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DNA methylation profiles in preeclampsia and healthy control placentas

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Yeung KR, Chiu CL, Pidsley R, Makris A, Hennessy A, Lind JM. DNA methylation profiles in preeclampsia and healthy control placentas. *Am J Physiol Heart Circ Physiol* 310: H1295–H1303, 2016. First published March 11, 2016; doi:10.1152/ajpheart.00958.2015.—Preeclampsia is a hypertensive disorder of pregnancy that affects 3–5% of all pregnancies. There is evidence to suggest that epigenetic mechanisms, such as DNA methylation, play a role in placental development and function. This study compared DNA methylation profiles of placentas from preeclampsia-affected pregnancies with placentas from healthy pregnancies to identify gene-specific changes in DNA methylation that may contribute to the development of preeclampsia. The methylation status of eight placental biopsies taken from preeclampsia-affected and 16 healthy pregnancies was analyzed using the Illumina Infinium Methylation 450 BeadChip array. Bisulfite pyrosequencing was used to confirm regions found to be differentially methylated between preeclampsia and healthy placentas. A total of 303 differentially methylated regions, 214 hypermethylated and 89 hypomethylated, between preeclampsia cases and controls were identified, after adjusting for gestational age (adjusted $P < 0.05$). Functional annotation found cell adhesion, wingless type MMTV Integration Site family member 2 (Wnt) signaling pathway, and regulation of transcription were significantly enriched in these gene regions. Hypermethylation of *WNT2*, sperm equatorial segment protein (*SPESP1*), NADPH oxidase 5 (*NOX5*), and activated leukocyte cell adhesion molecule (*ALCAM*) in preeclampsia placentas was confirmed with pyrosequencing. This study found differences in methylation in gene regions involved in cell signaling (*WNT2*), fertilization and implantation (*SPESP1*), reactive oxygen species signaling (*NOX5*), and cell adhesion (*ALCAM*). These results build on recently published studies that have reported significant differences in DNA methylation in preeclampsia placentas.

deoxyribonucleic acid methylation; placenta

NEW & NOTEWORTHY

This study identified novel gene-specific differences in DNA methylation in preeclampsia-affected placentas. These results improve our understanding of the pathophysiological disease process, including the involvement of cell signaling, fertilization and implantation, reactive oxygen species signaling, and cell adhesion.

PREECLAMPSIA IS A HYPERTENSIVE disorder of pregnancy that affects 3–5% of all pregnancies, and is one of the leading causes of maternal mortality and morbidity. It is a multisystem disorder primarily characterized by maternal hypertension from 20 wk gestation. The clinical phenotype varies, with symptoms ranging from increases in blood pressure, to more serious complications, including renal and liver dysfunction, and seizures. The exact mechanisms that cause preeclampsia remain unclear; however, genetic, immunological, endocrine, and environmental factors have all been implicated (13).

Numerous studies have reported gene expression changes in placentas from preeclampsia-affected pregnancies compared with placentas from healthy pregnancies (15, 31). Pathways that have been identified have included trophoblast motility and invasion, angiogenesis, cell adhesion and survival, the renin-angiotensin-aldosterone system, and immune response (8, 15, 27, 31, 34). Altered gene expression in preeclampsia-affected placentas would indicate a role for epigenetic modifications in the development of preeclampsia.

Epigenetic modifications regulate gene expression without changing the underlying DNA sequence. DNA methylation is the most commonly studied epigenetic modification. It primarily occurs in CpG dinucleotides and is associated with transcriptional silencing. There have been a few studies that have investigated the role of epigenetic modifications, particularly DNA methylation, in placentas from pregnancies complicated by preeclampsia. These studies have shown that a number of gene promoter regions are hyper- and/or hypomethylated in preeclampsia placentas compared with control placentas (3).

While these studies have provided evidence for an epigenetic role in the placental dysfunction associated with preeclampsia, further work is required to validate and confirm these findings. The aim of the current study is to compare the DNA methylation profiles of placentas from preeclampsia-affected pregnancies with placentas from healthy pregnancies to identify gene-specific changes in DNA methylation that may influence placental gene expression and contribute to the development of preeclampsia.

METHODS

Ethics statement. Ethics approval for this project was obtained from the Sydney Local Health District Human Research Ethics Committee (X13-0112), the South Western Sydney Local Health District (HREC/09RPAH/70), and Western Sydney University (H7019). Written informed consent was obtained from all study participants.

Sample collection. Women were recruited from Royal Prince Alfred Hospital and Campbelltown Hospital between November 1999 and January 2012. Preeclampsia cases ($n = 8$) were defined according to the criteria set by the Society of Obstetric Medicine of Australia and New Zealand (25). This included women who had a blood pressure ≥140 mmHg systolic and/or ≥90 mmHg diastolic after 20 wk gestation on two occasions at least 4 h apart; proteinuria (≥2+ on dipstick or 300 mg/24 h) or renal insufficiency (serum creatinine >0.09 mmol/l); liver disease (raised serum transami-

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nases or severe epigastric pain); neurological problems (including convulsions); and hematological disturbances (thrombocytopenia, disseminated intravascular coagulation, or hemolysis), all of which resolved within 3 mo postpartum. Control cases ($n = 16$) were defined as women without hypertension-related complications that presented for delivery at term (≥37 wk gestation). Immediately after delivery (≤30 min) placental biopsies were collected from five sites from the fetal side of the placenta. These samples were rinsed in phosphate buffered saline (PBS), snap-frozen in liquid nitrogen, and stored at −80°C until required.

DNA extraction. Placental tissue was incubated with proteinase K (Qiagen) at 56°C overnight. Genomic DNA was extracted from lysed placental tissue using the Qiagen DNA Mini kit according to the manufacturer's spin column protocol.

Illumina Infinium HumanMethylation450 BeadChip. Genomic DNA (150 ng) underwent sodium bisulfite conversion according to the manufacturer's protocol using the EZ DNA Methylation kit (Zymo Research). The bisulfite-converted DNA was hybridized to the Illumina Infinium HumanMethylation450 BeadChip (450K array) (Illumina, San Diego, CA), which provides genomewide coverage containing >450,000 methylation sites/sample. Amplification, hybridization, washing, labeling, and scanning of the 450K array was performed by the Australian Genome Research Facility (AGRF), a fee-for-service provider. The data obtained have been deposited into the publically accessible database, NCBI Gene Expression Omnibus (GEO) database, according to the MIAME guidelines (reviewer access: http://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE75196).

Raw IDAT files containing signal intensities for each probe were extracted using Illumina GenomeStudio software and imported into R Studio using the methylumi and minfi packages (2, 9). Multidimensional scaling plots of variably methylated probes on the sex chromosomes were used to check that the predicted sex corresponded with the reported sex for each placenta. Quality control and processing steps were conducted using the methylumi and wateRmelon packages (9, 30). The pfilter function was used to remove probes with missing β-values, a detection P value >0.01 in at least 1% samples, or/and a bead count less than three in 5% of samples. The 65 control probes on the array targeting single nucleotide polymorphisms (SNPs) were also removed. After quality control, $n = 483{,}615$ probes and all samples ($n = 24$) were retained. The dasen function was used to normalize the data as previously described (30). Probes containing a SNP with minor allele frequency >5% within 5 bp of the single base extension site were removed from all analyses based on the SNP annotation data provided by Illumina (5), the Bioconductor package minfi (2), and Chen and colleagues (5) (total probes removed = 23,520). Probes located on sex chromosomes (X: $n = 11{,}082$ and Y: $n = 403$) and probes targeting non-CpG sites ($n = 3{,}029$) were also removed from all subsequent analysis.

Statistical analysis. Analyses were performed to test for DNA methylation differences between preeclampsia cases and controls at the individual probe level. To model the effect of sample-specific variables, linear regression for each probe using gestational age and preeclampsia status as independent variables were performed using the limma package (32), and prior to analyses β-values were log-transformed to M-values to improve sensitivity. P values were adjusted for multiple testing according to the false discovery rate (FDR) procedure of Benjamini-Hochberg. The DMRcate (29a) package was then used to identify differentially methylated regions (DMRs), based on groups of probes that exhibited different methylation status (nominal $P < 0.05$) between preeclampsia cases and controls, where the next consecutive probe was within 1,000 nucleotides. Significant DMRs were selected at a 5% FDR.

To determine how our results compared with previous methylation array data in placentas affected by preeclampsia, the 450K methylation array dataset GSE44712 from NCBI GEO database (3) was extracted, and 20 cases of early onset preeclampsia and 20 normal cases were analyzed using the above pipelines. This dataset was selected, since information on the gestational age of each sample was also available.

Gene ontology. Database for Annotation, Visualization and Integrated Discovery (DAVID version 6.7) Bioinformatics Resources web-based software tool (19, 20) was used to perform functional annotation analysis and gene ontology (GO) enrichment analysis. Gene identifiers were uploaded, and functional annotation analysis was performed, against the human reference genome, using a Benjamini-Hochberg multiple-test adjustment threshold of $P < 0.05$. In addition, pathway enrichment analysis based on the protein annotation through evolutionary relationship and Kyoto Encyclopedia of Genes and Genomes classification databases was used to identify significant pathways.

Bisulfite pyrosequencing. Pyrosequencing assays were performed on five preeclampsia-associated DMRs [in the vicinity of sperm equatorial segment protein (*SPESP1*), wingless type MMTV Integration Site family member 2 (*WNT2*), activated leukocyte cell adhesion molecule (*ALCAM*), adenosine A2b receptor (*ADORA2B*), and ankyrin repeat and SOCS box containing 3 (*ASB3*)] to validate 450K array data. These regions were selected based on Δβ >10% and relevance to preeclampsia. All pyrosequencing assays were designed, optimized, performed, and analyzed by AGRF.

RESULTS

Clinical and demographic characteristics of the preeclampsia cases and controls are summarized in Table 1. Placental biopsies were obtained from 8 preeclampsia cases and 16 controls. All women were nulliparous and nonsmokers. Gestational age at delivery and mean birth weight were significantly lower in preeclampsia cases compared with controls. There was no significant difference in maternal age and fetal sex between the groups.

Whole genome methylation was performed using the Illumina 450K Beadchip array, and β-values were compared between placentas derived from preeclampsia ($n = 8$) and healthy control ($n = 16$) pregnancies. Initial analysis of all autosomes, using an adjusted P value of 0.05, identified 63 differentially methylated CpG sites, 8 hypermethylated and 55 hypomethylated, between preeclampsia cases and controls (Table 2). Previous studies have reported that gestational age at time of delivery is a major contributor toward altered DNA methylation (3). To overcome this we adjusted our analysis by gestational age. When we adjusted our analysis for gestational age, no single CpG sites were found to be differentially methylated between preeclampsia cases and controls (adjusted $P < 0.05$). Region analysis using the DMRcate package identified a number of regions containing multiple adjacent CpG sites that exhibited different methylation statuses between preeclampsia cases and controls. A total of 362 significant DMRs, 263 hypermethylated and 99 hypomethylated, were identified between preeclampsia cases and controls. After adjusting for gestational age, 303 DMRs, 214 hypermethylated

Table 1. *Clinical information of samples used in the study*

Characteristic	Control ($n = 16$)	PE ($n = 8$)	P Value
Maternal age, yr	32 ± 1.0	28 ± 2.0	0.40
Gestational age, wk	39 ± 0.2	35 ± 0.8	<0.001
Birth wt, g	3,509 ± 135	2,152 ± 186	<0.001
Fetal sex, %male	56	50	0.77

Data are expressed as means ± SE; n, no. of subjects. Clinical parameters were tested for between-group differences with a generalized linear model.

Table 2. *Significantly differentially methylated sites between preeclampsia cases and controls*

Probe	Associated Gene	Genomic Position	Adjusted *P* Value	Δβ
cg13757194	*MIR548H4*; *NOX5*; *SPESP1*	Body, 5′-UTR, body	0.0301	0.2822
cg26820811	*MIR548H4*; *NOX5*; *SPESP1*	Body, 5′-UTR, body	0.0301	0.2682
cg07775813	*MIR548H4*; *NOX5*; *SPESP1*	Body, 5′-UTR, body	0.0301	0.2043
cg09899989		Intragenic	0.0368	0.1754
cg18752527	*HECW2*	Body	0.0467	0.1334
cg06527166	*HIVEP3*	5′-UTR	0.0417	0.1115
cg01245776	*FAM66E*	Body	0.0364	0.0794
cg17194100		Intragenic	0.0301	0.0634
cg15605704	*AJAP1*	Body	0.0368	−0.2002
cg26625897		Intragenic	0.0364	−0.1960
cg10668363	*ZNF175*	Body	0.0301	−0.1830
cg25064552	*SLC39A1*	5′-UTR	0.0496	−0.1700
cg22305268		Intragenic	0.0416	−0.1684
cg10268811	*HUNK*	Body	0.0364	−0.1617
cg04365481	*MST1P9*	Body	0.0496	−0.1561
cg05459750	*C8orf42*	Body	0.0364	−0.1421
cg14703784	*RASA3*	Body	0.0368	−0.1388
cg20722088	*DUSP6*	3′-UTR	0.0307	−0.1361
cg19883388		Intragenic	0.0368	−0.1357
cg25539556		Intragenic	0.0364	−0.1339
cg11283860		Intragenic	0.0394	−0.1328
cg04930693	*ERRFI1*	5′-UTR	0.0301	−0.1309
cg20438445	*KCNH4*	3′-UTR	0.0364	−0.1306
cg13761949	*PARD3*	Body	0.0301	−0.1293
cg09244174	*BAIAP2L1*	Body	0.0301	−0.1278
cg10468845	*C3orf31*	TSS	0.0384	−0.1276
cg10341513	*MAPK8IP2*	Body	0.0367	−0.1198
cg12330703		Intragenic	0.0496	−0.1172
cg07177437	*ITLN1*	Body	0.0357	−0.1169
cg15282281	*MIR346*; *GRID1*	TSS, body	0.0496	−0.1128
cg03695871	*DKFZp761E198*	Body	0.0368	−0.1110
cg08622401	*UNKL*	5′-UTR	0.0364	−0.1073
cg01412654	*PPARG*	TSS	0.0496	−0.1069
cg09106587		Intragenic	0.0467	−0.1021
cg11808677	*USP5*; *CDCA3*	TSS, body	0.0364	−0.1000
cg03263613	*DGKD*	Body	0.0364	−0.0986
cg10103857	*LAMC2*	Body	0.0496	−0.0985
cg03721657	*HCCA2*	Body	0.0467	−0.0970
cg19042141	*RXRB*	Body	0.0342	−0.0960
cg20956548	*ZNF787*	5′-UTR	0.0496	−0.0938
cg10142436	*EHD4*	Body	0.0364	−0.0882
cg07284546	*FAM195A*	TSS	0.0467	−0.0875
cg13075295	*EHD4*	Body	0.0301	−0.0871
cg13003786	*CSNK1D*	TSS	0.0364	−0.0853
cg07127883		Intragenic	0.0496	−0.0834
cg03745114	*PNPO*	TSS	0.0364	−0.0831
cg15906733		Intragenic	0.0364	−0.0825
cg04007936	*CARHSP1*	5′-UTR	0.0496	−0.0819
cg26093966		Intragenic	0.0058	−0.0814
cg13614617	*IGDCC3*	Body	0.0364	−0.0805
cg14156446	*NR6A1*	3′-UTR	0.0467	−0.0793
cg00422714	*MAPK8IP3*	Body	0.0467	−0.0760
cg00840341		Intragenic	0.0364	−0.0756
cg11766468	*EVI5L*	Body	0.0301	−0.0746
cg03666203	*EPS8L1*	Body	0.0467	−0.0729
cg11461670	*PDLIM2*	3′-UTR	0.0369	−0.0725
cg14015401	*FOXK1*	Body	0.0496	−0.0698
cg00583304		Intragenic	0.0467	−0.0683
cg14418756	*STK32C*	Body	0.0467	−0.0676
cg13428217		Intragenic	0.0364	−0.0602
cg06218523	*KALRN*	Body	0.0457	−0.0523
cg15561501	*PLEKHF1*	5′-UTR	0.0368	−0.0437
cg24717159	*SLC27A2*	TSS	0.0496	−0.0291

TSS, transcription start site; 5′-UTR, 5′−untranslated region; 3′-UTR, 3′−untranslated region. Positive Δβ values indicate hypermethylation in preeclampsia cases; negative Δβ values indicate hypomethylation in preeclampsia cases.

Table 3. *Differentially methylated hypermethylated regions between preeclampsia cases and controls (Δβ ≥10%)*

Associated Gene(s)	Genomic Position	hg19 Coordinates	No. of Probes	Adjusted *P* Value	Δβ
CBR1	TSS, 5′-UTR, body	chr21:37442104-37442777	6	5.18^{-8}	0.21
MIR548H4, SPESP1, NOX5	TSS, 5′-UTR, body	chr15:69222400-69223895	7	5.07^{-4}	0.17
MYH15	Body	chr3:108125523-108125523	1	3.63^{-3}	0.15
CROT, TP53TG1	TSS, 5′-UTR, body	chr7:86974674-86975534	17	2.42^{-8}	0.14
	Intragenic	chr14:70690454-70690538	3	1.65^{-2}	0.13
GCSAML-AS1, OR2C3	TSS, body, 3′-UTR	chr1:247694041-247695328	8	4.81^{-6}	0.13
FLJ41603	TSS, 5′-UTR	chr5:148960831-148961536	8	5.51^{-16}	0.13
WNT2	TSS	chr7:116963492-116964841	9	7.70^{-3}	0.11
	Intragenic	chr7:64541193-64541923	4	2.55^{-7}	0.11
CSNK1A1P	TSS, body	chr15:37109747-37110854	8	9.68^{-4}	0.11
HOOK2	Body	chr19:12876846-12877188	4	8.68^{-3}	0.11
FAM184B	Body	chr4:17710804-17711202	4	4.07^{-2}	0.10
LHX4	Body	chr1:180202256-180205253	14	1.47^{-3}	0.10
HLA-DRB1	Body	chr6:32551749-32552453	7	3.25^{-3}	0.10
ALCAM	Body	chr3:105087718-105088546	6	1.57^{-2}	0.10

chr, Chromosome.

and 89 hypomethylated, between preeclampsia cases and controls were identified (Tables 3 and 4). Of the 303 significant DMRs, after adjusting for gestational age, 237 regions were associated with genes and 66 regions were in areas that did not contain genes. Of the 237 gene containing regions, 265 annotated genes were located within these regions and identified as being differentially methylated between preeclampsia cases and controls. Compared with genes within DMRs identified in the GSE44712 dataset, 92 of the 265 genes (34.7%) identified in our dataset were also present in GSE44712.

DAVID Bioinformatics Resources was used to perform functional annotation analysis on 265 genes and identified cadherin, cell adhesion, and cadherin signaling pathway and Wnt signaling pathway functional clusters as being the most significant (Table 5). GO analysis of the same genes identified terms associated with cell adhesion (GO:0007156, GO:0016337, GO:0007155, GO:0022610) and regulation of transcription, DNA dependent (GO:0006355) as the most significant terms for biological processes, and transcription factor activity (GO:0003700) and calcium ion binding (GO:0005509) as the most significant terms for molecular function (Table 6).

To confirm the array data, bisulfite pyrosequencing was used to validate five preeclampsia-associated DMRs in the vicinity of six genes within a subset of the original samples (preeclampsia $n = 8$ and control $n = 8$). These genes were selected based on the magnitude of hyper- or hypomethylation in preeclampsia cases compared with controls, if the gene was present within a functional annotation cluster, and/or if the function of the gene was relevant to the pathophysiology of preeclampsia. Genes selected for validation included *SPESP1*, NADPH oxidase 5 (*NOX5*), *WNT2*, activated leukocyte cell adhesion molecule (*ALCAM*), *ADORA2B*, and *ASB3*. We confirmed significant DNA methylation differences that were in the same direction as reported by the 450K array for three regions containing the following genes: *SPESP1*, *NOX5* ($P < 0.001$), *WNT2* ($P = 0.012$), and *ALCAM* ($P = 0.007$) (Fig. 1). These three genes were also shown to be significantly differentially methylated between cases and controls in our analysis of the GSE44712 dataset.

DISCUSSION

In the present study we investigated the role of placental DNA methylation in preeclampsia. We identified a number of regions that are differentially methylated between preeclampsia placentas and healthy controls, after adjusting for gestational age. Genes that had been previously associated with preeclampsia, as well as a number of novel genes, were located in these regions. GO analysis identified cell adhesion, transcriptional regulation, and Wnt signaling as significantly enriched terms. Pyrosequencing confirmed methylation differences at four genes (*SPESP1*, *NOX5*, *WNT2*, and *ALCAM*). The

Table 4. *Differentially methylated hypomethylated regions between preeclampsia cases and controls (Δβ ≥10%)*

Associated Gene(s)	Genomic Position	hg19 Coordinates	No. of Probes	Adjusted *P* Value	Δβ
SAR1B	TSS, 5′-UTR, body	chr5:133967994-133968668	9	3.76^{-3}	0.17
NAPRT1	TSS, 5′-UTR, body	chr8:144659627-144661051	11	4.82^{-3}	0.16
SDK1	Body	chr7:4244250-4244643	3	8.26^{-3}	0.15
CAPN8	Body	chr1:223747670-223747670	1	2.78^{-2}	0.14
ARID1B	Body	chr6:157198648-157198648	1	2.57^{-2}	0.14
PURA	TSS, body, 3′-UTR	chr5:139491579-139494937	18	2.66^{-4}	0.14
DIXDC1	TSS, 5′-UTR, body	chr11:111807548-111808394	10	5.71^{-8}	0.12
GPR75-ASB3, ASB3, CHAC2	TSS, 5′-UTR, body	chr2:53994559-53995338	9	1.25^{-7}	0.11
NKX1-2	TSS, body	chr10:126135810-126139435	16	3.63^{-7}	0.11
MST1P9	Body	chr1:17085860-17086558	6	4.63^{-3}	0.11
ADORA2B	TSS, 5′-UTR, body	chr17:15846926-15849556	14	6.69^{-6}	0.11
HKR1	TSS, 5′-UTR, body	chr19:37825009-37826008	7	2.78^{-7}	0.10
	Intragenic	chr21:15451242-15451401	3	3.23^{-2}	0.10
MECOM	TSS, 5′-UTR, body	chr3:169379554-169381574	8	1.39^{-3}	0.10

Table 5. *Functional annotation clusters containing differentially methylated genes between preeclampsia cases and controls*

Functional Annotation Cluster	No. of Genes in Cluster	Genes	Benjamini *P* Value
Cadherin	17	*PCDHGA-(1-10)*, *PCDHGB-(1-7)*	1.08^{-10}
Cadherin signaling pathway	19	*PCDHGA-(1-10)*, *PCDHGB-(1-7)*, *LEF1*, *WNT2*	1.99^{-9}
Wnt signaling pathway	23	*PCDHGA-(1-10)*, *PCDHGB-(1-7)*, *MYH15*, *LEF1*, *ARID1B*, *WNT2*, *SFRP5*, *HOXC4*	1.97^{-7}
Cell adhesion	22	*PCDHGA-(1-10)*, *PCDHGB-(1-7)*, *SDK1*, *NINJ2*, *ALCAM*, *CTGF*, *CHL1*	1.13^{-5}
Calcium	29	*PCDHGA-(1-10)*, *PCDHGB-(1-7)*, *PXDN*, *S100A6*, *TRPV3*, *RCVRN*, *KCNMA1*, *NOX5*, *SCUBE2*, *CAPN8*, *SNED1*, *CACNA1H*, *VCAN*, *TLL1*	8.50^{-5}
Homeobox	18	*IRX5*, *NOTO*, *OTX1*, *PAX6*, *VENTX*, *ISL1*, *HOXD11*, *HOXD9*, *ZFHX4*, *NKX1-2*, *LASS3*, *HOXC4*, *NKX6-2*, *LHX4*, *MKX*, *LHX8*, *NKX2-4*, *NKX2-2*	2.91^{-6}
Homeodomain related	16	*HOXD9*, *ZFHX4*, *IRX5*, *NOTO*, *NKX1-2*, *HOXC4*, *OTX1*, *NKX6-2*, *PAX6*, *LHX4*, *MKX*, *VENTX*, *NKX2-4*, *ISL1*, *NKX2-2*, *HOXD11*	4.18^{-5}
Developmental protein	27	*PAX6*, *PAX5*, *SPESP1*, *VENTX*, *HOXD11*, *WNT2*, *HAND2*, *NKX1-2*, *HOXC4*, *NKX6-2*, *ROBO2*, *MKX*, *ROBO3*, *NKX2-4*, *NKX2-2*, *SIM1*, *DIXDC1*, *NOTO*, *OTX1*, *DLL1*, *MECOM*, *ISL1*, *HOXD9*, *SFRP5*, *EBF2*, *CHL1*, *TLL1*	3.50^{-4}
DNA binding	47	*ZNF85*, *HKR1*, *IRX5*, *THAP3*, *ARNT2*, *PAX6*, *PAX5*, *VENTX*, *ZNF175*, *HOXD11*, *ZNF709*, *HAND2*, *NKX1-2*, *NKX6-2*, *ZFP90*, *HOXC4*, *ZNF442*, *LHX4*, *MKX*, *FOXB1*, *NKX2-4*, *LHX8*, *SIM1*, *NKX2-2*, *MAF*, *NOTO*, *OTX1*, *LEF1*, *ZNF792*, *ARID1B*, *ZNF793*, *ISL1*, *MECOM*, *TBR1*, *PURA*, *HOXD9*, *SALL3*, *ZFHX4*, *ZNF311*, *ZNF197*, *ZNF714*, *LASS3*, *ZNF417*, *ZIC4*, *EBF2*, *ZIK1*, *ZNF587*	2.94^{-4}
Transcription regulation	42	*ZNF85*, *HKR1*, *EIF2C1*, *ARNT2*, *PAX6*, *PAX5*, *ZNF175*, *HOXD11*, *TSC22D1*, *ZNF709*, *HAND2*, *ZFP90*, *HOXC4*, *ZNF442*, *LHX4*, *FOXB1*, *LHX8*, *SIM1*, *MAF*, *NOTO*, *OTX1*, *LEF1*, *ZNF792*, *ARID1B*, *ZNF793*, *ISL1*, *MECOM*, *TBR1*, *PURA*, *HOXD9*, *SALL3*, *ZFHX4*, *ZNF311*, *ZNF197*, *ZNF714*, *ZNF417*, *EBF2*, *HIVEP3*, *CDCA7L*, *ZIK1*, *VGLL4*, *ZNF587*	0.0479
Signal	64	*PCDHGA-(1-10)*, *PCDHGB-(1-7)*, *PXDN*, *MICA*, *FAM3B*, *NELL1*, *FGF10*, *VIPR2*, *LGR5*, *WNT2*, *ART5*, *CTGF*, *ROBO2*, *ROBO3*, *FAM150B*, *SCUBE2*, *PTPRN2*, *SDK1*, *DLL1*, *LYZL1*, *CRHR2*, *UGT2B17*, *THBD*, *C1ORF204*, *VSIG8*, *VCAN*, *PGCP*, *COL24A1*, *UGT2B15*, *CHL1*, *HLA-DRB1*, *EPDR1*, *SPESP1*, *NRN1*, *CCL28*, *SERPINH1*, *ALCAM*, *IGF1R*, *EMB*, *HLA-DPB1*, *LRFN4*, *GABRA1*, *MST1P9*, *GABRA5*, *NPR3*, *GRIA4*, *QPCT*, *SFRP5*, *SNED1*, *TLL1*	0.0071

results from this study build on recently published studies that have reported significant differences in DNA methylation in preeclampsia placentas (1, 3, 7, 18, 23).

Genes within the Wnt signaling pathway were found to be significantly enriched in our study, and the *WNT2* gene was found to be hypermethylated ($\Delta\beta = 10\%$) in preeclampsia placentas. The *WNT2* region was also identified as being hypermethylated in placentas from preeclampsia when using 450K methylation data from GSE44712 (3). The Wnt signaling pathway is involved in cell proliferation, differentiation, migration, and cell death. WNT2 is a glycoprotein that binds to the frizzled family of receptors, which activates gene expression via the Wnt/β-catenin pathway. Mice deficient in *Wnt2* exhibit placental defects, including reduced vascularization, which is associated with invasion of the spiral arteries, and their offspring had reduced birth weight and a high rate of fetal loss (28). In humans, *WNT2* is highly expressed in placentas throughout development. Hypermethylation of the *WNT2* promoter has been associated with reduced placental *WNT2* expression and low birth weight percentile in neonates (14). Furthermore, reduced *WNT2* expression has also been reported in third-trimester placentas in women with severe preeclampsia (38). In our study, significantly increased DNA methylation was observed in the *WNT2* promoter region in preeclampsia placentas. Whether increases in DNA methylation correlate to reduced expression in the preeclampsia placentas could not be

Table 6. *Top 10 GO terms for differentially methylated genes between preeclampsia cases and controls*

GO No.	GO Term	Count	Genes	Benjamini *P* Values
GO:0007156	Homophilic cell adhesion	18	*PCDHGA-(1-10)*, *PCDHGB-(1-7)*, *ROBO2*	3.22^{-9}
GO:0016337	Cell-cell adhesion	20	*PCDHGA-(1-10)*, *PCDHGB-(1-7)*, *NINJ2*, *CTGF*, *ROBO2*	5.12^{-6}
GO:0007155	Cell adhesion	30	*PCDHGA-(1-10)*, *PCDHGB-(1-7)*, *EPDR1*, *NELL1*, *NINJ2*, *ALCAM*, *CTGF*, *ROBO2*, *EMB*, *SDK1*, *LEF1*, *SNED1*, *VCAN*, *COL24A1*, *CHL1*	3.90^{-5}
GO:0022610	Biological adhesion	30	*PCDHGA-(1-10)*, *PCDHGB-(1-7)*, *EPDR1*, *NELL1*, *NINJ2*, *ALCAM*, *CTGF*, *ROBO2*, *EMB*, *SDK1*, *LEF1*, *SNED1*, *VCAN*, *COL24A1*, *CHL1*	3.02^{-5}
GO:0005509	Calcium ion binding	33	*PXDN*, *S100A6*, *EPDR1*, *NELL1*, *TRPV3*, *RCVRN*, *KCNMA1*, *NOX5*, *SCUBE2*, *CAPN8*, *DLL1*, *THBD*, *SNED1*, *CACNA1H*, *VCAN*, *TLL1*	3.21^{-4}
GO:0003700	Transcription factor activity	33	*ZNF85*, *IRX5*, *ARNT2*, *PAX6*, *PAX5*, *VENTX*, *ZNF175*, *HOXD11*, *TSC22D1*, *HAND2*, *NKX1-2*, *HOXC4*, *NKX6-2*, *LHX4*, *MKX*, *FOXB1*, *NKX2-4*, *LHX8*, *SIM1*, *NKX2-2*, *MAF*, *NOTO*, *OTX1*, *LEF1*, *ISL1*, *MECOM*, *TBR1*, *PURA*, *HOXD9*, *ZFHX4*, *ZNF197*, *LASS3*, *SCAND3*	5.71^{-4}
GO:0006355	Regulation of transcription, DNA dependent	47	*ZNF85*, *HKR1*, *IRX5*, *ARNT2*, *PAX6*, *PAX5*, *VENTX*, *ZNF175*, *HOXD11*, *TSC22D1*, *ZNF709*, *NKX1-2*, *HAND2*, *NKX6-2*, *ZFP90*, *HOXC4*, *ZNF442*, *LHX4*, *MKX*, *FOXB1*, *NKX2-4*, *LHX8*, *SIM1*, *NKX2-2*, *MAF*, *NOTO*, *OTX1*, *LEF1*, *ZNF792*, *ARID1B*, *ZNF793*, *ISL1*, *MECOM*, *TBR1*, *PURA*, *HOXD9*, *ZFHX4*, *ZNF311*, *ZNF197*, *ZNF714*, *LASS3*, *ZNF417*, *PFDN5*, *EBF2*, *ZIK1*, *SCAND3*, *ZNF587*	0.003
GO:0051252	Regulation of RNA metabolic process	47	*ZNF85*, *HKR1*, *IRX5*, *ARNT2*, *PAX6*, *PAX5*, *VENTX*, *ZNF175*, *HOXD11*, *TSC22D1*, *ZNF709*, *NKX1-2*, *HAND2*, *NKX6-2*, *ZFP90*, *HOXC4*, *ZNF442*, *LHX4*, *MKX*, *FOXB1*, *NKX2-4*, *LHX8*, *SIM1*, *NKX2-2*, *MAF*, *NOTO*, *OTX1*, *LEF1*, *ZNF792*, *ARID1B*, *ZNF793*, *ISL1*, *MECOM*, *TBR1*, *PURA*, *HOXD9*, *ZFHX4*, *ZNF311*, *ZNF197*, *ZNF714*, *LASS3*, *ZNF417*, *PFDN5*, *EBF2*, *ZIK1*, *SCAND3*, *ZNF587*	0.004
GO:0043565	Sequence-specific DNA binding	23	*MAF*, *IRX5*, *NOTO*, *OTX1*, *PAX6*, *LEF1*, *VENTX*, *ISL1*, *HOXD11*, *PURA*, *HOXD9*, *ZFHX4*, *HAND2*, *NKX1-2*, *LASS3*, *HOXC4*, *NKX6-2*, *LHX4*, *MKX*, *FOXB1*, *NKX2-4*, *LHX8*, *NKX2-2*	0.004
GO:0043167	Ion binding	83	*PCDHGA-(1-10)*, *PCDHGB-(1-7)*, *ZNF85*, *ADCY4*, *PXDN*, *S100A6*, *HKR1*, *NELL1*, *RNF39*, *TRPV3*, *RCVRN*, *ZFP90*, *ZNF442*, *ALOX12B*, *SAR1B*, *KCNMA1*, *SCUBE2*, *ZNF792*, *DLL1*, *ZNF793*, *MECOM*, *THBD*, *ZNF714*, *ZNF197*, *CA7*, *NBR1*, *GUCY1B3*, *VCAN*, *GLB1L3*, *PGCP*, *ZNF587*, *ADD2*, *ME3*, *EPDR1*, *ADHFE1*, *THAP3*, *AGMAT*, *RIMS1*, *ZNF175*, *ZNF709*, *LHX4*, *MT1E*, *LHX8*, *NOX5*, *GABRA1*, *MAP1D*, *RNF5P1*, *CAPN8*, *GABRA5*, *ANKMY1*, *PDZRN3*, *NPR3*, *ISL1*, *MARCH1*, *QPCT*, *SALL3*, *ZFHX4*, *ZNF311*, *SNED1*, *KCNJ8*, *RNF5*, *ZIC4*, *EBF2*, *ZNF417*, *HIVEP3*, *CACNA1H*, *DGKZ*, *ZIK1*, *TLL1*	0.013

GO, gene ontology.

examined, since we did not have access to mRNA from the original set of placental samples. However, given that previous studies have reported increased promoter methylation and reduced *WNT2* placental expression in preeclampsia placentas (14, 38), we would hypothesize that abnormal expression of *WNT2* in the placenta may lead to trophoblast dysfunction and the development of preeclampsia.

The region containing the genes *SPESP1* and *NOX5* exhibited the largest overall difference ($\Delta\beta$ = 5–28%) in methylation between preeclampsia cases and controls, with methylation levels being higher in preeclampsia placentas compared with controls. This region was also found to be significantly hypermethylated in the GSE44712 preeclampsia dataset. SPESP1 is a recently discovered human alloantigen that is required for successful fertilization (16). It is expressed abundantly in the testis and has been detected in the placenta (37). SPESP1 is expressed in the equatorial segment of sperm and is important in initiating fusion with the egg plasma membrane during fertilization (16). Male SPESP1 knockout mice have been shown to have significantly fewer offspring due to a reduced ability of their sperm to fuse with an egg while fertility in female SPESP1 knockout mice was unaffected (16). Most recently, SPESP1 has been reported to exhibit a sex-specific methylation profile with males having lower levels of methylation compared with females at the cg09886641 locus ($\Delta\beta$ = 0.06) (26). In our study, male and female placentas were

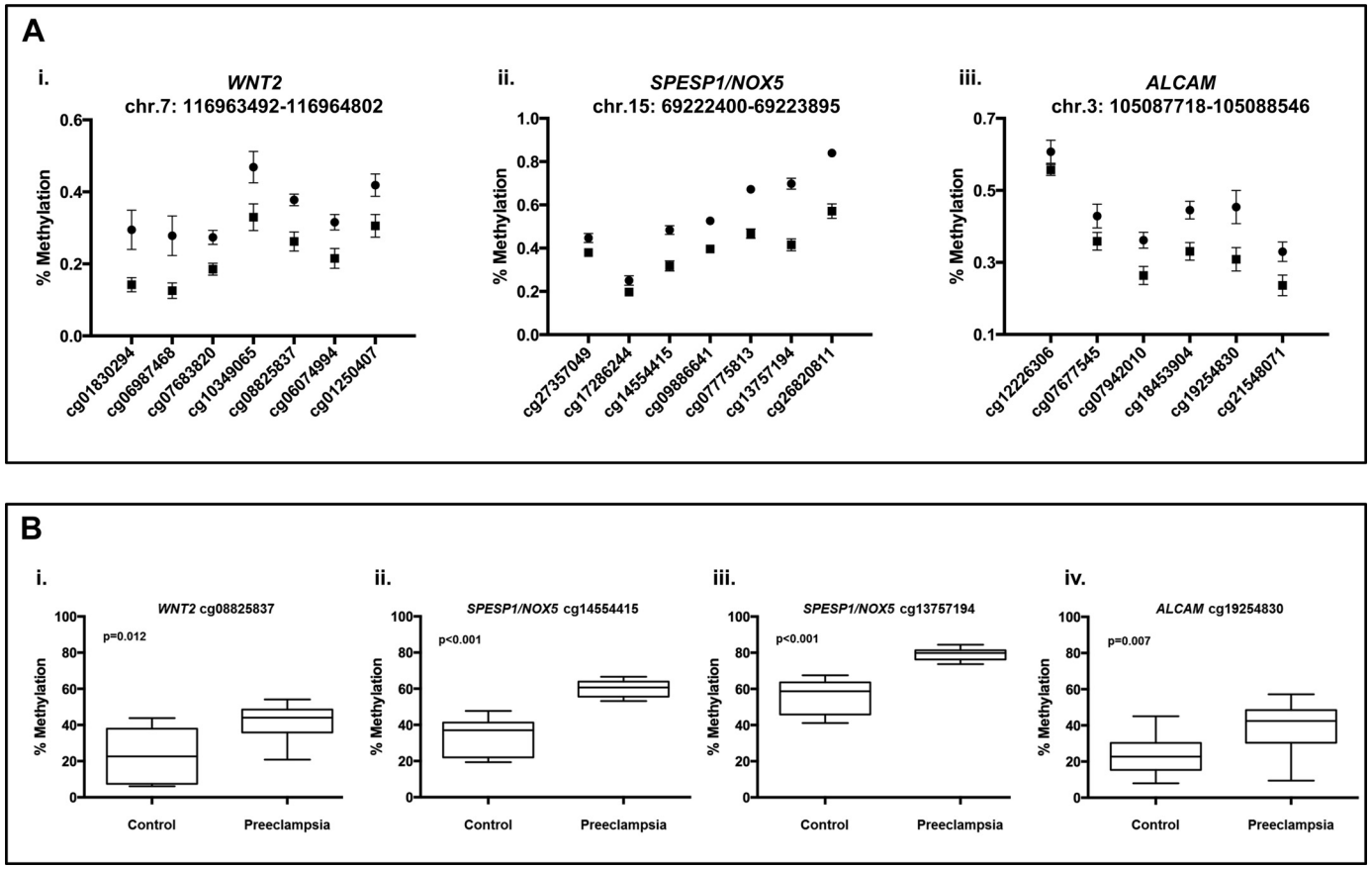

Fig. 1. Preeclampsia-associated differences in placental DNA methylation. *A*: the top-ranked differentially methylated regions of interest where there is coordinated hypermethylation across adjacent CpG sites in preeclampsia placentas (circle) compared with control placentas (square). β-Values (mean ± SE) from the 450K array at each probe site are presented. *i*, Chromosome (chr) 7: 116963492–116964802 containing wingless type MMTV Integration Site family member 2 (*WNT2*); *ii*, chr.15: 69222400–69223895 containing sperm equatorial segment protein (*SPESP1*) and NADPH oxidase 5 (*NOX5*); *iii*, chr.3: 105087718–105088546 containing activated leukocyte cell adhesion molecule (*ALCAM*). *B*: bisulfite pyrosequencing was carried out at selected CpG sites within each region. *i*, *WNT2* (cg08825837); *ii*, *SPESP1* and *NOX5* (cg14554415); *iii*, *SPESP1* and *NOX5* (cg13757194); *iv*, *ALCAM* (cg19254830).

equally represented in both the preeclampsia and control groups, which would indicate that the methylation differences observed in this study are not solely the result of sex differences. The role of SPESP1 expression in the placenta is unknown. We hypothesize that hypermethylation in the preeclampsia placentas would result in lowered *SPESP1* expression, which may be involved in the pathogenesis of preeclampsia. Furthermore, a gene expression study comparing healthy and preeclampsia-affected placentas has reported a 3.77-fold decrease in *SPESP1* expression in preeclampsia placentas (27). This is consistent with the increase in *SPESP1* promoter methylation in preeclampsia placentas that we observed. Further research is required to understand the role of this protein in placental function and therefore in the development of preeclampsia.

NOX5 is a major source of reactive oxygen species (ROS) and is the newest member of the NADPH oxidase family. ROS, derived by the NOX family, have roles in cell proliferation, transformation, differentiation, and apoptosis. *NOX5* has been shown to be highly expressed during embryonic development, and hypermethylation of the *NOX5* promoter region has been associated with congenital heart disease in human fetuses (39). Hypermethylation of *NOX5* leads to transcriptional silencing (39) and a reduction in the production of ROS. Because ROS signaling stimulates cell proliferation, reduced ROS may affect placentation via impaired cell proliferation and differentiation, and contribute to the development of preeclampsia.

Genes that clustered into the cell adhesion functional group were significantly enriched in our study. Of the genes in this cluster we were able to confirm the *ALCAM* gene was hypermethylated in the preeclampsia cases compared with controls. This gene was also found to be hypermethylated in the GSE44712 dataset. ALCAM is an immunoglobulin cell adhesion molecule that is expressed in a wide variety of cell types, including endothelial, hematopoietic, and epithelial cells (35), and is generally restricted to sites requiring cell proliferation and cell migration. The expression of ALCAM has been reported to be upregulated in a range of cancers, including melanoma, colon cancer, and gastric cancer where it has a role in promoting cell motility and migration, leading to more invasive phenotypes (11, 22, 36). In lung and pancreatic cancers, inhibition of ALCAM has been shown to have enhanced invasive and migratory properties of the cells (17, 33). In the context of pregnancy, developmental biology studies have shown that *ALCAM* expression generally occurs in cells undergoing proliferation and is required for implantation of blastocysts (17). *ALCAM* has previously been shown to be downregulated in placental tissue (24) and deciduas basalis

(12) from pregnancies complicated with preeclampsia. This is consistent with the hypermethylation observed in our study. Given the role ALCAM has in cell proliferation and migration, alterations in DNA methylation and gene expression may contribute to the development of preeclampsia via reduced trophoblast motility and invasion.

Gestational age is an important confounder when studying changes in placental DNA methylation. Some studies have attempted to match controls by gestational age; however, this results in using spontaneous preterm placentas that may have additional differences in methylation that are not found at term (≥37 wk gestation). A number of studies have demonstrated gestational age alters methylation profiles (4, 18, 29) while others assign the differences to the presence of preeclampsia and support the use of full-term placentas as controls (6). Due to the difficulties in matching for gestational age, we used late-onset preeclampsia and adjusted for gestational age in the analysis. This is a limitation of all studies of preeclampsia when collecting placental samples at birth and comparing them with samples from a healthy full-term pregnancy.

Another limitation is the small sample size of the present study, which reduced the power to detect smaller β differences. Therefore, there may be other DMRs that have differences in methylation between preeclampsia placentas and controls, and yet did not reach significance in the present study.

This study identified genomewide changes to the DNA methylation profiles in placentas from women with preeclampsia that may be associated with changes in placental development and function. We found differences in methylation in gene regions involved in cell signaling (*WNT2*), fertilization and implantation (*SPESP1*), ROS signaling (*NOX5*), and cell adhesion (*ALCAM*). Preeclampsia is not usually diagnosed until later in gestation when women develop the clinical signs and symptoms, and by this stage significant systemic injury has been established. We are yet to produce a biologically sound explanation for the development of preeclampsia, and this limits the ability to develop an early screening method for diagnosis in the clinical setting. Therefore, this research may lead to an improved understanding of the pathophysiological disease process and may assist in the establishment of biomarkers for early screening providing novel opportunities to improve the care of both the mother and her baby.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

K.R.Y. and C.L.C. performed experiments; K.R.Y., C.L.C., and R.P. analyzed data; K.R.Y., C.L.C., R.P., A.M., A.H., and J.M.L. interpreted results of experiments; K.R.Y. and C.L.C. drafted manuscript; C.L.C. prepared figures; C.L.C., R.P., and J.M.L. edited and revised manuscript; A.M., A.H., and J.M.L. approved final version of manuscript; J.M.L. conception and design of research.

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