The effect of pre-existing maternal obesity on the placental proteome: two-dimensional difference gel electrophoresis coupled with mass spectrometry

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Abstract

Our aim was to study the protein expression profiles of placenta obtained from lean and obese pregnant women with normal glucose tolerance at the time of term Caesarean section. We used two-dimensional difference gel electrophoresis (2D-DIGE), utilising narrow-range immobilised pH gradient strips that encompassed the broad pH range of 4–5 and 5–6, followed by MALDI-TOF mass spectrometry of selected protein spots. Western blot and quantitative RT-PCR (qRT-PCR) analyses were performed to validate representative findings from the 2D-DIGE analysis. Eight proteins were altered (six down-regulated and two up-regulated on obese placentas). Annexin A5 (ANXA5), ATP synthase subunit beta, mitochondria (ATPB), brain acid soluble protein 1 (BASP1), ferritin light chain (FTL), heterogeneous nuclear ribonucleoprotein C (HNRPC) and vimentin (VIME) were all lower in obese patients. Alpha-1-antitrypsin (A1AT) and stress-70 protein, mitochondrial (GRP75) were higher in obese patients. Western blot analysis of ANXA5, ATPB, FTL, VIME, A1AT and GRP75 confirmed the findings from the 2D-DIGE analysis. For brain acid soluble protein 1 and HNRPC, qRT-PCR analysis also confirmed the findings from the 2D-DIGE analysis. Immunohistochemical analysis was also used to determine the localisation of the proteins in human placenta. In conclusion, proteomic analysis of placenta reveals differential expression of several proteins in patients with pre-existing obesity. These proteins are implicated in a variety of cellular functions such as regulation of growth, cytoskeletal structure, oxidative stress, inflammation, coagulation and apoptosis. These disturbances may have significant implications for fetal growth and development.

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Introduction

There is no disputing the extent of the worldwide obesity epidemic (Chu *et al.* 2008, Colagiuri *et al.* 2010). The proportion of women who are obese at the beginning of pregnancy has dramatically increased (Heslehurst *et al.* 2010). Obesity in pregnancy is associated with substantially increased risks for both mother and infant (Heslehurst *et al.* 2008). Gestational diabetes, pre-eclampsia, Caesarean section, instrumental vaginal delivery, birth trauma and pelvic floor damage are increased in the mother (Sebire *et al.* 2001, Bhattacharya *et al.* 2007). Infants born to overweight and obese mothers are often macrosomic (Ehrenberg *et al.* 2004, Surkan *et al.* 2004) with increased adiposity (Hull *et al.* 2008). Perhaps of greatest importance for the health system as a whole is that these infants are at increased risk of later metabolic disease, including obesity, diabetes, cardiovascular disease and certain cancers (Boney *et al.* 2005, Drake & Reynolds 2010).

The placenta plays a vital role in the regulation of fetal growth and development during pregnancy. Roles include nutrient supply to the fetus, removal from the fetus of metabolic waste and hormone production. Placental phenotypes for intrauterine growth restriction (McCarthy *et al.* 2007, Struwe *et al.* 2010), pre-eclampsia (Hass *et al.* 2006, Dexlin-Mellby *et al.* 2010, Zhang *et al.* 2011) and diabetes in pregnancy (Radaelli *et al.* 2003, Zhao *et al.* 2011) have been described, but these have not specifically been studied in the context of maternal obesity. However, animal models have been crucial in delineating the impact of obesity on placental function. For example, maternal obesity and overnutrition alter fetal growth rate and cotyledonary vascularity and angiogenic factor expression in the ewe placenta (Ma *et al.* 2010). There is, however, a paucity of data on the effect of maternal

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obesity in humans. For this reason, we considered it of interest to determine the effect of pre-existing maternal obesity on the protein expression profiles of human placenta. Thus, the aim of this study was to use two-dimensional difference gel electrophoresis (2D-DIGE) to identify novel proteins associated with pre-existing maternal obesity in human placenta. Validation of the 2D-DIGE data will be done by quantitative RT-PCR (qRT-PCR) and western blotting.

Materials and methods

Tissue collection

Approval for this study was obtained from the Mercy Hospital for Women's Research (Heidelberg, Australia) and Ethics Committee and informed consent was obtained from all participating subjects. Human placenta was obtained from lean and obese pregnant women with normal glucose tolerance at the time of term Caesarean section before the onset of labour (Table 1). Indications for Caesarean section included repeat Caesarean section or breech presentation. Women with any adverse underlying medical condition (i.e. including asthma, diabetes and pre-eclampsia, polycystic ovary syndrome or thyroid or adrenal abnormalities) were excluded. None of the women conceived by artificial reproductive technology. Lean women were categorised as a body mass index (BMI) of <25 kg/m^2 and obese patients with a BMI of >30 kg/m^2. Placenta was collected from patients undergoing Caesarean section only to account for any effects of human labour and delivery on the protein profile. Placenta was obtained within 10 min of delivery and thoroughly washed in ice-cold PBS to remove any blood. Placental lobules (cotyledons) were obtained from various locations of the placenta; the basal plate and chorionic surface were removed from the cotyledon; and villous tissue was obtained from the middle cross-section. Placental tissue was blunt dissected to remove visible connective tissue and calcium deposits. Tissue samples were snap frozen in liquid nitrogen and immediately stored at −80 °C.

Sample preparation and DIGE labelling

Placenta (n=6 lean and n=6 obese) was homogenised into DIGE lysis buffer (30 mM Tris, 7 M urea, 2 M thiourea, 4% 3-(3-cholamidopropyl)dimethylammonio-1-propane sulphonate (CHAPS)). Proteins were extracted during 1 h at 4 °C, and lysates were clarified by centrifugation at 25 000 ***g*** at 4 °C for 20 min. The supernatant was precipitated with acetone, and protein extracts were then prepared following the general guidelines recommended for subsequent DIGE labelling. Briefly proteins were precipitated using the 2D clean-up kit (GE Healthcare, Piscataway, NJ, USA) and then buffer was exchanged against DIGE lysis buffer using Vivaspin-2 (3 kDa) concentrators (Sartorius Stedim, Goettingen, Germany) until conductivity could be reduced to below 200 μS/cm and the pH was adjusted to ∼8·5.

A lean pooled sample and an obese pooled sample were obtained (containing 50 μg of each sample; 300 μg total in each pool). This was then labelled with 400 pmol of Cy3 (obese samples) or Cy5 (lean samples; CyDye DIGE Fluors; GE Healthcare). Labelling was performed on ice and in the dark for 30 min. The reaction was then quenched by incubating with 1·5 μl of 10 mM lysine on ice and in the dark for 10 min. The Cy3-labelled obese samples were then combined with the Cy5-labelled lean samples and used for 2D gel electrophoresis analysis as detailed below.

OFF-GEL fractionation

To perform peptide fractionation according to their p*I*, the 3100 OFF-GEL fractionator and the OFF-GEL kit (both from Agilent Technologies, Waldbronn, Germany) were used following the manufacturer's

Table 1 Characteristics of the 2D-DIGE study group. Values represent mean ± S.E.M.

	2D-DIGE study		Validation study	
Clinical characteristic	Lean (n=6)	Obese (n=6)	Lean (n=10)	Obese (n=10)
Maternal age (years)	33·8 ± 1·3	30·8 ± 2·5	32·3 ± 1·6	31·3 ± 1·7
Maternal BMI at ∼12 weeks (kg/m^2)	20·1 ± 0·6	37·1 ± 1·1*	20·4 ± 0·8	39·0 ± 1·9*
Maternal BMI at delivery (kg/m^2)	23·3 ± 0·5	41·7 ± 1·5*	24·7 ± 0·8	44·2 ± 2·0*
Gestational age at birth (weeks)	39·0 ± 0·2	38·7 ± 0·2	38·8 ± 0·2	38·6 ± 0·2
Fetal birth weight (g)	3322 ± 220	3740 ± 171*	3295 ± 135	3781 ± 106*
Fetal gender	3 Females; 3 males	3 Females; 3 males	5 Females; 5 males	5 Females; 5 males
Gravida	2·8 ± 0·5	2·2 ± 0·4	2·8 ± 0·4	2·7 ± 0·5
Parity	2·3 ± 0·3	2·0 ± 0·4	2·1 ± 0·3	1·9 ± 0·3

*$P<0·05$ vs lean (Student's *t*-test).

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instructions. The device was set up for the 12 fractions separation by using a 13 cm long immobilised pH gradient (IPG) gel strip with a linear pH gradient ranging from 4 to 7. Six hundred micrograms of DIGE-labelled placenta (pooled placenta samples from six Cy3-labelled obese and six Cy5-labelled lean) were re-suspended with focusing buffer to a final volume of 1·8 ml. One hundred and fifty microlitres of this sample were loaded in each of the 12 wells. The sample was focused using the recommended method for OFF-GEL protein 12 wells fractionation with a maximum current of 50 μA. The focusing was stopped after total voltage reached 20 kVh. After focusing, 50–200 μl of the liquid upper phase sample were recovered for each well and pooled according to their p*I*. The two fractions used for this study were 4–5 and 5–6.

2D gel electrophoresis and imaging

The two fractions pools were precipitated with acetone, and protein extracts were then prepared following the general guidelines recommended for subsequent DIGE labelling. Briefly proteins were precipitated using the 2D clean-up kit (GE Healthcare) and then buffer was exchanged against DIGE lysis buffer using Vivaspin 2 (3 kDa) concentrators (Sartorius) until conductivity could be reduced to below 200 μS/cm and the pH was adjusted to ∼8·5. Proteins were focused on 11 cm, 3·9–5·1 and 4·7–5·9 IPG strips (Bio-Rad). Strips were rehydrated actively at 50 V and then focused overnight. IEF was performed for a total of ∼35 000 Vh at 20 °C. IPG strips were then equilibrated in equilibration buffer (50 mM Tris–HCl, 6 M urea, 30% glycerol, 2% SDS) supplemented with 1% dithiothreitol to maintain the fully reduced state of proteins, followed by 2·5% iodoacetamide to prevent re-oxidation of thiol groups during electrophoresis. Proteins were separated on 10·5–14% Criterion Tris–HCl gels (Bio-Rad Laboratories) at 15 mA/gel for 60 min, 30 mA/gel for 2 h and 45 mA/gel for 30 min at room temperature until the bromophenol blue dye-front had run off the bottom of the gels.

CyDye DIGE Fluor-labelled protein gels were scanned at 100 μm using a Typhoon Trio 9100 (GE Healthcare). The emission filters were Green 532 nm (Cy3) and Red 633 nm (Cy5). Gels were automatically aligned and relative protein quantification across lean and obese samples was performed using the Progenesis SameSpots Software 3.3.3420.25059 (Nonlinear Dynamics, Newcastle upon Tyne, UK). Normalisation was performed using the software algorithm. Student's *t*-test was used to calculate significant differences in relative abundances of protein spot features in placenta obtained from lean and obese pregnant women.

The 2D gels were fixed in 50% methanol, 3% phosphoric acid and then stained with Colloidal Coomassie stain and gels spots of interest were excised using a gel pen. Gel spots were then transferred into water for short-term storage at 4 °C. The water was subsequently removed and discarded from each gel plug. Gel pieces were destained by four sequential washes in destaining solution (10 mM ammonium bicarbonate (AmBic), 50% acetonitrile (ACN)), followed by two washes in 100% ACN, and then air dried at room temperature. The gel pieces were rehydrated by adding 10 μl of sequencing grade trypsin (0·02 μg/μl (Promega) in 10 mM AmBic) and left to incubate overnight at 37 °C. Digestion was quenched by addition of 2 μl of 2% formic acid (FA).

To avoid labelling bias arising from the varying fluorescence properties of gels at different wavelengths, samples from the same group were run twice in different gels but with the opposite dye labelling pattern. The Cy channels were individually imaged from each of the two gels using mutually exclusive excitation and emission wavelengths.

MALDI-TOF mass spectrometry and protein identification

Three microlitres of digest supernatant were applied to a Bruker Biosciences Anchorchip MALDI target, prepared with α-cyano-4-hydroxycinnamic acid (CHCA; see the thin layer affinity method for CHCA in the Bruker Anchorchip Manual). After 3 min, this solution was removed and the spots were air dried overnight at room temperature. MALDI-mass spectrometry (MS) was performed on a Bruker Microflex MALDI-TOF mass spectrometer (Bruker Daltonics, Bremen, Germany). Peaklists were generated using Flexanalysis (Bruker Daltonics) and were calibrated by utilising trypsin autolytic peptides. Biotools Software (Bruker Daltonics) and the Mascot search engine were used to interrogate the SwissProt database (release: July 2010, 517802 sequences; 182492287 residues) and proteins were identified by peptide mass fingerprinting. Search parameters were: taxonomy: human; MS tolerance, 100 ppm; missing cleavages, ≤1; enzyme, trypsin; fixed modifications, carbamidomethylation and variable modifications, oxidation (M). Identifications with Mascot expect probability values $<0·05$ were then manually verified by examination of spectra and/or resubmission of peak lists to Mascot. We took a conservative approach to protein identification and based acceptance on a number of criteria other than these scores. These included theoretical and experimental *M*r and p*I* being in accord, experimental peptide mass accuracy variation across the mass range and repeatability of identification across different gels. If multiple members of a protein family were identified those with the highest ranked hit were selected.

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RNA extraction and RT-PCR

Total RNA was extracted from ∼100 mg of tissue using Tri reagent according to the manufacturer's instructions (Sigma–Aldrich). RNA concentrations were quantified using a spectrophotometer (Smart Spec; Bio-Rad). RNA quality was determined via the A_{260}/A_{280} ratio. One microgram of RNA was converted to cDNA using the SuperScript VILO cDNA synthesis kit (Invitrogen) according to the manufacturer's instructions. The cDNA was diluted tenfold, and 2 μl of cDNA were used to perform RT-PCR using Sensimix Plus SYBR green (Quantace, Alexandria, NSW, Australia) and 100 nM of QuantiTect Primer Assays (Qiagen). Pre-validated primers for brain acid soluble protein 1 (BASP1; QT01672923) and GAPDH (QT01192646) were purchased from Qiagen. Average gene C_T values were normalised to the average GAPDH values of the same cDNA sample. The specificity of the product was assessed from melting curve analysis. RNA without reverse transcriptase during cDNA synthesis as well as PCR reactions using water instead of template showed no amplification. Gene expression levels were determined using the comparative threshold cycle (C_t) method.

Western blotting

For protein detection by western blot analysis, tissue was homogenised in radioimmuno precipitation assay buffer (1% SDS, 0·25% sodium deoxycholate, 1% Nonidet P-40, 150 mM NaCl, 50 mM Tris–HCl, pH 7·4), supplemented with protease inhibitors (1 mM EDTA, 0·5 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin and 5 μg/ml leupeptin). Cellular debris and lipids were eliminated by centrifugation of the solubilised samples at 25 000 ***g*** for 30 min (4 °C). Protein concentration was determined by the BCA Protein Assay (Pierce, Rockford, IL, USA).

Assessment of protein expression was analysed by western blotting as previously described (Lappas *et al*. 2003). Membranes were incubated with mouse monoclonal anti-alpha-1-antitrypsin (A1AT), Clone 1C2 (SAB4200198; Sigma) at 0·6 μg/ml; mouse monoclonal anti annexin A5 (ANXA5; WH0000308M1; Sigma) at 1 μg/ml; rabbit polyclonal anti ATP synthase subunit beta, mitochondria (ATPB; HPA001520; Sigma) at 0·2 μg/ml; rabbit polyclonal anti stress-70 protein, mitochondrial (GRP75; HPA000898; Sigma) at 0·1 μg/ml; mouse monoclonal anti vimentin (VIME; clone V9, N1421; Dako) at 2 μg/ml; rabbit polyclonal anti-ferritin light chain (FTL; F8556; Sigma) at 1 μg/ml; rabbit polyclonal anti-ferritin heavy chain (FTH; F8306; Sigma) at 1·8 μg/ml; and mouse monoclonal anti-β-tubulin (T8328; Sigma) at 1·2 μg/ml. The antibodies were diluted in blocking buffer (5% skim milk/tris-buffered saline–Tween (TBS–T; 0·05%)) for 24 h at 4 °C. Membranes were stripped and probed with mouse monoclonal anti-β-tubulin (T8328; Sigma) for normalisation of the data. Membranes were viewed and analysed using the Chemi-Doc system (Bio-Rad). Quantitative analysis of the relative density of the bands in western blots was performed using Quantity One 4.2.1 image analysis software (Bio-Rad). Data were corrected for background and then normalised to β-tubulin expression.

Immunohistochemistry

Placenta was placed in embedding cassettes fixed in buffered formaldehyde solution (4%) and embedded in paraffin. Serial sections (4 μm thick) were cut and mounted on sections onto superfrost plus slides. Slides were prepared consecutively for each sample. Each site was immunolabelled with each of the antibodies and one was used as a negative control slide. Sections were deparaffinised followed by an antigen retrieval step (boiled in 10 mM citrate buffer, pH 6·0 for 10 min followed by 20 min incubation). Endogenous peroxidase activity was removed using 3% H_2O_2 in methanol for 10 min. Sections were transferred to TBS (20 mM Tris pH 7·6, 150 mM NaCl). The sections were incubated in a humidity chamber for 1 h in antibody diluted in 1% BSA in TBS. The primary antibodies are detailed above (see Western blotting section). After incubation the binding sites were labelled with Dako Envision + polymer linked secondary reagent and visualised using Dako DAB + (DakoCytomation, Dako, Campbellfield, Vic, Australia). Nuclei were counterstained with Mayer's haematoxylin and the sections were dehydrated and cover slipped using a resinous mounting agent. Positive controls, which were composite slides with tonsil, breast tumour and ovarian tumour, were included in each run. Negative control slides, where primary antibody was replaced with normal mouse or rabbit IgG serum, were also included.

Statistical analysis

Statistical analyses were performed using a commercially available statistical software package (Statgraphics Plus version 3.1, Statistical Graphics Corp., Rockville, MD, USA). Student's *t*-test was used to assess statistical significance of the data. Statistical difference was indicated by a P value of $<0{\cdot}05$. Data are expressed as mean ± S.E.M.

Results

Analysis of the patient characteristics

Demographic data of the participants involved in this investigation are summarised in Table 1. All women in

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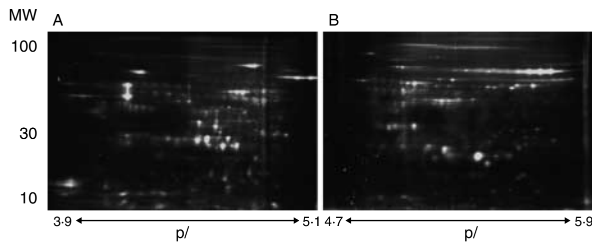

Figure 1 2D-DIGE analysis of placenta obtained from lean and obese women. (A) pH 3·9–5·1 and (B) pH 4·7–5·9 immobilised pH gradient strips were used for p*I* and 10·5–14% SDS-PAGE for the second dimension. The pH values of the IEF system and molecular mass standards (in kDa) of the 2D gels are indicated on the bottom and on the left of the panels respectively.

the lean group had a BMI of <25 kg/m^2, whereas all women in the obese group had a BMI of >30 kg/m^2. There were no significant differences in maternal age, fetal birth weight, gravid, parity and gestational age at delivery between the two sample groups.

Identification of differentially expressed proteins using 2D-DIGE

By using 2D-DIGE, we analysed the placenta proteome from a group of 12 individuals, six lean and six obese. Images for the two pH ranges are presented in Fig. 1. A total of 38 spots were positively identified; corresponding to 27 distinct proteins. Figure 2 shows the position of these spots in the 2D-DIGE gel. Table 2 displays detailed information about the corresponding proteins identified. This table summarises the protein accession number of identified muscle proteins, Mascot scores, the number of matched peptide sequences, the percentage sequence coverage, molecular mass, p*I* value, accession number, average normalised values and fold change of individual proteins affected by obesity.

ANXA5, ATPB, BASP1, FTL, heterogeneous nuclear ribonucleoprotein C (HNRPC), and VIME were all ≥1·5-fold higher in lean patients. On the other hand, A1AT and GRP75 were ≥1·5-fold higher in obese patients.

Validation of proteomic results by qRT-PCR and immunoblot analysis

Western blot and qRT-PCR analyses were used to validate representative findings from the 2D-DIGE analysis. In this study, we used the same six patients whose samples were used in the 2D-DIGE, plus an additional four lean and four obese pregnant women. For normalisation of the data, we used β-tubulin as the 2D-DIGE showed that this was unchanged between the two sample groups. As shown in Figs 3 and 4, the western blot analysis confirmed the 2D-DIGE data. That is, in obese placenta, the protein expression of cleaved A1AT (Fig. 3A) and GRP75 (Fig. 3B) was significantly higher than lean placenta. On the other hand, lower protein expression of ANXA5 (Fig. 4A), ATPB (Fig. 4B), FTL (Fig. 4C) and VIME (Fig. 4E) was observed in placentas obtained from obese women compared to placentas obtained from lean women. Given that ferritin consists of both a heavy and a light chain, we also chose to analyse FTH. As shown in Fig. 4D, FTH protein expression was also significantly lower in placenta obtained from obese women. For BASP1 and HNRPC, we could not find suitable antibodies, thus qRT-PCR was used to confirm the 2D-DIGE data. As shown in Fig. 5, *BASP1* mRNA expression was significantly lower in placentas obtained from obese women. Although the gene expression of *HNRPC* was lower in obese placentas, this just failed to reach significance ($P=0·07$).

Localisation of A1AT, FTL and FTH in human placenta

Immunohistochemistry was used to determine the cellular localisation of A1AT, FTL, FTH, ANXA5, ATPB, GRP75 and VIME in human placenta. Placenta exhibited extensive cytoplasmic A1AT staining, which was mainly localised to the syncytiotrophoblast layer and endothelial cells (Fig. 6A). Some A1AT staining was also observed within the villous stoma. FTL (Fig. 6B) and FTH (Fig. 6C) staining was predominantly found

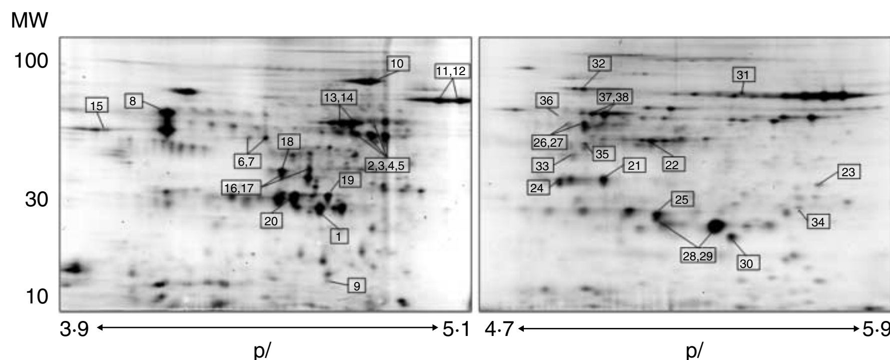

Figure 2 Numbered spots indicate proteins identified by MALDI-TOF MS. See Table 2 for a detailed listing of all proteins identified.

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Table 2 Human placental proteins identified by 2D-DIGE and by MALDI-TOF MS

Spot no.	**Protein name** (abbreviation)	Accession no.	Mascot score	MS coverage (%)	MW (kDa)	pI value	No. of matched peptides	Average Lean	Average Obese	Ratio
6	Brain acid soluble protein 1 (BASP1)	P80723	130	56	22·7	4·5	8	18·6	11·9	−1·56
7	BASP1	P80723	152	75	22·7	4·5	11	25·3	14·2	−1·78
30	Ferritin light chain (FTL)	P02792	109	46	20·1	5·4	8	11·7	6·6	−1·76
28	Chorionic somatomammotropin hormone (CSH)	P01243	150	50	25·3	5·2	14	96·0	55·8	−1·72
33	Heterogeneous nuclear ribonucleoprotein C (HNRPC)	P07910	55	20	33·7	4·8	6	4·7	2·7	−1·71
24	Annexin A5 (ANXA5)	P08758	235	63	36·0	4·8	19	5·8	3·4	−1·70
37	Vimentin (VIME)	P08670	384	65	53·7	4·9	32	12·2	7·8	−1·56
38	VIME	P08670	192	44	53·7	4·9	19	27·6	17·1	−1·64
26	ATP synthase subunit beta, mitochondria (ATPB)	P06576	230	44	56·5	5·1	22	9·1	5·8	−1·56
9	Protein dpy-30 homolog (DPY30)	Q9C005	59	34	11·2	4·7	4	7·2	5·0	−1·45
34	Heat shock protein beta-1 (HSPB1)	P04792	76	30	22·8	6·0	5	1·2	0·9	−1·31
32	78 kDa glucose-regulated protein (GRP78)	P11021	306	49	72·4	4·9	29	7·0	5·8	−1·20
27	ATPB	P06576	215	39	56·5	5·1	18	3·7	3·1	−1·20
23	ANXA4	P09525	147	41	36·1	5·8	12	2·5	2·2	−1·16
1	14-3-3 protein zeta/delta (1433Z)	P63104	207	52	27·9	4·6	13	112·3	104·0	−1·08
21	Breast carcinoma-amplified sequence 4 (BCAS4)	Q8TDM0	56	26	23·0	5·5	5	19·3	18·2	−1·06
29	CSH	P01243	103	38	25·3	5·2	10	8·6	8·2	−1·06
25	Apolipoprotein A-I (APOA1)	P02647	178	66	30·8	5·5	18	13·3	14·1	1·06
20	Tropomyosin alpha-4 chain (TPM4)	P67936	196	56	28·6	4·5	19	91·8	97·8	1·06
19	TPM3	P06753	121	30	32·9	4·5	13	50·0	53·8	1·08
35	Keratin, type I cytoskeletal 19 (K1C19)	P08727	448	81	44·1	4·9	34	2·3	2·5	1·09
36	Tubulin beta chain (TBB5)	P07437	137	27	50·1	4·6	10	0·6	0·6	1·10
11	GRP78	P11021	309	48	72·4	4·9	28	69·8	78·9	1·13
12	GRP78	P11021	319	52	72·4	4·9	32	86·0	97·6	1·13
8	Calreticulin (CALR)	P27797	167	33	48·3	4·1	14	64·1	81·6	1·27
22	Actin, cytoplasmic 1 (ACTB)	P60709	135	42	42·1	5·2	15	11·3	14·8	1·31
13	Protein disulphide-isomerase (PDIA1)	P07237	228	46	57·5	4·6	21	57·4	75·6	1·32
15	Polymerase I and transcript release factor (PTRF)	Q6NZI2	76	23	43·5	5·4	7	4·6	6·2	1·33
17	Tropomyosin alpha-1 chain (TMP1)	P09493	112	24	32·7	4·5	10	15·7	21·1	1·34
14	Protein disulphide-isomerase (PDIA1)	P07237	248	42	57·5	4·6	23	73·1	107·3	1·47
18	Tropomyosin beta chain (TMP2)	P07951	121	33	32·9	4·5	13	25·5	37·5	1·47
16	Tropomyosin alpha-1 chain (TMP1)	P09493	77	21	32·7	4·5	8	6·8	10·1	1·48
10	Endoplasmin (ENPL)	P14625	107	17	92·7	4·6	11	51·6	76·5	1·48
2	Alpha-1-antitrypsin (A1AT)	P01009	77	19	46·9	5·3	7	10·3	15·7	1·53
31	Stress-70 protein, mitochondrial (GRP75)	P38646	75	12	73·9	5·8	7	4·3	6·6	1·55
5	A1AT	P01009	103	34	46·9	5·3	10	19·6	48·0	2·44
4	A1AT	P01009	122	28	46·9	5·3	10	17·5	47·5	2·72
3	A1AT	P01009	103	25	46·9	5·3	9	12·2	48·8	4·01

within the villous stroma, although some staining was also observed in the endothelial cells and the syncytiotrophoblasts. For ANXA5 (Fig. 6D) and ATP5B (Fig. 6E), staining was observed in the syncytiotrophoblast layer. GRP75 was localised to the syncytiotrophoblast layer and the endothelial cells (Fig. 6F), and VIME expression was restricted to the villous stroma of the placenta (Fig. 6G). No staining was present in the negative controls (Fig. 6H).

Discussion

In this study, we have used 2D-DIGE to determine the effect of pre-existing maternal obesity on the protein profile of human placenta. Using MALDI-TOF MS, we were able to successfully identify 40 protein spots, which corresponded to 29 distinct proteins. Maternal obesity was associated with an up-regulation of three proteins; A1AT, serum albumin (ALBU) and GRP75. On the

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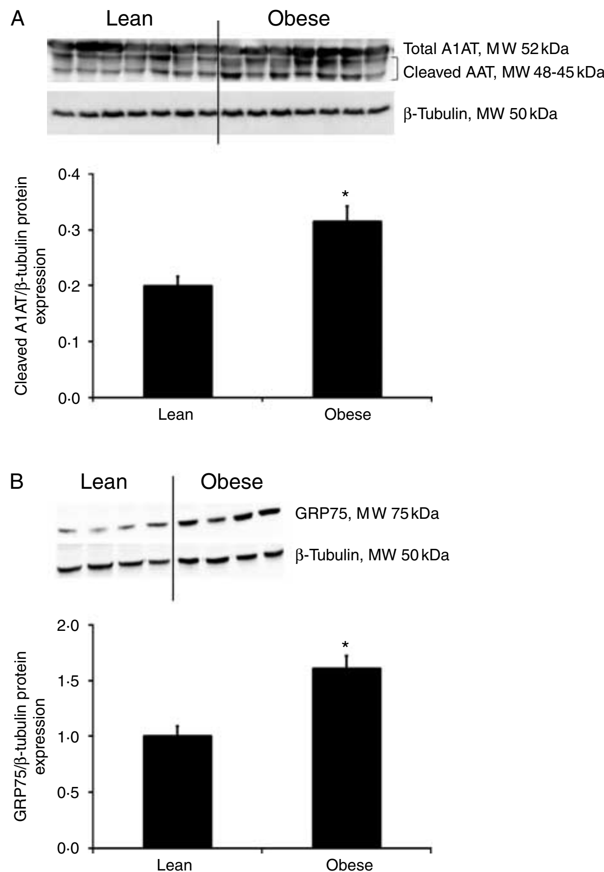

Figure 3 Maternal obesity up-regulates (A) A1AT and (B) GRP75 protein expression in human placenta. Data represent the mean ± S.E.M. (n=10 per group). *P<0·05 vs lean placenta. A western blot images is shown demonstrating the data for eight patients (four lean and four obese).

other hand, a total of six proteins were found to be down-regulated in the obese placenta. These were ANXA5, ATPB, BASP1, FTL, HNRPC and VIME. Western blot analysis and qRT-PCR analyses of proteins confirmed the 2D-DIGE data. Immunohistochemistry was also used to determine the localisation of protein in human placenta. The roles of these proteins in the regulation of placental function, and how they may influence fetus, are discussed below.

Inflammation

Exposure of the fetus to an intrauterine inflammatory environment may have short and long-term consequences, including developmental programming of obesity (Taylor & Poston 2007). In this study, we found evidence of increased inflammation in the placenta. This is consistent with previous studies demonstrating increased accumulation of a heterogeneous macrophage population and pro-inflammatory mediators in obese placentas (Challier *et al.* 2008).

A1AT is a 52 kDa protease inhibitor belonging to the serpin superfamily (Gettins 2002). It protects tissues from enzymes of inflammatory cells, especially neutrophil elastase, and as such its circulating levels rise upon acute inflammation. However, inactivation of A1AT is associated with cleavage of A1AT (∼4000 kDa lower). In this study, 2D-DIGE revealed a significant increase in cleaved A1AT in obese placenta, which was confirmed by western blotting. In previous studies, term placental syncytiotrophoblast and Hofbauer cells were positively stained for A1AT (Castellucci *et al.* 1994). Similarly, in this study, A1AT was localised to the syncytiotrophoblasts and the endothelial cells. Pro-inflammatory cytokines TNFα and interleukin 6 induce A1AT in human amnion (Izumi-Yoneda *et al.* 2009) and A1AT enhances the magnitude of LPS-induced specific cytokine/chemokine production (Subramaniyam *et al.* 2010) via the transcription factors NF-κB and AP-1 (Dichtl *et al.* 2000), which may play an important role in amplification of acute-phase inflammatory reactions. ANXA5 has anti-inflammatory, anti-thrombotic and anti-apoptotic properties (Leon *et al.* 2006, Ewing *et al.* 2011). It has previously been detected on trophoblasts in the placenta (Shu *et al.* 2000) and lower ANXA5 expression has been observed in placentas from pregnancies complicated with pre-eclampsia (Shu *et al.* 2000) and fetal growth restriction (Sifakis *et al.* 2010). Collectively, increased cleaved A1AT and lower ANXA5 expression in obese placentas is suggestive of increased inflammation in the placenta.

Oxidative stress

Oxidative stress disturbs placental function leading to perpetrations in fetal growth and development (Lappas *et al.* 2011). Animal models have been very useful to demonstrate that increases in reactive oxygen species (ROS), together with increases in reactive nitrogen species, are clearly related to the induction of malformations in the fetus (Lappas *et al.* 2011). In this study, we found increased expression of proteins that involved in the generation of ROS as well as increased expression of proteins with antioxidant properties.

Ferritin consists of both a light and a heavy chain (FTL and FTH respectively), which share about 50% sequence homology (Theil 1987). In this study, 2D-DIGE revealed a decrease in FTL expression in obese placenta, which was confirmed by immunoblotting. In addition, western blotting also revealed a significant decrease in FTH protein expression. The main function of ferritin is to keep iron in a soluble and non-toxic form and to transport it to areas where it is required (MacKenzie *et al.* 2008). Free iron, via the Fenton reaction, is involved in the formation of the highly damaging ROS hydroxyl radical (OH•; Bacic *et al.* 2008). FTL and FTH were mainly found in the villous

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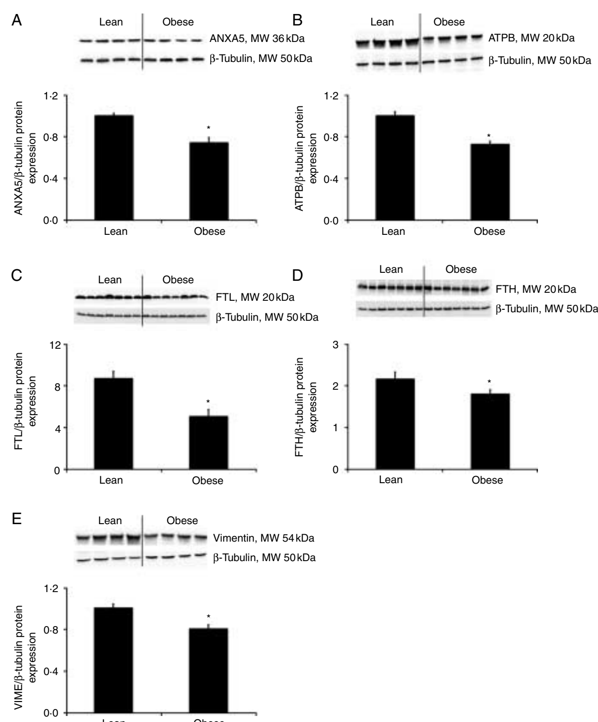

Figure 4 Maternal obesity down-regulates (A) ANXA5, (B) ATPB, (C) FTL, (D) FTH and (E) VIME protein expression in human placenta. Data represent the mean±S.E.M. (n=10 per group). *P<0·05 vs lean placenta. A western blot images is shown demonstrating the data for eight patients (four lean and four obese) or 14 patients (seven lean and seven obese).

stroma of the placenta suggesting an important role in the storage of iron in this tissue. The under-expression of ferritin in the placenta may increase free iron leading to increased oxidative stress. In support, studies have shown that there is increased oxidative stress in placentas obtained from obese women (Jarvie *et al.* 2010). Decreased ferritin expression in the placenta may also affect the rates of iron uptake from the maternal circulation and transfer to the fetal circulation. In support, recent studies have reported evidence of impaired iron status in newborns of women who were obese (Roy *et al.* 2009). Similarly, iron deficiency has also been reported in infants born to iron-sufficient diabetic mothers (Siddappa *et al.* 2004). Of clinical importance, iron deficiency in infancy is associated with impaired brain development (Lozoff *et al.* 1991, Siddappa *et al.* 2004).

ATP synthase is the universal enzyme that synthesises ATP from ADP and phosphate using the energy stored in a transmembrane ion gradient, with the mitochondrial oxidative phosphorylation machinery playing a crucial role in energy production, generation of ROS and apoptosis. Reduced content and functional capacity of mitochondria are involved in a wide range of human pathologies, including obesity and diabetes (Hojlund *et al.* 2003, Ritov *et al.* 2005). The decrease in ATPB expression in obese placentas may lead to mitochondrial dysfunction, leading to an accumulation of lipid metabolites (Vankoningsloo *et al.* 2006, Mailloux *et al.* 2007). An accumulation of lipid metabolites could, in turn, partially explain the insulin signalling defects reported in placentas from obese pregnant women (Colomiere *et al.* 2009). Of note, ATPB is also lower in syncytiotrophoblasts of pre-eclamptic placentas (Hache *et al.* 2011).

Heat shock proteins act as chaperones, thus having pivotal roles in the cellular stress responses (Prohaszka & Fust 2004). Of note, GRP75 protein, which is primarily localised to the mitochondria, is triggered by oxidative injury (Wadhwa *et al.* 2002). Increased GRP75 in obese placentas may be part of a stress-adaptive response that may be critical for the protection against oxidative damage, such as that which may be induced by low FTL and FTH or ATPB observed in this study.

Proliferation and differentiation

Changes in placental structure and thus function may adversely affect fetal development. We are not aware of any detailed studies that have examined the effect of maternal obesity on placental structure. However, in this study, we found decreased expression of proteins involved in cell integrity and structure.

BASP1 and VIME are proteins implicated in the maintenance of the cell integrity and the stabilisation of the cytoskeletal interactions (Goldman *et al.* 1996, Korshunova *et al.* 2008); as such, they play roles in adhesion, migration, survival and cell signalling. HNRPC is a nuclear pre-mRNA binding protein that has also been implicated in cell proliferation (Kim *et al.* 2003, Schepens *et al.* 2007) and DPY30 is a protein involved in differentiation (Jiang *et al.* 2011). All these

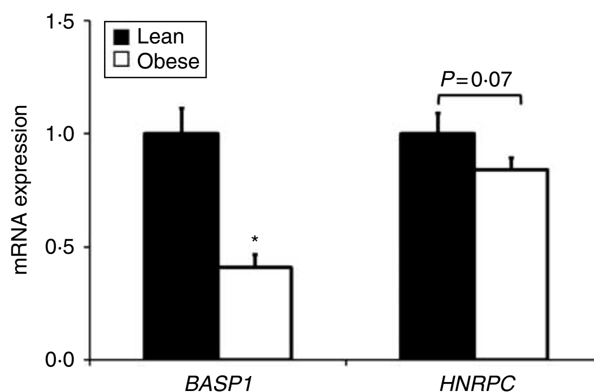

Figure 5 Maternal obesity down-regulates *BASP1* and *HNRPC* mRNA expression in human placenta. Data represent the mean±S.E.M. (n=10 per group). *P<0·05 vs lean placenta.

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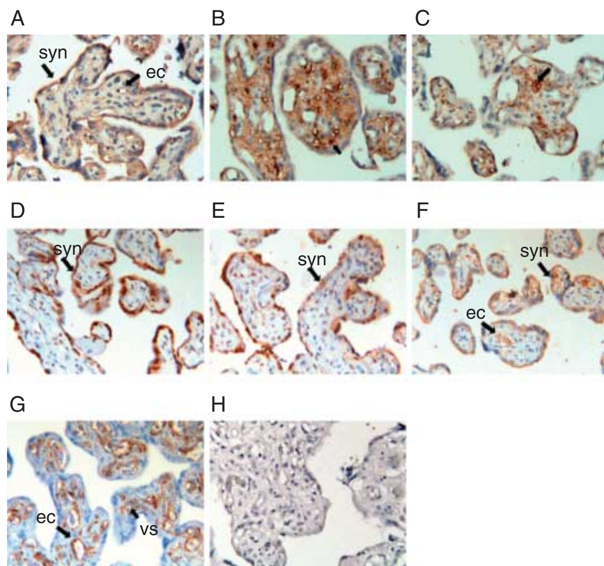

Figure 6 Immunohistochemical localisation of protein in human placenta. (A) A1AT staining was mainly contained in syncytiotrophoblasts (sy) and endothelial cells (ec). Some A1AT staining was also detected in the villous stoma (vs). (B) FTL and (C) FTH were detected mainly in the villous stroma (vs), with some staining detected in the endothelial cells (ec). No FTL staining was detected in the syncytiotrophoblasts (sy). (D) ANXA5 and (E) ATP5B staining was observed in the syncytiotrophoblast layer. (F) GRP75 was localised to the syncytiotrophoblast layer and the endothelial cells. (G) VIME expression was restricted to the villous stroma. (H) No specific staining is seen in the negative control for placenta. Magnification 250×.

proteins were down-regulated in the obese placenta, which is in keeping with recent studies suggesting that placental proliferation may be reduced in relation to increasing maternal early pregnancy BMI (Higgins *et al.* 2010).

Study limitations

There are a few limitations to this study. The initial 2D-DIGE data was not stratified according to gender, although equal numbers of males and females were included. However, when the western blotting data was stratified according to gender, there was no difference in expression between males and females (data not shown).

Concluding comments

In this study, we provide evidence demonstrating that maternal obesity at conception affects the protein profile of human placenta, which may have implications for fetal growth and development. Indeed, obesity reduces oxygen supply to unborn baby (El Baky *et al.* 2010) and neural tube defects and other developmental anomalies are more common in infants born to obese women (Stothard *et al.* 2009). Further to this, infants born to obese mothers have increased risks of developing the metabolic syndrome later in life (Boney *et al.* 2005, Taylor & Poston 2007). By further understanding the role of maternal diet and body composition on placental function, we may be able to prevent the intergeneration transmission of disease.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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Author contribution statement

M L conceived the idea, designed the study, analysed the data and wrote the manuscript. K O, G B, M J B and C R performed the 2D-DIGE experiments. G E R reviewed the manuscript. M P assisted in patient analysis.

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