Identification of Differential Gene Expression Profiles in Placentas from Preeclamptic Pregnancies Versus Normal Pregnancies by DNA Microarrays

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Abstract

The purpose of this study was to perform a comprehensive analysis of gene expression profiles in placentas from preeclamptic pregnancies versus normal placentas. Placental tissues were obtained immediately after delivery from women with normal pregnancies ($n=6$) and patients with preeclampsia ($n=6$). The gene expression profile was assessed by oligonucleotide-based DNA microarrays and validated by quantitative real-time RT-PCR. Functional relationships and canonical pathways/networks of differentially-expressed genes were evaluated by GeneSpring[™] GX 11.0 software, and ingenuity pathways analysis (IPA). A total of 939 genes were identified that differed significantly in expression: 483 genes were upregulated and 456 genes were downregulated in preeclamptic placentas compared with normal placentas (fold change ≥ 2 and $p < 0.05$ by unpaired *t*-test corrected with Bonferroni multiple testing). The IPA revealed that the primary molecular functions of these genes are involved in cellular function and maintenance, cellular development, cell signaling, and lipid metabolism. Pathway analysis provided evidence that a number of biological pathways, including Notch, Wnt, NF- κ B, and transforming growth factor- β (TGF- β) signaling pathways, were aberrantly regulated in preeclampsia. In conclusion, our microarray analysis represents a comprehensive list of placental gene expression profiles and various dysregulated signaling pathways that are altered in preeclampsia. These observations may provide the basis for developing novel predictive, diagnostic, and prognostic biomarkers of preeclampsia to improve reproductive outcomes and reduce the risk for subsequent cardiovascular disease.

Introduction

PREECLAMPSIA IS A PREGNANCY-SPECIFIC multisystem disorder which is characterized by gestational hypertension, proteinuria, and other systemic disturbances, occurring after the 20th week of pregnancy in a previously normotensive woman. Preeclampsia affects up to 8% of all pregnancies and contributes substantially to maternal and perinatal morbidity and mortality worldwide (Sibai et al., 2005; Steegers et al., 2010). Although several etiologic theories have been proposed and extensively investigated, the underlying pathophysiology of preeclampsia remains poorly understood, and progress in the development of reliable predictors and therapeutic interventions for preeclampsia has been hampered (James et al., 2010; Young et al., 2010). It is, however, generally accepted that the placenta is the principal contributor to the pathogenesis of preeclampsia, because the only effective therapy for preeclampsia is the delivery of the placenta, after which the symptoms vanish within days (Young et al., 2010). Therefore, comparative gene expression

profiling analysis of preeclamptic placentas and normal placentas is a prerequisite to understand the pathophysiology of this disease.

Microarray technology is a powerful tool that allows the simultaneous analysis of thousands of genes expressed in a single sample. To date, a number of microarray studies have been conducted on placental gene expression in preeclampsia and showed that obesity-related genes, cytokine-receptor genes, and apoptosis-related genes are involved in the development of preeclampsia (Centlow et al., 2011; Gack et al., 2005; Han et al., 2006; Heikkila et al., 2005; Herse et al., 2007; Kang et al., 2011; Lee et al., 2010a; Nishizawa et al., 2007; Pang and Xing, 2003a; Reimer et al., 2002; Sitras et al., 2009; Soleymanlou et al., 2005; Tsoi et al., 2003; Zhou et al., 2006; Zhu et al., 2002). However, comparison of different studies done using DNA microarrays has shown poor overlap, and only a minority of differentially-expressed genes are thus shared between them. The reason for this discrepancy may be the different maternal ethnicities, the use of different platforms and protocols, and the impact of individual differences on

expression signatures (Kang et al., 2011; Sitras et al., 2009). The search for genes involved in the development of preeclampsia therefore needs to continue in order to better understand the events leading to preeclampsia. In addition, most previous studies addressed the molecular profile of preeclamptic pregnancies by using low-density cDNA arrays that included a limited number of genes (Gack et al., 2005; Han et al., 2006; Herse et al., 2007; Pang and Xing, 2003a; Soleymannlou et al., 2005; Tsoi et al., 2003). Therefore, the comprehensive analysis of the gene expression profile of preeclamptic pregnancies is needed to identify novel aberrant expression genes, which may represent novel molecular targets for therapeutic intervention in this disease.

In order to explore novel genes involved in the development of preeclampsia, the present study investigated differential expression profiling between placentas from preeclamptic pregnancies and uncomplicated pregnancies using oligonucleotide arrays for whole genome gene expression analysis. Moreover, the expression of selected genes was further validated by quantitative real-time RT-PCR analysis to confirm the effectiveness of data obtained by DNA microarrays.

Materials and Methods

Patient characteristics and placental sample collection

Placental tissues were obtained with from nulliparous women who were admitted to the Department of Obstetrics, The First Affiliated Hospital of China Medical University (Shenyang, China). The clinical characteristics of the participants are summarized in Table 1. Placental tissues were collected from women with normal pregnancies (control group, $n=6$), and patients with preeclampsia (preeclampsia group, $n=6$). All women were delivered via elective cesarean delivery without labor to eliminate the effect of labor (Lee et al., 2010b). Preeclampsia was diagnosed when both pregnancy-induced hypertension and proteinuria were present, according to the International Society for the Study of Hypertension in Pregnancy (Perry and Beevers, 1994). Pregnancy-induced hypertension was defined as maternal systolic and diastolic blood pressure $>140/90$ mm Hg on at least two occasions separated by 6 h after 20 weeks of gestation. Proteinuria was defined as urinary protein $>2+$ on dipstick, or >0.3 g/day. Women who had a history of cardiovascular, renal, and other hypertension-associated diseases were excluded. Placental

samples were obtained immediately after delivery, and tissue blocks (approximately 1 cm^3 each) were dissected from the standard locations on the maternal face of the placentas as previously described (Sood et al., 2006). Villous portions were harvested by dissecting free of blood vessels and connective tissue and washing off adherent blood clots. Villous tissues were immediately immersed in liquid nitrogen overnight and then stored at -80°C until needed. Written informed consent was obtained from each participant, and the study protocol was approved by the Institutional Review Board of China Medical University.

Isolation of total RNA

Total RNA was extracted from placental tissues with TRIzol reagent (Life Technologies, Inc., Rockville, MD, USA) according to the manufacturer's instructions. The concentration and purity of the RNA in each sample were determined by measuring the absorbance at 260 and 280 nm. RNA integrity was confirmed by electrophoresis on 1% agarose gels.

Microarray procedures

Amplification and labeling of complementary RNA (cRNA) with biotin were performed using the Illumina® TotalPrep™ RNA amplification kit (Ambion, Austin, TX, USA), on an aliquot of 440 ng total RNA of each sample as input material. *In vitro* transcription of cDNA to cRNA was performed overnight using biotin-11-deoxyuridine triphosphate (biotin-11-dUTP) to label the cRNA product. The cRNA yields were quantified with a spectrophotometer. Labeled cRNA (500 ng) was then hybridized to HumanHT-12 V4 BeadChip™ arrays (Illumina, San Diego, CA, USA) containing 47,231 transcripts (targeting approximate 31,000 annotated genes) at 55°C overnight following staining with $1\text{ }\mu\text{g/mL}$ streptavidin-Cy3 (Amersham Biosciences, Piscataway, NJ, USA) for visualization. Washing of the arrays was performed using Illumina high-stringency wash buffer for 30 min at 55°C , followed by scanning according to standard Illumina protocols. Probe intensity and detection data were obtained using Illumina BeadStudio™ software, and further processed with GeneSpring™ GX 11.0 software (Agilent Technologies, Santa Clara, CA, USA). Quality standards for hybridization, labeling, staining, background signal, and basal level of housekeeping gene expression for each chip were verified. The microarray data have been deposited in NCBI's Gene Expression Omnibus (<http://www.ncbi.nlm.nih.gov/geo>), and are accessible through GEO series accession number GSE30186.

Analysis of microarray data

Raw intensity data were background subtracted and quantile normalized using BeadStudio software, and then imported into the GeneSpring GX 11.0 software. Unpaired *t*-test analysis with Bonferroni multiple testing correction was utilized to obtain genes whose fold change between preeclamptic placentas and normal placentas was ≥ 2.0 (with a *p* value cut-off of <0.05). Average linkage hierarchical clustering was performed, and the Pearson centered distance metric was used as a measure of similarity between the gene expression profile samples based on log-transformed signal values across the differentially-expressed genes. The differential expression gene list derived from unpaired *t*-tests was

TABLE 1. CLINICAL CHARACTERISTICS OF THE STUDY POPULATION

Variables	Control (n=6)	Preeclampsia (n=6)	p Value
Maternal age (years)	28.5 $\pm$ 1.87	26.0 $\pm$ 4.34	0.224
Gestational age (days)	273 $\pm$ 5	255 $\pm$ 6	<0.01
Systolic blood pressure (mm Hg)	111 $\pm$ 11.1	163 $\pm$ 12.1	<0.01
Diastolic blood pressure (mm Hg)	73 $\pm$ 5.2	108 $\pm$ 4.1	<0.01
Birthweight (g)	3557 $\pm$ 255	2472 $\pm$ 401	<0.01
Proteinuria (g/L)	Not detected	3.15 $\pm$ 1.8	

Values are expressed as mean $\pm$ standard deviation. Statistical significance for differences in means among groups was assessed by Student's *t*-test.

plotted on a volcano plot, and the intersection among the two comparisons of the two groups was submitted to principal component analysis (PCA). The BioPAX pathways/networks exchange format from public databases (Reactom, Biocyc and Pathway Commons, available online: <http://www.biopax.org>) was imported into the GeneSpring GX 11.0 software, and the Find Significant Pathway tool was then used to identify the biological pathway for which there was significant enrichment in the differential expression gene list. In addition, the gene set analysis (GSA; Broad Institute, Cambridge, MA, USA) developed by Subramanian and associates was applied to interpret expression profiles from microarrays of preeclampsia (Subramanian et al., 2005). The gene sets were downloaded and imported from the Broad Institute website: <http://www.broadinstitute.org/gsea/downloads.jsp> into the GeneSpring GX 11.0 software. The designated cutoffs were a minimum of 15 genes and a *p* value cutoff of 0.05.

The web-based pathway analysis tool IPA 5.5 (Ingenuity Systems, www.ingenuity.com) was used to identify biological and molecular networks in preeclamptic pregnancies. Differentially-expressed genes identified by DNA microarrays were uploaded into IPA along with the gene identifiers and corresponding fold change values. Each identifier was mapped to its corresponding gene object in the Ingenuity knowledge base. Networks were then algorithmically generated

based on their connectivity and a score was assigned. The score is a numerical value used to rank networks according to how relevant they are to the genes in the input dataset. The network identified is then presented as a graph indicating the molecular relationships between genes/gene products. Genes are represented as nodes, and the biological relationship between two nodes is represented as an edge (line). The intensity of the node color indicates the degree of upregulation (red) or downregulation (green) of a given gene. Genes in uncolored nodes were not identified as differentially expressed in our experiment, and were integrated into the computationally-generated networks on the basis of the evidence stored in the IPA knowledge memory indicating a relevance to this network. Nodes are displayed using various shapes that represent the functional class of the gene product. Edges are displayed with various labels that describe the nature of the relationship between the nodes.

Canonical pathway analysis identified the pathways, from the IPA library of canonical pathways, which were most significant to the input data set. The significance of the association between the genes from the dataset and the canonical pathway was determined based on two parameters as described in the IPA documentation: (1) a ratio was calculated of the number of genes from the dataset in a given pathway divided by the total number of molecules that make up the

TABLE 2. PRIMERS USED FOR QUANTITATIVE REAL-TIME REVERSE TRANSCRIPTASE POLYMERASE CHAIN REACTION

<i>Gene symbol</i>	<i>Sequences</i>	<i>Product size (bp)</i>
HMBS	Forward: 5'-CTGCAAGCGGGAAAACCT-3' Reverse: 5'-CTCCAGATGCGGGAACCTTCT-3'	148
ULBP1	Forward: 5'-AGTTGTTTAGAGTGACAGGTGGAA-3' Reverse: 5'-ATGAGCGAAGGTAATGAGTGG-3'	212
LEP	Forward: 5'-CTGTGCGGATTCTTGTTGG-3' Reverse: 5'-GTGACTTTCTGTTTGGAGGA-3'	158
FLT1	Forward: 5'-GAATAGGGAGACAGGGTAGG-3' Reverse: 5'-GTGGCACATAAGAACAGAGG-3'	281
GPR144	Forward: 5'-AGTTCTCTGGACAGCGACTGA-3' Reverse: 5'-CGTCCTCTCGTCGAACACC-3'	206
GPR115	Forward: 5'-TTTAAGGACTCAACTGGTGCATC-3' Reverse: 5'-ACACTCTCAATGGTCTCTGGAG-3'	98
GPR149	Forward: 5'-GCTTACAAAAGTCGTCCTTTGGC-3' Reverse: 5'-GGACAAGACAAACACTGGGGT-3'	145
CXCL9	Forward: 5'-ATCTTGCTGGTTCTGATTGGAGTG-3' Reverse: 5'-AAGGTCTTCAAGGATTGTAGGTG-3'	111
TMCC1	Forward: 5'-ATGATAATTGAAGGGTACTTCTGTC-3' Reverse: 5'-TTGGACATGGCTAGTGAGGAA-3'	227
HPDL	Forward: 5'-CTAGATGGTGATAAAGGCAAGTT-3' Reverse: 5'-GTTCCCTCAGTTCTGTGCTGGTCT-3'	245
INSL6	Forward: 5'-GTGGCTTGGACTCCTGCTGGTTC-3' Reverse: 5'-ACTGGTATGGGCTGTAGGCTTCG-3'	209
KCNN1	Forward: 5'-GGGTTCGGAAACACCAGCGTAAG-3' Reverse: 5'-TGGGTCTTGGCTAGGTCGGTAAG-3'	127
RMRP	Forward: 5'-ATACTCCAAAGTCCGCCAAGAAGC-3' Reverse: 5'-TAGAGAATGAGCCCCGTGTGGTTG-3'	127
ANKS1B-1	Forward: 5'-CAGTTTATGGGAAGCAATGTTATGG-3' Reverse: 5'-CAATGGGTCTCATCTTTGGAAGGAG-3'	121
CYP1A2	Forward: 5'-CACTCCTCCTTCTTGCCCTTCAC-3' Reverse: 5'-GTTGACCTGCCACTGGTTTACGA-3'	114
WNT10A	Forward: 5'-GGGCTCTAGGACTGACTGGGTT-3' Reverse: 5'-TAAATGAATGATGAAGGGAATGGTG-3'	279
β -actin	Forward: 5'-CCATCGTCCACCGCAAAT-3' Reverse: 5'-GCTGTACCTTCACCGTTC-3'	194

canonical pathway; (2) Fisher's exact test was used to calculate a p value determining the probability that there is an association between the genes in the dataset and the canonical pathway that cannot be explained by chance alone.

Quantitative real-time RT-PCR

To validate the results of the microarrays, several selected genes were analyzed by quantitative real-time RT-PCR. cDNA was synthesized from 1 μ g of placental total RNA using the Superscript first-strand synthesis kit system according to the manufacturer's recommended protocol (Tiangen Biotechnology Co. Ltd., Beijing, China). Quantitative real-time PCR reactions were carried out in 20 μ L of reaction mixture containing 10 μ L of SYBR-Green Master mix (Tiangen), 0.5 μ M of forward and reverse primers, and 1.5 μ L template cDNA on a Exicycler 96 real-time PCR system (Bioneer, Daejeon, South Korea). PCR primers were designed using Primer Express 2.0 software (Applied Biosystems, Foster City, CA, USA), and are listed in Table 2. The specificity of the amplified products was analyzed through dissociation curves generated by the

equipment yielding single peaks. β -Actin was used as an internal control to normalize samples (Centlow et al., 2011; Lee et al., 2010a). PCR reactions of each sample were conducted in triplicate. Data were analyzed through the comparative threshold cycle (C_T) method (Livak and Schmittgen, 2001).

Results

Clinical parameters

Clinical characteristics of the studied population are presented in Table 1. Maternal age of preeclamptic women was similar to that of controls ($p = 0.224$). The systolic and diastolic blood pressures and proteinuria were significantly higher in the preeclamptic women, but the gestational age and birth-weight were markedly lower than those in the control group (all $p < 0.01$).

Global differences in gene expression

To explore novel genes involved in the development of preeclampsia, genome-wide expression profiles of placentas

FIG. 1. Principal component analysis (PCA) was applied to 12 placental samples that were characterized by the gene expression of all probes on the HumanHT-12 V4 BeadChip arrays, including the control group (blue) and the preeclampsia group (red).

from the preeclampsia group and the control group were investigated. As shown in Figure 1, the relationship between the two groups is visually apparent in PCA. The PCA is an exploratory tool to characterize the predominant gene expression patterns, derived from a matrix of the measurements of the 47,231 genes in different subjects. Using PCA, it is possible to identify whether samples from the same conditions have similar patterns of gene expression. The patterns that are grouped in similar areas within the three-dimensional condition scatterplot demonstrate similarity between members of each sample group, as well as differences between groups (Fig. 1), suggesting that the samples used in this study were appropriately prepared and selected.

Using Illumina HumanHT-12 V4 BeadChip arrays (composed of 47,231 probe sets to measure human mRNA), the expression profiles of placentas from the preeclampsia group and the control group were compared. After performing unpaired *t*-test analyses with Bonferroni correction, 939 genes whose expression differed significantly at $p < 0.05$ with a fold change ≥ 2 were identified. Among these, 483 genes were upregulated and 456 genes were downregulated in preeclamptic placentas compared with normal placentas. The complete list of these genes is shown in Supplementary Material 1 (see online supplementary material at <http://www.liebertonline.com>). Genes differing in expression were represented in a volcano plot (Fig. 2), and then subjected to

FIG. 2. Volcano plot of probe sets differing between preeclamptic placentas and normal placentas. Fold change (x axis) is plotted against statistical significance (y axis) for each probe set. Genes upregulated with a fold change ≥ 2 and $p < 0.05$ are depicted in red, and those downregulated with a fold change ≥ 2 and $p < 0.05$ are shown in green. Grey represents genes in the arrays that were not found to differ significantly between preeclamptic placentas and normal placentas.

FIG. 3. Cluster analysis of the probes that were increased and decreased significantly in preeclamptic placentas compared with normal placentas ($p < 0.05$, fold change ≥ 2). A dendrogram of the cluster correlation is shown on the right. Pseudocolors indicate differential expression (red indicates transcript levels greater than the median; black indicates transcript levels equal to the median; green indicates transcript levels below the median; distance metric, Pearson centered; linkage rule, average).

hierarchical clustering analysis, revealing that preeclamptic placentas are distinctly different from normal placentas (Fig. 3). The data on the top 10 genes upregulated and 10 genes downregulated in preeclamptic placentas compared with normal placentas are listed in Tables 3 and 4, respectively.

Find Significant Pathway, IPA, and GSA

Using the Find Significant Pathway tool in GeneSpring GX 11.0 and public pathway databases, we found a total of 18 pathways or networks closely associated with the genes that

exhibited significant differential expression between preeclamptic placentas and normal placentas ($p < 0.05$; Supplementary Material 2; see online supplementary material at <http://www.liebertonline.com>), and the following three pathways were found to be the most highly associated networks: (1) hemoglobins chaperone; (2) transport of vitamins, nucleosides, and related molecules; and (3) transport of nucleosides and free purine and pyrimidine bases across the plasma membrane (all $p < 0.01$). By GSA, the neuroactive ligand-receptor interaction gene set was significantly enriched in the 939 gene sets containing at least 15 matching genes.

TABLE 3. TOP 10 GENES FOUND TO BE SIGNIFICANTLY UPREGULATED IN PREECLAMPTIC PLACENTAS COMPARED WITH NORMAL PLACENTAS

Probe ID	p Value	log 2 (fold change)	Gene symbol	Accession no.
2060364	0.001538	5.86	BTNL9	NM_152547.3
4730403	0.009155	4.61	HMBS	NM_001024382.1
2970128	0.001627	4.16	ULBP1	NM_025218.2
4120717	0.007952	4.02	CHRNA1	NM_000079.2
10026	0.001281	3.81	RMRP	NR_003051.2
6480026	0.015168	3.61	HPDL	NM_032756.2
1850634	0.002688	3.54	LOC100133277	XR_039146.1
2690674	0.011646	3.52	POLR2J4	NR_003655.1
5690592	0.026544	3.43	TLE6	NM_024760.1
130204	0.00559	3.33	LOC643955	NR_003952.1

TABLE 4. TOP 10 GENES FOUND TO BE SIGNIFICANTLY DOWNREGULATED IN PREECLAMPTIC PLACENTAS COMPARED WITH NORMAL PLACENTAS

Probe ID	p Value	log 2 (fold change)	Gene symbol	Accession no.
2900301	0.004259	5.21	INSL6	NM_007179.2
5570278	0.017253	4.76	CXCL9	NM_002416.1
7040603	0.001575	4.34	CATSPERB	NM_024764.2
6760471	0.001962	4.29	TMCC1	NM_001017395.1
1240195	0.017154	4.27	PAGE2	NM_207339.2
1340240	4.62E-04	4.19	LOC100134067	XM_001716369.1
4250064	5.14E-04	3.94	KCNN1	NM_002248.3
6660463	0.001067	3.69	ANKS1B	NM_020140.2
4860494	0.020812	3.65	C3	NM_000064.1
1300300	0.18719	3.64	ACOXL	NM_018308.1

The 939 genes obtained from unpaired *t*-tests were uploaded to IPA, revealing that the networks involved in cellular function and maintenance, cellular development, cell signaling, and lipid metabolism, were particularly enriched by genes in our set of differentially-expressed genes (Supplementary Material 3; see online supplementary material at <http://www.liebertonline.com>). This most significantly enriched network generated from this comparison was Cell-To-Cell Signaling and Interaction, Hematological System Development and Function, and Immune Cell Trafficking. The score for this network was 39 and is shown in Figure 4. In addition, assessment with IPA also showed that 194 canonical pathways were represented in the differential expression gene list (Supplementary Material 4; see online supplementary material at <http://www.liebertonline.com>), and the top five pathways are: (1) G protein-coupled receptor signaling ($p=1.05E-03$, ratio=0.047); (2) natural killer cell signaling ($p=1.08E-03$, ratio=0.082); (3) atherosclerosis signaling ($p=3.96E-03$, ratio=0.069); (4) calcium-induced T lymphocyte apoptosis ($p=4.37E-03$, ratio=0.086); and (5) altered T-cell and B-cell signaling in rheumatoid arthritis ($p=5.63E-03$, ratio=0.076; Fig. 5). Ratio refers to the number of genes in the differential expression gene list over the total number of genes in the respective canonical pathway.

Validation of microarray results by quantitative real-time RT-PCR

Quantitative real-time RT-PCR was performed to validate a number of candidate genes (Table 2). These genes were selected on the basis of previous associations with preeclampsia (LEP and FLT1), their expression levels in microarrays (HMBS, ULBP1, CXCL9, TMCC1, INSL6, KCNN1, RMRP, ANKS1B, HPDL, CYP1A2, and WNT10A), or were supervised by our pathway analysis (GPR144, GPR115, and GPR149). Triplicate reactions were conducted on each sample, and the data were analyzed using the delta delta C_T method (Livak and Schmittgen, 2001). Fold-change differences that compared PCR gene expression measurements between preeclamptic placentas and normal placentas were not as pronounced as those observed in the microarray study. However, when the log of the fold change in gene expression as determined by real-time RT-PCR was plotted against the log of the fold change as determined by DNA microarray analysis, there was an excellent correlation between the two technologies ($R^2=0.933$; Fig. 6), suggesting that the results of real-time RT-PCR were consistent with those of microarray analysis.

Discussion

The present study represents a comprehensive analysis of gene expression profiling in preeclamptic pregnancies with the use of microarray technology, which identifies 483 genes upregulated and 456 genes downregulated in preeclamptic placentas versus normal placentas. The IPA revealed that these genes are involved in cellular function and maintenance, cellular development, cell signaling, and lipid metabolism. Quantitative real-time RT-PCR analysis verified the results of the microarray analysis.

The Illumina Expression BeadChip arrays are the preferred system for array-based gene expression studies because of their low input requirement, high sensitivity, low cost, and high throughput. The latest content on the HumanHT-12 v4 Expression BeadChip provides more biologically meaningful results through genome-wide transcriptional coverage of well-characterized genes, gene candidates, and splice variants. A unique feature distinct from previous microarray studies of preeclamptic pregnancies is that we used the HumanHT-12 v4 Expression BeadChip, followed by a thorough bioinformatics analysis using GeneSpring software and IPA. In the present study, we identified a large number of novel genes and some biological pathways that were aberrantly regulated in preeclampsia. These findings not only add to a growing body of literature demonstrating dysregulated genes and biological pathways in preeclamptic pregnancies, but also provided the basis for identification of new biomarkers in preeclampsia, and are worthy of future in-depth studies to elucidate their roles in preeclampsia.

Since the clinical symptoms associated with preeclampsia are rapidly resolved once the placenta is delivered, the placenta is generally believed to play a central role in the pathogenesis of preeclampsia (Young et al., 2010). Therefore, defining the gene expression of preeclamptic placentas has been the focus of numerous studies (Damiano, 2011; Ornaghi et al., 2011; Shin et al., 2011; Wang et al., 2011). To our knowledge, a number of comprehensive analyses have been conducted on the gene expression in preeclamptic placentas using DNA microarrays (Kang et al., 2011; Pang and Xing, 2003a, 2003b, 2004; Reimer et al., 2002; Sitras et al., 2009; Tsoi et al., 2003; Zhou et al., 2006). These studies revealed a substantial number of molecular players involved in the process of preeclampsia development, and helped defined the role of some of the signaling pathways involved in the pathogenesis of preeclampsia. However, their results,

Score = 39

FIG. 4. The most significantly enriched network that was identified with ingenuity path analysis (score=39).

FIG. 5. The top five canonical pathways that were identified with ingenuity path analysis in preeclamptic placentas.

together with the present results that identified genes in preeclamptic placentas, are inconsistent concerning the genes and pathways of interest, which reflects the complexity of preeclampsia. Despite these inconsistencies, our results also showed overlapping of some genes, such as leptin and FLT1, and of some pathways, including the Notch, Wnt, NF- κ B, and TGF- β signaling pathways, as evidenced by the study from Sitras and colleagues (Sitras et al., 2009). These findings suggest that the differentially-expressed genes in preeclamptic placentas were related to dysregulation of various signaling

pathways, which further contributes to the pathogenesis of preeclampsia.

Insulin resistance has long been known as a risk factor for preeclampsia, which could help to explain the link with future cardiovascular disease (Carty et al., 2010; Hauth et al., 2011). Furthermore, experimental and epidemiological evidence has indicated that fatty acids can improve glucose tolerance and prevent insulin resistance (Ebbesson et al., 2005; Ghafoor-unissa et al., 2005). Using Find Significant Pathway, we provided supporting evidence for some of these defined

FIG. 6. Comparison of microarray and quantitative real-time RT-PCR expression measurements for the selected genes. Graph shows log of fold changes for array data and log of fold changes for quantitative real-time RT-PCR data.

relationships in our networks, such as regulation of insulin secretion by fatty acids bound to GPR40 (FFAR1) and free fatty acid receptors (Supplementary Material 2; see online supplementary material at <http://www.liebertonline.com>). In addition, preeclamptic patients are characterized by increased vascular responsiveness to angiotensin II, and dysregulation of G protein-coupled receptor signaling has been demonstrated to be a key event in the etiology of preeclampsia (Rocheville et al., 2000; Stepan et al., 2006). In the present study, IPA consistently showed G protein-coupled receptor signaling to be the most highly associated network represented in the differential expression gene study. Another interesting observation of the present microarray analysis is the significantly exhibited network hemoglobins chaperone. In support of this result, May and colleagues have recently shown that the *ex vivo* hemoglobin perfusion of human placenta resulted in physiological and morphological changes, and a gene expression profile similar to what is observed in preeclamptic placentas, suggesting the potentially important role of hemoglobin in the pathogenesis of preeclampsia (May et al., 2011).

Some limitations of the present study need to be pointed out, especially regarding the placenta sample collection. Although we used placental samples from a standardized location, it is still uncertain whether the observed gene expression profile represents the global placental gene expression changes seen in preeclampsia, and which cells within the placenta are the sources responsible for these transcripts. In addition, the gene expression differences observed in the present study probably result from the disease process instead of disease onset and/or pathogenesis. Nonetheless, it is indeed ethically unacceptable to conduct a longitudinal study taking serial placental biopsies at different time points in gestation.

In summary, in this microarray study, we present a comprehensive list of placental gene expression profiles, and various dysregulated signaling pathways that are altered in preeclampsia. The observed differences in gene expression may provide the basis for developing novel predictive, diagnostic, and prognostic biomarkers of preeclampsia, to improve reproductive outcomes, and to reduce the risk of subsequent cardiovascular disease development. Further studies are being conducted by our group to unravel the functions of the differentially-expressed genes in preeclampsia.

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Author Disclosure Statement

The authors declare that no conflicting financial interests exist.

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