Molecular Human Reproduction Vol.12, No.3 pp. 169–179, 2006
Advance Access publication March 23, 2006 doi:10.1093/molehr/gal011

Gene expression profiling of human placentas from preeclamptic and normotensive pregnancies

S.R.Hansson[1,6], Y.Chen[2], J.Brodszki[1], M.Chen[3], E.Hernandez-Andrade[1], J.M.Inman[3,4], O.A.Kozhich[3,4], I.Larsson[1], K.Marsál[1], P.Medstrand[5], C.C.Xiang[3] and M.J.Brownstein[3]

[1]Department of Obstetrics and Gynecology, Lund University Hospital, Lund, Sweden, [2]Cancer Genetics Branch, National Human Genome Research Institute, [3]Laboratory of Genetics, National Institutes of Mental Health, National Institutes of Health, Bethesda, [4]SAIC Frederick, National Cancer Institute at Frederick, Fredrick, MD, USA and [5]Department of Cell and Molecular Biology, Lund University, Lund, Sweden

[6]To whom correspondence should be addressed at: Department of Obstetrics and Gynecology, BMC-C14, Lund University, University Hospital Lund, S-221 85 Lund, Sweden. E-mail: stefan.hansson@med.lu.se

The aim of this study was to investigate patterns of gene expression in placental samples from patients with preeclampsia (PE), persistent bilateral uterine artery notching (without PE), and normal controls. This study included placental tissue from nine women with PE, seven with uncomplicated pregnancies and five with bilateral uterine artery notching in Doppler velocimetry tracings. Human cDNA microarrays with 6500 transcripts/genes were used and the results verified with real-time PCR and *in-situ* hybridization. Multidimensional scaling method and random permutation technique demonstrated significant differences among the three groups examined. Within the 6.5K arrays, 6198 elements were unique cDNA clones representing 5952 unique UniGenes and 5695 unique LocusLinks. Multidimensional scaling plots showed 5000 genes that met our quality criteria; among these, 366 genes were significantly different in at least one comparison. Differences in three genes of interest were confirmed with real-time PCR and *in-situ* hybridization; *acid phosphatase 5* was shown to be overexpressed in PE samples and c*almodulin 2* and *v-rel reticuloendotheliosis viral oncogene homolog A* (RELA) were downregulated in PE and uterine artery notch placentas. In conclusion downregulation of RELA and calmodulin 2 might represent an attempt by the placenta to compensate for elevations in intracellular calcium, possibly caused by hypoxia and/or apoptosis, in both pregnancies with uterine artery notching and preeclampsia.

Key words: Doppler ultrasound/hypertension/microarray/placenta/pregnancy

Introduction

Preeclampsia (PE), characterized by hypertension and proteinuria during pregnancy, has been called the disease of theories (Roberts and Cooper, 2001); no single mechanism satisfactorily explains its etiology. PE was recognized as an important problem by the ancient Egyptians 3000 years ago (Stevens, 1975), and remains a leading cause of perinatal and maternal morbidity and mortality. Worldwide PE occurs in 3–7% of pregnancies. Mainly primigravidae are affected and conditions such as diabetes, obesity, essential hypertension and renal disease are all recognized as predisposing factors. Hyperplacentation (multiple pregnancy, hydatidiform mole) also increases the risk of developing PE (Chun *et al*., 1964). Treatment of PE is symptomatic and consists of antihypertensive therapy. It has not changed dramatically for some time. Removal of the placenta generally leads to disappearance of patients' symptoms, and is the only curative therapy available (Roberts and Cooper, 2001). This suggests that a factor produced by the placenta and released into the bloodstream might be responsible for the symptoms seen in PE.

Positive maternal and paternal histories of PE have been correlated with an increased risk of PE development (Lie *et al*., 1998), and recently, evidence for linkage of PE to markers on chromosome 2 has been obtained (Lachmeijer *et al*., 2001). Monozygotic twins can be discordant for PE, indicating that one's genetic make-up may contribute to the disorder but cannot completely explain it (Roberts and Cooper, 2001).

PE typically becomes clinically manifest in midgestation, but the underlying pathophysiological processes probably begin as early as the time of implantation. In PE there is general damage to the maternal vascular endothelium. Although the etiology of PE is still unknown, several hypotheses have been put forward to explain its occurrence (Dekker and Sibai, 1998). These hypotheses are not mutually exclusive. During placentation it is generally believed that PE is associated with a defect in invasion by trophoblasts in the maternal spiral arteries leading to maladaption and abnormal villous development (Page, 1939; Goldman-Wohl and Yagel, 2002; Chaddha *et al*., 2004). The maternal arterioles are responsible for providing the fetus with nutrients and enable gas exchange via the established uteroplacental blood circulation. In the majority of PE cases, compensation occurs and fetal perfusion is adequate, but in 25% of cases intrauterine growth restriction (IUGR) develops (Kaufmann *et al*., 2003).

Pulsed Doppler techniques have been used to study placental blood flow during pregnancy. The diseased placenta has a high pulsatility index (PI), and abnormal waveforms (notching) in the Doppler shift spectrum recorded from the uterine arteries. In fact, persistent bilateral notching early in pregnancy is associated with an increased risk of developing PE and/or IUGR (Thaler *et al*., 1992).

Inadequate perfusion and suboptimal oxygen delivery are especially stressful to the placenta because of its high energy demand. Oxidative stress and an imbalance between reactive oxygen species

and endogenous antioxidants (Roberts and Hubel, 1999) can cause a further impairment of placental vascular function by damaging endothelial cells (Hung *et al*., 2002).

Placental injury seems to trigger damage to the entire vascular system. This is undoubtedly mediated by the immunological system (Saito and Sakai, 2003). PE differs from patient to patient because in any given individual one or more vascular beds are affected preferentially (Strevens *et al*., 2003). Thus, while vasoconstriction/hypertension and renal leakage/proteinuria are common features of PE, other problems are often also observed: hepatic injury, headache, blurred vision, etc.

In the present study we used cDNA arrays to compare patterns of gene expression in placental samples from patients with PE, persistent bilateral uterine artery notching (without PE), and normal controls.

Materials and methods

Doppler velocimetry

The Doppler ultrasound examinations and collection of placenta tissue were performed at the Department of Obstetrics and Gynecology, Lund University Hospital. Uterine artery Doppler velocimetry was done at 18 weeks of gestation in the majority of subjects from whom placental tissue later was obtained, using an Aspen Acuson system fitted with a 3.5 MHz curved array probe (Acuson, Mountain View, Ca). The uterine arteries were localized in a parasagital view of the uterus, using colour Doppler, the sample gate was placed one cm above the crossing point with the external iliac artery and the Doppler shift signals of uterine artery blood flow velocity were recorded. The PI according to Gosling (Gosling *et al*., 1971) was computed for the left and right uterine arteries. The presence or absence of an early diastolic notch in the Doppler shift waveform was assessed visually according to Bower *et al*. (Bower *et al*., 1993). A normal uteroplacental circulation was defined as a PI < 1.2 (Hofstaetter *et al*., 1996) and absence of the notch. Presence of an early diastolic notch at 18 weeks of gestation defined persisting bilateral uterine artery notching. The mean PI values ± SD in the control, notch and PE groups were not significantly different.

Collection of placental tissue

Placental tissue was collected at the Department of Obstetrics and Gynecology, Lund University Hospital. Sampling after informed consent was approved by the Ethics Committee Review Board for studies in human subjects. Placental tissue from nine women with PE, seven controls, and five women with notching were included in the study. All included patients were Caucasian except for one healthy control that was black. The groups did not differ in maternal age or parity (Table I) based on non-parametric tests, Anova and Bonferroni/Dunn. Cesarean section was performed in two controls, five women with PE, and one woman with bilateral notching. PE was defined as blood pressure = 140/90 mmHg or a rise in blood pressure = 20 mmHg as compared with the first trimester of pregnancy, and proteinuria = 0.3 g/L (Sibai, 2003). Patients with essential hypertension and renal or other systemic diseases were excluded from the study.

Tissue sampling and handling

Tissue samples were obtained immediately after delivery of the placenta. A 10 mm^3 cube of tissue consisting mainly of villi from the central part of the placenta was quickly frozen on dry ice and stored at –80°C until the RNA was extracted. Areas with macroscopic evidence for necrosis and infarctions were not used. The samples were pulverized, but not thawed, prior to extraction.

RNA extractions

Total RNA was extracted from frozen tissue using 1ml Trizol® per 50 mg of tissue according to the manufacturer's instructions (Invitrogen). Proteoglycans and polysaccharides were removed by performing a high-salt precipitation with 0.8 M sodium citrate and 1.2 M sodium chloride. The integrity of the RNA samples was determined by denaturing 1.5% agarose gel electrophoresis with 2% formalin and 1X MOPS buffer. The samples to be analysed were dissolved in RNA loading mix (GenHunter, Nashville, TN). Only samples with 28S/18S ratios of 2 were used for expression profiling.

Arrays

Human cDNA microarrays with 6500 elements were printed on poly-L-lysine coated glass slides using an OmniGrid arrayer (GeneMachines, San Carlo, CA). The methods used have been described elsewhere (Xiang and Brownstein, 2003). The authors may be contacted for the complete list of cDNAs employed.

Probe labelling

Probes were prepared from total RNA using an indirect labelling method driven by modified random hexamer primers (Xiang *et al*., 2002). Briefly, 10 μg of total RNA 4μg of modified P2 primer, and 5 units of RNase inhibitor

Table I. Clinical characteristics of pregnant women at delivery and their offsprings of preeclampsia, asymptomatic women with bilateral notch and controls

	Preeclampsia	Notch	Controls
N	9	5	7
Maternal age (years)	32 (23–40)	37 (32–43)	32 (28–38)
Gestational age (days)	260 (187–281)	250 (219–284)	278 (258–286)
Systolic pressure (mmHg)	140.7 ± 9.8*	120.0 ± 14.1†	118.7 ± 10.2
Diastolic pressure (mmHg)	98.1 ± 8.3‡	77.5 ± 12.6§	77.1 ± 8.1
Proteinuria (g/l)	2.8 ± 2.6¶	0.2 ± 0.5**	ND
Birthweight (g)	2766 ± 1166††	3142 ± 479	3723 ± 425
SGA	0	1‡‡	0
PI	1.20 ± 0.47§§	0.91 ± 0.19	0.88 ± 0.14¶¶
Bilateral Notch	2	5[a]	0
Gender F:M	1:8	2:3	3:4
Apgar score (1 min)	9 (8–10)	9 (3–10)	9 (9–10)
Apgar score (5 min)	10 (9–10)	9 (9–10)	10 (10–10)
Mode of delivery			
VD/PS/ES	4/1/4	4/0/1	5/0/2

ND = not detected, F =female, M = male, VD = vaginal delivery, PS = planned Cesarean section, ES = emergency Cesarean section, SGA = small for gestational age (birthweight below mean –2SD of the reference population), PI = pulsatility index.
The results 1–2 and 11–12 are expressed as median (range).
The results 3–6 and 8 are expressed as mean ± SD.
Uterine artery Doppler velocimetry was performed at 18 weeks of gestation in all subjects from which placental tissue was later obtained.
Anova and Bonferroni/Dunn test showed a significant difference between the controls and preeclampsia groups, *P<0.0009, $^{\ddagger}P$<0.0003, $^{\P}P$<0.0096 and between the notch and preeclampsia, $^{\dagger}P$<0.0054, $^{\S}P$<0.0016, **P<0.0202.
Anova and Bonferroni/Dunn test showed a significant difference between controls and preeclampsia, $^{\dagger\dagger}P$<0.0418.
‡‡The subject with SGA showed a grade III flow, §§N=7, ¶¶N=4.

in a volume of 13 µl were incubated at 70°C for 10 min and cooled on ice for an additional 10 min. The solution was added to a reverse transcriptase mix consisting of 6 µl 5× transcription buffer, 0.6 µl 50× aa-dUTP/dNTPs (25mM dATP, dGTP and dCTP, 15mM dTTP and 10mM aminoallyl-dUTP) and 3 µl 0.1 M DTT. Three µl of Superscript II reverse transcriptase (Invitrogen) were added to this solution and the tubes were incubated for 2 hrs at 42°C. The reaction was stopped with 10 µl 0.5 M EDTA, and the RNA was hydrolysed by addition of 10 µl 1M NaOH and incubation at 65°C for 30 min. The resulting solution was then neutralized with 10 µl 1M HCl, and the cDNA was purified using a Microcon PCR purification Kit (Millipore). The product was dried in a vacuum and resuspended in 4.5 µl distilled water for 30 min at room temperature. Monofunctional NHS-ester dyes (Cy3 and Cy5, Amersham) were added to the cDNAs along with 4.5 µl 0.1 M sodium bicarbonate, pH 9.0. The coupling reaction was allowed to proceed in the dark for 1 h at room temperature. Cy3- (experimental sample) and Cy5- (a reference placental extract common to all of the analyses) labelled probes were combined; unincorporated dyes were removed using a Qia-quick PCR purification kit (Quiagen), and the solutions were concentrated in a vacuum centrifuge and adjusted to 23 µl with water.

Hybridization

Prior to hybridization, the labelled cDNA probes were denaturated at 100°C for 2 minutes after the addition of 4.5 µl 20× saline-sodium citrate (SSC), 2 µl 8mg/ml polyA and 0.6 µl 10% (wt/vol) SDS. The probes were pipetted (12µl) onto arrays, coverslipped, placed in hybridization chambers (Corning, Corning, NY) and incubated in a 65°C water bath for 16–24 hrs. The arrays were washed with 0.5× SSC, 0.01% (wt/vol) SDS, followed by 0.06× SSC at room temperature for 10 min each on a shaker (60 rpm). The slides were placed in a slide rack and dried in a centrifuge with horizontal rotor for 5 min at 1200 g. Each probe was applied to two separate arrays.

Array scanning and expression ratio extraction

Hybridized arrays were read using a GenePix 4000A scanner (Axon, Foster City, CA) and resulting TIFF images were analysed with IPLab software (Fairfax, VA) ArraySuite developed at National Human Genome Research Institute, NHGRI (Chen *et al*., 2002). The ratios of sample intensities to the reference intensities for all targets were computed, and then ratio normalization was performed to set the center of the ratio distribution to 1.0. To assess the reliability of each ratio measurement, a quality score ranging from 0 (low) to 1 (high) was determined for each spot location.

Statistical analysis

Cross-array normalization and ratio averaging: Probes prepared from each RNA sample listed in Table I were applied to a minimum of two arrays. To reduce the noise level, eliminate some missing values and maintain statistical independence while performing other statistical analyses, we chose to perform cross-array normalization and then average the ratios for each clone across all replicated samples. We adopted the procedure employed by Cunliffe (Cunliffe *et al*. 2003). In short, we first equalized the dynamic range of data from replica arrays via a linear normalization procedure, and then clone by clone weighted averages (based on measurement quality and averaged fluorescent intensities from both channels) were calculated yielding average log-ratios. These were used in all of the analyses presented below.

Multidimensional scaling

To visualize and compare all the samples' gene expression profiles, a multidimensional scaling method was employed (Hedenfalk *et al*., 2001). Similarities among the 21 samples (9 PE, 5 Notch, 7 Controls) were assessed by Pearson correlation coefficients of log-transformed expression ratios. As shown in Figure 1, the distance between each pair of samples approximates the dissimilarity (1 minus Pearson correlation coefficient between two samples). In other words, samples with similar gene expression profiles are placed close to each other in the MDS plot and separated from other dissimilar samples.

Differentially expressed genes—identification and significance assessment: To identify genes with differential expression levels across two or more categories based on the pathological reports, we utilized a distance-based method in which a discriminative weight for each gene was established from between-cluster and within-cluster distances defined by $w = d_B / (d_w + \alpha)$, where d_B is the center-to-center distance (between-cluster Euclidean distance), while d_w is the averaged within-cluster Euclidean distances. α is a small constant (0.01 in our study) to prevent the zero denominator case (Bittner *et al*., 2000). The advantage of the method is the direct extension to multiple categories (three in this study). To assess the significance of each weight, we adopted the random permutation technique commonly applied to many gene expression profiling studies (Tusher *et al*., 2001). In short, samples' category labels are randomly permuted (10,000 times) and reassigned, and weights for genes are then reevaluated. The weights derived from all random permutations were polled to assess the probability, *P*-value, for each gene [see supplemental information from (Hedenfalk *et al*., 2001)]. The discriminative weight for each comparison was picked corresponding to a *P*-value of 10^{-4}.

Bioinformatics analysis

Three databases with information about the functions of genes were used to determine whether certain classes of genes preferentially changed their expression profiles in the samples that we studied. The Gene Ontology (GO) database was used to classify genes according to their biological process or molecular function. GO annotations were obtained using GoMiner (Zeeberg *et al*., 2003). Annotations of functional protein domains were downloaded from the Interpro (IPR) database (http://www.ebi.ac.uk/interpro/) and metabolic pathway analysis was performed by KEGG (http://www.genome.ad.jp/kegg/). We first determined the associated GO, IPR and KEGG terms associated with all of the genes represented on the microarray. Duplicate loci were only counted once. This served as the background distribution for subsequent analyses. The same terms were retrieved for genes identified as being significantly changed (see above) in a given set of microarray experiments (Notch vs. Controls, PE vs. Controls and PE vs. Notch). In Table III, the *P*-value cut-off calculated for each term was set to <0.01, an expression of the probability of finding such a count at random in the background distribution assuming a Poisson distribution.

Real-time PCR amplification

cDNA synthesis

RNA was reverse transcribed according to protocols from Applied Biosystems. We used a 50 µl reaction containing 0.5µg total RNA, 1X TaqMan RT buffer, 5.5mM $MgCl_2$, 500 µM dNTPs, 2,5 µM random hexamers, 0.4 U/µl RNase inhibitor and 1.25 U/µl MultiScribe Reverse Transcriptase. The reactions were incubated at 25°C for 10 min, at 48°C for 30 min and finally 5 min 95°C. They were stored at –20°C until analysis was performed.

Analysis

Gene transcripts were quantified by means of real-time PCR with an ABI PRISM® 7000 sequence detection system (Applied Biosystems). Primers and probes were designed using the Primer Express® software program or ordered from Assays on-Design/Demand™ (Applied Biosystems). The primers in each pair targeted different exons of the genes of interest to avoid amplifying contaminating genomic DNA (Table II). Oligonucleotide probes were labelled with fluorogenic dye, 6 carboxyfluorescein (Fam), and quenched with 6 carboxy-tetramethylrhodamine (Tamra). PCR reactions were carried out in a 25 µl volume 1× Universal PCR Master Mix (Applied Biosystems), 0.5 µM TaqMan probe, 0.9 µM of forward and reverse primers respectively and 10 ng DNA. For transcripts analysed with pre-manufactured probes the reactions were carried out in a 25 µl of 1× Universal PCR Master Mix (Applied Biosystems), 1× Assaymix (Applied Biosystems), 0.25 µM probe, 0.9 µM of forward and reverse primers respectively and 10 ng of DNA. The thermal cycling conditions were initiated by UNG activation at 50°C for 2 min and an initial denaturation at 95°C for 10 min. Then 40 cycles were run: 95°C for 15 s, 60°C for 1 min. Two negative controls (no template) were included in every set of amplifications. Each sample was assayed two to three times. β-actin and 18S RNAs were used as references to normalize the signal from the sample. Quantitation was achieved by making a calibration curve using serial 10-fold dilutions of the template DNA (0.08–80ng). Results are expressed as ratios; β-actin or 18S RNA levels serve as the denominator.

Methodological considerations

Using real-time PCR to confirm array data can be difficult because of sample-to-sample variability in levels of any given reference RNA used. This appeared

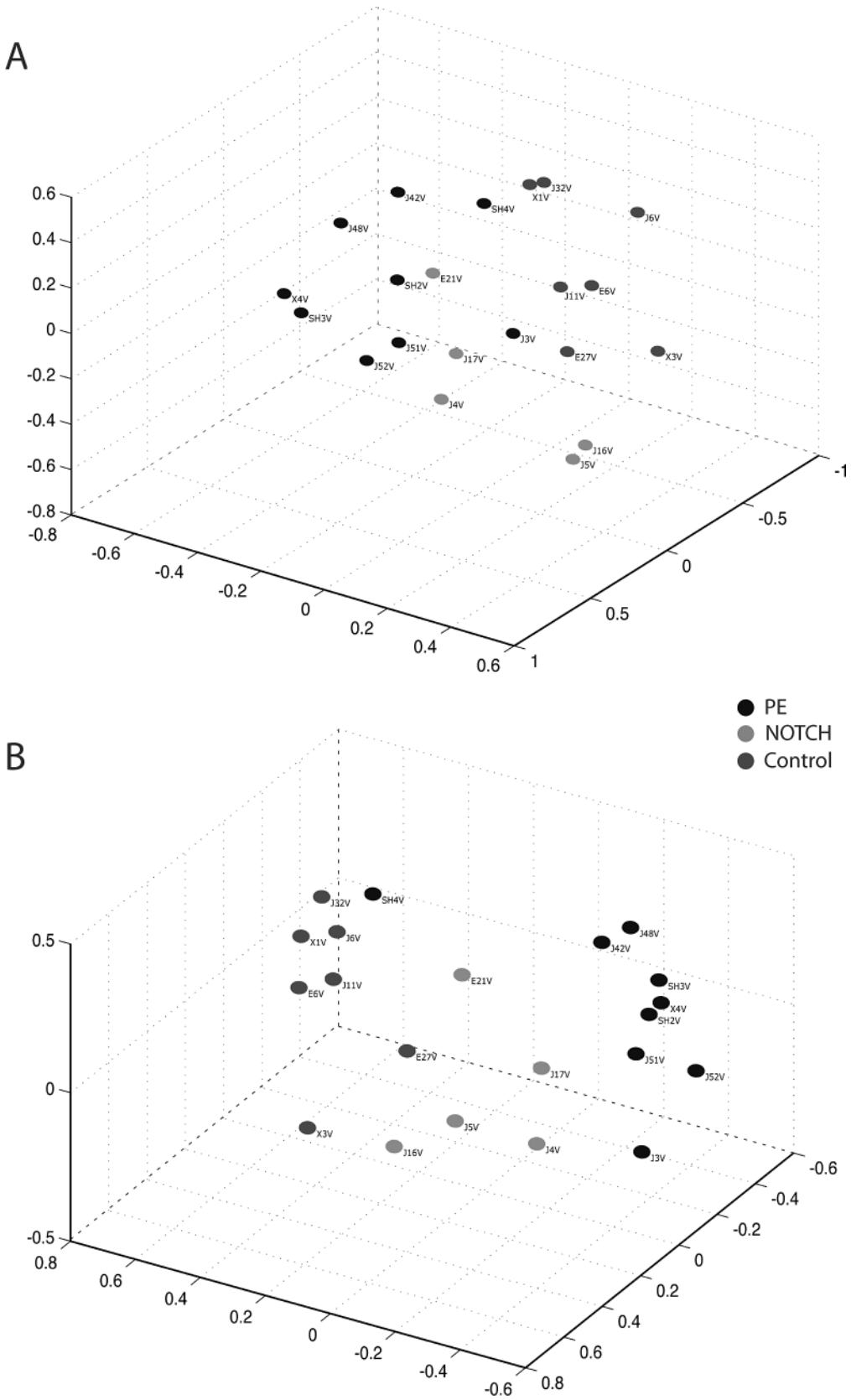

Figure 1. Multidimensional scaling plots for all samples. (**A**) With about 5000 genes that satisfied measurement quality requirement (quality measurement greater than 0.5). (**B**) With 366 genes that satisfied measurement quality requirement (greater than 0.5) and were significantly differentially expressed at least in one of the comparisons shown in Fig. 2. Note that the scale of MDS plots represents the relative distance between samples. The distance between samples was measured by 1 minus Pearson correlation coefficient (the range of correlation coefficient is from –1–1). In other words, if two samples have similar gene expression profiles, they will be placed close together by MDS program.

Table II. Real time PCR probes

mRNA	Accession number	Size (NT)	Primers, forward (F) and reverse (R)/Assay on demand number	Probe/part of amplicon sequence
β-actin		<150	4333762F	
18S rRNA			Hs99999901_s1	
APC5	NM_001611	<150	Hs00356261–1	
CALM2	NM_001743.3	<150	F: CAG AAG CAG AGT TAC AGG ACA TGA T R: TCA TTG TCA GAA ATT CAG GGA AGT CAA	CCA TTA CCA TCA GCA TCT AC
RELA	NM_021975.1	<150	Hs00153294–1	

to be the case in the present study. Nonetheless, we were able to confirm the changes in four genes selected for validation using β-actin and/or 18S RNA references. We feel that the global normalization employed in our analysis of the array data may have been more reliable than normalization based on a single reference. Even though GADPH is commonly used to normalize real-time PCR data (Tricarico *et al*., 2002), we elected not to use it because this enzyme is known to be induced by hypoxia (Yamaji *et al*., 2004) and changes during pregnancy (Cale *et al*., 1997).

Statistics

Real-time PCR results are presented as box plots. The Mann–Whitney U test was used to evaluate the significance of differences between groups. $P<0.05$ was considered statistically significant.

In situ *hybridization*

For the human *acid phosphatase 5 (ACP5)* mRNA, a 360 base probe was used corresponding to NT 40–40, Genbank accession # NM_001611. For the human *Calmodulin 2 (Calm2)* mRNA, a 360 base probe was used corresponding to NT 70–430, Genbank accession # NM_001743.3 and for *v-rel reticuloendotheliosis viral oncogene homolog A, (RELA) mRNA*, a 260 base probe was used corresponding to NT 270–530, Genbank accession # NM_021975.1.

DNA templates were generated by polymerase chain reaction (PCR) from cDNA and sequenced as described previously (Bottalico *et al*., 2003, 2004). Complementary RNA (cRNA) probes were transcribed from 25 ng of gel-purified DNA template using ^{35}S-UTP (Dupont NEN, 1300 Ci/mmol) and either T3 or T7 RNA polymerase according to manufacturer's instructions (Ambion MAX-Iscript) to generate sense and antisense probes, respectively.

Cryostat sections (12 μm) were thaw mounted onto sialinized slides, and stored at –80°C until use. To ensure best possible tissue integrity, thawing of tissue did not occur prior to mounting. Fresh frozen tissue rather than fixative-treated tissue was used in order to maximize mRNA detection. The sections were fixed, dehydrated, dilapidated and hybridized as previously described (Bradley *et al*., 1992; Bottalico *et al*., 2003, 2004). Sections were hybridized (20–24 hrs, 55°C) with 2×10^6 cpm of denatured ^{35}S-cRNA probe per 80 μl hybridization buffer—20 mM Tris-HCl (pH 7,4), 1mM EDTA (pH 8,0), 300mM NaCl, 50% formamide, 10% dextran sulphate, 1× Denhardt's, 25 mg/ml yeast tRNA, 100 μg/ml salmon sperm DNA, 250 μg/ml total yeast RNA (fraction XI, Sigma), 150 mM dithiothreitol (DTT), 0.15% sodium thiosulfate (NTS) and 0.15% sodium dodecyl sulphate (SDS). Following washes, slides were apposed to Kodak Hyperfilm Biomax MR for 2 days, then coated with nuclear track emulsion (NTB-3, Kodak). After a 4-week exposure at 4°C, slides were developed in Dektol (Kodak), fixed and counterstained with a Giemsa stain.

Results

Within the 6.5K arrays, 6198 elements are unique cDNA clones representing 5952 unique UniGenes and 5695 unique LocusLinks. Multidimensional scaling plots show 5000 genes that met our quality criteria (Figure 1a). Among these, a total of 366 genes were significantly different in at least one comparison (Figure 1b). It is evident that, with a single exception, samples with the same clinical classification tend to group together (or have higher correlation coefficient values).

Ten thousand random permutations of the data set were performed, and the corresponding weights found were 3.0 at $P<0.0001$, 4.0 at $P<0.0002$, 3.8 at $P<0.0001$ and 2.4 at $P<0.0001$, without multiple-test correction. At the above levels of statistical significance, we obtained 80 genes (PE vs Controls), 192 genes (Notch vs Controls), 47 genes (PE vs Notch) and 210 genes (all three groups), for a total of 366 (not necessarily unique) genes (Figure 2). After these genes were identified, a new multidimensional scaling plot was drawn (Figure 1b). Clinical groups are separated even more on this plot than on the original one, showing that the genes selected are likely to account for the differences observed. To demonstrate the stringency of each grouping, we removed samples derived from patients with gestational age less than 30 weeks (J42V and J52V), as well as samples with less than 35 weeks of gestation (J42V, J52V, J3V and J48V), i.e. early onset PE. For PE vs Notch comparison at $P<0.001$, we have $w > 2.3$ and $w > 2.4$ for removing two samples and four samples, respectively. For PE vs Control comparison at $P<0.0005$, we have $w > 2.0$ and $w > 2.3$ for removing two samples and four samples, respectively. An expression profile combining 90 significant genes (expression ratios > 1.5) from the three groups is shown in Figure 3. The results of removing two samples or four samples from PE groups and comparing to both Notch and Control groups are also listed in the figure.

The functions of the genes depicted in Figure 3 are listed in Table III. These were ascertained by looking at gene ontology (GO) terms associated with each gene. The functions included cell–cell signalling (gene ontology—GO 7267), apoptosis inhibitor activity (GO 8189), calcium ion binding (GO 5509) and isomerase activity (GO 16853). Pathway analysis (KEGG) placed many of the genes in the cell cycle (hsa4110), MAPK signalling pathway (hsa4010) and ATP synthesis (hsa193) groups. Three genes of interest were selected from the 90 unique genes (Figure 3) and analysed with real-time PCR. When β-actin was used to normalize the PCR data, *acid phosphatase 5, (ACP5)*, NM_001611 (Figure 4a) was shown to be overexpressed in PE samples. Levels of β-actin mRNA were not significantly different between the study groups (data not shown). When 18S RNA was used for normalization, *Calmodulin 2 (Calm2)*, NM_001743.3 (Figure 4b) and *v-rel reticuloendotheliosis viral oncogene homolog A, (RELA)*, NM_021975.1 (Figure 4c) were confirmed to be downregulated in PE and Notch placentas. Removing two samples or four samples from PE group did not change the significance for RELA and calm2, but the change in ACP5 was no longer significant after removal of the four samples.

In situ hybridization on placental tissue sections revealed a general mRNA expression in cytotrophoblast cells for ACP5, RELA and Calm2. In addition, Calm2 mRNA was expressed at higher levels in extravillous trophoblasts and in vascular endothelium (Figure 5). No difference in qualitative expression pattern was seen in any gene among any of the samples studied (data not shown). Sense probes did not show any specific expression (data not shown).

Discussion

Failure to develop a low-resistance vascular bed in the placenta, reflected in the finding of diastolic notch in the Doppler spectrum recorded from the uterine artery, appears to be associated with a

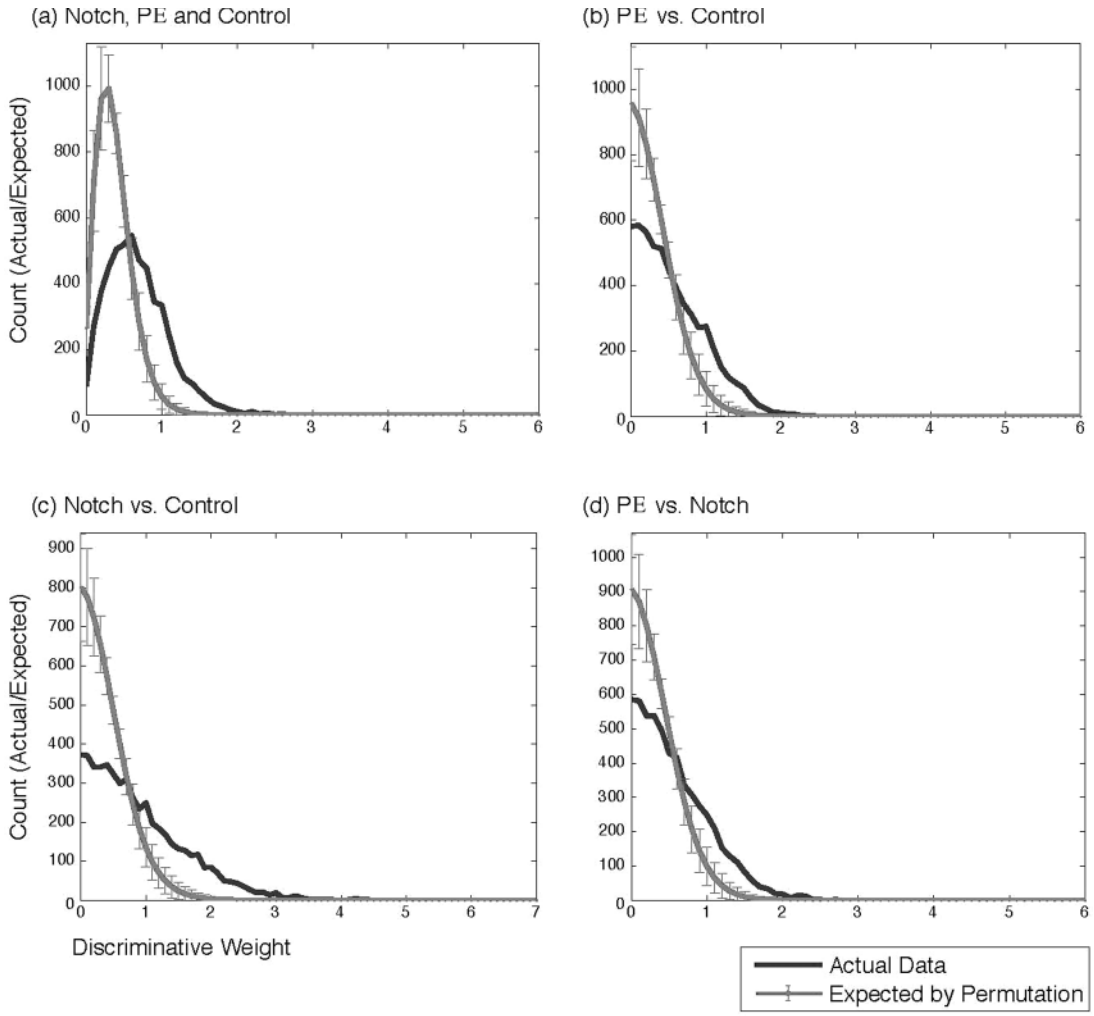

Figure 2. Significance assessment via random permutation. In all following figures, *x*-axis is the discriminative weights, and *y*-axis is the number of genes (either from actual data set—blue colour, or random permutation—red colour) counted at each weight value with a span of 0.1. (**a**) Distance-based method for three groups: PE, Notch and Control samples. Distance weight >1.7 for $P < 0.0005$, for a total of about 114 clones. (**b**) Distance-based method for PE vs Control samples. Weight >1.9 at $P < 0.0005$, for a total of 40 clones. (**c**) Distance-based method for Notch vs Control samples. Weight >2.5 at $P < 0.0005$, for a total of 191 clones. (**d**) Distance-based method for PE vs Notch samples. Weight >2.2 at $P < 0.001$, for a total of 92 clones.

number of gene expression changes. Since the women with bilateral notches in our series did not develop PE, some of the changes in their placentas may have been compensatory and may have protected them and their fetuses from harm. Women with notching who develop PE (n=2), on the other hand, might be expected to induce harmful genes or repress beneficial ones. Our results showed that the expression profiles in PE and Notch groups were similar. The Notch and PE placentas may differ in many ways, but significant changes in gene expression were hard to detect. There were only 27 elements with PE/notch ratios >3 (Figure 3) and 8 with ratios <0.5 (Figure 3), and our bioinformatics analysis identified only one significant category, isomerase activity (GO 16853) (Table III), in this gene set.

Interpreting placental expression profiles, contrary to those of cell lines, is made more difficult by the fact that the placenta is not a homogenous tissue. The samples that we studied comprised mainly trophoblasts, but they also contained blood vessels and cells, macrophages and fibroblasts (Demir *et al*., 1997). Furthermore, placentas from PE patients have a higher number of inflammatory cells (Salafia *et al*., 2000) than normal placentas. Mode of delivery may influence placental gene expression. A majority of the included subjects were delivered vaginally. In the PE group half of the group was delivered by Caesarian section, but the expression profile of the group did not appear to be affected by the mode of delivery.

Among the samples studied, all were from Caucasian subjects except one—a placenta from a woman from Zaire who had a normal pregnancy. Black women are known to have a higher risk of developing PE than Caucasians, and the fact that the black woman's placental expression profile is similar to that of PE placentas is interesting. It would undoubtedly be worth repeating a study similar to ours using placentas from black women. A smaller number of particularly important genes might be found.

Placentas from women with asymptomatic uterine artery notching clearly differed from those of controls. In fact, placentas in the Notch group had many expression changes in common with placentas in the PE group (Figure 3, Table III). The genes separating the two groups could either protect the notch placentas from developing PE or be responsible for the progression from notch to PE. Consequently, they are particularly interesting and should be studied in more detail.

No particular categories of genes comprise the up- and downregulated sets seen in the samples studied. In Figure 3, the transcripts of genes involved in oxidative and reductive processes (GO cell–cell signalling), isomerase activity (GO 16853) and fatty acid metabolism were more abundant in the control group. Apoptosis inhibitor activity

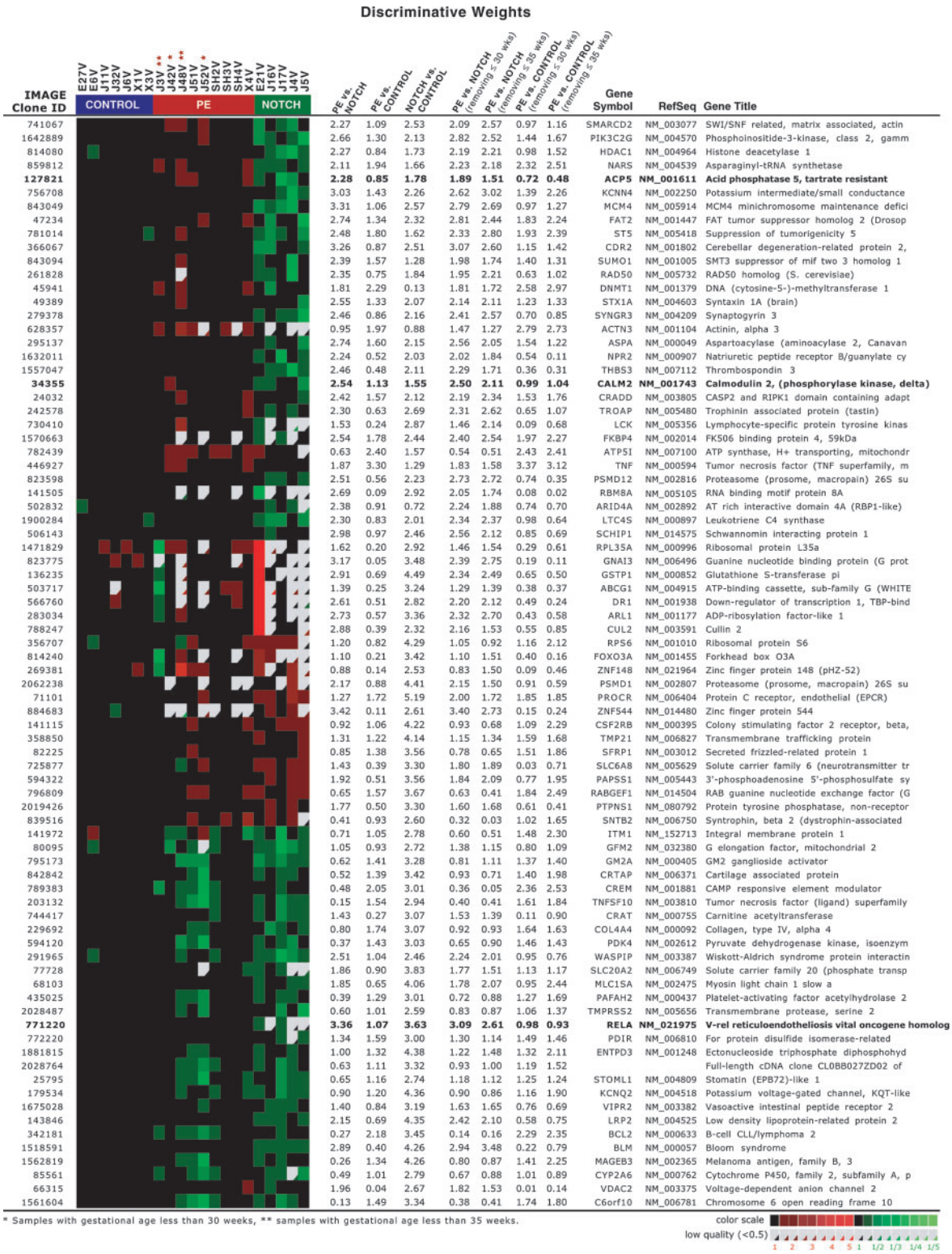

Figure 3. Expression Profiles from significant genes. Three differential gene lists were generated and shown in one combined profile. Significance weights from three comparisons were listed under the heading of PE vs Notch, PE vs Control and Notch vs Control. Total of 292 clones were selected when at least one *P*-value criteria (listed in Figure 2) was satisfied. We further required that average expression ratio (fold-change) between one of two groups must be greater than 1.6 fold, leaving only 85 clones satisfied with this additional requirement. After removing array locations without annotation and duplicated clone ID, we obtained 80 unique genes shown in the figure. In the figure, genes are further sorted based on mean expression ratio in each group following the order of PE, Notch and Control. As illustrated in the figure, clones from 741067 to 356707 have higher expression ratio in PE group; clones from 81420 to 839516 have higher expression in Notch group (but many of them are not unique to the Notch group), and the rest of the genes have higher expression in the Control group. To demonstrate the stringency of each grouping, we removed samples derived from patients with gestational age less than 30 weeks (J42V and J52V), as well as samples with less than 35 weeks of gestation (J42V, J52V, J3V and J48V). The results of removing two samples or four samples from PE group and comparing to both Notch and Control groups were also listed in the figure. For PE vs Notch comparison at $P < 0.001$, we have $w > 2.3$ and $w > 2.4$ for removing two samples and four samples, respectively. For PE vs Control comparison at $P < 0.0005$, we have $w > 2.0$ and $w > 2.3$ for removing two samples and four samples, respectively.

Table III. Functional classification of genes found significantly changed in microarray experiments

ID	Name	Total[†]	Changed[‡]	Array[§]
Gene ontology (GO)				
GO:0030246/ GO:0005529	Carbohydrate binding/Sugar binding	46	6 (1.4)	NvC
GO:0008189	Apoptosis inhibitor activity	25	4 (0.8)	NvC
GO:0007267	Cell–cell signalling	228	14 (7.1)	NvC
GO:0000502	Proteasome complex	26	3 (0.8)	NvC
GO:0005254/GO:0005253	Chloride/Anion channel activity	17	3 (0.3)	PvC
GO:0005509	Calcium ion binding	202	8 (3.1)	PvC
GO:0003779	Actin binding	95	5 (1.4)	PvC
GO:0016853	Isomerase activity	48	2 (0.2)	PvN
InterPro (IPR)				
IPR001304	C-type lectin	28	4 (0.7)	NvC
IPR002957	Keratin, type I	19	3 (0.5)	NvC
IPR001664	Intermediate filament protein	24	3 (0.6)	NvC
IPR001715	Calponin-like actin-binding	28	3 (0.5)	PvC
IPR001589/IPR002017	Actin-binding, actinin-type/Spectrin repeat	19	2 (0.3)	PvC
Pathway analysis (KEGG)				
hsa04110	Cell cycle	74	6 (1.9)	NvC
hsa04010	MAPK signalling pathway	27	4 (0.4)	PvC
hsa01510	Neurodegenerative Disorders	15	2 (0.2)	PvC
hsa00193	ATP synthesis	27	2 (0.4)	PvC
hsa00510	N-Glycans biosynthesis	29	2 (0.4)	PvC

Cases where the observed number differs from the expected with $P<0.01$ (see methods). Only categories containing at least 15 genes are listed.
[†]Total number of genes belonging to the category.
[‡]Observed number of genes belonging to the category, among the genes which were significantly changed in expression (determined from microarray experiments), and in parentheses, expected number of changed genes assuming random background distribution.
[§]Microarray experiment; NvC = Notch vs Controls; PvC = Preeclampsi vs Controls; PvN = Preclampsi vs Notch.

(GO 8189) and genes in the MAPK signalling pathway (hsa4010) were more abundant in the Notch group, and calcium ion binding (GO 5509), cell cycle (hsa 4110), ATP synthesis (hsa193) and MAPK signalling pathway genes were relatively abundant in the PE group. It is striking that many of the changes observed reflect oxidative stress, metabolic disturbances and alterations in chemical signalling associated with cellular proliferation or apoptosis.

We selected three genes for validation by real time PCR: acid phosphatase 5, tartrate resistant (ACP5), which was overexpressed in placentas from patients with preeclampsia, and RELA and calmodulin 2, which were underexpressed in placentas from patients with notching and preeclampsia. It seemed that using the beta-actin mRNA or 18S RNA to normalize the PCR results may not have been as reliable as global normalization of the microarray data. In spite of this, we were able to confirm the changes that we had seen in the genes examined, and we would like to discuss their potential significance. Poor placental perfusion associated with notching and preeclampsia, particular in combination with IUGR, may result in chronic or intermittent fetal hypoxia and malnutrition (Sibley *et al*., 2002; Chaddha *et al*., 2004). To compensate for the latter problem, certain transporters may be induced, among them ACP5, which is also known as uteroferrin and which has been suggested to play a part in iron transfer to the fetus. Thus, the increase in ACP5 may be compensatory in nature, and its absence in the Notch group could indicate that placentas with this vascular defect provide the fetus with more adequate nutrient levels than those provided by preeclamptic placentas. However, placentas from patients with notching, as well as those with PE, may still suffer from hypoxia. In fact, a recent study provides molecular evidence that placental gene expression changes seen in PE may be due to reduced oxygenation (Soleymanlou *et al*., 2005). This is also consistent with results from earlier studies showing global overexpression of the hypoxia-inducible transcription factor, HIF-2alpha (Rajakumar and Conrad, 2000), or glycogen phosphorylase (Tsoi *et al*., 2003), and of lactate dehydrogenase-A4 in (Tsoi *et al*., 2001) placentas from women with preeclampsia. These changes all reflect an ischemic/hypoxic state. In parallel with increases in transcripts of genes that promote anaerobic metabolism, there seems to be a decrease in the level of cytochrome C oxidase mRNA (He *et al*., 2004). This enzyme is found in the mitochondrial inner membrane, and it is required for aerobic metabolism. Since anaerobic metabolism produces ATP much less efficiently than mitochondrial metabolism, the ATP levels in preeclamptic placental cells should be lower than those in normal placentas. We here show a significant change in ATP synthase gene (Figure 3), an important enzyme in the ATP metabolism.

Hypoxia and apoptosis have been shown to cause increases in intracellular calcium in a number of cell types. The mechanisms responsible for this have been debated. They potentially include energy (ATP) depletion resulting in reduced calcium removal from the cytoplasm by plasma membrane and sarco/endoplasmic reticulum calcium ATPases, calcium mobilization from internal storage sites and calcium influx from the extracellular medium. In fact, the calcium-ATPase activity of trophoblastic tissue from placentas of preeclamptic patients has been reported to be only half that of controls (Carrera *et al*., 2003).

Increase in intracellular calcium would activate calmodulin, which in turn, affects RELA. Recall that NFKB1 or NFKB2 bind to RELA, RELB or REL to form the NFKB family of transcription factors. These heterodimers participate in controlling a wide variety of genes, and are important in embryonic development, apoptosis or protection from apoptosis depending on the cellular context, immune, inflammatory and stress responses. The NFKB1/RELA complex is the most abundant form of NFKB. In neurons, at least, Ca(2+)/calmodulin-dependent kinase activates NFKB in the periphery of cells, permitting the activated species to move to the nucleus and act on genes there. Similarly, in HeLa cells, calmodulin-dependent kinase IV phosphorylates the serine-535 residue of RELA resulting in increased transcription of NFKB target genes and inhibiting apoptosis. Thus, it could be argued that the parallel decreases in RELA and calmodulin 2 are functionally

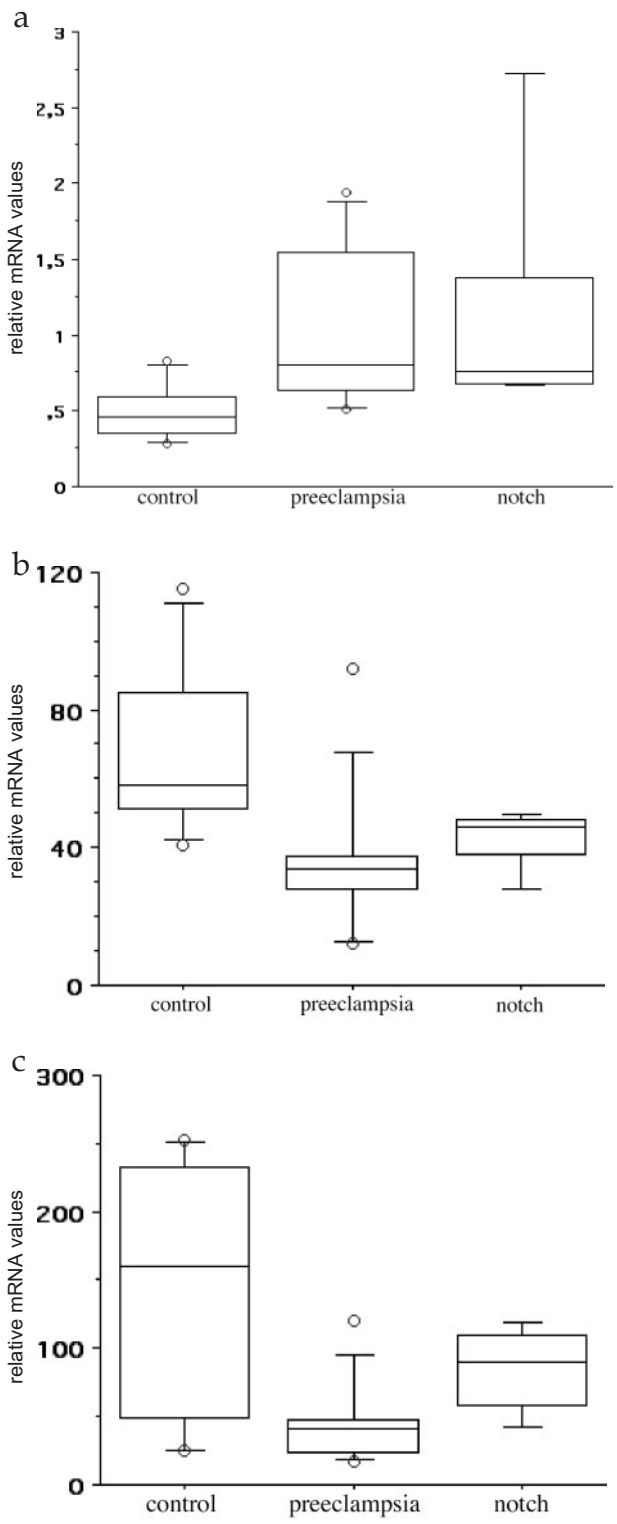

Figure 4. Real-time PCR quantification of ACP5, Calm2 and RELA. (**a)** The amount of ACP5 mRNA normalized to the amount of β-actin mRNA and the relative values are presented in a box plot diagram. Significant changes were obtained between Controls (*n*=7) and PE groups (*n*=9). *P*-value =0.03. (**b)** The amount of Calm2 mRNA normalized to the amount of 18S mRNA and the relative values are presented in a box plot diagram. Significant changes were observed between Controls (*n*=7) and PE (*n*=9) and between Controls versus Notch (*n*=5). *P*-value =0.005 and 0.05. (**c**) The amount of Rela mRNA normalized to the amount of 18S mRNA and the relative values are presented in a box plot diagram. Significant changes were observed between Controls (*n*=7) and PE (*n*=9) and Notch (*n*=5). *P*-value =0.04.

related, and they could represent an attempt by the placenta to compensate for elevation in intracellular calcium in both uterine artery notching and preeclampsia.

In this case, agents that inhibit the activity of NFKB might prove useful in the treatment of PE. An example is curcumin (Aggarwal *et al*., 2004). Similarly, drugs that reduce intracellular calcium levels in the placenta could be helpful. Magnesium sulfate, which remains one of the effective drugs to prevent eclampsia (Altman *et al*., 2002) may act this way. Magnesium, which is actively transported into trophoblasts, is a competitive antagonist of calcium channels. In addition, Mg^{2+} is thought to compete with Ca^{2+} for binding sites on calmodulin, and Okhi *et al*. (Ohki *et al*., 1997) have shown that the affinity of calcium-saturated calmodulin for some of its targets is decreased in the presence of excess Mg^{2+}. Thus, Mg^{2+} may reduce the entry of Ca^{2+} into the cells and also blunt its activation of calmodulin. The net effect of these actions might be the reduction in secretion of harmful factors into the peripheral circulation by the placenta. Should this suggestion be correct, one might also predict that calcium channel antagonists could be used alone or in combination with magnesium sulfate to prevent preeclampsia. In fact, Khandelwal *et al*. (Khandelwal *et al*., 2002) reported that diltiazem, a non-dihydropyridine calcium channel antagonist, appeared to decrease proteinuria, preserve renal structure and function and reduce blood pressure in pregnant women with chronic renal disease. It seems possible that the drug's efficacy could be related, in part at least, to placental effects. Against this hypothesis is the fact that inhibition of L-type calcium channels had no measurable effect on basal uptake of calcium by trophoblasts *in vitro* (Moreau *et al*., 2002) while magnesium had. This suggests that the effect of diltiazem described above may indeed be on the kidney, and that another class of calcium channel blocker would have to be used to decrease calcium's entry into placental cells. Before jumping to the conclusion that this would be useful, however, it is worth noting that primary villous trophoblasts from preeclamptic placentas are much more sensitive to hypoxia-induced apoptosis than those from normal placentas (Kilani *et al*., 2003). It is possible that the reductions in RELA and calmodulin 2 gene expression may contribute to this and be harmful rather than helpful. *In vitro* experiments have shown that magnesium induced apoptosis of placental cells, an effect that was prevented by antioxidants (Black *et al*., 2001). In addition, since the trophoblast supplies the fetus with calcium, it could be detrimental to the baby to reduce calcium levels in trophoblasts too much, and any effort to do this would have to be undertaken with care. High levels of cadmium in tobacco smoke have been shown to reduce the Ca handling ability of trophoblastic cells (Lin *et al*., 1997). In line with our hypothesis above, this may explain the fact that smoking has been associated with a reduced risk of developing PE.

In conclusion our findings suggest that placentas from women with uterine artery notching and those from women with PE may be affected by different degrees of hypoxia and apoptosis. Intermittent placental ischemia may result in a long-term increase in intracellular calcium that activates calmodulin and RELA. Down-regulation of RELA and calmodulin 2 might represent an attempt by the placenta to compensate for elevation in intracellular calcium in both pregnancies with uterine artery notching and PE.

Acknowledgements

This work was supported by the Swedish Research Council: 14358, 14187, 5980, Anna Lisa & Sven Erik Lundgrens foundation for Medical Research, Crawford foundation, Magnus Bergvalls foundation, Swedish Society for Medical Research, Tegger and Wenner-Gren foundation for postdoctoral research.

This project was partly supported by federal funds from the National Cancer Institute, National Institutes of Health, under contract number NO1-CO-12400. The content of this publication does not necessarily reflect the views or policies of the Department of Health and Human Services, nor does mention of trade

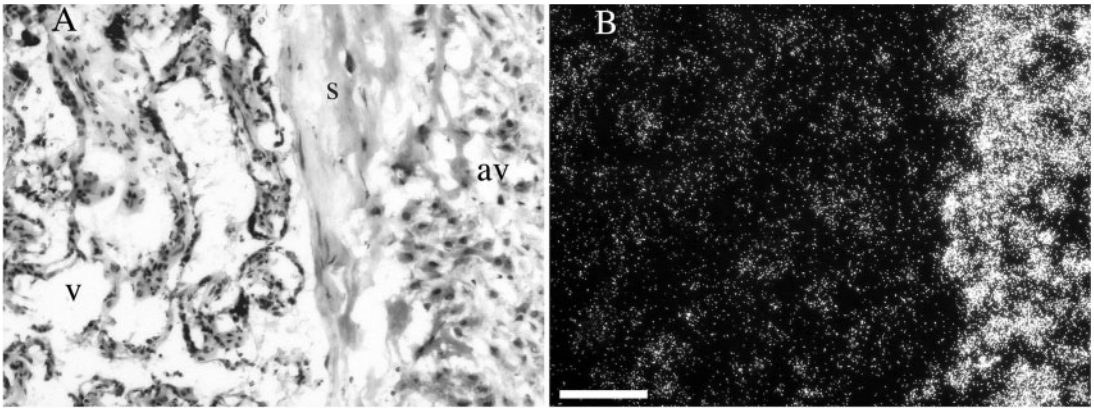

Figure 5. *In-situ* hybridization showing Calm2 mRNA in normal placenta tissue. Brightfield (**A**) and darkfield (**B**) views Calm2 mRNA expression in chorionic villi (v) from the central part of the placenta and in anchoring villi (av). Septae (s). Scale bar: B= 100 μm.

names, commercial products or organizations imply endorsement by the US government. This work is dedicated to my dear mentor and friend, Dr Eva Mezey, for her never-ending support and for being an endless source of inspiration.

References

Aggarwal S, Takada Y, Singh S, Myers JN and Aggarwal BB (2004) Inhibition of growth and survival of human head and neck squamous cell carcinoma cells by curcumin via modulation of nuclear factor-kappaB signaling. Int J Cancer 111,679–692.

Altman D, Carroli G, Duley L, Farrell B, Moodley J, Neilson J, Smith D and MTC Group (2002) Do women with pre-eclampsia, and their babies, benefit from magnesium sulphate? The Magpie Trial: a randomised placebo-controlled trial. Lancet 359,1877–1890.

Bittner M, Meltzer P, Chen Y, Jiang Y, Seftor E, Hendrix M, Radmacher M, Simon R, Yakhini Z, Ben-Dor A et al. (2000) Molecular classification of cutaneous malignant melanoma by gene expression profiling. Nature 406,536–540.

Black S, Lee H, Yu J, Sachchithananthan M and Medcalf RL (2001) Physiologic concentrations of magnesium and placental apoptosis: prevention by antioxidants. Obstet Gynecol 98,319–324.

Bottalico B, Pilka R, Larsson I, Casslen B, Marsal K and Hansson S (2003) Plasma membrane and vesicular monoamine transporters in normal endometrium and early pregnancy decidua. Mol Hum Reprod 9,389–394.

Bottalico B, Larsson I, Casslen B, Broszki J, Hernandez-Andrade E, Marsal K and Hansson RS (2004) Norepinephrine (NET), Serotonin (SERT), vesicular monoamine (VMAT2) and organic cation transporters (OCT1,2 and EMT) in human placenta from preeclamptic and normotensive pregnancies. Placenta 25,518–529.

Bower S, Bewley S and Campbell S (1993) Improved prediction of preeclampsia by two-stage screening of uterine arteries using the early diastolic notch and color Doppler imaging. Obstet Gynecol 82,78–83.

Bradley DJ, Towle HC and Young WS III (1992) Spatial and temporal expression of α- and β-thyroid hormone receptor mRNAs, including the β_2-subtype, in the developing mammalian nervous system. J Neurosci 12,2288–2302.

Cale JM, Millican DS, Itoh H, Magness RR and Bird IM (1997) Pregnancy induces an increase in the expression of glyceraldehyde-3-phosphate dehydrogenase in uterine artery endothelial cells. J Soc Gynecol Investig 4,284–292.

Carrera F, Casart YC, Proverbio T, Proverbio F and Marin R (2003) Preeclampsia and calcium-ATPase activity of plasma membranes from human myometrium and placental trophoblast. Hypertens Pregnancy 22,295–304.

Chaddha V, Viero S, Huppertz B and Kingdom J (2004) Developmental biology of the placenta and the origins of placental insufficiency. Semin Fetal Neonatal Med 9,357–369.

Chen Y, Kamat V, Dougherty ER, Bittner ML, Meltzer PS and Trent JM (2002) Ratio statistics of gene expression levels and applications to microarray data analysis. Bioinformatics 18,1207–1215.

Chun D, Braga C, Chow C and Lok L (1964) Clinical observations on some aspects of hydatidiform moles. J Obstet Gynaecol Br Commonw 71,180–184.

Cunliffe HE, Ringner M, Bilke S, Walker RL, Cheung JM, Chen Y and Meltzer PS (2003) The gene expression response of breast cancer to growth regulators: patterns and correlation with tumor expression profiles. Cancer Res 63,7158–7166.

Dekker GA and Sibai BM (1998) Etiology and pathogenesis of preeclampsia: current concepts. Am J Obstet Gynecol 179,1359–1375.

Demir R, Kosanke G, Kohnen G, Kertschanska S and Kaufmann P (1997) Classification of human placental stem villi: review of structural and functional aspects. Microsc Res Tech 38,29–41.

Goldman-Wohl D and Yagel S. (2002) Regulation of trophoblast invasion: from normal implantation to pre-eclampsia. Mol Cell Endocrinol 187,233–238.

Gosling RG, Dunbar G, King DH, Newman DL, Side CD, Woodcook JP, FitzGerald DE, Keates JS and MacMillian D (1971) The quantitative analysis of occlusive peripheral arterial disease by a non-intrusive ultrasound technique. Angiology 22,52–55.

He L, Wang Z and Sun Y (2004) Reduced amount of cytochrome c oxidase subunit I messenger RNA in placentas from pregnancies complicated by preeclampsia. Acta Obstet Gynecol Scand 83,144–148.

Hedenfalk I, Duggan D, Chen Y, Radmacher M, Bittner M, Simon R, Meltzer P, Gusterson B, Esteller M, Kallioniemi OP et al. (2001) Gene-expression profiles in hereditary breast cancer. N Engl J Med 344,539–548.

Hofstaetter C, Dubiel M, Gudmundsson S and Marsal K (1996) Uterine artery color Doppler assisted velocimetry and perinatal outcome. Acta Obstet Gynecol Scand 75,612–619.

Hung TH, Skepper JN, Charnock-Jones DS and Burton GJ (2002) Hypoxia-reoxygenation: a potent inducer of apoptotic changes in the human placenta and possible etiological factor in preeclampsia. Circ Res 90,1274–1281.

Kaufmann P, Black S and Huppertz B (2003) Endovascular trophoblast invasion: implications for the pathogenesis of intrauterine growth retardation and preeclampsia. Biol Reprod 69,1–7.

Khandelwal M, Kumanova M, Gaughan JP and Reece EA (2002) Role of diltiazem in pregnant women with chronic renal disease. J Matern Fetal Neonatal Med 12,408–412.

Kilani RT, Mackova M, Davidge ST and Guilbert LJ (2003) Effect of oxygen levels in villous trophoblast apoptosis. Placenta 24,826–834.

Lachmeijer AM, Arngrimsson R, Bastiaans EJ, Frigge ML, Pals G, Sigurdardottir S, Stefansson H, Palsson B, Nicolae D, Kong A et al. (2001) A genome-wide scan for preeclampsia in the Netherlands. Eur J Hum Genet 9,758–764.

Lie RT, Rasmussen S, Brunborg H, Gjessing HK, Lie-Nielsen E and Irgens LM (1998) Fetal and maternal contributions to risk of pre-eclampsia: population based study. BMJ 316,1343–1347.

Lin FJ, Fitzpatrick JW, Iannotti CA, Martin DS, Mariani BD and Tuan RS (1997) Effects of cadmium on trophoblast calcium transport. Placenta 18,341–356.

Moreau R, Daoud G, Bernatchez R, Simoneau L, Masse A and Lafond J (2002) Calcium uptake and calcium transporter expression by trophoblast cells from human term placenta. Biochim Biophys Acta 1564,325–332.

Ohki S, Ikura M and Zhang M (1997) Identification of $Mg2^+$-binding sites and the role of $Mg2^+$ on target recognition by calmodulin. Biochemistry 36,4309–4316.

Page EW (1939) The relation between hydatid moles, relative ischemia of the gravid uterus, and the placental origin of eclampsia. Am J Obstet Gynecol 37,291–293.

Rajakumar A and Conrad KP (2000) Expression, ontogeny, and regulation of hypoxia-inducible transcription factors in the human placenta. Biol Reprod 63,559–569.

Roberts JM and Cooper DW (2001) Pathogenesis and genetics of pre-eclampsia. Lancet 357,53–56.

Roberts JM and Hubel CA (1999) Is oxidative stress the link in the two-stage model of pre-eclampsia? Lancet 354,788–789.

Saito S and Sakai M (2003) Th1/Th2 balance in preeclampsia. J Reprod Immunol 59,161–173.

Salafia CM, Thorp J and Starzyk KA (2000) Placental pathology in spontaneous prematurity. In Kingdom JCP, Jauniaux ERM and O'Brien SPM (eds), The Placenta: Basic Science and Clinical Practice, RCOG Press, London, pp. 169–186.

Sibai BM (2003) Diagnosis and management of gestational hypertension and preeclampsia. Obstet Gynecol 102,181–192.

Sibley CP, Pardi G, Cetin I, Todros T, Piccoli E, Kaufmann P, Huppertz B, Bulfamante G, Cribiu FM, Ayuk P et al. (2002) Pathogenesis of intrauterine growth restriction (IUGR)-conclusions derived from a European Union Biomed 2 Concerted Action project 'Importance of Oxygen Supply in Intrauterine Growth Restricted Pregnancies' – a workshop report. Placenta 23,S75–S79.

Soleymanlou N, Jurisica I, Nevo O, Ietta F, Zhang X, Zamudio S, Post M and Caniggia I (2005) Molecular evidence of placental hypoxia in preeclampsia. J Clin Endocrinol Metab 90,4299–4308.

Stevens JM (1975) Gynaecology from ancient Egypt: the papyrus Kahun: a translation of the oldest treatise on gynaecology that has survived from the ancient world. Med J Aust 2,949–952.

Strevens H, Wide-Swensson D, Hansen A, Horn T, Ingemarsson I, Larsen S, Willner J and Olsen S (2003) Glomerular endotheliosis in normal pregnancy and pre-eclampsia. Br J Obstet Gynaecol 110,831–836.

Thaler I, Weiner Z and Itskovitz J (1992) Systolic or diastolic notch in uterine artery blood flow velocity waveforms in hypertensive pregnant patients: relationship to outcome. Obstet Gynecol 277–282.

Tricarico C, Pinzani P, Bianchi S, Paglierani M, Distante V, Pazzagli M, Bustin SA and Orlando C (2002) Quantitative real-time reverse transcription polymerase chain reaction: normalization to rRNA or single housekeeping genes is inappropriate for human tissue biopsies. Anal Biochem 309,293–300.

Tsoi SC, Zheng J, Xu F and Kay HH (2001) Differential expression of lactate dehydrogenase isozymes (LDH) in human placenta with high expression of LDH-A(4) isozyme in the endothelial cells of pre-eclampsia villi. Placenta 22,317–322.

Tsoi SC, Cale JM, Bird IM and Kay HH (2003) cDNA microarray analysis of gene expression profiles in human placenta: up-regulation of the transcript encoding muscle subunit of glycogen phosphorylase in preeclampsia. J Soc Gynecol Investig 10,496–502.

Tusher VG, Tibshirani R and Chu G (2001) Significance analysis of microarrays applied to the ionizing radiation response. Proc Natl Acad Sci USA 98,5116–5121.

Xiang CC and Brownstein MJ (2003) Fabrication of cDNA microarrays. Methods Mol Biol 224,1–7. Available from http://www.ncbi.nlm.nih.gov/entrez/query.fcgi?cmd=Retrieve&db=PubMed&dopt=Citation&list_uids=12710662

Xiang CC, Kozhich OA, Chen M, Inman JM, Phan QN, Chen Y and Brownstein MJ (2002) Amine-modified random primers to label probes for DNA microarrays. Nat Biotechnol 20,738–742.

Yamaji R, Fujita K, Nakanishi I, Nagao K, Naito M, Tsuruo T, Inui H and Nakano Y (2004) Hypoxic up-regulation of triosephosphate isomerase expression in mouse brain capillary endothelial cells. Arch Biochem Biophys 423,332–342.

Zeeberg BR, Feng W, Wang G, Wang MD, Fojo AT, Sunshine M, Narasimhan S, Kane DW, Reinhold WC, Lababidi S et al. (2003) GoMiner: a resource for biological interpretation of genomic and proteomic data. Genome Biol 4,R28.

Submitted on December 1, 2005; resubmitted December 28 2005; accepted on January 9, 2006