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Maternal protein restriction with or without folic acid supplementation during pregnancy alters the hepatic transcriptome in adult male rats

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Feeding pregnant rats a protein-restricted (PR) diet induces altered expression of candidate genes in the liver of the adult offspring, which can be prevented by supplementation of the PR diet with folic acid (PRF). We investigated the effect of maternal nutrition during pregnancy on the liver transcriptome in their adult male offspring. Pregnant rats were fed control, PR or PRF diets. Male offspring were killed on day 84. The liver transcriptome was analysed by microarray (six livers per maternal dietary group) followed by *post hoc* analysis of relative mRNA levels and gene ontology. These results were confirmed for selected genes by real-time RT-PCR. There were 311 genes that differed significantly ($\geq$1·5-fold change; $P<0{\cdot}05$) between PR offspring (222 increased) and control offspring, while 191 genes differed significantly between PRF offspring (forty-five increased) compared with offspring of control dams. There were sixteen genes that were significantly altered in both PR and PRF offspring compared with controls. Ion transport, developmental process, and response to reactive oxygen species (RROS) and steroid hormone response (SHR) ontologies were altered in PR offspring. Folic acid supplementation prevented changes within RROS and SHR response pathways, but not in ion transport or developmental process. There was no effect of maternal PR on mRNA expression of imprinted genes. Insulin 1 and Pleckstrin homology-like domain family A member 2 were increased significantly in PRF compared with PR offspring. The present findings show that the pattern of induced changes in the adult liver transcriptome were dependent on maternal protein and folic acid intakes during pregnancy.

Transcriptome: Maternal diet: Microarray analysis: Protein restriction: Folate

Environmental cues acting through developmental plasticity induce altered expression of the genome which, in turn, changes the phenotype of the offspring[1]. In human subjects a poor *in utero* environment is associated with reduced prenatal growth and increased risk of chronic diseases including cardiometabolic disease in later life[2]. These findings have been replicated in animal models where restricted nutrition during pregnancy induces dyslipidaemia, obesity, hypertension, hyperinsulinaemia and hyperleptinaemia in the offspring[3,4].

The mechanism by which cues about nutrient availability in the prenatal environment are transmitted to the fetus and the process by which different phenotypes are induced are poorly understood. However, induction of an altered phenotype in the offspring that persists throughout the lifespan implies stable changes to gene transcription which then result in altered activities of metabolic pathways and homeostatic control processes[5]. A number of studies in animal models have demonstrated that poor maternal nutrition during pregnancy does indeed induce persistent changes in gene expression in the offspring. Feeding pregnant rats a protein-restricted (PR) diet during pregnancy induces increased expression of the glucocorticoid receptor (GR) in the liver, lung, kidney and brain of the offspring during fetal, neonatal and adult life[6–8] which can be transmitted, at least in liver, to a second generation[9]. In the liver, increased GR expression is associated with increased expression of its target gene phosphoenolpyruvate carboxykinase[7,8] and up-regulation of gluconeogenesis[10]. Expression of PPAR-α is also increased in the liver of offspring born to dams fed a PR diet during pregnancy and is accompanied by the increased expression of its target gene acyl-CoA oxidase which is rate limiting in peroxisomal β-oxidation[7]. However, increasing the folic acid content of the maternal PR diet prevented altered expression of PPARα and GR[7].

Abbreviations: GR, glucocorticoid receptor; *Ins1*, insulin 1; *Phlda2*, Pleckstrin homology-like domain family A member 2; PR, protein restricted; PRF, protein-restricted diet with folic acid.

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In contrast, 70 % global undernutrition reduced hepatic PPARα and GR expression in the liver of the adult offspring[11], which suggests that different nutritional exposures during pregnancy induce different phenotypes in the offspring. This is consistent with the hypothesis that the fetus adapts to environmental cues in a manner which predicts the future environment, thus potentially conferring a Darwinian fitness advantage[12]. Other examples of persistent changes in gene expression induced by poor maternal diet during pregnancy include 11β-hydroxysteroid dehydrogenase-2, glucokinase, fatty acid synthase, acetyl-CoA carboxylase, angiotensin 1b and 2 receptors and carnitine palmitoyl transferase-1[6,13–15]. For at least some of the genes which show altered mRNA expression in response to poor prenatal nutrition, altered transcriptional activity has been shown to be associated with changes in their epigenetic regulation by DNA methylation and/or histone modifications[7,8,14,16], although there are exceptions such as glucokinase[13].

To date, studies on long-term changes in gene expression induced by maternal protein restriction have used a candidate gene approach. While this has produced useful findings, this approach does not allow assessment of the specificity of the changes induced by maternal dietary constraint in the transcriptome of the offspring or which gene networks are affected. We have used a genome-wide microarray to determine the specificity of the effect of differences in maternal nutrition during pregnancy on gene expression in their offspring. We investigated the effect of feeding pregnant rats a PR diet or the PR diet supplemented with folic acid on hepatic gene expression in their adult male offspring.

Materials and methods

Animals and tissues

All animal procedures were carried out in accordance with the Home Office Animals (Scientific Procedures) Act (1986). The tissues reported here were from the same animals as described previously[17]. Virgin female Wistar rats (about 250 g) (six rats per diet) were mated and fed one of three diets from conception until delivery (each group contained six females): control, a PR diet, or the PR diet supplemented with additional folic acid (PRF) (Table 1). Diets were manufactured by Special Diets Services (Witham, Essex, UK). Dams were allowed to deliver spontaneously at about 21 d and litters were reduced to eight rats, equal males and females, within 24 h after birth. Dams were fed the standard semi-purified American Institute of Nutrition (AIN)-76G during lactation. Offspring were weaned at 28 d onto a nutritionally adequate diet and were killed by asphyxiation with CO_2 after food had been withdrawn for 6 h on postnatal day 84. Livers were removed immediately, frozen in liquid N_2 and stored at −80°C. Six livers from male offspring, one per litter, were selected in each dietary group for analysis. One additional liver from a male offspring of a control dam was collected as the reference for the microarray analysis.

RNA isolation and measurement of the expression of the liver transcription by Agilent oligonucleotide array hybridisation

RNA was extracted from each liver using the SV Total RNA Extraction Kit (Promega, Madison, WI, USA). RNA was quantified by absorbance at 260 nm and the integrity of the 28s and 18s ribosomal RNA was verified by agarose gel electrophoresis. In all cases the absorbance ratio at 260 and 280 nm was greater than 2. Microarray hybridisation and analysis was carried out by Oxford Gene Technology (OGT, Oxford, UK) in accordance with the company's quality-control procedures using standard protocols for labelling, hybridisation and washing (Two Colour Microarray Based Gene expression analysis version; Agilent Technologies, Inc., Palo Alto, CA, USA). Six microarrays were performed per dietary group. Briefly, total RNA (300 ng) extracted from the liver of each rat was transcribed into cDNA. After denaturation of the RT enzyme, samples were then transcribed

Table 1. Composition of diets fed to pregnant and lactating dams, and to the offspring after weaning

	Diet fed during pregnancy				
	Control	PR	PRF	Diet fed during lactation: AIN-76A	Post-weaning diet
Casein (g/kg)	180	90	90	200	180
Folic acid (mg/kg)	1	1	5	2	1
Maize starch (g/kg)	425	485	485	150	455
Sucrose (g/kg)	213	243	243	500	243
Choline (g/kg)	2	2	2	2	2
Methionine (g/kg)	5	5	5	3	5
Vitamin mix (g/kg)*	5	5	5	5	5
Mineral mix (g/kg)†	20	20	20	20	20
Cellulose (g/kg)	50	50	50	50	50
Maize oil (g/kg)	0	0	0	50	0
Soyabean oil (g/kg)	100	100	100	0	4
Lard (g/kg)	0	0	0	0	36
Total metabolisable energy (MJ/kg)	17·3	17·5	17·5	15·5	16·1

PR, protein restricted; PRF, protein restricted with folic acid; AIN, American Institute of Nutrition.

* Vitamin mix: thiamin hydrochloride, 2·4 mg/kg; riboflavin, 2·4 mg/kg; pyridoxine hydrochloride, 2·8 mg/kg; nicotinic acid, 12·0 mg/kg; D-calcium pantothenate, 6·4 mg/kg; biotin, 0·01 mg/kg; cyanocobalamin, 0·003 mg/kg; retinyl palmitate, 6·4 mg/kg; DL-α-tocopheryl acetate, 79·9 mg/kg; cholecalciferol, 1·0 g/kg; menaquinone, 0·02 mg/kg.

† Mineral mix: calcium phosphate dibasic, 11·3 g/kg; sodium chloride, 1·7 g/kg; potassium citrate monohydrate, 5·0 g/kg; potassium sulfate, 1·2 g/kg; magnesium sulfate, 0·5 g/kg; magnesium carbonate, 0·1 g/kg; ferric citrate, 0·1 g/kg; zinc carbonate, 36·2 mg/kg; cupric carbonate, 6·8 mg/kg; potassium iodate, 0·2 mg/kg; sodium selenite, 0·2 mg/kg; chromium potassium sulfate, 12·5 mg/kg.

into cRNA and labelled with the fluorescent dye Cy (test sample Cy3, reference sample Cy5). Each test sample together with a reference sample (prepared from an additional control animal) was hybridised to an Agilent 014879 whole rat genome array (4 × 44K) G4131F. This array contains 45 018 features with 41 012 unique probes. Microarray slides were scanned at 5 μm resolution using the extended dynamic range (Hi 100 %, Low 10 %). The slides were then feature extracted using Agilent feature extraction software 9.5.3.1. All arrays were uploaded into Genespring GX V 7.3 (Silicon Genetics Inc., Palo Alto, CA, USA) for data normalisation, quality control and first-pass analysis. All arrays were normalised per spot and per chip using an intensity-dependent normalisation (Lowess normalisation) using Genespring (http://stat-www.berkeley.edu/users/terry/zarray/Html/normspie.html). This is a within-slide normalisation that adjusts for intensity-dependent variation due to dye properties. The expression ratios were calculated for each probe by dividing the Cy3 processed signal by Cy5 processed signal. The identification of the genes showing increased or decreased expression was performed using GeneSifter™ software (www.genesifter.net; VizX Labs LLC, Seattle, WA, USA) by averaging the expression ratios from the six samples and running a Wilcoxon rank sum test (significance set at $P<0{\cdot}05$) with a Benjamini and Hochberg false discovery rate correction.

Ontology report

Gene ontology reports (Biological Process and Molecular Function), based on the Gene Ontology Consortium (http://www.geneontology.org/GO.doc.html)[18], including *z*-score analyses, were generated using GeneSifter™ software. A *z*-score is a statistical rating of the relative expression of gene ontologies and indicates how much each ontology is over-represented (positive *z*-score) or under-represented (negative *z*-score) in a gene list. Positive *z*-scores (>2) reflect gene ontology categories with a greater number of genes meeting the criterion than is expected by chance, while negative *z*-scores (>-2) identify gene ontology categories with a lower number of genes meeting the criterion by chance.

Real-time RT-PCR

To verify the changes in gene expression detected by microarray analysis, we analysed the expression of six genes – TNFα, inducible NO synthase, c-Fos, NFκB, uncoupling protein (UCP)-3 and thyroid receptor (TR)-1β – which differed in microarray analysis between control and PR offspring. Liver samples (approximately 100 mg) were pulverised under liquid N_2 and the resulting powder was divided and used to prepare mRNA[8]. mRNA expression was measured by real-time RT-PCR[8]. Briefly, total RNA was isolated from cells with TRIzol reagent (Invitrogen, Paisley, UK), and 1 μg was used as a template to prepare cDNA with 100 units of Moloney murine leukaemia virus RT. cDNA was amplified with real-time RT-PCR primers specific for the products of the TNFα, inducible NO synthase, c-Fos, NFκB, uncoupling protein 3 and TR-1β (Table 2). The reaction was performed in a total volume of 25 μl with SYBR Green Jumpstart Ready Mix (Sigma, Poole, Dorset, UK).

Statistical methods

From the microarray data the identification of the genes showing increased or decreased expression was performed using GeneSifter™ software (www.genesifter.net; VizX Labs LLC) by averaging the expression ratios from six samples and running a Wilcoxon rank sum test (significance set at $P<0{\cdot}05$) with a Benjamini and Hochberg false discovery rate correction. For RT-PCR verification of the expression array data statistical significance was determined using a one-way ANOVA with a Bonferroni's *post hoc* analysis.

Results

Effects of maternal dietary exposure on the hepatic transcriptome of the offspring

The mRNA expression of 311 genes differed by ≥ 1·5-fold ($P<0{\cdot}05$) between PR and control offspring. The expression of 222 genes was increased in the PR offspring compared with control offspring, while eighty-nine genes were down-regulated (Supplemental Table 1). The twenty genes which showed the greatest difference in expression between PR offspring and controls are shown in Table 3.

The mRNA expression of 191 genes was significantly different (≥ 1·5-fold; $P<0{\cdot}05$) between PRF and control offspring. Of these, forty-five genes showed higher expression and 146 genes showed lower expression compared with controls (Table 4 and Supplemental Table 2). Only sixteen of the 311 genes which differed significantly between PR and control offspring differed significantly between PRF and control offspring (Table 5).

There were 480 genes that differed significantly (≥ 1·5-fold; $P<0{\cdot}05$) between PRF and PR offspring. Of these, 463 genes showed greater expression and 230 genes showed lower expression in PRF than PR offspring (Table 6 and

Table 2. Primers for analysis of mRNA expression by real-time RT-PCR

Gene	Forward primer	Reverse primer
c-Fos	5′-GGCAAAGTAGAGCAGCTATCTCCT-3′	5′-TCAGCTCCCTCCTCCGATTC-3′
iNOS	5′-CAGCGGGATGACTTTCCAAG-3′	5′-AGGCAAGATTTGGACCTGCA-3′
TNFα	QuantiTect Primer Assay QT00178717	
NFκB	QuantiTect Primer Assay QT01577975	
TR-1β	QuantiTect Primer Assay QT00193690	
UCP3	5′-CTGGACTCTCACCTGTTCAC-3′	5′-GCCACCATCCTCAGCATAC-3′

iNOS, inducible NO synthase; TR-1β, thyroid receptor 1β; UCP3, uncoupling protein 3.

Table 3. Genes with higher or lower expression (top twenty) in liver of the offspring of dams fed a protein-restricted (PR) diet compared with controls*

Gene name	Gene identifier	Ratio	Direction
UDP glucuronosyltransferase precursor	J02589	26·94	Down
Interferon-inducible GTPase	NM_001024884	20·71	Up
UDP glycosyltransferase 2 family, polypeptide B	NM_031533	17·28	Down
Cyclin B3	XM_228779	4·53	Down
Olfactory receptor 1326	NM_001000474	3·89	Down
Unknown	TC558741	3·64	Down
Unknown	TC563179	3·61	Down
Unknown	XM_223906	3·50	Up
Nebulin	XM_229925	3·38	Up
Periostin, osteoblast specific factor	XM_342245	3·37	Up
Solute carrier family 13 (sodium/sulfate symporters), member 1	NM_031651	3·33	Down
Centaurin, α1	NM_133567	3·29	Up
Signal peptide, CUB domain, EGF-like 3	XM_228030	3·25	Down
Gap junction membrane channel protein α7	XM_001081521	3·20	Up
3-Oxoacid CoA transferase 1	XM_001073523	3·14	Up
Fc receptor, IgE, low affinity II, α polypeptide	NM_133550	3·11	Up
Unknown	AW142860	3·08	Down
Mucin 13	AI044457	3·02	Down
Adenylate kinase 5 isoform 1	XM_001080050	3·01	Up
Chemokine (C-X-C motif) ligand 14	NM_001013137	3·00	Down

* The ratio is calculated as the average expression ratio between the offspring of dams fed a PR (n 6) or a control (n 6) diet. Direction denotes whether the change in expression between PR and control offspring is increased or decreased. All genes showed a significant change in expression between PR and control offspring ($P<0·05$). P values were calculated using the Wilcoxon ranking feature with the Benjamini and Hochberg correction.

Supplemental Table 3). Of the 311 genes whose expression was significantly altered in PR compared with control offspring, ninety-two were also significantly altered in PRF compared with control offspring (Supplemental Table 4).

Pathway analysis by gene ontology

Data were categorised on two independent gene ontology terms, Biological Process and Molecular Function. In the Biological Processes ontology, the major pathways affected in the PR offspring compared with control offspring (z-score greater than 2; $P>0·05$) among the up-regulated genes were ion transport, developmental process and response to stress, in particular response to oxidative stress (Fig. 1). For the down-regulated genes, response to steroid hormone stimulus was significantly over-represented. In PRF *v.* control offspring, the pathways significantly over-represented were ion transport, developmental process, fatty acid and steroid metabolic process amongst the up-regulated genes. No pathways were significantly affected amongst the down-regulated genes.

Table 4. Genes that were up- or down-regulated (top twenty) in the liver of the offspring of dams fed a protein-restricted diet supplemented with folic acid (PRF) compared with controls*

Gene name	Gene identifier	Ratio	Direction
UDP glucuronosyltransferase precursor, gene	J02589	22·61	Down
UDP glycosyltransferase 2 family, polypeptide B	NM_031533	11·35	Down
Casein kinase II, α 1	BF551036	8·13	Down
Anterior pharynx defective 1a homolog	NM_001014255	5·92	Down
E74-like factor 2 (Elf2), transcript variant 2	NM_001012181	5·18	Down
Copine IV	XM_001070003	4·54	Down
RIKEN cDNA 4932415L06 gene	XM_237289	3·94	Down
Chemokine (C-C motif) ligand 11	NM_019205	3·72	Down
Class VI alcohol dehydrogenase	S79716	3·53	Down
Olfactory receptor 1337	NM_001000480	3·51	Down
Small nuclear ribonucleoprotein D3	XM_001079870	3·26	Down
Carnitine acetyltransferase	NM_001004085	3·19	Up
Growth hormone 1	NM_001034848	2·93	Down
Sema domain, transmembrane domain (TM), and cytoplasmic domain, (semaphorin) 6A	XM_341612	2·91	Down
B-box and SPRY domain containing	NM_022261	2·89	Up
Zinc finger, MYND domain containing 19	NM_198770	2·88	Down
Unknown	BF552300	2·84	Down
Unknown	BQ196294	2·79	Down
Unknown [BF550568]	BF550568	2·68	Up
Jumonji domain containing 4	XM_220561	2·58	Down

* The ratio is calculated as the average ratio in expression levels between the offspring of dams fed a PRF diet (n 6) and controls (n 6). Direction denotes whether the change in expression between PRF and control offspring is increased or decreased. All genes showed a significant change in expression between PRF and control offspring ($P<0·05$). P values were calculated using the Wilcoxon ranking feature with the Benjamini and Hochberg correction.

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Table 5. Genes significantly altered in the liver of the offspring of dams fed a protein-restricted (PR) diet and protein-restricted diet supplemented with folic acid (PRF) compared with a control diet*

Gene name	Gene identifier	PR *v.* control ratio	Direction	PRF *v.* control ratio	Direction
UDP glucuronosyltransferase precursor	J02589	26·94	Down	22·61	Down
UDP glycosyltransferase 2 family, polypeptide B	NM_031533	17·28	Down	11·35	Down
Jumonji domain containing 4	XM_220561	1·8	Down	2·58	Down
Leucine rich repeat containing 2	XM_001056081	1·96	Down	2·52	Down
Thyroid hormone receptor β	NM_012672	2·51	Down	2·51	Down
Unknown	AW142860	3·08	Down	2·22	Down
Olfactory receptor 276	NM_001000227	1·99	Down	2·14	Down
Butyrylcholinesterase	NM_022942	1·77	Down	1·92	Down
Rous sarcoma oncogene	NM_031977	1·9	Down	1·91	Down
Unknown	DV728362	1·54	Down	1·72	Down
KIAA0157 gene product	XM_219441	1·88	Down	1·71	Down
Natural cytotoxicity triggering receptor 3	NM_181822	1·91	Down	1·68	Down
Transmembrane glycoprotein A33 antigen	XM_341149	2·37	Up	1·67	Up
Tudor domain containing 3	NM_001012043	1·61	Down	1·64	Down
Solute carrier family 4, member 4	NM_053424	1·75	Down	1·59	Down
Unknown	XM_573395	1·76	Down	1·59	Down

* The ratio is calculated as the average ratio in expression levels between either the offspring of dams fed a PR diet (n 6) and the control diet (n 6), or between offspring of dams fed a PRF diet (n 6) and the control diet (n 6). Direction indicates whether the change in expression between PR or PRF and control offspring is increased or decreased. All genes showed a significant change in expression between PR and control offspring and between PRF and control offspring ($P<0·05$). P values were calculated using the Wilcoxon ranking feature with the Benjamini and Hochberg correction.

Comparison of PRF *v.* PR offspring revealed that the major pathways affected amongst the over-expressed genes were response to steroid hormone, steroid metabolic process and fatty acid metabolic process and among the down-regulated genes response to oxidative stress.

In the Molecular Function ontology, the major categories altered among the up-regulated genes in the PR *v.* control offspring were receptor binding, tetrapyrrole binding, and cation transmembrane transporter activity and among the down-regulated genes pathways affected were UDP-glycosyltransferase activity, anion transmembrane transporter activity, growth factor activity and ATPase activity (Fig. 2). No pathways were over-represented in PRF offspring compared with controls. In PRF *v.* PR offspring, receptor binding, oxido-reductase activity, transcription factor cofactor activity were among the up-regulated genes and acetyl transferase activity and tetrapyrrole binding were among the down-regulated genes.

Imprinted genes

There was no effect of maternal protein restriction on mRNA expression of imprinted genes, although insulin-1 (*Ins1*)

Table 6. Genes that were up- or down-regulated (top twenty) in the liver of the offspring of dams fed a protein-restricted diet supplemented with folic acid (PRF) compared with offspring of dams fed a protein-restricted (PR) diet*

Gene name	Gene identifier	Ratio	Direction
RT1 class Ib	AW142896	11·86	Down
Camello-like 3	XM_575585	5·75	Down
Cyclin B3	XM_228779	4·9	Up
X-linked gene	NM_019203	4·68	Up
ATPase, H^+ transporting, V0 subunit D, isoform 2	NM_001011972	4·57	Down
Q96AL3 PHA4 protein	TC522828	4·27	Up
Unknown	TC525504	3·95	Up
Similar to Eph receptor A4	XM_244186	3·9	Up
Unknown	TC558494	3·77	Up
Copine IV	XM_001070003	3·74	Down
Phosphorylase kinase γ1	NM_031573	3·38	Down
Unknown	TC557723	3·29	Down
Chemokine (C-C motif) ligand 11	NM_019205	3·21	Down
N-acetyltransferase 8	NM_022635	3·2	Down
Unknown	TC544566	3·18	Down
Protein tyrosine phosphatase, receptor type, J	NM_017269	3·16	Up
Hypothetical protein FLJ10901	XM_213984	3·14	Up
Unknown	TC560462	3·11	Down
Rho/rac guanine nucleotide exchange factor (GEF) 18	XM_221775	3·11	Up
RNAJ3232 mRNA for MHC class II RT1-D β1 chain haplotype k	AJ003232	3·1	Up

* The ratio is calculated as the average ratio in expression levels between the offspring of PRF (n 6) and PR offspring (n 6) dams. Direction indicates whether the change in expression between PRF and PR offspring is increased or decreased. All genes showed a significant change in expression between PRF and PR offspring ($P<0·05$). P values were calculated using the Wilcoxon ranking feature with the Benjamini and Hochberg correction.

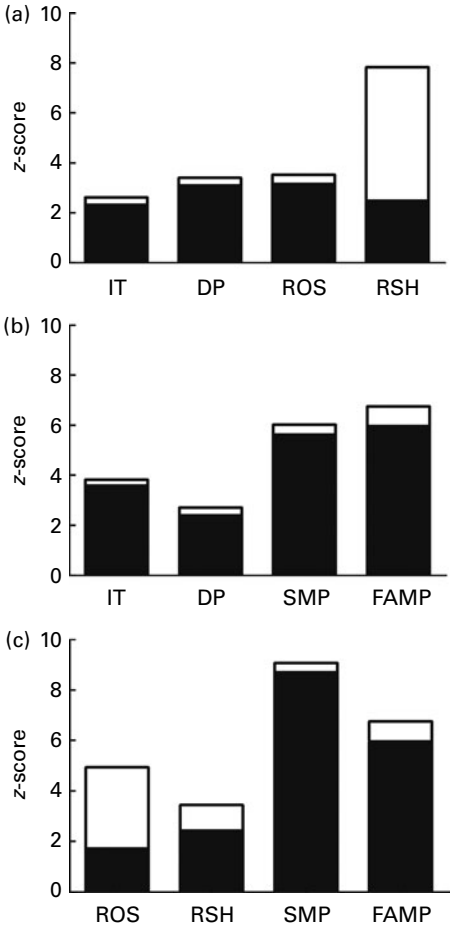

Fig. 1. Pathways over-represented in the gene ontology Biological Processes category. Values are based on six offspring in each of the maternal dietary groups: control, protein-restricted (PR) and PR supplemented with folic acid (PRF). (a) Control *v.* PR; (b) control *v.* PRF; (c) PR *v.* PRF. Pathways with *z*-scores above 2 are plotted for both up-regulated genes (■) and down-regulated (□) genes. IT, ion transport; DP, developmental processes; ROS, response to oxidative stress; RSH, response to steroid hormone; SMP, steroid metabolic processes; FAMP, fatty acid metabolic processes.

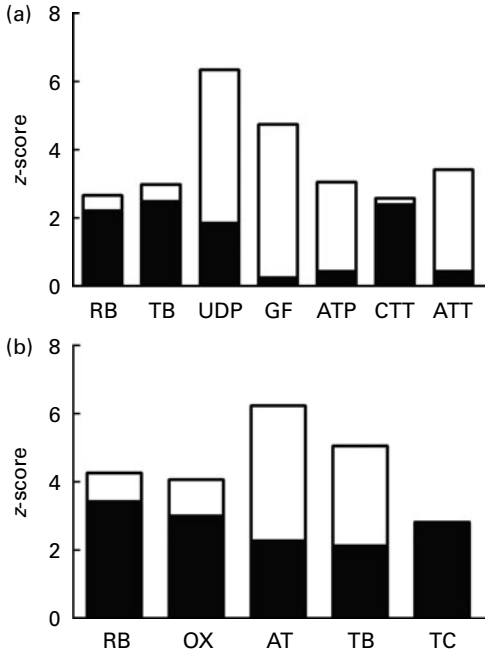

Fig. 2. Pathways over-represented in the gene ontology Molecular Function category. Values are based on six offspring in each of the maternal dietary groups: control, protein-restricted (PR) and PR supplemented with folic acid (PRF). (a) Control *v.* PR; (b) PR *v.* PRF. Pathways with *z*-scores above 2 are plotted for both up-regulated genes (■) and down-regulated (□) genes. RB, receptor binding; TB, tetrapyrrole binding; UDP, UDP-glycosyltransferase activity; GF, growth factor activity; ATP, ATPase activity; CTT, cation transporter activity; ATT, anion transporter activity; OX, oxidoreductase activity; AT, acetyl transferase activity; TC, transcription cofactor activity.

(2·2-fold increase) and Pleckstrin homology-like domain family A member 2 (*Phlda2*) (1·7-fold increase) were significantly altered in PRF compared with PR offspring (Supplemental Table 3).

Validation of array by real-time RT-PCR

The mRNA expression of TNFα, iNos, c-Fos, NFκB, uncoupling protein 3 and thyroid receptor-1β agreed with the corresponding data from the array (Fig. 3).

Discussion

Maternal dietary protein restriction during pregnancy has been reported to induce long-term changes in the expression of a number of candidate genes within the offspring[5]. However, the range of genes reported to date reflects the interests of the researchers and does not provide an indication of the proportion of the transcriptome which is affected or which gene networks and pathways show the greatest or least susceptibility. The present results show for the first time that a small proportion of genes are affected in adult liver by variation in the protein and folic acid content of the maternal diet. The microarray allows measurement of the mRNA expression of approximately 39 000 transcripts (Agilent). Based on this estimate, maternal dietary protein restriction during pregnancy induced the altered expression of approximately 1·3 % of the liver transcriptome which was reduced to 0·7 % by increasing the amount of folic acid in the maternal PR diet. These data suggest that as the changes in gene expression are limited to a relatively small subset of genes within the genome this may reflect an adaptive response induced in response to maternal protein restriction. A similar proportion of genes was reported to have significantly changed in the placenta (E17.5) of mice in response to maternal protein restriction[19]. Genes with a wide range of functions were found to be altered in PR *v.* control offspring but this is consistent with the studies to date which have shown that offspring from PR dams show a number of the metabolic and physiological alterations including increased fat deposition and altered feeding behaviour[20–22], impaired glucose homeostasis[10], vascular dysfunction[20,23], impaired immunity[24] and increased susceptibility to oxidative stress[25] and tumorigenesis[26]. For instance, although the genes showing the greatest change in expression between control and PR offspring have not previously been demonstrated to be changed in response to maternal diet, changes in their expression is consistent with some of the phenotypic changes reported

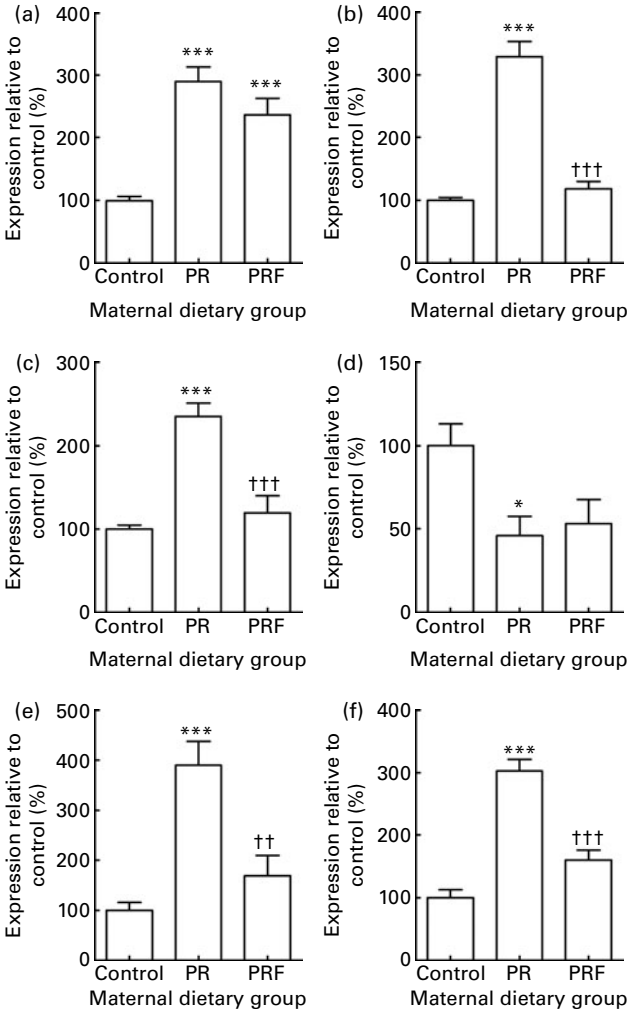

Fig. 3. mRNA expression of c-Fos (a), inducible NO synthase (iNOS) (b), TNFα (c), thyroid receptor 1β (TR-1β) (d), NFκB (e) and uncoupling protein (UCP)-3 (f) in liver from day 84 male offspring of dams fed either a control, protein-restricted (PR) or PR supplemented with folic acid (PRF) diet during pregnancy. Values are means for six offspring, with standard errors represented by vertical bars. Statistical comparisons are by one-way ANOVA with Bonferroni's *post hoc* analysis. Only statistically significant differences are shown. Mean value was significantly different from that of the control group: * $P<0{\cdot}05$, *** $P<0{\cdot}0001$. Mean value was significantly different from that of the PR group: †† $P<0{\cdot}01$, ††† $P<0{\cdot}0001$. Overall probabilities were c-FOs, $P<0{\cdot}0001$; iNOS, $P<0{\cdot}0001$; TNFα, $P<0{\cdot}0001$; TR-1β, $P=0{\cdot}022$; NFκB, $P=0{\cdot}002$; UCP3, $P<0{\cdot}0001$.

to be induced in PR offspring. UDP-glucuronosyltransferase precursor, which shows a 26·94-fold decrease in expression in PR offspring, plays a major role in conjugation and subsequent elimination of potentially toxic xenobiotics and endogenous compounds. Decreased expression and function of this enzyme have been linked to a number of human cancers[27–29]. Thus, this decrease in PR offspring may in part explain the increased susceptibility of PR offspring to carcinogenesis[26]. Interferon-γ-induced GTPase showed a 20-fold increase in expression in PR offspring. Interferon-γ GTPase is important for host immunological defence[30] which is consistent with an increased tendency towards inflammation in PR offspring[31]. However, it may be that not all of the changes in gene expression have functional consequences; some of the changes may simply reflect the process of adaptation. For instance, the olfactory receptor 1326 is up-regulated in PR offspring. Olfactory receptors are known to play roles in odour sensing[32] and in environmental sensing in non-olfactory tissues including the kidney[33]. Thus, although their role in any adaptive process in this nutritional model is unclear, they may play an as-yet uncharacterised role in regulating cell function which may contribute to the induced phenotype.

Previous studies have shown that supplementation of the maternal PR diet with folic acid or the methyl donor glycine prevents the induction of hypertension, dyslipidaemia and altered hepatic PPARα and GR expression in the offspring[7,34,35]. The present findings are in part consistent with these studies. We also compared gene expression changes in PRF offspring as well. We found that, while maternal protein restriction induced the altered expression of 1·3 % of the genome, this was reduced to 0·7 % of the genome in PRF compared with control offspring. However, in the present study only 7 % of genes which differed in expression between control and PRF offspring also differed between control and PR offspring. Within this set of genes altered in response to both maternal PR and PRF, we found that the maternal PR diet tended to induce increased gene expression while the PRF diet reversed this trend. Together, these findings indicate that although increasing the folic acid content of the maternal PR diet prevented some of the effects on the transcriptome induced by the PR diet alone, increasing the level of folic acid relative to protein did not simply correct for the PR diet. This is consistent with the finding that the maternal PRF diet decreased weight gain in the offspring, which suggests that the apparently beneficial effect of increased folic acid intake on some metabolic processes may be at the expense of others[17]. Supplementation of the diet of the offspring of dams fed control or PR diets with folic acid after weaning induced specific changes in the mRNA expression of specific genes and in fat metabolism[35]. Furthermore, while the maternal PRF diet prevented hypomethylation of specific CpG dinucleotides in the liver PPARα promoter, it also increased the methylation level of two others which suggests a shift, albeit modest, in gene control[16].

Ontology analysis showed altered maternal diet induced different changes in individual pathways in the liver of the adult offspring depending on the nature of the maternal diet. Comparison of control *v.* PR offspring showed that ion transport, developmental process, and response to steroid hormone and reactive oxygen species were the pathways significantly altered. Alterations in these pathways are consistent with previous phenotypic studies which show that ion transport[36], cell commitment in blastocysts[37], response to reactive oxygen species[25] and steroid hormones[6,38] are altered by maternal diet. Response to steroid hormone and reactive oxygen species pathways were not altered in PRF offspring, suggesting that folate supplementation corrected these changes induced by PR. However, ion transport and developmental process pathways remained altered in PRF offspring, demonstrating that folate supplementation does not reverse all gene expression changes induced by protein restriction. Interestingly we also observed that fatty acid and steroid metabolic process pathways were altered in PRF offspring but not in PR offspring, suggesting that folate

supplementation induces distinct changes in gene expression. Thus, the results of these analyses are consistent with the findings of measurements of individual genes in that although the PRF diet prevented changes in gene expression in some pathways, it either did not affect others or changed the expression of genes in pathways not affected by the PR diet. This is in contrast to previous studies which show that for a limited number of phenotypic outcomes increasing the folic acid content of the PR diet prevents the induction of an altered phenotype[17,23].

Changes in the epigenetic regulation of imprinted genes results in a range of developmental abnormalities and changes in metabolic processes in humans[39–41] and agricultural animals[42], and in some animal models of metabolic disease[43]. It has been proposed that altered regulation of imprinted genes changes the partitioning of nutrient resources and so may represent one mechanism for the induction of impaired nutrient homeostasis in the offspring by prenatal environmental constraint[44]. There were no differences in the expression in the liver of imprinted genes between control and PR offspring, although the imprinted genes *Ins1* and *Phlda2* were significantly altered in PRF compared with PR offspring. This suggests that induction of an altered phenotype in the offspring by modest variations in maternal protein restriction does not involve changes in imprinted genes. This is consistent with the differences in the patterns of disease associated with prenatal environmental constraint and those associated with impaired imprinting[39,45]. However, interestingly, folate supplementation did alter the expression of *Ins1* and *Phlda2*. Folate supplementation of patients with hyperhomocysteinaemia has also been reported to alter the methylation of imprinted genes[46], suggesting that imprinted genes may be susceptible to changes in dietary folate intake.

Together, the present findings show that the long-term regulation of the transcriptome reflects the nature of the prenatal nutritional environment. It has been suggested that phenotypes induced by variations in the intra-uterine environment may have adaptive value[12]. Whether the induced changes in this relatively small subset of genes are adaptive or not requires further experimentation, including more detailed characterisation of the induced phenotype which accompanies such alterations in mRNA levels.

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G. C. B., K. A. L., P. D. G. and M. A. H. designed the study. K. A. L., J. R., E. S. G. and J. S.-J. carried out the laboratory procedures and analysed the data. K. A. L. and G. C. B. wrote the manuscript with input from all authors.

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