabolism* 1 1–9.