

Induction of altered epigenetic regulation of the hepatic glucocorticoid receptor in the offspring of rats fed a protein-restricted diet during pregnancy suggests that reduced DNA methyltransferase-1 expression is involved in impaired DNA methylation and changes in histone modifications

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Prenatal nutritional constraint induces an altered metabolic phenotype in the offspring which in humans confers an increased risk of non-communicable disease. Feeding a protein-restricted (PR) diet to pregnant rats causes hypomethylation of specific gene promoters in the offspring and alters the phenotype. We investigated how altered epigenetic regulation of the hepatic glucocorticoid receptor (GR) 1_{10} promoter is induced in the offspring. Rats were fed a control (180 g casein/kg) or a PR (90 g casein/kg) diet throughout pregnancy, and chow during lactation. Offspring were killed at postnatal day 34 (n 5 per maternal dietary group). Methylation-sensitive PCR showed that GR 1_{10} promoter methylation was 33 % lower ($P < 0.001$) and GR expression 84 % higher ($P < 0.05$) in the PR offspring. Reverse transcription-PCR showed that DNA methyltransferase-1 (Dnmt1) expression was 17 % lower ($P < 0.05$) in PR offspring, while Dnmt3a/b and methyl binding domain protein-2 expression was not altered. Thus hypomethylation of the GR 1_{10} promoter may result from lower capacity to methylate hemimethylated DNA during mitosis. Histone modifications which facilitate transcription were increased at the GR 1_{10} promoter (147–921 %, $P < 0.001$), while those that suppress methylation were decreased (54 %, $P < 0.01$) or similar to controls. In human umbilical cord (n 15), there was a 2-fold difference between the highest and lowest level of GR1-C_{Total} promoter methylation. Dnmt1, but not Dnmt3a, expression predicted 49 % ($P = 0.003$) of the variation in GR1-C_{Total} promoter methylation. These findings suggest that induction in the offspring of altered epigenetic regulation of the hepatic GR 1_{10} promoter, and hence metabolic phenotype, may be due to reduced Dnmt1 expression.

Fetal programming: Epigenetic: Rat: Glucocorticoid receptor: Histones: Human

There is an increasing awareness that aspects of the prenatal environment such as maternal nutrition and stress levels provide cues that alter the phenotype of the fetus without overt reductions in fetal growth (Bateson *et al.* 2004). Such nutritional cues may operate within the normal range for the human population and contribute to the early origins of risk of chronic diseases such as the metabolic syndrome and CVD (Godfrey & Barker, 2001). In rats, variations in phenotype can be induced by maternal undernutrition (Langley & Jackson, 1994; Vickers *et al.* 2005) or increased intake of specific nutrients (Armitage *et al.* 2005). As in humans (Ravelli *et al.* 1998), the phenotype which is induced is

dependent upon the timing of nutrient restriction during pregnancy or lactation (Remacle *et al.* 2004).

Induced changes to the phenotype which persist throughout the life span are likely to involve stable alterations to the expression of the genome. The offspring of rats fed a diet with a moderate reduction in protein content (protein-restricted (PR) diet) during pregnancy show tissue-specific alterations in the expression of transcription factors which regulate a wide range of developmental and metabolic processes, specifically the glucocorticoid receptor (GR) (Bertram *et al.* 2001; Lillycrop *et al.* 2005) and peroxisomal proliferator-activated receptors (PPAR) (Burdge *et al.* 2004; Lillycrop

Abbreviations: ChIP, chromatin immunoprecipitation; Dnmt, DNA methyltransferase; GR, glucocorticoid receptor; HDAC, histone deacetylase; HMT, histone methyltransferase; MBD, methyl binding domain protein; MeCP2, methyl CpG-binding protein; PEPCK, phosphoenolpyruvate carboxykinase; PPAR, peroxisomal proliferator-activated receptor; PR, protein restricted; RT-PCR, reverse transcription-PCR; UC, umbilical cord.

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et al. 2005), and changes to the expression of genes associated with fatty acid metabolism (Maloney *et al.* 2003; Lillycrop *et al.* 2005) and carbohydrate homeostasis (Burns *et al.* 1997; Desai *et al.* 1997). Little is known about how information about the availability of nutrients in the extra-uterine environment is transmitted to the offspring or how different phenotypes are induced.

The methylation of CpG dinucleotides clustered at the 5' promoter regions of genes established during early life confers stable silencing of transcription and is critical for cell differentiation (Bird, 2001). Following fertilisation, maternal and paternal genomes undergo extensive demethylation followed by *de novo* methylation by the activities of DNA methyltransferases (Dnmt) 3a and 3b around the time of implantation (Bird, 2001; Reik *et al.* 2001). Patterns of DNA methylation are maintained through mitosis by Dnmt1 activity (Bird, 2001). Activities of Dnmt1 and Dnmt3a are modified by folic acid and homocysteine (James *et al.* 2002; Ghoshal *et al.* 2006). The timing of gene silencing during early development differs between genes and tissues (Grainger *et al.* 1983; Benvenisty *et al.* 1985; Gidekel & Bergman, 2002; Hershko *et al.* 2003). In addition, the phenotype of an embryo can be modified by manipulation of Dnmt1 expression, and hence maintenance of patterns of DNA methylation (Stancheva & Meehan, 2000; Stancheva *et al.* 2001; Biniszkiwicz *et al.* 2002).

DNA methylation can induce transcriptional silencing by blocking transcription factor binding and/or through the methyl CpG-binding protein (MeCP2) that binds to methylated cytosines and which, in turn, recruits the histone deacetylase-histone methyltransferase (HDAC-HMT) complex to the DNA (Fuks *et al.* 2003). Covalent modifications to histones, such as acetylation and methylation of specific lysine residues in the N-terminal regions of histones, influence chromatin structure and hence the ability of the basal transcriptional machinery to gain access to the DNA (Strahl *et al.* 1999; Turner, 2000; Lachner *et al.* 2001; Litt *et al.* 2001; Nakayama *et al.* 2001; Zegerman *et al.* 2002).

Since epigenetic regulation of gene promoters which is established during development and is retained throughout the life span of the organism confers patterns of transcriptional expression and silencing, perturbations to such processes represent one possible molecular mechanism for induction of an altered phenotype. Feeding a PR diet to rats during pregnancy induces hypomethylation and increased expression of the GR and PPAR α promoters in the liver of the adult offspring (Lillycrop *et al.* 2005), but was prevented by supplementation of the PR diet with folic acid. Supplementation of the PR diet with glycine or folic acid prevented induction of an altered phenotype (Jackson *et al.* 2002; Torrens *et al.* 2006). Thus 1-carbon metabolism is central to the induction of an altered phenotype in this model, which is consistent with the transient increase in plasma homocysteine, a marker of impaired 1-carbon metabolism, in early pregnancy when rats were fed a PR diet (Petrie *et al.* 2002).

We have tested the hypothesis that the transmission to the fetus of information regarding maternal nutrition and induction of altered DNA methylation involves modulation of Dnmt action. We investigated the effect of altered maternal protein intake during pregnancy on the epigenetic regulation of the hepatic GR promoter in the adult offspring. Specifically,

we determined the effect of feeding a PR diet to pregnant rats on the methylation status and the level of histone modification at the hepatic GR promoter. In order to determine the mechanism which modifies the epigenetic regulation of the GR promoter, we investigated whether prenatal undernutrition alters the expression of enzymes that catalyse DNA methylation *de novo*, methylation of hemimethylated DNA or active demethylation of DNA. As a result of our findings, we also investigated the relationship between the expression of Dnmt and the methylation of the GR promoter that is expressed in human umbilical cord (UC).

Materials and methods

Animal procedures

Virgin Wistar rats (n 5 per dietary group) were fed isocaloric diets containing either 180 g/kg casein and 1 mg/kg folic acid (control) or 90 g/kg casein and 1 mg/kg folic acid (PR). In some experiments, livers from the offspring of rats fed 90 g/kg casein and 5 mg/kg folic acid were studied (Lillycrop *et al.* 2005) (Table 1). Dams were fed standard chow (AIN 76A) from delivery (Lillycrop *et al.* 2005). Litters were reduced to eight at birth, equal numbers of males and females, and offspring were weaned onto standard chow (AIN 76A) at 28 d and were killed 6 d later. Livers were excised immediately, frozen in liquid nitrogen and stored at -80°C . One liver from each litter was selected for analysis, male to female ratio 3:2. All animal procedures were conducted in accordance with the UK Home Office Animals (Scientific Procedures) Act (1986).

Measurement of DNA methylation of the GR $_{10}$ promoter in rat

DNA methylation was carried out essentially as described (Lillycrop *et al.* 2005). Genomic DNA (5 mg) isolated from the livers of rats (n 5 per maternal dietary group) was incubated with the methylation-sensitive restriction endonucleases *Aci*I and *Hpa*II, as instructed by the manufacturer (New England Biolabs, Hitchin, Hertfordshire, UK). The resulting DNA was amplified using real-time PCR, which was performed in a total volume of 25 ml with SYBR[®] Green Jumpstart Ready Mix (Sigma, Poole, Dorset, UK) as described by the manufacturer. Cycle parameters were 55 $^{\circ}\text{C}$ for 5 min, 95 $^{\circ}\text{C}$ for 10 min, then 40 cycles of 95 $^{\circ}\text{C}$ for 30 s, 60 $^{\circ}\text{C}$ for 1 min and 72 $^{\circ}\text{C}$ for 1 min. Single bands of the correct size were verified by gel electrophoresis. Primers were designed to amplify the CpG island spanning the GR $_{10}$ promoter used in rat liver (McCormick *et al.* 2000). Primer sequences are listed in Table 2. The promoter region from the rat PPAR γ 2 promoter which does not contain *Aci*I or *Hpa*II cleavage sites was used as an internal control (Lillycrop *et al.* 2005). All C_t values were normalised to the internal control and each sample analysed in duplicate.

Measurement of mRNA expression in rat

Total RNA was isolated from liver (n 5 per maternal dietary group) samples using Tri[®] Reagent (Sigma) according to the manufacturer's instructions. cDNA was prepared as described (Lillycrop *et al.* 2005) and amplified using real-time PCR

Table 1. Composition of the diets fed during pregnancy, lactation and after weaning

	Diets fed during pregnancy			Diet fed to dams during lactation and to offspring after weaning
	Control	Protein restricted	Protein restricted, supplemented with folic acid	AIN-76A
Casein (g/kg)	180	90	90	200
Folic acid (mg/kg)	1	1	5	2
Cornstarch (g/kg)	425	482	482	150
Sucrose (g/kg)	213	243	243	500
Choline chloride (g/kg)	2	2	2	2
DL-Methionine (g/kg)	5	5	5	3
Vitamin mix* (g/kg)	5	5	5	5
Mineral mix† (g/kg)	20	20	20	20
Cellulose (g/kg)	50	50	50	50
Corn oil (g/kg)	100	100	100	50
Total metabolisable energy (MJ/kg)	20.2	19.9	19.9	15.5

* Vitamin mix: thiamine 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 -tocopherol acetate 79.9 mg/kg; cholecalciferol 1.0 mg/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 sulphate 1.2 g/kg; magnesium sulphate 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 sulphate 12.5 mg/kg.

(Harris *et al.* 2002) which was performed in a total volume of 25 ml with SYBR[®] Green Jumpstart Ready Mix (Sigma) as described by the manufacturer. PCR primers are listed in Table 2. Samples were analysed in duplicate, and the expression of GR1₁₀ promoter transcript, Dnmt1, Dnmt3a, Dnmt3b, MeCP2 and methyl binding domain protein-2 (MBD-2) was normalised to the housekeeping gene cyclophilin (Bustin, 2000). Dnmt1, 3a and 3b were measured using primer kits from QIAGEN Ltd UK (Crawley, Sussex, UK). Cycle parameters were 55°C for 5 min, 95°C for 10 min, then 40 cycles of 95°C for 30 s, 60°C (cyclophilin, phosphoenolpyruvate carboxylase (PEPCK), Dnmt1, Dnmt 3a, Dnmt3b, MeCP2 and MBD2) or 65°C (GR) for 1 min and 72°C for 1 min. Single bands of the correct size were verified by gel electrophoresis (data not shown).

Measurement of histone acetylation and methylation at the GR promoter in rat

Histone modification, and MeCP2 and Dnmt1 binding at the GR1₁₀ promoter was analysed by chromatin immunoprecipitation

(ChIP) assay (Boyd & Farnham, 1999). A 100 mg aliquot of liver tissue (*n* 5 per maternal dietary group, one per litter) was ground in liquid nitrogen and fixed with formaldehyde (1% (v/v)) for 10 min. The chromatin was sonicated to yield DNA fragments of 100–400 bp in length. The sonicated chromatin was quantified on the basis of DNA content at A_{260 nm}. The chromatin equivalent of 40 µg of DNA was used in each immunoprecipitation. The sonicated supernatant was diluted 10-fold in ChIP dilution buffer (0.01% (w/v) SDS, 1.1% (v/v) Triton X-100, 1.2 mM-EDTA, 16.7 mM-NaCl, 20 mM-Tris-HCl pH 8.1) and pre-cleared with salmon sperm DNA–protein A agarose (50% (w/v) slurry). Pre-cleared chromatin was then incubated overnight with 2 mg of antibody at 4°C. Anti- β -galactosidase (c-20) antibodies were obtained from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Anti-acetyl histone H3 (06–599), anti-acetyl H4 (06–866), di-methylated H3K4 (07–030), di-methylated H3K9 (07–441) were from Upstate Biotechnology (Dundee, UK) and tri-methylated H3K9 (ab8898), Dnmt1 (ab5208) and MeCP2 (ab3752) were from Abcam (Cambridge, UK). The immunocomplexes were collected by the addition of salmon sperm DNA–protein A agarose slurry, washed with 0.1% (w/v) SDS, 1% (v/v)

Table 2. PCR primers for analysis of mRNA expression and promoter methylation

Gene	Forward primer	Reverse primer
Methylation-sensitive PCR		
GR1 ₁₀	TCCTCCATTTTTGCGAGCTC	CCACCCGAGCCAGATAAAC
PPAR- γ ₂	GTCTCTGCTCTGGTAATTC	AAGGCTTGTTGGTCATTGAG
hGR1-C _{Total}	ATTTTTCGAGCTCGTGTCTG	CGCAGCCGAGATAAACAACT
hPPAR- α	CGGAGTTTATGAGGCCATATTC	AGGGAGATATCACTGTCTATCCAG
mRNA expression		
GR	TGACTTCCTTCTCCGTGACA	GGAGAATCCTCTGCTGCTTG
Dnmt1	QIAGEN Quantitect [®] primer assay QT00493577	
Dnmt3a	QIAGEN Quantitect [®] primer assay QT00429380	
Dnmt3b	QIAGEN Quantitect [®] primer assay QT00436002	
MeCP2	CAGCTCCAACAGGATTCATGGT	TGATGTCTCTGCTTTGCCTGCCT
MDB2	GGCAAGAGCGATGTCTACTA	CTGGACCGACTCCTTGAAGA
Cyclophilin	TTGGGTGCGTCTGCTTCGA	GCCAGGACTGTATGCTTCA
hGR1-C _{Total}	GCTCCTTGCCAGAGTTGAT	CAGTGGATGCTGAACTCTTGG

GR1₁₀, glucocorticoid receptor 1₁₀ promoter; hPPAR α , human peroxisomal proliferator-activated receptor- α ; hGR1-C_{Total}, human GR1-C_{Total} promoter; Dnmt1, DNA methyltransferase-1; Dnmt3a, DNA methyltransferase-3a; Dnmt3b, DNA methyltransferase-3b; MeCP2; methyl CpG-binding protein-2; MDB2; methyl binding domain protein-2.

Triton X-100, 2 mM-EDTA, Tris-HCl pH 8.1, 150 mM-NaCl, Tris-HCl pH 8.1 and then with 0.1% (w/v) SDS, 1% (v/v) Triton X-100, 2 mM-EDTA, 500 mM-NaCl, 20 mM-Tris-HCl pH 8.1 followed by 0.25 M-LiCl, 1% (v/v) NP-40, 1% (w/v) sodium deoxycholate, 1 mM-EDTA, 10 mM-Tris-HCl pH 8.1 and twice with 10 mM-Tris-HCl pH 7.5, 1 mM-EDTA. DNA was eluted by the addition of a solution of 1% (w/v) SDS and 0.1 M-NaHCO₃, the cross-links reversed, and the DNA purified. The ChIP-precipitated DNA and the input DNA were subjected to real-time PCR using SYBR[®] Green Jumpstart Ready Mix (Sigma). The PCR primers used for the GR1₁₀ promoter were 5'-CGTCTTGTCCACCCACT-3' and 5'-CCTTGCAGTTGC-CGACAG. All values were normalised with respect to the input DNA and expressed as a percentage of the control group. To demonstrate the specificity of the immunoprecipitation reactions, an antibody directed against β -galactosidase and a negative antibody control were used in each experiment (data not shown).

Human umbilical cord samples

These studies utilised samples of UC from a stratified random sample of fifteen term infants in the Birthright Fetal Growth Rates Study. This study of nutrition during pregnancy and fetal growth recruited Caucasian women with singleton pregnancies and known menstrual dates who registered in early pregnancy with participating obstetricians at the Princess Anne Maternity Hospital, Southampton UK (Godfrey *et al.* 2001). Samples were stored at -80°C. Birthweights of the infants were in the normal range (range 2665–4430 g, mean 3426 g). Collection and analysis of human UC samples was carried out with written informed consent from all subjects and under IRB approval from the Southampton and South West Hampshire Joint Research Ethics Committee.

DNA methyltransferase mRNA expression in human umbilical cord

Total RNA was isolated from human UC using Tri[®] Reagent (Sigma, Poole, Dorset, UK) according to the manufacturer's instructions. cDNA was prepared as described (Lillycrop *et al.* 2005) and amplified using real-time reverse transcription-PCR (RT-PCR), which was performed in a total volume of 25 ml with SYBR[®] Green Jumpstart Ready Mix (Sigma) as described by the manufacturer. Human Dnmt1 shows 80% identity to rat Dnmt1, and human Dnmt3a shows 91% identity to rat Dnmt3a. There was 100% identity between rat and human Dnmt1 and between rat and human Dnmt3a within the primer sequences. Therefore, we used the same RT-PCR primers to measure Dnmt1 and Dnmt3a expression in human UC and rat liver. PCR primers are listed in Table 2. Samples were analysed in duplicate and the expression of Dnmt1 and Dnmt3a was normalised to the housekeeping gene cyclophilin (Bustin, 2000). Cycle parameters were 94°C for 2 min, then 40 cycles of 95°C for 30 s, 60°C (cyclophilin and Dnmt1) or 62°C (Dnmt3a) for 1 min and 72°C for 1 min.

Measurement of DNA methylation of the GR1-C_{Total} promoter in human umbilical cord

To confirm that the GR1-C_{Total} promoter is expressed in human UC, total RNA was isolated from UC and human

blood using Tri[®] Reagent (Sigma) as described by the manufacturers. The size of the GR1-C_{Total} promoter RT-PCR transcript from UC on agarose gel electrophoresis was compared with the blood reference transcript (Turner & Muller, 2005) (Fig. 2A). PCR primers are listed in Table 2. Cycle parameters were 94°C for 2 min, then 40 cycles of 95°C for 30 s, 62°C for 1 min and 72°C for 1 min.

For analysis of GR1-C_{Total} promoter methylation, genomic DNA (400 ng) was incubated with the methylation-sensitive restriction endonucleases *Aci*I and *Hpa*II as instructed by the manufacturer (New England Biolabs, Hitchin, Hertfordshire, UK). The resulting DNA was amplified using real-time PCR, which was performed in a total volume of 25 ml with SYBR[®] Green Jumpstart Ready Mix (Sigma) according to the manufacturer's instructions. A fragment of the human PPAR α exon 7 which does not contain *Aci*I or *Hpa*II cleavage sites was used as an internal control gene. Primers were designed to amplify the CpG island spanning the GR1-C_{Total} promoter (Turner & Muller, 2005). Primer sequences are listed in Table 2. Cycle parameters were 94°C for 2 min, then 40 cycles of 95°C for 30 s, 59.3°C (GR1-C_{Total}) or 66°C (PPAR α) for 1 min and 72°C for 1 min. All C_t values were normalised to the internal control and each sample analysed in duplicate. Single bands of the correct size were verified by gel electrophoresis (not shown).

Statistical analysis

Data are presented as mean (SEM). Statistical comparisons were by Student's unpaired *t* test or one-way ANOVA with Bonferroni's *post hoc* analysis as indicated in the text. The relationship between DNA methylation and gene expression in human UC was determined by calculation of Pearson's correlation coefficient.

Results

GR promoter methylation, and expression of GR and PEPCK in rat liver

Hepatic GR1₁₀ promoter methylation was 33% lower (Table 3) at postnatal day 34 in the offspring of dams fed a PR diet compared with offspring of the control group. Hypomethylation of the GR1₁₀ promoter was associated with higher mRNA expression of GR1₁₀ (84%). The expression of the GR target gene PEPCK was also increased by 16% in the offspring of the PR group (Table 3).

MeCP2 expression in rat liver

Having shown GR hypomethylation in PR offspring, we then investigated whether the expression and recruitment of MeCP2 to the GR1₁₀ promoter was also altered in response to maternal diet. MeCP2 mRNA expression was 29% lower in the liver of the PR offspring versus controls (Table 4). Binding of MeCP2 to the GR promoter in the liver of control and PR offspring was assessed using a ChIP assay. Binding of MeCP2 to the GR1₁₀ promoter was reduced by 43% ($P < 0.05$) in PR offspring compared with control offspring (Table 4). No signal was obtained when chromatin was precipi-

Table 3. Glucocorticoid receptor promoter methylation, and glucocorticoid receptor and phosphoenolpyruvate carboxykinase mRNA expression in the liver of day 34 offspring

(Mean values with their standard errors of the mean)

	Relative to the control group (%)				<i>P</i>
	Control		PR		
	Mean	SEM	Mean	SEM	
GR1 ₁₀ methylation	100.0	3.5	67.3	1.5	<0.001
GR1 ₁₀ mRNA expression	100.0	22.0	184.0	11.0	<0.05
PEPCK mRNA expression	100.0	2.3	116.0	4.0	<0.05

PR, protein-restricted; GR1₁₀, glucocorticoid receptor 1₁₀ promoter; PEPCK, phosphoenolpyruvate carboxykinase.

Values are the results of RT-PCR analysis for *n* 5 samples/maternal dietary group. Statistical comparison was by Student's unpaired *t* test.

pitated with an anti-β-galactosidase antibody or with a negative antibody control. This demonstrates that the immunoprecipitation reactions were specific.

Analysis of histone modifications at the hepatic GR1₁₀ promoter in rats

In order to determine whether the hypomethylation of the GR1₁₀ promoter, and reduced expression and binding of MeCP2 were associated with altered covalent modifications of histones bound to the GR promoter, ChIP assays were used to measure the acetylation and methylation of specific histone lysine residues at the GR1₁₀ promoter. The level of histone modifications which facilitate transcription was higher at the hepatic GR1₁₀ promoter in the PR versus control offspring, namely acetylation of H3K9 (174%) and H4K9 (302%), and methylation of H3K4 (925%) (all *P*<0.001) (Table 4). Di-methylation and tri-methylation of H3K9, which are associated with suppression of transcription, were 54% lower (*P*<0.01) or not statistically significantly

Table 4. MeCP2 mRNA expression, binding of MeCP2 and histone modifications at the glucocorticoid receptor promoter

(Mean values with their standard error of the mean)

	Relative to the control group (%)				<i>P</i>
	Control		PR		
	Mean	SEM	Mean	SEM	
MeCP2 mRNA expression	100.0	9.3	71.4	7.3	<0.05
MeCP2 binding	100	2	57	2	<0.05
Histone modifications					
H3K9 acetylation	100	12	274	20	<0.0001
H4K9 acetylation	100	18	402	33	<0.0001
H3K9 methylation	100	20	1025	87	<0.0001
H3K9 dimethylation	100	22	19	16	<0.001
H3K9 trimethylation	100	60	103	11	NS

MeCP2, methyl CpG-binding protein; PR, protein-restricted.

Values are for *n* 5 samples/maternal dietary group. Statistical comparison was by Student's unpaired *t* test. Expression of MeCP2 and binding of MeCP2 at the glucocorticoid receptor 1₁₀ (GR1₁₀) promoter in liver from 34-day-old offspring of rats fed either a control or PR diet during pregnancy. Binding of MeCP2 and the extent of histone modifications at the GR1₁₀ promoter were determined by chromatin immunoprecipitation assay.

different, respectively, in the PR offspring versus controls at the GR1₁₀ promoter (Table 4).

Analysis of the expression of genes which regulate DNA methylation in the liver of rats

There are three possible mechanisms underlying hypomethylation of the GR1₁₀ promoter: (1) impaired methylation *de novo* by Dnmt 3a and 3b; (2) failure to maintain CpG methylation through mitosis by Dnmt1; or (3) active demethylation by the putative DNA demethylase MBD2 (Detich *et al.* 2002). To examine which of these mechanisms may be responsible for the hypomethylation of GR, the expression of Dnmt1, Dnmt3a/b and the demethylase MBD2 was measured using real-time RT-PCR. Expression of Dnmt1 mRNA was 17% lower (*P*<0.05) in PR versus control offspring (Fig. 1A). There were no significant differences between PR and control offspring in the expression of Dnmt 3a and 3b, or MBD2 (Fig. 1B–D). ChIP assays using an anti-Dnmt1 antibody showed that binding of Dnmt1 at the GR1₁₀ promoter was significantly lower (12%, *P*<0.05) in the PR offspring compared with controls (Fig. 1E).

Since 1-carbon metabolism appears to be involved centrally for the induction of an altered phenotype by variations in maternal protein intake during pregnancy (Petrie *et al.* 2002; Torrens *et al.* 2006), and hypomethylation of the hepatic GR is prevented by supplementation of the PR diet with folic acid (Lillycrop *et al.* 2005), we next investigated the effect of supplementation of the PR diet with 5-fold more folic acid than was present in the control or PR diet. Supplementing the PR diet with folic acid prevented reduced Dnmt1 expression compared with the control group (Fig. 1A), but did not alter Dnmt 3a or 3b expression (Fig. 1B, C).

Analysis of GR promoter methylation and Dnmt1 expression in human umbilical cord

Since Dnmt1 mRNA expression appeared to be related to the level of methylation of the hepatic GR promoter, we investigated whether Dnmt1 expression was also related to the level of GR methylation in fetal human tissue. The human GR1-C_{Total} promoter, like the rat GR1₁₀ promoter, contains a CpG island which spans the 5'-untranslated region of the gene and which contains eleven different heterogeneous non-coding first exons (Turner & Muller, 2005). In the human fetal UC samples, we examined the methylation status of the GR1-C_{Total} promoter, which shows 70.6% homology with the rat GR1₁₀ promoter (Turner & Muller, 2005). We showed that transcripts from the GR1-C_{Total} promoter are expressed in human UC (Fig. 2A). Using a methylation-sensitive restriction enzyme PCR assay, we found that there was variation in the level of methylation of the GR1-C_{Total} promoter between human fetal cord samples such that the highest level of methylation was approximately 2-fold greater than the lowest (Fig. 2D).

Single bands of the correct size for the PCR products of the Dnmt1 and Dnmt3a primers in human and rat tissue were verified by gel electrophoresis (Fig. 2B, C). Dnmt1 expression predicted 49% (*P*=0.003) of the variation in GR1-C_{Total} methylation (Fig. 2D), while Dnmt3a expression was not related to GR1-C_{Total} methylation (Fig. 2E).

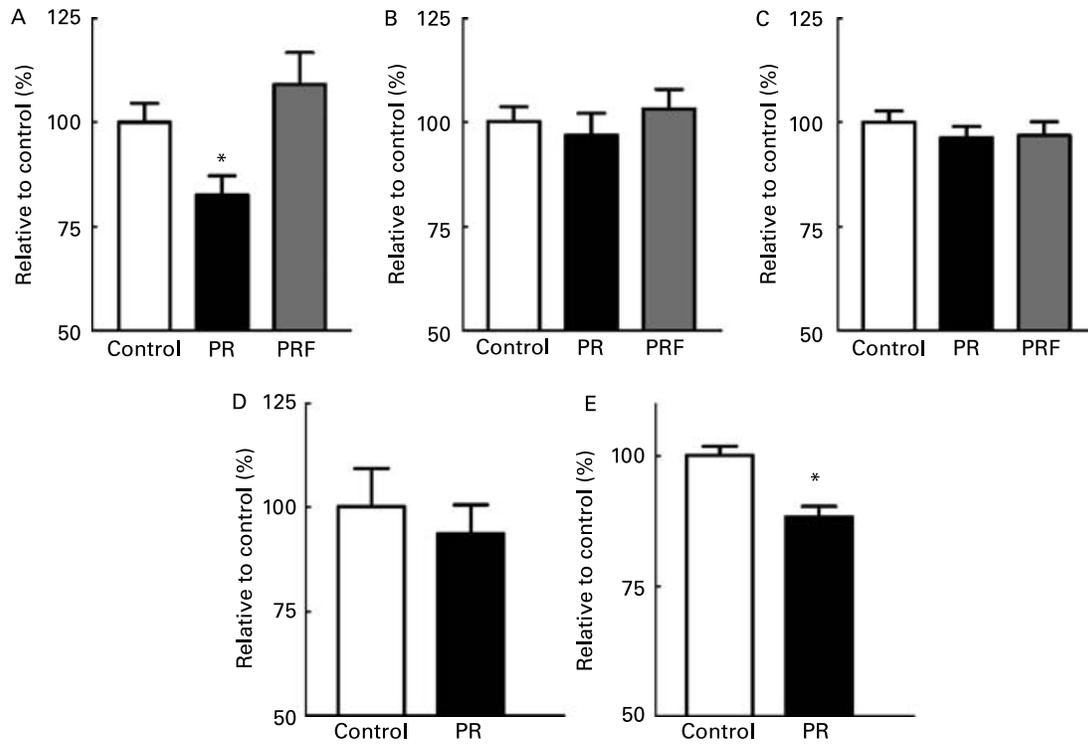


Fig. 1. mRNA expression of DNA methyltransferases (Dnmt) in liver from 34-day-old offspring of rats fed either a control or protein-restricted (PR) diet, or the PR diet supplemented with folic acid (PRF) during pregnancy. Data from RT-PCR analysis are means (with SEM) relative to the control group (n 5 per maternal dietary group). (A) Dnmt1 expression. (B) Dnmt3a expression. (C) Dnmt3b expression. Statistical comparisons were by one-way ANOVA with Bonferroni's *post hoc* analysis. One-way ANOVA showed a significant difference ($P < 0.05$) between groups for Dnmt1, but not for the other DNA methyltransferases. * $P < 0.05$ compared with the control group. (D) Expression of the methyl binding domain protein-2 from 34-day-old offspring of rats fed either a control or PR diet. Data are means (with SEM) relative to the control group (n 5 per maternal dietary group). Statistical analysis was by Student's unpaired t test. (E) Binding of Dnmt1 at the glucocorticoid receptor 1₁₀ promoter in liver from 34-day-old offspring of rats fed either a control or PR diet measured by chromatin immunoprecipitation assay. Data are means (with SEM) relative to the control group (n 5 per maternal dietary group). Statistical analysis was by Student's unpaired t test. * $P < 0.05$ compared with the control group.

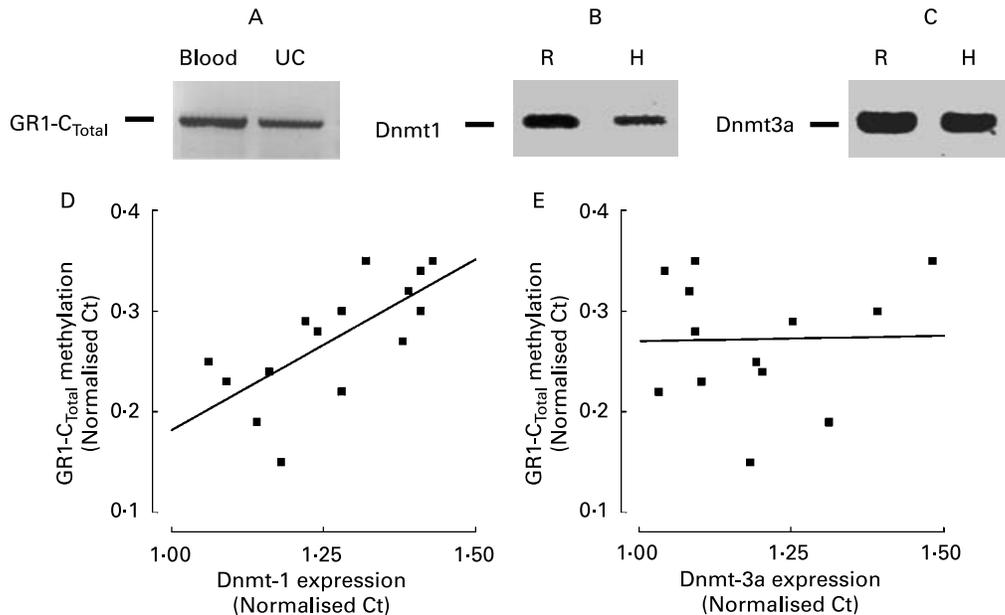


Fig. 2. Methylation status and mRNA expression of glucocorticoid receptor (GR) 1-C_{Total} promoter and mRNA expression of DNA methyltransferase (Dnmt)-1 and Dnmt3a in human umbilical cord (UC). (A) Expression of the transcript of the GR1-C_{Total} in human UC by real-time RT-PCR using adult human blood as reference. The same primers were used to detect (B) Dnmt1 and (C) Dnmt3a in human (H) UC as used with rat (R) liver. Transcripts of the appropriate size were identified by analysis of the RT-PCR products by agarose gel electrophoresis. The methylation status of the GR1-C_{Total} promoter and the mRNA expression of Dnmt1 and Dnmt3a were measured in human UC samples from fifteen pregnancies. The association between GR1-C_{Total} promoter methylation and Dnmt expression was analysed by calculation of Pearson's correlation coefficient. (D) The relationship between Dnmt1 expression and GR1-C_{Total} promoter methylation, $r = 0.70$, $P = 0.03$. (E) The relationship between Dnmt3a expression and GR1-C_{Total} promoter methylation, $r = 0.001$, $P = 0.89$.

Discussion

The results of this study show for the first time that hypomethylation and increased expression of the GR₁₀ promoter induced in the liver of the offspring of rats fed a PR diet during pregnancy are associated with reduced Dnmt1 expression and altered covalent modifications to histones at the GR promoter.

Feeding a PR diet to pregnant rats increases hepatic gluconeogenesis in the adult offspring (Burns *et al.* 1997; Desai *et al.* 1997), which reflects increased expression of hepatic GR and PEPCK expression (Bertram *et al.* 2001, Lillycrop *et al.* 2005). This is consistent with increased corticosteroid activity in the offspring of rats fed a PR diet during pregnancy (Langley-Evans *et al.* 1996). Our current and previous findings (Lillycrop *et al.* 2005) show that increased hepatic GR expression in the PR offspring is associated with hypomethylation of the GR₁₀ promoter. The livers of these rats also show hypomethylation of the PPAR α promoter and increased expression of its target gene acyl-CoA oxidase (Lillycrop *et al.* 2005). Together, these findings suggest that feeding a PR diet during pregnancy induces in the liver of the offspring a metabolic phenotype characterised by altered regulation of energy balance, with increased capacity for gluconeogenesis and peroxisomal fatty acid β -oxidation. Moreover, since promoter methylation status may be modified at different stages of development in a gene- and tissue-specific manner (Grainger *et al.* 1983; Benvenisty *et al.* 1985; Gidekel & Bergman, 2002; Hershko *et al.* 2003), this suggests a mechanism by which timing of nutritional constraint may influence the induced phenotype (Ravelli *et al.* 1998; Rémacle *et al.* 2004).

The stable silencing of genes by DNA methylation is critical for cellular differentiation (Bird, 2001; Hershko *et al.* 2003) and for the developmental regulation of the activities of metabolic pathways (Grainger *et al.* 1983; Benvenisty *et al.* 1985). Induction of hypomethylation of the hepatic GR promoter in the PR offspring may occur by either impaired methylation *de novo* during cell differentiation in the early embryo, failure to maintain DNA methylation during mitosis or active demethylation. Failure to maintain methylation or active demethylation may result in the activation of genes not normally expressed in adult tissues, or accelerated or more extensive demethylation of genes that are induced during tissue maturation. Our findings show that, while the expression of Dnmt3a and 3b and the demethylase MBD2 was not altered, Dnmt1 expression was significantly lower in the liver of the PR offspring compared with controls. Thus hypomethylation of the GR₁₀ promoter, and in turn altered regulation of hepatic glucose metabolism, may be induced by reduced capacity to methylate hemimethylated DNA during mitosis rather than failure of DNA methylation *de novo* or active demethylation. This conclusion is supported by lower binding of Dnmt1 at the GR₁₀ promoter in the PR offspring. These findings agree with those showing induction of DNA hypomethylation and altered phenotype by depletion of xDnmt1 in *Xenopus* embryos (Stancheva & Meehan, 2000; Stancheva *et al.* 2001) and promoter demethylation by Dnmt1 knockdown (Leu *et al.* 2003).

Altered 1-carbon metabolism plays a central role in phenotype induction in this model (Jackson *et al.* 2002; Petrie *et al.* 2002; Lillycrop *et al.* 2005; Torrens *et al.* 2006). Our findings

show that lowering of Dnmt1 expression by the PR diet was prevented by maternal folic acid supplementation, while expression of Dnmt3a was unaltered. Hence Dnmt1 expression in the offspring is modified by maternal folic acid intake, which is consistent with the modulation of Dnmt1 expression in adult rats by folic acid intake (Ghoshal *et al.* 2006) and the inhibition of Dnmt1 and induction of DNA hypomethylation by hyperhomocysteinaemia (James *et al.* 2002). Thus, the effects of differences in maternal folic acid intake, and in turn capacity for metabolism of methyl groups, during pregnancy could explain the induction or prevention of GR₁₀ hypomethylation in the liver of the offspring. These findings also emphasise the importance of adequate dietary intake of folic acid during pregnancy for optimal fetal development.

One possible mechanism for induction of an altered metabolic phenotype in the liver of the offspring of rats fed a PR diet during pregnancy is impairment of 1-carbon metabolism leading to down-regulation of Dnmt1 expression and progressive loss of methyl groups from the GR₁₀ promoter. Reduced expression of Dnmt1 may be expected to result in decreased methylation of all promoters containing CpG dinucleotides. However, studies in cells lacking Dnmt1 show only a 20% decrease in genomic methylation and no changes to the methylation status of specific genes (Rhee *et al.* 2000). This suggests that Dnmt1 activity is targeted to specific genes, possibly through binding to transcription factors such as E₂F1 (Robertson *et al.* 2000). Thus because binding of Dnmt1 to the GR promoter was reduced in addition to an overall reduction in Dnmt1 expression, the proposed pathway is consistent with gene-specific hypomethylation in the liver in the offspring of dams fed a PR diet (Lillycrop *et al.* 2005). Dnmt1 activity is also required for progression through mitosis (Milutinovic *et al.* 2003). Thus it is possible that suppression of Dnmt1 activity or expression by altered 1-carbon metabolism in the pre-implantation period could also account for the reduction in cell number during the early development in this model (Kwong *et al.* 2000).

The specific link between reduced protein intake and altered 1-carbon metabolism cannot be deduced from the present data. However, we offer two possible explanations. First, it is possible that it may result from decreased availability of glycine, leading to altered flux of methyl groups between different metabolic fates. Secondly, increased maternal corticosteroid activity (Langley-Evans *et al.* 1996), possibly as a result of stress induced by constrained nutrient availability, may reduce folic acid availability (Terzolo *et al.* 2004). The second mechanism could explain how maternal corticosteroid blockade prevents induction of hypertension in the PR offspring (Langley-Evans *et al.* 1997) as well as prevention of altered phenotype by folic acid administration (Lillycrop *et al.* 2005; Torrens *et al.* 2006).

Analysis of GR₁-C_{Total} in human UC samples showed for the first time that among individuals within the normal birth-weight range, there is considerable variation in the methylation status of a gene expressed in human fetal tissue. Dnmt1 expression predicted 49% of the variation in methylation of the GR₁-C_{Total} promoter expressed in human UC. Because the UC were frozen, we were unable to dissect and measure Dnmt1 expression and GR₁-C_{Total} methylation in specific tissues. However, these data suggest that methylation of the GR₁-C_{Total} promoter in human UC is associated with

the capacity of Dnmt1 to maintain methylation of CpG dinucleotides rather than the capacity for DNA methylation *de novo*. The extent to which maternal diet during pregnancy determines Dnmt1 expression cannot be examined within this small group of infants. However, these findings are consistent with our observations in the rat and so suggest the hypothesis that induction of different phenotypes in humans by prenatal nutrition may involve variations in Dnmt1 expression and, in turn, DNA methylation.

Covalent modifications of specific residues in the N-terminal domain of histones also confer epigenetic regulation of transcription (Turner, 2000). Such modifications are closely linked to promoter methylation as methylated CpG nucleotides are required for binding of MeCP2 and recruitment of the HDAC–HMT complex. Binding of MeCP2–HDAC–HMT to methylated CpGs causes histone deacetylation and methylation of specific lysine residues, leading to suppression of transcription. We found higher levels of histone modifications which facilitate transcription, while modifications that suppress transcription were reduced, at the GR₁₀ promoter in the offspring of rats fed the PR diet compared with controls. One possible explanation is that hypomethylation of the GR₁₀ promoter reduced binding of MeCP2 and, in turn, the HDAC–HMT complex. If so, this suggests that lower

Dnmt1 expression is the primary process in inducing increased GR₁₀ expression, and altered histone modifications are a secondary effect. This may be exacerbated by lower hepatic MeCP2 expression in these offspring. Such changes to the regulation of transcription of the GR₁₀ promoter are consistent with the higher level of transcription in the PR offspring.

We therefore propose a mechanism for the induction of an altered metabolic phenotype in the offspring of rats fed a PR diet during pregnancy based upon our findings for the GR₁₀ promoter (Fig. 3). Promoter methylation is induced in a gene-specific manner during the development of the early embryo by the activities of Dnmt 3a and 3b. Impaired 1-carbon metabolism, either as a direct result of constrained maternal nutrition or by increased corticosteroid activity, reduces Dnmt1 expression, resulting in progressive hypomethylation of specific genes during successive mitotic cycles. The hypomethylated GR₁₀ promoter would result in the reduced binding of the MeCP2–HDAC–HMT complex which facilitates persistence of histone modifications that permit transcription. In turn, gluconeogenesis increases due to upregulation of PEPCK expression by the action of the GR. The pathway may also explain hypomethylation of PPAR α and increased expression of acyl-CoA oxidase in the liver of the PR offspring (Lillicrop *et al.* 2005). If this pathway acts primarily

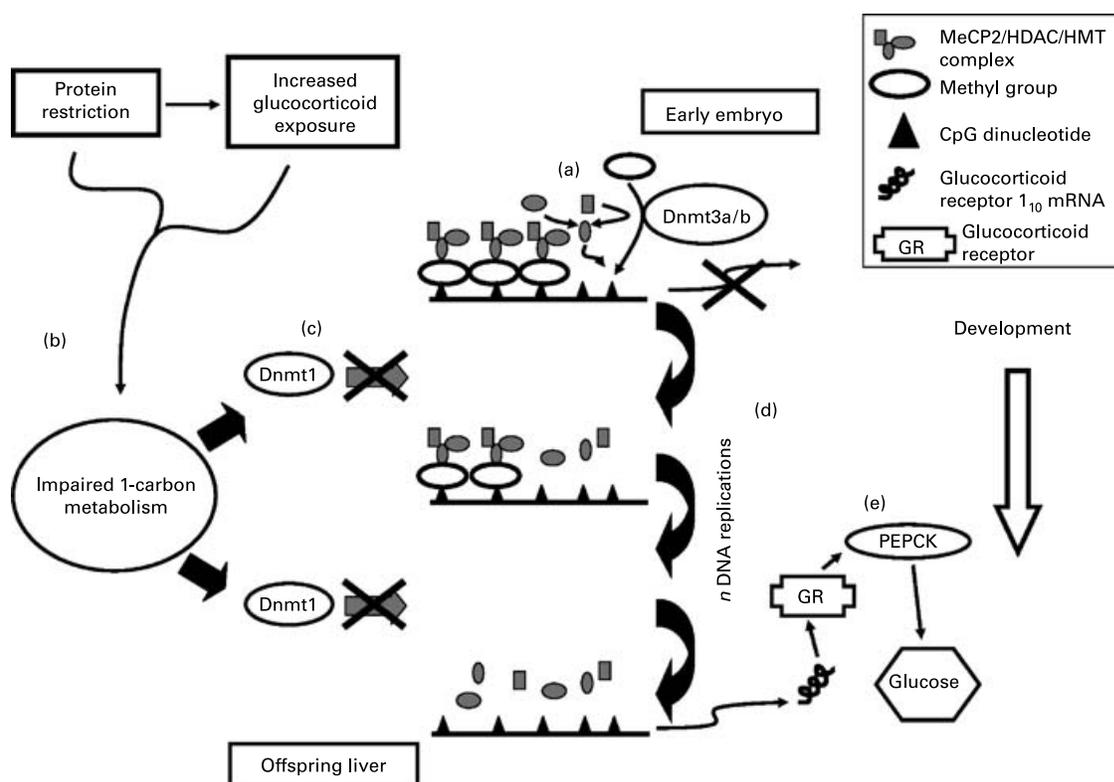


Fig. 3. A diagram of the proposed pathway for induction of altered glucose homeostasis in the offspring of rats fed a protein-restricted (PR) diet during pregnancy. A full explanation is given in the text. (a) Glucocorticoid receptor (GR) expression is silenced in the early embryo in cells destined to become hepatocytes by the activities of DNA methyltransferases (Dnmt) 3a and 3b. (b) In the offspring of rats fed a PR diet, 1-carbon metabolism is impaired either as a direct consequence of the restricted diet or by increased glucocorticoid exposure. This downregulates Dnmt1 expression (c). Lower Dnmt1 expression results in a impaired capacity to methylate hemimethylated DNA during mitosis (d). After sequential mitotic cycles, this results in hypomethylation of the GR promoter and loss of epigenetic memory of gene silencing, such that, in the liver of the adult offspring, expression of GR is induced in cells which do not express GR in control animals. The increased expression of GR is facilitated by lower binding and expression of methyl CpG-binding protein (MeCP)-2 and reduced recruitment of the histone deacetylase (HDAC)–histone methyltransferase (HMT) complex, resulting in higher levels of histone modifications which permit transcription. (e) This results in increased phosphoenolpyruvate carboxykinase (PEPCK) expression and increased gluconeogenesis.

by altering the epigenetic regulation of specific transcription factors, then changes in the activities of a number of metabolic pathways may be induced. However, the proposed pathway does not exclude the possibility that other genes may also be hypomethylated by this process. This pathway also explains how administration of glucocorticoids during pregnancy induces persistent changes to gluconeogenic enzymes in the offspring as a result of increasing GR expression (Nyirenda *et al.* 1998).

In conclusion, the present findings suggest a pathway for induction of an altered phenotype or fetal programming. In humans, this may provide a basis for interventions in early life to reduce risk of later metabolic disease.

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