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Sex-specific differences in placental global gene expression in pregnancies complicated by asthma

A. Osei-Kumah^a, R. Smith^b, I. Jurisica^{c,d,e}, I. Caniggia^f, V.L. Clifton^{a,*}

^a Robinson Institute, School of Paediatrics and Reproductive Health, Lyell McEwin Hospital, Haydown Rd, Elizabeth Vale, Adelaide, SA 5112, Australia

^b Mothers and Babies Research Centre, Department of Endocrinology, Hunter Medical Research Institute, Newcastle, NSW, Australia

^c Department of Medical Biophysics, University of Toronto, Canada

^d Department of Computer Science, University of Toronto, Canada

^e Ontario Cancer Institute and the Campbell Family Institute for Cancer Research, University Health Network, Toronto, Canada

^f Samuel Lunenfeld Research Institute, Mt Sinai Hospital, Toronto, Canada

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ABSTRACT

Background: Chronic maternal asthma is associated with reduced growth of the female fetus and normal growth of the male fetus. The mechanisms that control the differential effects of maternal asthma on the fetus have not been fully elucidated but alterations in placental function may play a role. In the current study we have used microarray platform to examine fetal sex-specific global changes in placental gene expression in pregnancies complicated by asthma as compared to non-asthmatic subjects.

Methods: Placental RNA was extracted from 11 control subjects and 38 asthmatic subjects. Labeled cDNA was hybridized to an oligonucleotide chip with 1700 double spotted well-characterized human genes. Global gene expression data analysis and visualization were performed using the Binary Tree-Structured Vector Quantization (BTSVQ) software. Functional relationships of differentially expressed genes were assessed using protein–protein interaction database I2D, network analysis and visualization software NAViGaTOR and Ingenuity Pathway Analysis software.

Results: Overall, 65 genes were found to be altered in placentae of pregnancies complicated by asthma. Of these, only 6 genes were altered in male placentae. There were 59 gene changes in female placentae of asthmatic mothers relative to control placentae. Some of the sex-specific genes were associated with growth, inflammation and immune pathways.

Conclusion: There are sex-specific alterations in placental gene expression in the presence of maternal asthma. Given that many of the identified genes in the female placentae were associated with or involved in cellular growth and tissue development, these may contribute to the sexually dimorphic difference in fetal growth in response to maternal asthma.

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1. Introduction

Asthma is the most common disease to affect human pregnancy [21]. Maternal asthma during pregnancy is associated with a poor fetal outcome such as preterm delivery, intrauterine growth restriction (IUGR) and low birth weight [10,12,14,22]. Studies have suggested that the effects of asthma on fetal outcome are related to asthma severity and the intensity of glucocorticoid treatment [11]. Murphy et al. [24,25] have shown that there was significantly reduced birth weight centiles in female neonates from asthmatic women who did not control their asthma with inhaled steroids

compared to female neonates from women who controlled their asthma with inhaled glucocorticoids during pregnancy and control women. The low birth weight observed in pregnancies complicated by untreated asthma was only associated with the female fetus while the male fetus was of normal birth size [24]. These findings suggest male and female fetuses institute different strategies to cope with maternal asthma during pregnancy. Since the placenta has a central role in fetal growth and development, it is possible that variations in gene expression may lead to functional differences that contribute to the sex-specific alterations in fetal development.

We have previously identified sex-specific differences in placental cytokine mRNA expression. Specifically, we have shown that there are significant increases in tumor necrosis factor (TNF)- α , interleukin (IL)-1 β , IL-6, IL-8 and IL-5 mRNA expression in female placentae compared with males [29]. In addition we have shown

* Corresponding author. Tel.: +61 8 8133 2133; fax: +61 8 8182 9337.
E-mail address: vicki.clifton@adelaide.edu.au (V.L. Clifton).

Table 1
Maternal and Neonatal Characteristics.

	Inhaled Glucocorticoid Intake		
	Control	Glucocorticoids	No glucocorticoids
Total number of subjects	11	23	15
Maternal Age (years)	28.63 ± 1.83	26.91 ± 1.31	24.47 ± 1.02
Parity	1.75 ± 0.65	1.22 ± 0.23	0.87 ± 0.29
Gravida	3.00 ± 0.78	2.61 ± 0.26	2.31 ± 0.36
FEV ₁ (liter)	3.28 ± 0.16	3.10 ± 0.11	3.16 ± 0.10
Vital Capacity (VC)	3.74 ± 0.08	3.94 ± 0.11	3.76 ± 0.11
FEV ₁ :VC	0.88 ± 0.05	0.81 ± 0.02	0.84 ± 0.02
Birth weight centile	68.13 ± 5.81	55.70 ± 6.18	51.79 ± 6.35
Gestational age at delivery	40.03 ± 0.51	39.51 ± 0.25	39.83 ± 0.30
Labor	9/2	18/5	19/0
Spontaneous/Induced			
Mode of delivery	10/1	22/1	14/1
Vaginal/Caesarean Section			
Nonsmokers/Smokers	11/0	17/6	11/4

Data expressed as mean ± SEM (standard error of mean).

a decrease in placental 11 β -HSD 2 activity in female placenta [25]. This suggests that male and female fetuses have differential placental gene regulation during development. The aim of the current study was to examine global changes in placental gene expression using microarray in the presence and absence of maternal asthma, taking into account the sex of the fetus.

Microarrays are used as a high-throughput tool for the identification of differentially expressed genes. Gene array studies have been employed to examine placental gene profile in normal pregnancies [32] and from mothers who smoked during pregnancy [18]. It has also been used to show differences in global gene expression in the placentae of pregnancy complications, such as preeclampsia [30,31] and gestational diabetes [13]. However the application of gene array studies in the pathogenesis of asthma during pregnancy has not been examined. Furthermore, only a few studies have examined the differential expression of placental genes in relation to fetal sex, despite a number of emerging studies showing sex-specific differences in placental function in normal and pathological conditions [15,29]. This is the first study to use a global approach to examine the gene expression profile in placentae of pregnancies

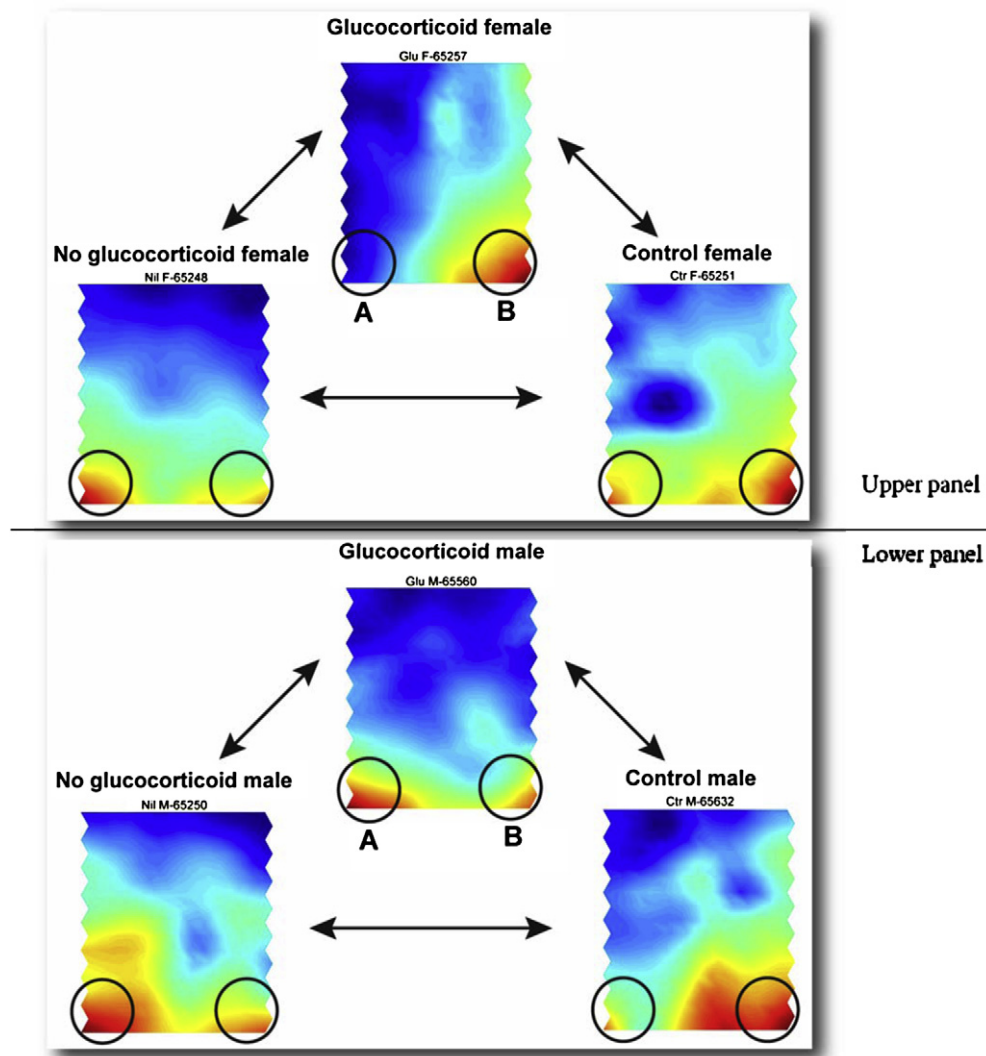


Fig. 1. Heat maps identifying genes uniquely expressed (areas A and B) in study groups (control non-asthmatic, asthmatics using inhaled glucocorticoids or no inhaled glucocorticoids). Regions appearing as red shows highly up-regulated genes and blue depict highly down-regulated genes. Differentially expressed genes in areas A and B are compared from the study groups in the no glucocorticoid female versus control female and glucocorticoid female (upper panel). Glucocorticoid male was also compared with no glucocorticoid male and control male (lower panel). For example, in the upper panel, glucocorticoid female and control female have up-regulated genes in area B compared with no glucocorticoid female.

Table 2

Genes that changed with asthma in the placenta.

Unigene ID	Gene name	Description	Location	Family	Regulation
Male (no glucocorticoid treatment)					
Hs.75452	HSPA1A	Heat shock 70 kDa protein 1A	Cytoplasm	Other	Up
Hs.2540	CHRNA7	Cholinergic receptor, nicotinic, alpha 7	Plasma Membrane	Transmembrane receptor	Up
Hs.79345	F8	Coagulation factor VIII, procoagulant component	Extracellular Space	Other	Up
Hs.430150	FTL	Ferritin, light polypeptide	Cytoplasm	Other	Down
Hs.286161	UTRN	Utrophin	Plasma Membrane	Transmembrane receptor	Up
Hs.169476	GAPDH	Glyceraldehyde-3-phosphate dehydrogenase	Cytoplasm	Enzyme	Down
Male (glucocorticoid treatment)					
None					
Female (no glucocorticoid treatment)					
Hs.198365	BPGM ^a	2,3-bisphosphoglycerate mutase	Unknown	Phosphatase	Down
Hs.227913	API5	Apoptosis inhibitor 5	Cytoplasm	Other	Down
Hs.343521	MDH2 ^a	Malate dehydrogenase 2, NAD (mitochondrial)	Cytoplasm	Enzyme	Down
Hs.240013	COMT	Catechol-O-methyltransferase	Cytoplasm	Enzyme	Down
Hs.78056	CTSL1	Cathepsin L1	Cytoplasm	Peptidase	Down
Hs.43910	CD164	CD164 molecule, sialomucin	Plasma Membrane	Other	Down
Hs.278573	CD59	CD59 molecule, complement regulatory protein	Plasma Membrane	Other	Down
Hs.301921	CCR1	Chemokine (C–C motif) receptor 1	Plasma Membrane	G-protein coupled receptor	Down
Hs.89866	CPOX	Coproporphyrinogen oxidase	Cytoplasm	Enzyme	Up
Hs.75294	CRH	Corticotropin releasing hormone	Extracellular Space	Other	Up
Hs.76111	DAG1	Dystroglycan 1 (dystrophin-associated glycoprotein 1)	Plasma Membrane	Transmembrane receptor	Up
Hs.74047	ETFB	Electron-transfer-flavoprotein, beta polypeptide	Cytoplasm	Transporter	Down
Hs.118804	ENO3	Enolase 3 (beta, muscle)	Cytoplasm	Enzyme	Up
Hs.7195	GABRG2	Gamma-aminobutyric acid (GABA) A receptor, gamma 2	Plasma Membrane	Ion channel	Up
Hs.386793	GPX3	Glutathione peroxidase 3 (plasma)	Extracellular Space	Enzyme	Up
Hs.119651	GPC3	Glypican 3	Plasma Membrane	Other	Down
Hs.77326	IGFBP3	Insulin-like growth factor binding protein 3	Extracellular Space	Other	Down
Hs.80828	KRT1 ^a	Keratin 1	Cytoplasm	Other	Up
Hs.348401	LCAT	Lecithin-cholesterol acyltransferase	Extracellular Space	Enzyme	Down
Hs.2605	CD300C	CD300c molecule	Plasma Membrane	Transmembrane receptor	Down
Hs.155324	MMP11	Matrix metalloproteinase 11 (stromelysin 3)	Extracellular Space	Peptidase	Down
Hs.239018	RAB11B ^a	RAB11B, member RAS oncogene family	Cytoplasm	Enzyme	Down
Hs.151051	MAPK10	Mitogen-activated protein kinase 10	Cytoplasm	Kinase	Down
Hs.73090	NFKB2	Nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (p49/p100)	Nucleus	Transcription regulator	Down
Hs.83920	PAM	Peptidylglycine alpha-amidating monooxygenase	Plasma Membrane	Enzyme	Down
Hs.2894	PGF	Placental growth factor	Extracellular Space	Growth factor	Down
Hs.179657	PLAUR	Plasminogen activator, urokinase receptor	Plasma Membrane	Transmembrane receptor	Down
Hs.22584	PDYN	Prodynorphin	Extracellular Space	Transporter	Up
Hs.26045	PTPRA	Protein tyrosine phosphatase, receptor type, A	Plasma Membrane	Phosphatase	Down
Hs.74267	RPL15	Ribosomal protein L15	Cytoplasm	Other	Down
Hs.28491	SAT1	Spermidine/spermine N1-acetyltransferase 1	Cytoplasm	Enzyme	Down
Hs.178137	TOB1	Transducer of ERBB2, 1	Nucleus	Transcription regulator	Down
Hs.23582	TACSTD2	Tumor-associated calcium signal transducer 2	Plasma Membrane	Other	Down
Hs.75103	YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide	Cytoplasm	Enzyme	Down
Hs.23019	ZNF16	Zinc finger protein 16	Nucleus	Other	Down
Hs.2076	COMMD5	COMM domain containing 5	Nucleus	Other	Down
Female (glucocorticoid treatment)					
Hs.198365	BPGM ^a	2,3-bisphosphoglycerate mutase	Unknown	Phosphatase	Up
Hs.432659	OAS2	2'-5'-oligoadenylate synthetase 2, 69/71 kDa	Cytoplasm	Enzyme	Down
Hs.183153	ARL4D	ADP-ribosylation factor-like 4D	Nucleus	Enzyme	Up
Hs.75249	ARL6IP1	ADP-ribosylation factor-like 6 interacting protein 1	Cytoplasm	Other	Up
Hs.69771	CFB	Complement factor B	Extracellular Space	Peptidase	Up
Hs.20644	BCKDK	Branched chain ketoacid dehydrogenase kinase	Cytoplasm	Kinase	Up
Hs.75562	DDR1	Discoidin domain receptor tyrosine kinase 1	Plasma Membrane	Kinase	Up
Hs.120844	FOXJ2	Forkhead box J2	Nucleus	Transcription regulator	Down
Hs.59242	FURIN	Furin (paired basic amino acid cleaving enzyme)	Cytoplasm	Peptidase	Up
Hs.22554	HOXB5	Homeobox B5	Nucleus	Transcription regulator	Up
Hs.80828	KRT1 ^a	Keratin 1	Cytoplasm	Other	Up
Hs.308026	HLA-DRB1	Major histocompatibility complex, class II, DR beta 1	Plasma Membrane	Transmembrane receptor	Up
Hs.343521	MDH2 ^a	Malate dehydrogenase 2, NAD (mitochondrial)	Cytoplasm	Enzyme	Up
Hs.155455	PFKL	Phosphofructokinase, liver	Cytoplasm	Kinase	Up
Hs.32505	KCNJ4	Potassium inwardly-rectifying channel, subfamily J, member 4	Plasma Membrane	Ion channel	Up
Hs.251850	PSG5	Pregnancy specific beta-1-glycoprotein 5	Extracellular Space	Other	Up
Hs.1519	PRKAR1B	Protein kinase, cAMP-dependent, regulatory, type I, beta	Cytoplasm	Kinase	Up
Hs.239018	RAB11B ^a	RAB11B, member RAS oncogene family	Cytoplasm	Enzyme	Up
Hs.281564	ROM1	Retinal outer segment membrane protein 1	Plasma Membrane	Other	Up
Hs.82109	SDC1	Syndecan 1	Plasma Membrane	Other	Up
Hs.110837	TUBB4	Tubulin, beta 4	Cytoplasm	Other	Up
Hs.75275	UBE4A	Ubiquitination factor E4A (UFD2 homolog, yeast)	Cytoplasm	Enzyme	Up
Hs.379466	UBE2A	Ubiquitin-conjugating enzyme E2A (RAD6 homolog)	Cytoplasm	Enzyme	Up

^a Genes identified with or without use of inhaled glucocorticoid use.

complicated by asthma and to examine the sex-specific differences in placental gene expression with the aim of identifying placental pathways that are altered by maternal asthma.

2. Methods

2.1. Subjects

Asthmatic women ($n = 38$) and control non-asthmatic women ($n = 11$) were recruited to the study from the antenatal clinic at the John Hunter Hospital during the first trimester of their pregnancy, following written, informed consent. Women were assessed throughout pregnancy, according to their asthma severity and medication usage as previously described by our group [25]. Placenta was collected following delivery. Maternal and neonatal characteristics of patients are summarized in Table 1.

2.2. Tissue collection

Placenta was collected within 45 min of delivery and sections were snap frozen in liquid nitrogen and kept at -80°C . Multiple specimens were sampled from central and peripheral regions of maternal side of all the placentae collected. Five term normal healthy placentae were collected at Mount Sinai Hospital (Toronto, Canada). These placentae were used as a neutral internal reference control allowing for normalization of microarray data across various chips used. The term reference group were normal and within healthy physiologic range for all maternal and fetal clinical parameters. Placenta was grouped based on maternal asthma status and fetal sex. There were 5 male and 6 female in the control non-asthmatic group. In the untreated asthma group, there were 10 males and 13 females. There were 7 male and 8 female placentae in the group of mothers that treated their asthma with inhaled glucocorticoids.

2.3. RNA extraction

Total RNA was extracted from frozen placental samples using the trizol method (Life Technologies, Inc., Frederick, MD) as previously described in Refs. [23,29]. RNA quality and integrity was assessed on denaturing 1.2% agarose gel. Only RNA samples demonstrating 28S to 18S rRNA ratio of 2:1 were included in study.

2.4. Microarray experiment

RNA from male and female placenta within each group of control and asthma were pooled. Each pooled experimental sample was labeled with cyanine-3 dye and compared to the same neutral pooled cyanine-5 labeled RNA sample, obtained from five term placentae acquired from vaginal deliveries (reference RNA). The labeling and hybridization procedures were according to University Health Network Microarray Centre's indirect labeling protocol ([http://www.microarrays.ca/support/](http://www.microarrays.ca/support/proto.html)

<http://www.microarrays.ca/support/glists.html>) as previously described in Ref. [31]. Briefly, equal amounts of RNA (10 μg) from each individual sample in each experimental condition were pooled. Fluorophorelabeled cDNA was hybridized to 1.7k version 4 human microarrays, containing 1700 double spotted well-characterized human genes (<http://www.microarray.ca/support/glists.html>; for a complete list of genes and related information). Labeled cDNA was hybridized on 3 different arrays. Arrays were scanned using a GenePix 4000 Microarray Scanner and spot density was analyzed using the GenePix Pro 4.0 Acquisition and Analysis software (Axon Instruments, CA).

2.5. Quantitative real-time RT-PCR

Quantitative real-time RT-PCR was performed on samples used in the array experiments to quantify mRNA abundance of selected confirmatory genes. Primer and probe sets were purchased from Applied Biosystems (Applied Biosystems, CA). Analysis was performed using the DNA Engine Opticon[®] 2 System (MJ Research). The Comparative CT method (where CT is the threshold cycle) was used to derive a relative quantitative measure of the test gene expression compared with 18S ribosomal RNA, as previously described in Ref. [31].

3. Results

3.1. Characteristics of study population

Maternal characteristics were not significantly different between the study groups as shown in Table 1. Within the asthmatic group, 53% of the patients were classified as mild, 8% as moderate and 39% as severe asthmatics. Most of the patients who were not using inhaled glucocorticoid were mild asthmatics, whereas the majority of the patients who were using inhaled glucocorticoid were severe asthmatics (Table 1).

3.2. Gene expression analysis from heat maps

Global gene expression data analysis and visualization was performed using the Binary Tree-Structured Vector Quantization (BTSVQ) software as previously described in Ref. [33]. BTSVQ performs unsupervised clustering of full gene expression profiles, and supports intuitive visualization of obtained clusters, both as a binary tree and component planes of Self-Organizing Maps (SOMs). Data analysis was focused on two areas (A and B) as depicted by the heat maps where we observed differentially expressed gene clusters between the groups (Fig. 1). Two interesting observations

Table 3
Gene networks identified by ingenuity pathway analysis in relation to asthma.

Network ID	Molecules in network	Score	Focus molecules	Top Functions
1	API5 , CCL5, CCR1 , CFB , CTSC, DUSP10, FCGR1A, FGF2, FKBP1A, FURIN , GOS2, GAPDH (includes EG:2597), GPX3 , IFNB1, IL13, IRAK3, KRT1 , LCN2, LTb, MMP9, NAMPT, NFKB2 , NID1, OAS2 , PGF , PLAUR , PSMB8, RFTN1, SAT1 , SDC1 , SNCA, SQLE, TBP, TLR1, TNF	20	13	Genetic Disorder, Immunological Disease, Inflammatory Disease
2	ARL6IP1 , BAX, CD59 , CENPF, COMT , CPOX , DDR1 , ENO3 , ENPP1, ESR1, HAS1, HINT1 (includes EG:3094), HSPA1A , IGFBP3 , IL1B, ITPA, LAMA3, MDH2 , MLL2, MMP11 , MMP12, MMP1 (includes EG:4312), NEK2, POLE2, PRIM1, S100A4, SMAD2, SMAD6, TADA3L, TGFB1, TMEM97, TOB1 , TP53, TPM1, YWHAZ	18	12	Cancer, Cellular Growth and Proliferation, Reproductive System Disease
3	DAG1 , LARGE	2	1	Infection Mechanism, Nervous System Development and Function, Tissue Development
4	HLA-DRA, HLA-DRB1	2	1	Antigen presentation, Dermatological Conditions and Diseases, Genetic Disorder
5	PA2G4, RPL15	2	1	Cancer, Cellular Growth and Proliferation, Endocrine System Disorders
6	A2M, LCAT	2	1	Lipid Metabolism, Small Molecule Biochemistry, Carbohydrate Metabolism
7	F8 , F9, VWF	2	1	Hematological Disease, Cell-To-Cell Signaling and Interaction, Hematological System Development and Function

Score is based on Fisher exact test and a score of >3 correspond to $P < 0.001$ These networks were developed using genes identified to be differentially expressed in asthma. Genes in **bold** are those identified from our differentially expressed gene list. For full names of all genes involved in the network please see supplementary Table E1.

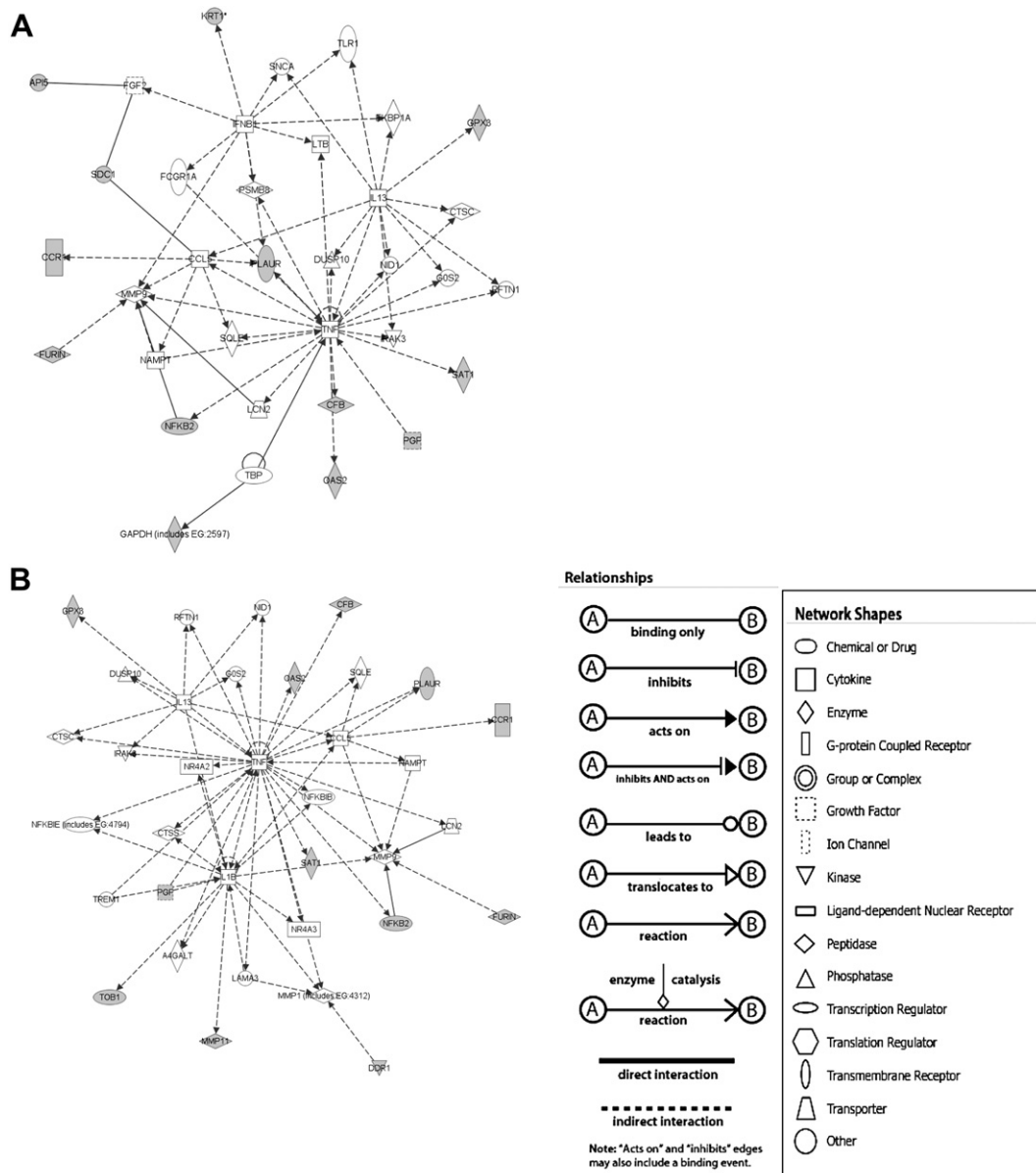


Fig. 2. (A): Network 1 identified by ingenuity pathway analysis using differentially expressed genes in asthma in both male and female placentae. Molecules shaded in gray are those genes included in our uploaded gene list. The unshaded molecules are those that have been added from the Ingenuity Knowledge Base to have interaction or association with our uploaded gene list. The shapes and the relationships in the network are explained in the legend below. For full names of all genes involved in the network please see supplementary Table E1. (B): Network 2 identified by ingenuity pathway analysis using differentially expressed genes in asthma in male and female placentae. Molecules shaded in gray are those genes included in our uploaded gene list. The unshaded molecules are those that have been added from the Ingenuity Knowledge Base to have interaction or association with our uploaded gene list. The shapes and the relationships in the network are explained in the legend below. For full names of all genes involved in the network please see supplementary Table E1.

were made from these arrays. First, in the mothers that were not using inhaled glucocorticoid for treatment and were pregnant with a female, there was down regulation of genes in areas A and B compared to placentae from the control group and asthmatic mothers using inhaled glucocorticoid (Fig. 1, upper panel). Second, in male placentae collected from asthmatic mothers that were not using inhaled glucocorticoid for treatment and those using inhaled glucocorticoid treatment, there were several genes down-regulated in area B when compared with control male placentae (Fig. 1, lower panel). The list of differentially and commonly expressed genes between areas in various experimental conditions were then selected based on 3 standard deviations of the mean; significance

defined as *P* value less than 0.05. The lists of differentially expressed placental genes in the presence of asthma are presented in Table 2.

3.3. Genes differentially expressed between male and female placenta from pregnancies complicated by asthma

When genes were analyzed based on fetal sex using BTSVQ only a few genes (six) were found to change in male compared to female placentae. Thirty six placental genes were altered in female placentae of pregnancies complicated by asthma and not treated with inhaled glucocorticoids compared with normal pregnancy. Twenty three genes were significantly different in female placentae of pregnancies

complicated by asthma and treated with inhaled glucocorticoids. Eleven genes were found to be commonly altered in placenta from both males and females of pregnancies complicated by asthma, [Table 2](#).

3.4. Effect of maternal inhaled glucocorticoid treatment on placental gene expression

There were no gene changes in male placenta with the use of inhaled glucocorticoid treatment ([Table 2](#)). However in the female placenta, there were nineteen out of twenty three gene changes associated with inhaled glucocorticoid treatment. The other four genes depicted by an asterisk in [Table 2](#), including 2,3-bisphosphoglycerate mutase (BPGM), malate dehydrogenase 2, NAD (MDH2), keratin 1 (KRT1) and member RAS oncogene family (RAB11B) changed with inhaled glucocorticoid treatment but were also identified in the placenta of female babies whose mothers did not use inhaled glucocorticoid treatment. These four genes may be due to asthma-related changes in the female placenta.

3.5. Gene networks identified by pathway analysis

Similarly and differentially expressed genes identified from placenta of male and female fetuses of asthmatic pregnancies and the specific pathways that microarray genes are associated with were identified using the Interologous Interaction Database (I2D ver. 1.72; <http://ophid.utoronto.ca/i2d>, PMID 17535438) [2]. I2D is designed to explore known and predicted protein–protein interactions. NAViGaTOR (<http://ophid.utoronto.ca/navigator>) ver. 2.1.14 [3] was used to narrow down target genes and identify key proteins and corresponding pathways which may be targets for further *in vitro* studies. Furthermore, gene pathway analysis was conducted using Ingenuity Pathway Analysis (IPA) Software to identify possible biological functions of the differentially expressed genes between experimental groups (Ingenuity, Redwood City, CA, USA). IPA generates large global molecular network based on thousands of curated directed and indirect physical and functional

interactions between mammalian genes from published peer-reviewed content in the Ingenuity Knowledge Base.

Ingenuity Pathway analysis was conducted with all 65 genes (listed in [Table 2](#)) that changed with asthma, irrespective of inhaled glucocorticoid treatment. Seven networks were identified as shown in [Table 3](#). Networks involved in cellular growth, proliferation, tissue development, inflammatory disease and immunological dysfunction were enriched by our differentially expressed genes. The top two networks had 13 and 12 of our identified genes involved in the network respectively (Fischer *T*-test, $p < 0.001$) ([Table 3](#)). In the first network ([Fig. 2a](#)), TNF expression is directly or indirectly associated with our differentially expressed genes including placental derived growth factor (PGF), complement factor B (CFB), spermidine/spermine N1-acetyltransferase 1 (SAT1), 2'-5'-oligoadenylate synthetase 2 (OAS2), glyceraldehyde-3-phosphate dehydrogenase (GAPDH), plasminogen activator, urokinase receptor (PLAUR) and nuclear factor of kappa light polypeptide gene enhancer in B-cells 2 (NFKB2). Interestingly in network two ([Fig. 2b](#)), TNF, IL13, IFN, MMP9 and CCL5 are all highly connected genes, indicating that they are hub genes (i.e., genes with >5 connections). This suggests that they may be key regulatory molecules. Similarly in this network, the main regulatory molecules predicted by our identified genes are IL-1 β , TGF- β and tumor protein p53 (TP53).

Ingenuity pathway analysis was also used to identify sex-specific networks. In male placenta, three networks were identified and in female placenta 8 networks were identified ([Table 4](#)). The molecules identified in the male network genes were mostly associated with acute phase response signaling and oxidative stress (ferritin light chain, F8), gene expression (GAPDH) and glucocorticoid receptor signaling (HSPA1A = HSP70). In the female network, genes were associated with cellular growth, proliferation and tissue development (IGFBP3, PGF). There were also many inflammatory and immune related gene pathways observed in the female placental network as well as genes associated with cell death ([Table 4](#)). IPA analysis also showed that the molecules identified in the female placenta were involved in signaling pathways of IGF, HIF,

Table 4
Sex-specific gene networks identified by ingenuity pathway analysis in relation to asthma.

Network ID	Molecules in network	Score	Focus molecules	Top Functions
Male				
1	F8 , F9, VWF	3	1	Hematological Disease, Cell-To-Cell Signaling and Interaction, Hematological System Development and Function
2	GAPDH (includes EG:2597), GTF2B, ILF3, TAF6, TBP	2	1	Gene Expression, Infection Mechanism, Cellular Assembly and Organization
3	HSF1, HSF2, HSPA1A , IFNG, NANOG, TP53	2	1	Antigen Presentation, Antimicrobial Response, Cancer
Female				
1	API5 , ARL6IP1 , BAX, CD59 , COMT , CPOX, CTNNB1, DDX17, EIF2B1, ENO3, ESR1, FGF2, GPR64, HINT1 (includes EG:3094), IFNB1, IGFBP3 , KRT1 , LIMK1 , MDH2 , MLL2, MYC, PLS3 , POLE2, PPP3CA, RAD23A, RXRRA , SDC1 , SMAD6, TGFB1, TMEM97, TMSB4X, TMSL8, TP53, UBE2C, VWHAZ	21	14	Inflammatory Disease, Connective Tissue Disorders, Skeletal and Muscular Disorders
2	A4GALT, CCL5, CCR1 , CFB , CTSC, CTSS, DDR1 , DUSP10, FURIN , GOS2, GPX3 , IL13, IL1B, IRAK3, LAMA3, LCN2, MMP9, MMP11 , MMP1 (includes EG:4312), NAMPT, NFKB2 , NFKBIB, NFKBIE (includes EG:4794), NID1, NR4A2, NR4A3, OAS2 , PGF , PLAUR , RFTN1, SAT1, SQLE, TNF, TOB CYP11A1 , TRERF1	17	12	Inflammatory Disease, Connective Tissue Disorders, Skeletal and Muscular Disorders
3		2	1	Endocrine System Development and Function, Lipid Metabolism, Small Molecule Biochemistry
4	DAG1 , LARGE	2	1	Infection Mechanism, Nervous System Development and Function, Tissue Development
5	HLA-DRA, HLA-DRB1	2	1	Antigen Presentation, Dermatological Diseases and Conditions, Genetic Disorder
6	PA2G4, RPL15	2	1	Cancer, Cellular Growth and Proliferation, Endocrine System Disorders
7	A2M, LCAT	2	1	Lipid Metabolism, Small Molecule Biochemistry, Carbohydrate Metabolism
8	ENAH, LATS1, TES, ZYX	1	1	Nervous System Development and Function, Organ Development, Cancer

For full names of all genes involved in the network please see supplementary [Table E1](#). Genes in bold represent hub genes as identified by Ingenuity Pathway Analysis.

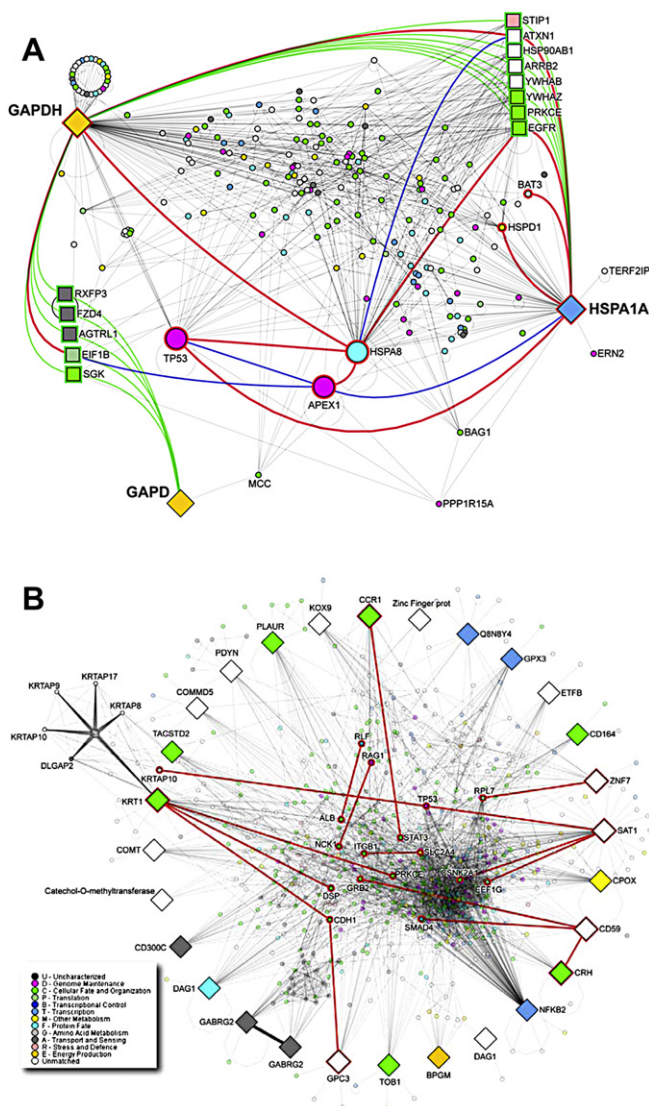


Fig. 3. (A): Protein–protein interaction network for genes changed in male placenta in the presence of asthma. I2D version 1.72 was used to identify direct protein interactions for the male-related targets (highlighted as diamond-shape nodes; $n = 3$). The resulting network of 214 proteins and 892 interactions was visualized using NAViGaTOR 2.1.14 (<http://ophid.utoronto.ca/navigator>). Color of nodes corresponds to Gene Ontology biological function; shape of nodes represents different types of important proteins; color of edges represents different interactions among targets. Highlighted square-shaped nodes represent proteins interacting with at least two male-specific differential asthma targets. Highlighted circular-shape nodes represent central proteins, and red edges show direct interactions linking them to the male-specific differential asthma targets. Remaining nodes and edges were faded out to reduce network complexity. (B): Protein–protein interaction network for genes changed in female placenta in the presence of asthma. I2D version 1.72 was used to identify direct protein interactions for the female-related targets (highlighted as diamond-shape nodes). The resulting network of 668 proteins and 3735 interactions was visualized using NAViGaTOR 2.1.14 (<http://ophid.utoronto.ca/navigator>)¹⁹. Color of nodes corresponds to Gene Ontology biological function; shape of nodes represents different types of important proteins; color of edges represents different interactions among targets, as described in the legend. Highlighted square-shaped nodes represent proteins interacting with at least two female-specific differential asthma targets. Highlighted circular-shape nodes represent central proteins, and red edges show direct interactions linking them to the female-specific differential asthma targets. Remaining nodes and edges were faded out to reduce network complexity.

IL-10, IL-13, IL-6, IL-8 and PPAR. Using a complementary pathway analysis tool (I2D and NAViGaTOR), GAPDH and HSP70 were also determined to be central proteins in the male placenta (Fig. 3a). Similarly, multiple central proteins involved in growth and inflammation were identified in the female placenta (Fig. 3b), which supports the pathways predicted by ingenuity pathway analysis.

3.6. PCR data validation on pooled and individual mRNA samples

To determine if pooling RNA sample for array analysis affected gene expression, selected candidate genes was analyzed in individual samples using real-time RT-PCR. There were no significant differences in either the pooled and individual mRNA measurements of CRH in the control group (Fig. 4A), asthmatic group not using inhaled glucocorticoid (Fig. 4B) and asthmatic group using inhaled glucocorticoid. (Student's *t*-test $P > 0.05$).

4. Discussion

Using cDNA array with over 1700 human encoding genes together with pathway analysis, we have shown that there are sex-specific alterations in placental gene expression in pregnancies complicated by asthma. We have also shown that within the asthmatic group there are sex-specific differences in placental gene expression with maternal inhaled glucocorticoid treatment. A higher proportion of placental gene alteration was observed in the female placenta and was strongly related to regulation of immune pathways and cellular growth. In contrast, there were minor gene alterations in male placentae in response to maternal asthma or inhaled glucocorticoid treatment. These altered genes were associated predominantly with cell–cell signaling (including acute phase and glucocorticoid receptor signaling) and gene expression. These results suggest that male and female fetuses have differential gene regulatory networks in the placenta in response to asthma and its treatment during pregnancy and this may influence the sexually dimorphic difference in growth and morbidity of the human fetus.

We have previously shown that untreated maternal asthma results in reduced female fetal growth [24] and this sexually dimorphic response to maternal asthma was associated with adaptations to placental function [25] and cytokine mRNA expression [29]. These findings are supported by the current gene array network analysis where we identified that in the female placenta the genes altered were involved in cytokine network regulation (TNF- α , IL13, IFN) and pro-inflammatory transcription factors such as NFkB. Pathway analysis also showed that the molecules identified in the female placenta were involved in signaling pathways of IL-10, IL-6 and IL-8. The use of network analysis has confirmed some of our in-vitro findings of increased cytokine mRNA expression in the female placenta [29].

Genes that have been previously identified to be associated with asthma, including the chemokine ligand CCL5 (RANTES), heat shock protein 70 and chemokine receptor 1 [1,7,28] were also identified by microarray analysis. Genes expressed in normal placenta including placental derived growth factor [17] and CRH mRNA [25] were also identified in our current analysis. The altered genes that were identified with asthma had strong associations with immune system processes, including 2,3-bisphosphoglycerate mutase, CD59 molecule, complement regulatory protein, chemokine (C–C motif) receptor 1 and nuclear factor of kappa light polypeptide gene enhancer in B-cells 2. These genes were directly or indirectly related to the regulation of TNF, IL13, IFN and CCL5 (RANTES). Components of the innate immune system including complement factor B (CFB) and spermidine/spermine N1-acetyltransferase 1

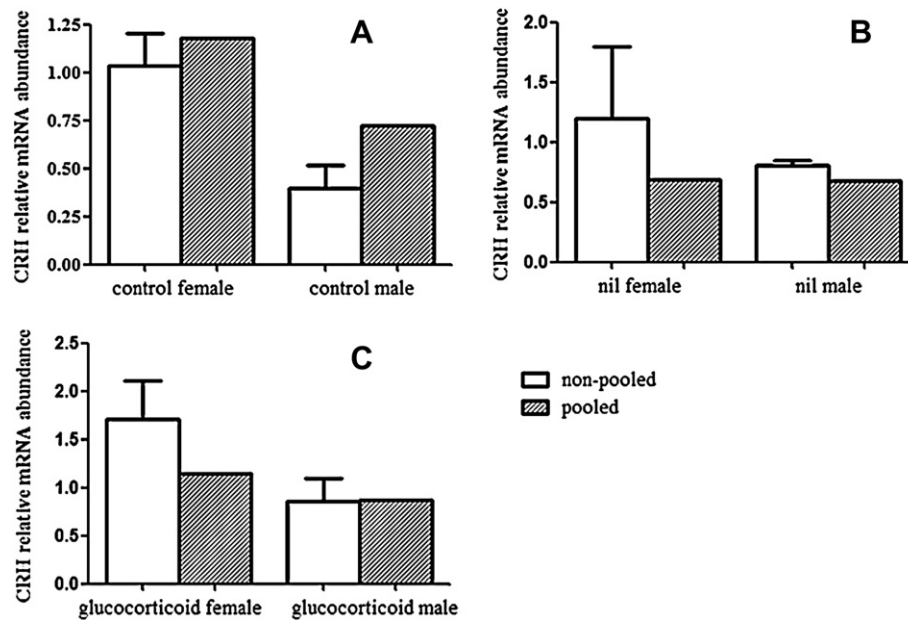


Fig. 4. The relative abundance of CRH mRNA from pooled placenta samples (shaded bars) and samples measured individually in (A): control non-asthmatic group; (B): asthmatic group not using inhaled glucocorticoid (nil) and (C): asthmatic group using inhaled glucocorticoid.

(SAT1) were also altered in the presence of maternal asthma and are known to be regulated by TNF- α [20]. This suggests that changes in cytokine mRNA expression in response to maternal asthma may be mediated by multiple genes that impact on the pro-inflammatory cytokine pathway. The network analysis has also identified novel target pathways and potential gene interaction from the list of numerous identified genes that need to be confirmed.

Using cDNA arrays to examine placenta from term healthy pregnancies, Sood et al. [32] identified sets of genes that were changed with the sex of the fetus. They also showed that these genes were involved in immune related pathways, which support our findings in the female placentae from pregnancies complicated by asthma. However, there was no overlap in the list of genes identified in the current study and the study conducted by Sood and colleagues. This may be due to differences in the type of cDNA array chip used. One of the limitations of comparing gene lists from gene array studies even of the same tissue is the use of different array platforms (e.g., in-house printed slides versus commercial chips such as Affymetrix). Therefore, the network comparisons may be more useful, and may be an important indirect way of validating the involvement of a pathway shown in several studies.

Another variation in the comparison of array dataset could be due to regional variation in placental gene expression [32]. The strength of the current study was the use of pooled sample from different sections of the maternal side of the placenta to give a more homogenous representation of the tissue. This controls for regional variation that has been identified in the placenta in recent studies [4–6,32]. This technique also increases the signal to noise ratio in array samples which helps reduce the number of false positives often observed within microarray expression dataset. This helps in diminishing the false positive discovery rate for clusters of differentially or similarly expressed genes across different experimental conditions [27].

One of the main concerns of the enormous amount of data generated from microarray methods is how to select target genes of biological significance for further studies. Using another complementary pathway analysis tool, the Interologous Interaction

Database (I2D) [2], we have identified target nodal genes and generated hypothetical protein–protein interaction pathways that need to be confirmed with *in vitro* studies. I2D is a web-based database of known and predicted protein–protein interactions [19]. Whilst the predictions of I2D should be thought of as hypotheses until experimentally validated, there is increasing evidence that interactions are conserved through evolution [26,35]. The nodal genes identified by I2D, such as HSP70, were also identified by Ingenuity Pathway analysis. Using different pathway tools, we have shown key regulatory genes involved in cytokine expression and immune responses in the female placenta and glucocorticoid signaling in male placenta.

A number of candidate genes from the microarray analysis were selected for validation based on their known roles in pregnancy, placental function and growth regulation. There were significant alterations in growth regulatory genes such as placental growth factor (PGF) and insulin-like growth factor binding protein-3 (IGFBP-3). PGF and IGFBP3 were decreased in a sex-specific manner. IGFBP3 has previously been shown to be down-regulated in placenta from other pathologies of pregnancy including preeclampsia [16]. Our previous studies have shown that male fetal growth is not affected by maternal asthma or inhaled glucocorticoid treatment [24] and we hypothesize that males may institute a state of glucocorticoid resistance to continue to grow in the presence of maternal asthma. In our microarray studies heat shock protein 70 (HSP70) was altered in the male placentae and interestingly this gene is involved in the regulation of glucocorticoid receptor signaling and glucocorticoid regulatory pathways in asthma [1,34]. The global gene changes observed in the current study support our previous mechanistic investigations of changes in placental function in response to maternal asthma [8,9].

In combination, the data presented indicates that there are significant alterations in placental global gene expression from pregnancies complicated by asthma. In addition, there are sex-specific differences in placental gene expression that vary with maternal asthma and its treatment. Interestingly, the male placenta had fewer adjustments in global gene expression in response to a complication during pregnancy, which may explain the sexually

dimorphic growth response to maternal asthma. Further studies are required to validate the protein–protein interactions and the role of corresponding networks in regulating fetal growth.

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Appendix. Supplementary material

Supplementary data related to this article can be found online at doi:10.1016/j.placenta.2011.05.005

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