GENETICS

Global placental gene expression in gestational diabetes mellitus

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OBJECTIVE: Gestational diabetes mellitus (GDM) is thought to modify the pattern of placental transcriptome. In a microarray study and a confirmatory quantitative real-time reverse transcription–polymerase chain reaction study, we investigated global placental gene expression in GDM.

STUDY DESIGN: Ribonucleic acid was extracted from placental samples collected from 19 GDM cases and 21 controls. Oligonucleotide probes representing 22,000 genes were used to measure gene expression. Differential gene expression was evaluated using the Student *t* test, fold change assessment, and significance analysis of microarrays. Path analysis was used to assess functions and functional relationships of differentially expressed genes.

RESULTS: Sixty-six genes participating in cell functions involving cell activation, immune response, organ development, and regulation of cell death were differentially expressed in GDM placentas. These genes include previously described candidate genes (eg, LEP, CEBPA, and MIF), genes with related functions (eg, ADFP), and novel genes (eg, AQP3).

CONCLUSION: Expression of genes responsible for diverse biologic processes are modified in GDM.

Key words: gestational diabetes mellitus, global gene expression, microarray, placenta

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Gestational diabetes mellitus (GDM), a disorder of glucose metabolism that complicates 4-7% of pregnancies in the United States,[1,2] is associated with short- and long-term morbidity in both the offspring and the mother.[3-11] Short-term adverse infant outcomes,[3] such as macrosomia, and long-term complications, such as obesity, abnormal glucose tolerance, and diabetes in adolescence or early adulthood, were observed among children of mothers with gestational diabetes, compared with offspring of euglycemic women.[4,5] Women with GDM experience an increased risk of developing other pregnancy complications, such as preeclampsia, and are more likely to develop overt diabetes after pregnancy.[5-10]

In addition to exaggerated glucose intolerance and insulin resistance, GDM is characterized by chronic systemic inflammation,[11] elevated leptin,[12,13] and reduced adiponectin concentrations.[13-16] Gene expression studies suggest that GDM is characterized by changes in placental gene expression that include up-regulation of inflammatory mediators and adipocytokines.[16,17] Although global gene expression profiling tools have been used to understand the molecular basis for adverse perinatal outcomes, including preeclampsia[18,19] and intrauterine growth restriction,[20] we are aware of only 1 such study that has focused on GDM.[17] In their study of 8 GDM case and 8 controls, Radaelli et al[17] reported that GDM elicits substantial changes in the expression profiles of placental genes regulating inflammatory responses and endothelial reorganization, reflecting a state of chronic systemic inflammation and endothelial activation.

Given the clinical and public health significance of GDM, the potentially useful information that can be obtained using gene expression studies and, that to date, only 1 other investigative team has used global gene expression methods to evaluate genes differentially expressed in GDM placenta, we conducted a microarray study and a confirmatory quantitative real-time polymerase chain reaction (QRT-PCR) study to identify global placental gene expressions among 19 GDM cases and 21 controls. We used applied network and path analysis to identify differentially expressed genes in GDM to evaluate potential pathways involved in GDM pathogenesis.

Materials and Methods

Study population and data collection

Study methods, described earlier,[16,21,22] are briefly as follows. The study was con-

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TABLE 1
Characteristics of study population

Characteristics	GDM cases (n = 19)	Controls (n = 21)
Maternal age, y[a]	32.8	34.2
Race, %		
White	47.4	81.0
African American	5.3	0.0
Asian	26.3	14.3
Other	15.8	4.7
Gestational age at delivery, wks[a]	37.7	38.4
Delivery mode, %		
Vaginal	36.8	33.3
Cesarean	63.2	66.7
Labor during delivery, %	68.4	38.1
Preterm labor, %	10.5	9.5
Preeclampsia, %	10.5	0.0
Chronic hypertension, %	26.0	0.0
Chorioamnionitis, %	5.3	0.0
Nulliparous, %	42.1	23.8
Preterm delivery (< 37 wks), %	63.2	52.4
Pregestational BMI, kg/m^2[a]	28.5	25.9

Only history of chronic hypertension was statistically significantly different between cases and controls.
BMI, body mass index (kg/m^2).

[a] Mean.

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ducted as part of a pilot Placenta MicroArray study designed to examine differential placental gene expression associated with pregnancy complications.[21,22] Participants were recruited among women who delivered at Swedish Medical Center (Seattle, WA).

GDM was defined by the presence of 2 or more of the following 4 oral glucose tolerance test results based on the American Diabetes Association criteria:[23] fasting > 5.3 mmol/L (95 mg/dL); 1 hour > 10.0 mmol/L (180 mg/dL); 2 hours > 8.6 mmol/L (155 mg/dL); 3 hours > 7.8 mmol/L (140 mg/dL). Controls, frequency matched to cases for gestational age and mode of delivery, were selected among participants who had normal pregnancy. Women with a history of pregestational diabetes and those with a nonsingleton index pregnancy were excluded from this study.

Among eligible women, 19 cases (9 diet-controlled and 10 insulin-taking GDM cases) and 21 controls consented and provided placental samples at delivery. Information on risk factors, pregnancy history, and outcome was obtained from medical records. All study procedures were approved by the Institutional Review Board of Swedish Medical Center. All participants provided written informed consent.

Placental sample collection

Placenta specimen were weighed, double bagged, and transported in coolers. The chorionic plate and overlying membranes were removed and tissue biopsies (approximately 0.5 cm^3 each) were obtained from 16 sites (8 maternal and 8 fetal) using a grid system.[21,22] For this analysis, biopsy samples taken from the fetal side, which consisted of the intervillous tissues and chorionic villi, were evaluated. Biopsies were placed in cryotubes containing RNAlater (Qiagen Inc, Valencia, CA), at 10 μL per 1 mg of tissue and stored at -80°C.

RNA extraction

A pooled sample (240 mg) representing each placenta, composed of four 60-mg tissue biopsies, was homogenized using a Tissue Tearor (Biospec Products Inc, Bartlesville, OK) or Mini-Beadbeater 8 (Biospec Products) in a lysis buffer from the RNeasy fibrous Midi kit (Qiagen) with added β-mercaptoethanol to disrupt any proteins that might be destroying nucleic acid. RNA was extracted using a standardized protocol adapted from RNeasy Fibrous Tissue Midi Handbook (Qiagen).

Total RNA concentration was calculated by determining absorbance at 260 nm (Spectramax Plus 384 spectrophotometer; Molecular Devices, Sunnyvale, CA) in 10 mM Tris-HCl. Protein contamination was evaluated using the A260/A280 ratio. All samples had A260/A280 ratios > 1.8. Samples were aliquoted at 10 μL for storage at -80°C.

Quality control was conducted using an Agilent 2100 Bioanalyzer capillary electrophoresis system (Agilent Technologies Inc, Palo Alto, CA) and a spectrophotometry scan. Samples were amplified using Ambion's MessageAmp I kit (Ambion Inc, Austin, TX), and subsequent amplified RNA was labeled with a fluorescent dye tag. All RNA samples, including reference RNAs, underwent a quality control check and were labeled using the same standardized protocols.

Microarray experiment

Arrays were manufactured by depositing genome-wide 70 mer oligonucleotide microarray probes (representing ~22,000 genes) from Operon's Human Genome Array Ready Oligo Set version 2.1 (Operon Biotechnologies Inc, Huntsville, AL), onto Corning UltraGAP microarray slides (Corning, NY) using an OmniGrid 300 high-capacity microarray printer (Genomic Solutions, Ann Arbor, MI). Arrays were processed using GeneTAC hybridization station (Genomic Solutions) and imaged using an Axon GenePix 4000B microarray scanner (Molecular Devices). Array images were quantified using GenePix Pro 6.0 image extraction software

FIGURE 1
Volcano plot of placental gene expression

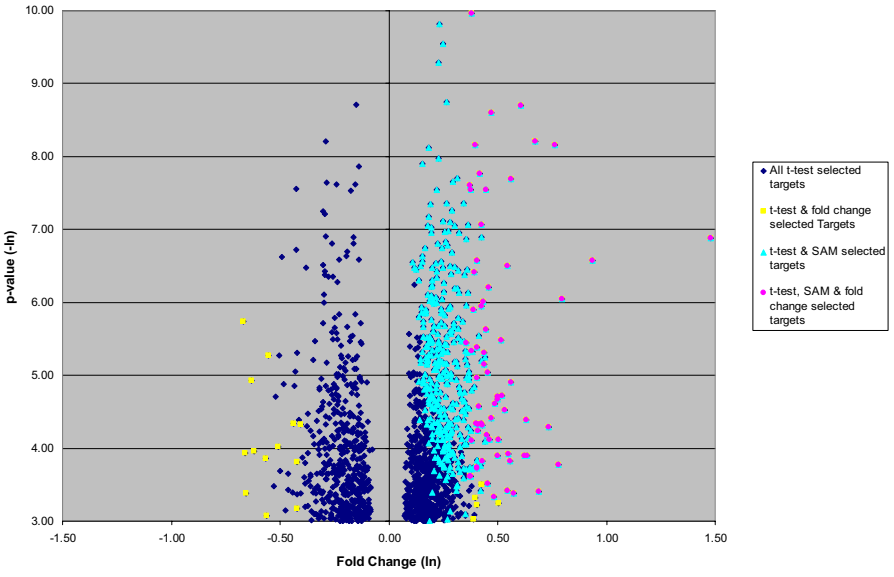

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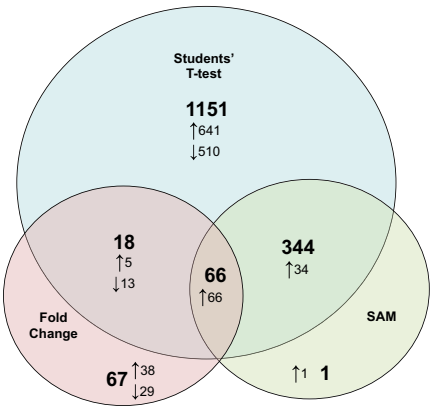
FIGURE 2
Venn diagram summary of distribution of differentially expressed genes



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(Molecular Devices). Data were subsequently preprocessed through a custom-built quality-control filter (GDFilter) based on spot-level signal quality. Raw data and preprocessed results were stored in an Iobion GeneTraffic relational database (Stratagene Corp, La Jolla, CA). Intraarray normalization was performed using a lowess algorithm to correct for intensity-dependent ratio biasing.[24]

QRT-PCR experiment

The confirmatory QRT-PCR experiment to measure expression of selected differentially expressed genes in our microarray study was conducted in duplicates using assays (information available on request) developed by Clontech (Mountain View, CA). First-strand complementary DNA (cDNA) was synthesized using the high capacity cDNA archive kit (Applied Biosystems, Foster City, CA). Reactions were run on an ABI PRISM 7000 real-time PCR machine (Applied Biosystems) using the default cycling conditions. Threshold cycle (Ct) values of the duplicates differing by $>$ 0.5 times the SD were retested. Ct value duplicates differing by $<$ 0.5 times the SD were averaged for analysis. Raw measurements were normalized using the geometric mean of SDHA, TBP, and YWHAZ genes as previously described by our group.[22]

Statistical analysis

Analysis was conducted on natural log-transformed data. Genes with available information on $<$ 20% of samples in either test groups were dropped from further analysis. Differential gene expression among cases and controls was evaluated using the Student *t* test (2 sample, unequal variances). In addition, genes that met the following criteria in case-control comparisons constituted the final set of differentially expressed genes in GDM placenta. These criteria were based on fold change differences[25] and significance analysis of microarrays (SAM)[26] analysis. Absolute fold change differences greater than the mean plus 3 times the SD for the specific window (determined by expression measurement among controls)[25] and false discovery rates (FDR) $\leq$10% in SAM were considered significant. A phylogenetic tree of differentially expressed genes, using a Pearson correlation coefficient–based hierarchical clustering scheme, was constructed using Cluster and TreeView software.[27]

In path analysis, relationships between differentially expressed genes were investigated using 2 independent tools: DAVID (Database for Annotation, Visualization, and Integrated Discovery)[28] and Ingenuity Pathway Analysis (IPA) software (Ingenuity, Redwood City, CA). Gene enrichment of annotation clusters (enrichment score) or networks (network score) was measured in DAVID or IPA, respectively, using a modified Fisher exact test. These were used to rank the biologic significance of gene function clusters or networks. Correlations between microarray and QRT-PCR expression measurements were evaluated using Spearman correlation coefficients. In addition, expression fold change results from microarray and QRT-PCR experiments were compared for those selected genes with both measurements.

RESULTS

Selected characteristics of GDM cases and controls are summarized in Table 1. Cases were more likely to self-identify as nonwhite and were more likely to be heavier than controls. Expression measurements were determined for 21,713 genes (98.7%) represented on the spotted microarray platform. Expressions of 14,453 genes, measured for at least 80% of GDM cases and 80% of controls, were further analyzed.

A volcano plot of Student *t* test *P* values (Y-axis) against fold change differ-

TABLE 2
List of selected differentially expressed genes in GDM placenta[a]

Gene symbol	Gene name	Gene ontology (molecular function)	Location	Fold[a]	*P*[a]	FDR[a]
LEP	Leptin	Growth factor and hormone activity, protein binding	7q31.3	4.40	.00103	0.00
RFNG	RFNG O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase	O-fucosylpeptide 3-beta-N-acetylglucosaminyltransferase activity, transferase activity, transferring glycosyl groups	17q25	2.22	.00238	0.00
C1D	C1D nuclear DNA-binding protein	DNA binding	2p13-p12	2.18	.02290	6.45
ADFP	Adipose differentiation–related protein	Adipocyte differentiation	9p22.1	2.08	.01374	6.45
AQP3	Aquaporin 3	Transporter activity	9p13	1.99	.03330	7.48
BHLHB2	Basic helix-loop-helix domain containing, class B, 2	Protein binding, transcription factor, and transcription repressor activity	3p26	1.89	.02038	7.48
FLT1	fms-related tyrosine kinase 1	ATP, identical protein and nucleotide binding; receptor, transferase, and vascular endothelial growth factor receptor activity	13q12	1.88	.01243	6.45
PPIB	Peptidylprolyl isomerase B	Isomerase and peptidyl-prolyl cis-trans isomerase activity; protein and unfolded protein binding	15q21-q22	1.87	.02031	7.48
TUSC3	Tumor suppressor candidate 3	Contribute to dolichyl-diphosphooligosaccharide-protein glycotransferase activity	8p22	1.83	.00017	0.00
CD93	CD93 molecule	Calcium ion, protein, sugar, and complement component C1q binding; receptor activity	20p11.21	1.75	.00740	5.23
UTS2	Urotensin 2	Hormone activity	1p36	1.75	.00046	0.00
RPS7	Ribosomal protein S7	RNA and protein binding; structural constituent of ribosome	2p25	1.75	.02199	7.48
MAGEA9	Melanoma antigen family A, 9	Unclear molecular function	Xq28	1.73	.01973	8.93
AGPAT4	1-acylglycerol-3-phosphate O-acyltransferase 4	1-acylglycerol-3-phosphate O-acyltransferase, acyltransferase and transferase activity	6q26	1.73	.00150	3.47
HLA-DRA	Major histocompatibility complex, class II, DR alpha	Major histocompatibility complex class II receptor activity	6p21.3	1.72	.03298	9.47
GALNT2	UDP-N-acetyl-alpha-D-galactosamine: polypeptide N-acetylgalactosaminyltransferase 2	Calcium ion, manganese ion and, sugar binding; polypeptide N-acetylgalactosaminyltransferase and transferase activity	1q41-q42	1.71	.01092	5.23
RPS29	Ribosomal protein S29	RNA, metal ion, zinc ion, and protein binding	14q	1.69	.00895	6.45
FERMT1	Fermitin family homolog 1	Protein binding	20p12.3	1.67	.00416	4.02
IFI30	Interferon γ–inducible protein 30	Oxidoreductase activity	19p13.1	1.66	.01638	8.93
ADAM12	ADAM metallopeptidase domain 12	Metal ion, protein, and zinc ion binding; metalloendopeptidase activity	10q26.3	1.65	.00899	6.45
PROCR	Protein C receptor, endothelial	Receptor activity	20q11.2	1.65	.02034	6.45
UCK2	Uridine-cytidine kinase 2	ATP and nucleotide binding; kinase, phosphotransferase, and uridine kinase activity	1q23	1.65	.00937	6.45
FXYD5	FXYD domain containing ion transport regulator 5	Actin and cadherin binding, ion channel activity	19q12-q13.1	1.63	.00991	6.45

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(continued)

TABLE 2
List of selected differentially expressed genes in GDM placenta[a] *(continued)*

Gene symbol	Gene name	Gene ontology (molecular function)	Location	Fold[a]	*P*[a]	FDR[a]
NDRG1	N-myc downstream regulated gene 1	Protein binding	8q24.3	1.62	.03574	9.47
DYNLL1	Dynein, light chain, LC8-type 1	Microtubule motor activity, protein binding	12q24.23	1.60	.00018	0.00
RNASE4	Ribonuclease, RNase A family, 4	Endonuclease, hydrolase, and pancreatic ribonuclease activity; nucleic acid binding	14q11.1	1.60	.01211	7.48
GDF15	Growth differentiation factor 15	Cytokine and growth factor activity	19p13.11	1.59	.01630	7.48
SRI	Sorcin	Calcium channel regulator activity, calcium ion, and receptor binding	7q21.1	1.59	.00203	3.47
ARS2	Arsenate resistance protein 2	Protein binding	7q21	1.57	.00651	5.23
CPVL	Carboxypeptidase, vitellogenic-like	Peptidase and serine carboxypeptidase activity	7p15-p14	1.57	.02977	9.47
PRO1843	Hypothetical protein PRO1843	Protein coding	12q13.13	1.57	.01537	8.93
HDGF	Hepatoma-derived growth factor	Growth factor activity; heparin binding	1q21-q23	1.56	.00053	4.02
BDNF	Brain-derived neurotrophic factor	Growth factor activity; protein binding	11p13	1.56	.00360	4.02
INHA	Inhibin, alpha	Activin inhibitor, growth factor, cytokine, and hormone activity	2q33-q36	1.55	.00579	4.02
CLDN7	Claudin 7	Identical protein binding, structural molecule activity	17p13	1.55	.00492	6.45
KCNIP3	Kv channel interacting protein 3	DNA, calcium ion, and potassium ion binding; potassium channel, transcription corepressor, and voltage-gated ion channel activity	2q21.1	1.54	.00246	3.47
COL17A1	Collagen, type XVII, alpha 1	Structural molecule activity	10q24.3	1.54	.01336	6.45
FOSL2	FOS-like antigen 2	Protein dimerization and transcription factor activity; sequence-specific DNA binding	2p23.3	1.54	.02178	8.93
CEBPA	CCAAT/enhancer binding protein alpha	RNA polymerase II transcription factor and protein dimerization activity; enhancer, sequence-specific DNA and transcription factor binding	19q13.1	1.53	.00085	3.47
CRHSP-24	Calcium regulated heat stable protein 1	DNA, phosphatase, and protein binding	16p13.2	1.53	.00263	6.45
CD63	CD63 molecule	Positive regulation of endocytosis	12q12-q13	1.52	.00042	0.00
KIF1C	Kinesin family member 1C	ATP and nucleotide binding; microtubule motor activity	17p13.2	1.51	.01035	6.45
SMPD1	Sphingomyelin phosphodiesterase 1	Hydrolase and sphingomyelin phosphodiesterase activity	11p15.4-1	1.51	.01442	6.45
ADD2	Adducin 2 (beta)	Actin, calmodulin, and metal ion binding	2p14-p13	1.51	.01321	6.45
ANXA4	Annexin A4	Calcium ion and calcium-dependent phospholipid binding; phospholipase inhibitor activity	2p13	1.50	.02369	9.47
CALM1	Calmodulin 1	Growth and the cell cycle; signal transduction	14q24-q31	1.50	.00702	5.23

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TABLE 2
List of selected differentially expressed genes in GDM placenta[a] *(continued)*

Gene symbol	Gene name	Gene ontology (molecular function)	Location	Fold[a]	*P*[a]	FDR[a]
POLG2	Polymerase (DNA directed), gamma 2, accessory subunit	ATP, DNA, identical protein, nucleotide, and single-stranded DNA binding; gamma DNA-directed DNA polymerase, glycine-transfer RNA ligase activity, nucleotidyltransferase and transferase activity	17q	1.49	.01309	8.93
STEAP4	STEAP family member 4	FAD, copper ion, iron, and metal ion binding; oxidoreductase activity	7q21.12	1.47	.00273	4.02
GNG7	Guanine nucleotide binding protein gamma 7	Signal transducer activity	19p13.3	1.47	.01648	7.48
HRASLS3	HRAS-like suppressor 3	Protein binding	11q12.3-13.1	1.47	.00482	5.23
TMEM4	Canopy 2 homolog	Protein binding	12q15	1.46	.00005	0.00
SPCS3	Signal peptidase complex subunit 3	Signal peptidase activity	4q34.2	1.46	.02684	9.47
MIF	Macrophage migration inhibitory factor	Cytokine, isomerase, and phenylpyruvate tautomerase activity; protein binding	22q11.23	1.46	.00053	0.00
GBA	Glucosidase, beta	Catalytic, hydrolase, and glucosylceramidase activity; cation binding	1q21	1.45	.00050	3.47

ATP, adenosine triphosphate; *DNA*, deoxyribonucleic acid; *FDR*, false discovery rate.

[a] Selected genes differentially expressed in GDM placenta in order of fold change values (fold), *P* value: Student *t* test *P* value.

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ences (X-axis) comparing gene expressions for GDM cases and controls depicts signal in the data because there were more genes with extreme *P* values than would have been expected by chance (Figure 1). For instance, there were 3 and > 30 genes with *P* values below 1 per 14,453 ($-\ln[P\text{ value}] = 9.58$) and 10 per 14,453 ($-\ln[P\text{ value}] = 7.28$), respectively, whereas we expected to see only 1 and 10 computed *P* values (corresponding to 1 and 10 false positives).

Overall, a total of 1579 genes (10.9%) were differentially expressed among GDM cases vs controls in Student *t* test ($P < .05$) comparisons (Figure 2). Of these, expression of 66 genes (all up-regulated), comprising 0.5% of the 14,453 evaluated, also had absolute fold change differences greater than mean plus 3 SD within each window of expression and FDR ≤ 10% in SAM analysis.

These 66 genes constituted our list of differentially expressed genes in GDM (Table 2). This list includes previously identified candidate genes involved in GDM pathogenesis (such as LEP, MIF, FLT1, and UTS2), potential candidates with limited previous evidence of direct involvement but that are known to play important roles in regulating GDM-related functions (such as ADFP, STEAP4) and novel genes (such as AQP3).

The heat map shows hierarchical clustering results of GDM cases (18/19) and controls (17/21) based on expression measurements of selected differentially expressed genes (Figure 3). In path analysis using DAVID, differentially expressed genes belonged to clusters of genes involved in lysosome/vacuole functions, cytokine activity, immune cell activation, response to external pathogens, hemopoiesis, calcium metabolism, and cell death regulation (Table 3).

In IPA, networks involving vascular system development and function, cell injury, and cell death were enriched by our differentially expressed genes (Table 4). Three significantly enriched networks (scores of 31, 26, and 23) are shown in Figure 4. In network 1 (Figure 4, A), Ca2+ is directly or indirectly associated with CALM1, KCNIP3, NDRG1, SRI, and ADAM12, genes up-regulated in our study. In network 2 (Figure 4, B), tumor necrosis factor is directly or indirectly associated with FOSL2, DYNLL1, STEAP4, HLA-DRA, and BHLHB2, also up-regulated in our GDM cases. Lastly, in network 3 (Figure 4, C), CEBPA is up-regulated and is directly or indirectly associated with ADFP, FLT1, and LEP, which were up-regulated in our study.

Nine genes (ADFP, AQP3, CEBPA, FLT1, INHA, ITGAX, MIF, STEAP4, and TUSC3) were selected among differentially expressed genes based on a priori evidence for involvement in pathways involved in GDM pathology for the confirmatory QRT-PCR study (Table 5 and Figure 5). Comparable results and similar patterns of expression differences between cases and controls were observed, regardless of gene expression measurement method used. However, fold change differences were more pronounced in the microarray experiment,

and within-group comparison of microarray and QRT-PCR gene expression results show high correlations for 6 of the 9 genes evaluated.

Comment

In this microarray study, we compared global gene expression profiles of placental tissue from GDM cases and controls. Of the more than 14,000 genes evaluated, 66 genes were differentially expressed in GDM. These genes include those with strong a priori evidence for involvement in GDM pathogenesis (such as LEP, MIF, CD63, UTS2, and FLT1), those that are involved in putative pathways (such as CEBPA, ADFP, and STEAP4),[29] and novel genes (such as AQP3). Genes involved in a diverse set of cellular functions including cell activation, immune response, organ development, and regulation of cell death were represented in our set of differentially expressed genes.

The only other microarray study that conducted global profiling of messenger RNA expression in placental samples among GDM cases and controls identified 435 genes involved in inflammation, endothelial differentiation, substrate metabolism, translation, transport and trafficking, and signal transduction, among others.[17] A significant cluster of genes was related to inflammatory responses including LEP, its receptor (LEPR), TGFB1I1, IL1, PTX3, and MIG-6.[17] For example, LEP and FL1 were upregulated 2.3- and 2.0-fold, respectively, in GDM placental samples, compared with control placentas, similar to our findings.[17]

Several genes involved in inflammatory pathways (such as ARTS-1) and endothelial differentiation (such as ACTG2), reported by Radaelli et al,[17] were differential expressed in GDM placenta in our study, although expression differences between cases and controls were weak to marginal (Table 6). In their study, Radaelli et al reported differential expressions of genes such as VEGF and other genes coding for structural and contractile proteins, which we did not replicate (Table 6).

On the other hand, in our microarray study, other genes functioning in immune response and cytokine activity

FIGURE 3
Heat map illustration of phylogenetic tree of samples and selected differentially selected genes

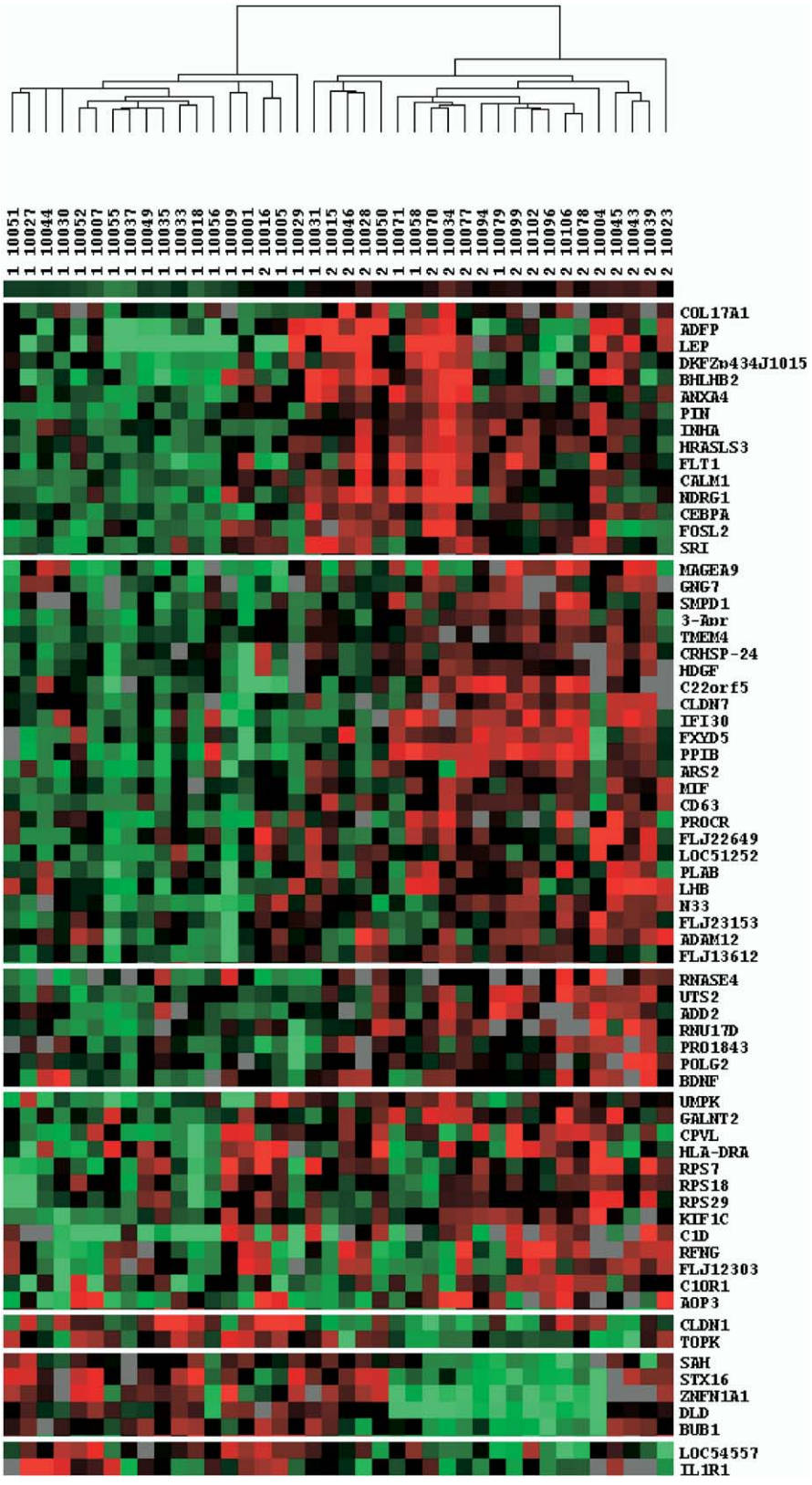

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TABLE 3
DAVID mapping of genes differentially expressed in GDM placenta[a]

Gene list	Enrichment score	Cluster
CD63, GBA, HLA-DRA, IFI30, SMPD1	2.86	Lysosome/vacuole
INHA, INHBA, MIF, PLAB	2.02	Transforming growth factor-β and cytokine activity
INHA, INHBA, MIF, SRI	1.81	Regulation of cell activation, physiologic processes, and immune response
CEBPA, INHA, INHBA	1.75	Myeloid differentiation, hemopoiesis, and hemopoietic or lymphoid organ development
CALM1, CSEN, FLJ13612, SRI	1.68	Calcium binding EF-hand
C1QR1, INHA, INHBA, MIF, POLG2, PROCR	1.32	Immune cell activation and response to wounding, external stimulus, pathogen, or stress
ANXA4, CSEN, FOSL2, INHA, INHBA, MIF	1.09	Regulation of cell death
ADAM12, ADD2, ADFP, AGPAT4, AQP3, C1QR1, C2orf28, C22orf5, CALM1, CD63, CLDN7, COL17A1, FLJ22649, FLT1, FXYD5, GALNT2, GBA, HLA-DRA, HRASLS3, PROCR, RFNG, SC5DL, STEAP4, TMEM4, TUSC3	1.07	Transmembrane

[a] Genbank accession numbers were mapped using functional annotation clustering in the DAVID 2007 pathway analysis tool. For each group, the processes or functions are tabulated with the gene list and enrichment score. Enrichment score is calculated as the geometric mean (in log scale) of members' *P* values in a corresponding annotation cluster. Clusters shown here are those with enrichment scores > 1.0.

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TABLE 4
Gene clusters identified using Ingenuity Path Analysis in GDM placenta[a]

Genes in network[a]	Score	Focus genes	Functions
ADAM12, **ANXA4**, ANXA7, **AQP3**, beta-estradiol, BTG1, **C20ORF42**, Ca2+, **CALM1**, **CD63**, CD40LG, CYB5A, FOS, **FXYD5**, GAL, GCLC, GNB1, GNG3, **GNG7**, **GRSF1**, GYPC, **HDGF**, IL15, ITGB3, **KCNIP3**, MPP1, **NDRG1**, NTS (includes EG:57303), PIK3CG, RCVRN, **RPS7**, Ryr, **SRI**, **UTS2**, ZFP36	31	15	Cardiovascular system development and function, organismal development, gene expression
AKAP12, AKR1B1, ASS1, **BHLHB2**, **C1D**, **CARHSP1**, **CD93**, CFD, CPB2, **DYNLL1**, F2, **FOSL2**, **GALNT2**, GBP1, GLRX, GPAM, **HLA-DRA**, HOXA9 (includes EG:3205), IGFBP4, MBL2, NFYB, NOTCH1, ORM2, PRKDC, **PROCR**, **RFNG**, RFX1, RFX5, **RNASE4**, **STEAP4**, THBD, TNF, TP53, **UCK2 (includes EG:7371)**, ZIC2	26	13	Gene expression, cancer, organismal injury and abnormalities
5-Hydroxyindol-3-acetic acid, **ADD2**, **ADFP**, ADRB3, Ap1, **BDNF**, C3AR1, carbon monoxide, **CEBPA**, CFD, CPS1, Creb, CYP3A5, **FLT1**, **FOSL2**, FYN, **GDF15**, GRN, **INHA**, Jnk, **KCNIP3**, **LEP**, Mapk, **MIF**, NTS (includes EG:57303), ORM2, PDE3B, PER2, PMCH, PTPRH, SCP2, SHC2 (includes EG:25759), **SMPD1**, UCN, VEGFB (includes EG:7423)	23	12	Organismal development, organ development, behavior
ATF1, CCL2, CDC34 (includes EG:997), CEACAM1, Ck2, **CLDN7**, **COL17A1**, DDIT3, DIABLO, EGF, ELA2A, FASN, Gsk3, HLA-A, **HRASLS3**, **IFI30**, IFNG, IGFBP4, IL1RN, ITGAL, ITGB4, JAK1, JUND, **KIF1C**, MBP, MMP9, MYH9, PIK4CA, **PPIB**, SOCS3, Stat, STAT5a/b, STAT5B, TRH, ZFP36	10	6	Cancer, cell death, cellular growth and proliferation

[a] The networks were generated through the use of Ingenuity Pathways Analysis (Ingenuity Systems, www.ingenuity.com). Each gene identifier was mapped to its corresponding gene object in the Ingenuity Pathways Knowledge Base (IPKB). These genes were overlaid onto a global molecular network developed from information contained in the IPKB. Network enrichment is then assessed using a network score (negative log of *P* values of Fisher tests). Focus genes (**in bold**) are genes identified in our list of differentially expressed genes. Networks shown here are those with network scores > 3.0.

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FIGURE 4
Pathway networks (n = 3) identified using Ingenuity Pathway Analysis

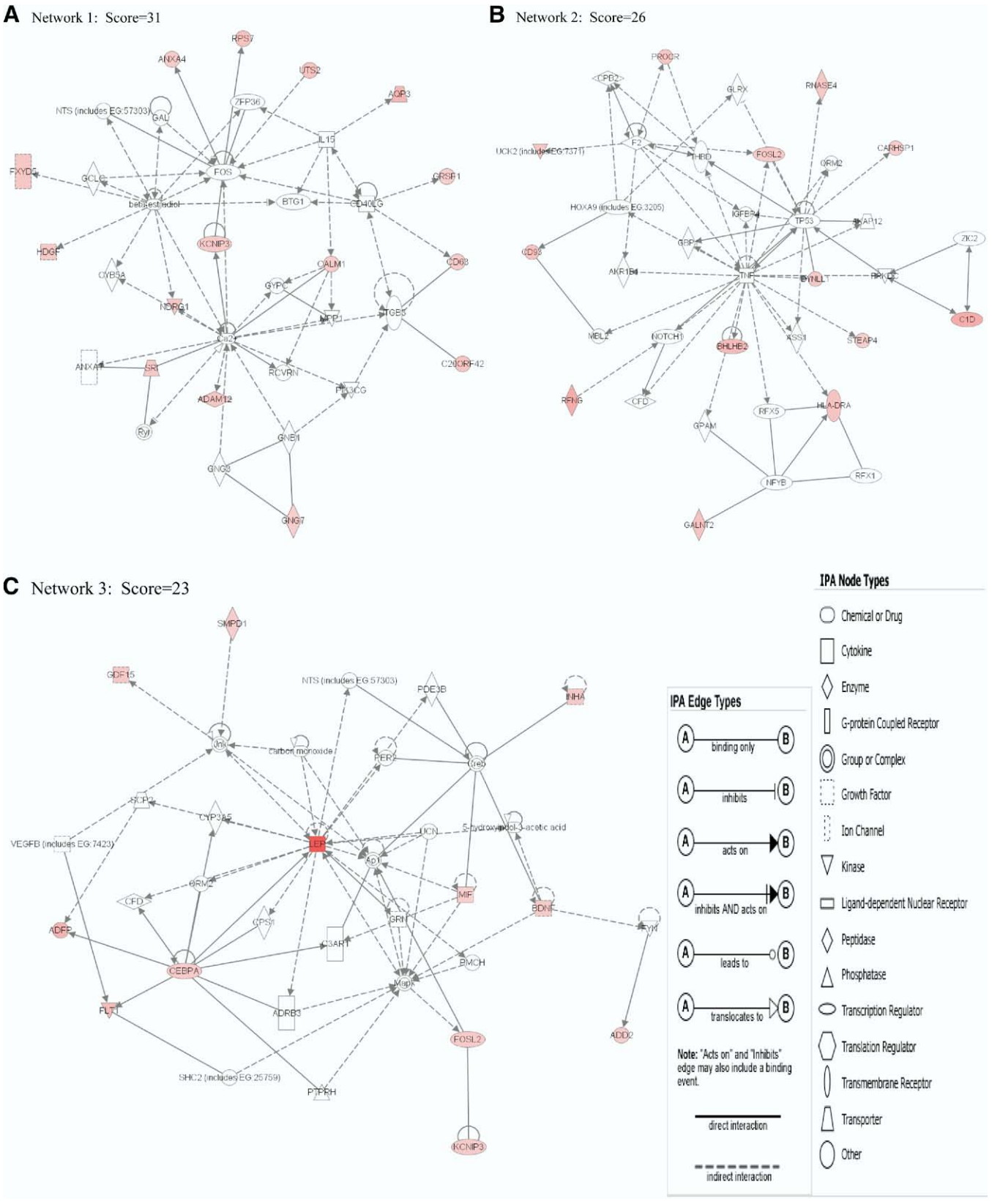

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TABLE 5
Comparison of microarray and QRT-PCR expression measurements for selected genes among GDM cases and controls

	Correlations[a]					
	Cases (n = 19)		Control (n = 21)		Fold change differences	
Gene symbol	ρ	P	ρ	P	Microarray	QRT-PCR
ADFP	0.77	< .001	0.87	< .001	2.0849	1.5555
AQP3	0.96	< .001	0.68	< .001	1.9923	1.5244
CEBPA	0.68	.001	0.65	.001	1.5321	1.1005
FLT1	0.73	< .001	0.73	< .001	1.8840	1.2558
INHA	0.63	.004	0.46	.04	1.5516	1.3581
ITGAX	0.11	.67	-0.21	.38	1.3052	1.1099
MIF	0.29	.22	0.25	.28	1.4557	1.0030
STEAP4	-0.008	.97	0.34	.13	1.4726	1.2139
TUSC3	0.55	.02	0.66	.002	1.8343	1.2071

[a] Spearman rank correlations and *P* values between microarray and QRT-PCR gene expression measurements.

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such as INHA and INHBA were differentially expressed. Neither of these studies reported differential expression of genes (such as TNFα) that regulate peripheral concentrations of proteins altered in GDM.[14-16,30] However, Radaelli et al[17] reported changes in TNFα-induced protein (DKFZP434F0318), and our study showed gene expression changes in a number of targets that indirectly interact with TNFα (Figure 4, B).

Our path analysis extends the 2-way comparison of placental gene expressions of GDM cases and controls and helps to identify potential candidate genes even when these particular genes are not either up- or down-regulated in GDM placenta in our study. Previous evidence for known candidate genes, such as TNFα, implies that other genes, such as FOS (Figure 4, A), TP53 (Figure 4, B), and Mapk (Figure 4, C) with similar strong relationships with other differentially expressed genes in our list are potential candidate genes that deserve further investigations. Similar findings from analysis using DAVID and IPA on involvement of genes participating in response to cellular injury and cell death indicate the potential significant role these pathways play in GDM pathogenesis.

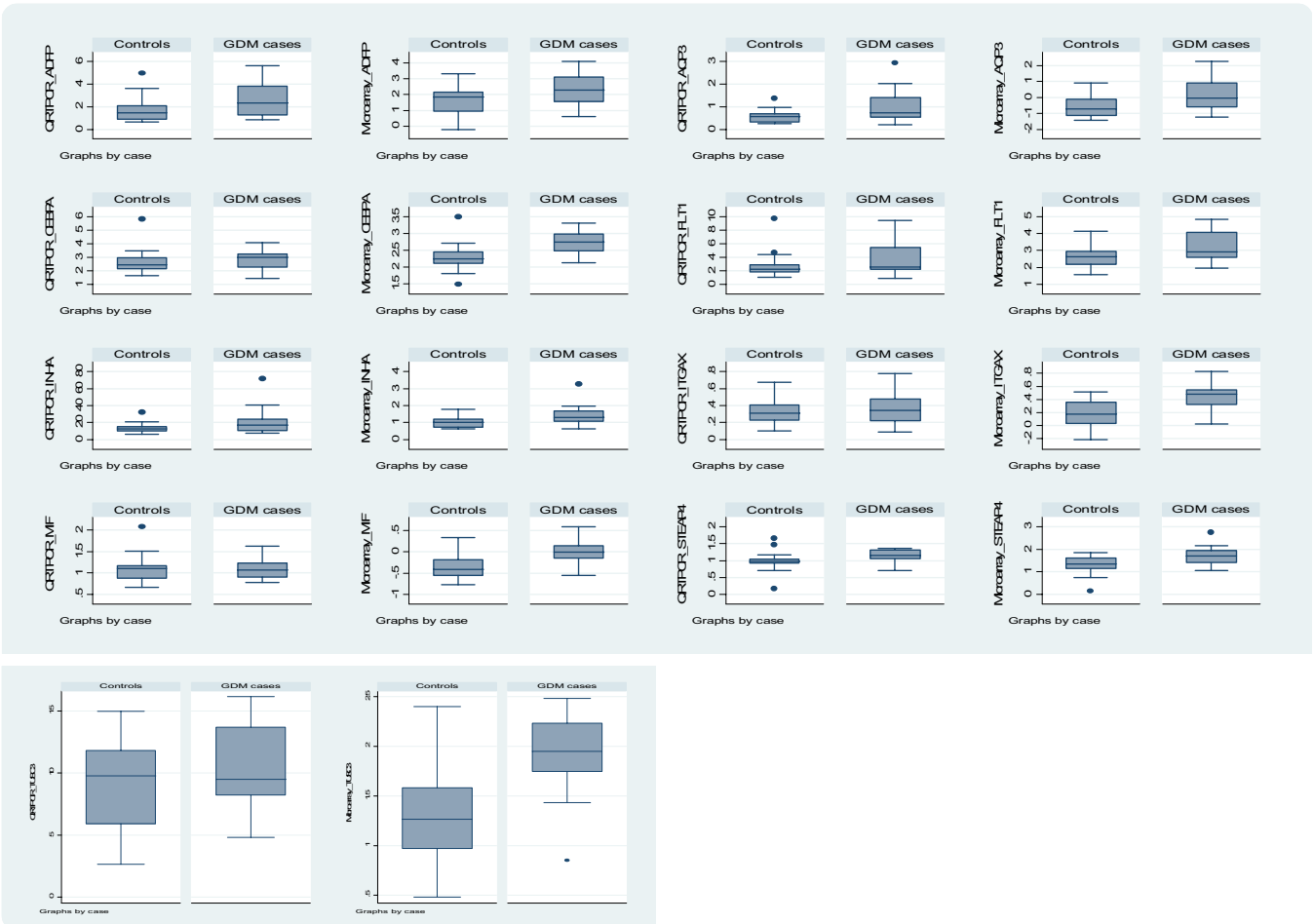
Previous evidence from experimental and population studies supports associations of GDM with genes and pathways identified in our study. Destruction or impairment of the pancreatic β cell, increased insulin degradation, and decreased tissue sensitivity to insulin that involve inflammatory and oxidative stress markers contribute to the pathogenesis of GDM.[31] MIF may function in the placenta to control trophoblast growth and modulate the maternal immune reaction.[32] MIF is also known to affect glucose metabolism by acting as a positive regulator of insulin secretion.[33]

Gestational diabetes is closely linked with LEP dynamics.[34] Evidence exists supporting LEP up-regulation either as a cause or result of glucose uptake in placentas among mothers with GDM.[12,34] Some genes that were differentially expressed in GDM in our study, such as CD63 (a platelet activation antigen),[35] UTS2 (a potent vasoactive hormone),[36] and FLT1 (vascular growth factor involved in vascular development),[37] have been either previously described in association with GDM or linked to pathogenesis of type 2 diabetes. Finally, genes involved in adipogenesis and decreased insulin sensitivity and secretion, such as CEBPA, ADFP, and STEAP, may play a role in impaired glucose tolerance, affecting the risk of GDM and postpartum diabetes.[38]

Potential novel candidate genes were also identified in our study. For example, AQP3, 1 of a group of genes coding for aquaporins (AQPs), which are channel-forming integral proteins functioning as water channels,[39] was up-regulated in GDM placentas. Previous evidence exists in support of the role of AQPs in the control of blood glucose level through regulation of glycerol (an important substrate for hepatic gluconeogenesis and triglyceride synthesis) release from adipocytes and intake by the liver.[39,40] AQP3 is thought to contribute to the hypoglycemic effect of insulin,[40] and regulation of AQP3 expression has been shown to be tightly linked to blood glucose and insulin regulation.[40] Furthermore, experimental evidence exists supporting dysregulation of AQP3 expression in diabetes mellitus.[41]

In our confirmatory QRT-PCR study, gene expression measurement of 6 of the 9 genes evaluated was significantly correlated with microarray expression measurements. Discordance between measurements using the 2 methods may have resulted from differences in assays in terms of location in the gene sequence, presence of messenger RNA transcripts of multiple isoforms because of splicing, or other posttranscriptional–processing. We investigated whether mRNA tran-

FIGURE 5
Comparison of selected gene expression measurements using microarray platform and QRT-PCR



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scripts amplified by the QRT-PCR primer/probe sets directly corresponded to the messenger RNA transcripts that would have been hybridized to the microarray sequences[42] and found that all 3 genes with discordance between microarray and QRT-PCR measurements lack concordance of transcripts identified by QRT-PCR and those identified by microarray. In addition, all 3 genes have described splice variants. Therefore, in future studies, we recommend evaluating concordance of transcript identification between the 2 assays and evaluating the presence of splice variants before performing QRT-PCR analysis for confirmation.

Our present study has several important strengths. First, our study population was well characterized. We used strict case and control definitions to enable us to evaluate differences in gene expressions. Second, our study population comprising 19 cases and 21 controls is considerably larger than the only previous similar study[17] and in fact most previous placental microarray studies. Specimens for this study were collected from the fetal side of the placental tissue that mainly consisted of villous and other fetal tissue with previous evidence for significant involvement in GDM pathogenesis.[43] In sample collection, we used a mapping scheme to achieve uniformity and adequate sampling of the whole placenta.[44] We applied 3 separate screens to identify differentially expressed genes and minimize false-positive association results. One of these criteria, SAM, uses permutations for multiple testing correction.[26] In our fold change analysis, we used windows of expressions among controls to reduce expression level-dependent noise and increase study efficiency to evaluate genes with a low fold change profile.[25] It has been reported that physiologic or pathologic significance of a gene is not necessarily determined by the absolute magnitude of the expression profile change.[45]

Some limitations of our study merit mention. In our study, we found low fold changes in gene expression, compared with the previous GDM microarray study.[17] Cases in the previous study required chronic insulin therapy for glucose control, whereas our study included a more heterogeneous group of GDM cases (9 on diet therapy and 10 on insulin therapy).

Some differences across the 2 studies may be attributable to differences in the

TABLE 6
Differentially expressed genes in GDM placenta in the study by Radaelli et al[17] and the current study[a]

		Fold change	
Gene symbol	**Gene name**	**Radaelli et al[17]**	**Current study**
ACTG2	Actin, gamma 2, smooth muscle, enteric	2.1	1.3
ADD3	Adducin 3 (gamma)	2.3	-1.3
ARTS1	Type 1 tumor necrosis factor receptor shedding aminopeptidase regulator	18	1.2
CALD1	Caldesmon 1	2.3	-1.9
CAPN3	Calpain 3 (p94)	-2.6	1.3
F11	Coagulation factor XI	-2.6	1.3
FBN2	Fibrillin 2	2.3	-1.4
FLT1	Fms-related tyrosine kinase 1 (VEGF receptor)	2	1.9
FN1	Fibronectin 1	-2.5	-1.4
GNGT1	Guanine nucleotide binding protein (G protein)	2	1.2
GPNMB	Glycoprotein (transmembrane)	2.4	1.6
ICAP-1A	Integrin cytoplasmic domain-associated protein 1	2.3	1.2
IFI30	Interferon, gamma-inducible protein 30	-3	1.7
IL8RB	Interleukin 8 receptor beta	5.3	1.2
ITGB5	Integrin beta 5	4.8	-1.3
KRT6A	Keratin 6A	-7.8	1.2
LAMB1	Laminin beta 1	2.4	-1.4
LEP	Leptin	2.3	4.4
MIF	Macrophage migration inhibitory factor	-2.1	1.5
NRIP1	Nuclear receptor interacting protein 1	2.2	1.4
PRG1	Proteoglycan 1, secrotory granule	2.2	1.3
PTX3	Pentaxin-related gene, rapidly induced by IL-1 β	5	1.2
STXBP3	Syntaxin binding protein 3	2.1	-1.2
WSX1	Class I cytokine receptor	2.1	1.3

[a] Selected genes differentially expressed in GDM placenta in the report by Radaelli et al[17] and with absolute fold changes $>$ 1.20 in the current study.

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composition of the GDM case population. We conducted a sensitivity analysis that compares cases on insulin therapy with controls excluding cases on diet therapy and found generally similar findings reported in our study, indicating the robust nature of our findings. In addition, we conducted post hoc sensitivity analysis among cases and controls who had elective cesarean section to assess whether disproportionate presence/absence of labor among cases and controls influenced our findings and found no significant differences in findings to those included in this report.

GDM cases in our study had higher rates of comorbidities (such as preeclampsia), compared with controls. Because these conditions are associated with GDM, we did not adjust for them in analysis. We did not match cases and controls on these variables to avoid overrepresentation of complicated pregnancies among our controls.

Finally, inferences regarding temporality of association of altered placental gene expression profile and GDM are limited by the cross-sectional nature of the study. Expression studies of RNA extracted from blood collected before GDM diagnosis may overcome some of these limitations.

In conclusion, our study provides supportive evidence for involvement of potential candidate genes in GDM pathogenesis. When corroborated by other studies, these findings could advance understanding of GDM pathogenesis that leads to early diagnosis, treatment, and improved outcome. ■

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