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Imprinted gene expression in the rat embryo–fetal axis is altered in response to periconceptional maternal low protein diet

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Abstract

In our previous study, we have shown that maternal low protein diet (LPD, 9% casein vs 18% casein control) fed exclusively during the rat preimplantation period (0–4.25 day postcoitum) induced low birth weight, altered postnatal growth and hypertension in a gender-specific manner. In this study, we investigated the effect of maternal LPD restricted only to the preimplantation period (switched diet) or provided throughout gestation on fetal growth and imprinted gene expression in blastocyst and fetal stages of development. Male, but not female, blastocysts collected from LPD dams displayed a significant reduction (30%) in *H19* mRNA level. A significant reduction in *H19* (9.4%) and *Igf2* (10.9%) mRNA was also observed in male, but not in female, fetal liver at day 20 postcoitum in response to maternal LPD restricted to the preimplantation period. No effect on the blastocyst expression of *Igf2R* was observed in relation to maternal diet. The reduction in *H19* mRNA expression did not correlate with an observed alteration in DNA methylation at the *H19* differentially methylated region in fetal liver. In contrast, maternal LPD throughout 20 days of gestation did not affect male or female *H19* and *Igf2* imprinted gene expression in fetal liver. Neither LPD nor switched diet treatments affected *H19* and *Igf2* imprinted gene expression in day 20 placenta. Our findings demonstrate that one contributor to the alteration in postnatal growth induced by periconceptional maternal LPD may derive from a gender-specific programming of imprinted gene expression originating within the preimplantation embryo itself.
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Introduction

Epidemiological studies in human populations have revealed that low birth weight and reduced intra-uterine and early postnatal growth lead to the increased risk of developing adult chronic diseases including coronary heart disease, hypertension, type 2 diabetes and osteoporosis (Barker 1993, Phillips 1998). Several animal models have provided support for an early origin to the disease susceptibility. Thus, in different mammalian species, global food restriction (Woodall *et al.* 1996), manipulation of maternal nutrition (Langley & Jackson 1994, Kwong *et al.* 2000), uterine ligation (Jansson & Lambert 1999) or increased maternal exposure to synthetic glucocorticoid (Nyirenda *et al.* 1998) during gestation have been shown to alter postnatal growth and/or physiology into adult life. These observations have led to the 'Developmental Origin of Health and Disease' (DOHaD) hypothesis which proposes that the level of nutrition available during gestation influences the developmental programme through 'predictive adaptive responses' to set appropriate postnatal growth and metabolic criteria (Gluckman *et al.* 2005, McMillen & Robinson 2005). However, a mismatch between gestational and postnatal nutrition will lead to adverse phenotypic consequences and increased the disease susceptibility.

The DOHaD concept may be extended by the identification of developmental 'windows' of plasticity when nutrient availability may act decisively to alter the pattern of future development. One such potential window is the preimplantation embryo when embryonic and extra-embryonic lineages are first established and segregated, and which is known to be sensitive to the environmental conditions with lasting consequences (reviewed in Khosla *et al.* 2001*a*, Thompson *et al.* 2001, Fleming *et al.* 2004*a*,*b*). Thus, we have shown that feeding pregnant rats with low protein diet (9 vs 18%

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casein) exclusively during the preimplantation period led to male offspring with altered postnatal growth rate and organ sizes and elevated systolic blood pressure, with less severe changes evidence in female offspring (Kwong *et al.* 2000).

In vitro culture conditions for preimplantation embryos have also been shown to alter incipient gene expression (Niemann & Wrenzycki 2000), fetal development rate after transfer (Khosla *et al.* 2001*b*, Thompson *et al.* 2001, Lane & Gardner 2003) and postnatal physiology (Young *et al.* 2001). In sheep and cattle, early embryo culture conditions can give a rise to large offspring syndrome (LOS) after transfer, associated with increased perinatal malformations and mortality rate (Walker *et al.* 1996). In addition, mouse offspring derived from cultured embryos have been shown to display altered behaviour with respect to anxiety and spatial memory compared with *in vivo*-derived counterparts (Ecker *et al.* 2004, Fernández-Gonzalez *et al.* 2004). Although a variety of mechanisms may associate preimplantation environment with future developmental changes, the aberrant expression of imprinted genes has been proposed to play a significant role (Young & Fairburn 2000, Fleming *et al.* 2004*a*,*b*).

Imprinted genes are only expressed from either maternal or paternal alleles due to epigenetic modifications such as DNA methylation to CpG islands in regulatory domains (Delaval & Feil 2004, Dean *et al.* 2005). Several studies have identified the changes in imprinted gene expression throughout the development associated with culture composition during the preimplantation period when, through zygotic genome activation mechanisms, the DNA methylation pattern may be sensitive to environmental conditions (Doherty *et al.* 2000, Thompson *et al.* 2001, Jaenisch & Bird 2003). Thus, exposure of sheep embryos to serum leading to LOS induced the decreased fetal expression of imprinted insulin-like growth factor 2 receptor (*IGF2R*) transcript and the loss of methylation on the normally active maternal allele (Young *et al.* 2001). In mouse, fetuses produced from blastocysts cultured in the presence of fetal calf serum were lighter at embryonic day 14 and exhibited decreased insulin-like growth factor 2 (*Igf2*), *H19* and *Grb7* imprinted gene expression and an increase in *Grb10* expression (Khosla *et al.* 2001*b*). Mouse preimplantation embryos cultured in Whitten's medium displayed the aberrant expression of *H19* from the normally silent paternal allele due to altered methylation pattern (Doherty *et al.* 2000). This aberrant *H19* expression persisted in placental tissues in mid-gestation (Mann *et al.* 2004). Furthermore, the culturing of mouse zygotes to the blastocyst stage in the presence of ammonium increased the level of *H19* gene transcription (Lane & Gardner 2003).

Fetal growth is largely controlled by the complex IGF system. Several imprinted genes are shown to be related to expression/function of IGF2, which is paternally expressed in several tissues. The deletion of maternal *H19* allele gives rise to pups that are 27% heavier than their wild type littermate (Leighton *et al.* 1995*a*). The level of IGF2 protein can also be regulated by IGF2R (Lau *et al.* 1994). Previously, studies have shown that *H19* is very sensitive to preimplantation culture condition (Doherty *et al.* 2000, Lane & Gardner 2003, Mann *et al.* 2004) and maternal dietary restriction altered *H19* and *IGF2* gene expression in fetal sheep (Brameld *et al.* 2000, Naimeh *et al.* 2001). The culturing of sheep embryos to blastocyst stage led to reduction in *IGF2R* transcript and protein in late gestation (Young *et al.* 2001). However, it is unknown whether embryo environment *in vivo*, through maternal LPD treatment, may similarly alter the pattern of embryonic imprinted gene expression with lasting consequences. Therefore, we examined the effect of maternal LPD fed solely during preimplantation period (switched diet) or throughout gestation (LPD) on fetal growth and the pattern of embryonic and fetal expression on these imprinted genes. For the first time, we show that maternal LPD alters blastocyst imprinted gene expression with reduced *H19* mRNA evident in male embryos. A reduced expression level for *H19* and *Igf2* transcripts is also evident in day 20 male fetal liver. These changes in gene expression precede alteration in growth which is not evident until the postnatal period.

Materials and Methods

Animals and treatments

Virgin female Wistar rats were maintained in a temperature-controlled room with 12 h light:12 h darkness cycle. At 13–14 weeks of age (200–240 g), they were mated overnight with males of the same strain and the presence of a vaginal plug was identified the following morning (1100=day 0 of development). Plug-positive animals were assigned randomly to either 9% casein (low protein diet, LPD) or 18% casein (control diet) fed *ad libitum* (Langley & Jackson 1994, Kwong *et al.* 2000). Dams were either sacrificed during the preimplantation development (day 2–4) (for blastocysts collection, total dams=24; 18%, $n=11$; 9%, $n=13$) or were maintained until day 20 before analysis of fetal or placental tissues (total dams=35; 18%, $n=12$; 9%, $n=11$; switched diet, $n=12$). During this time, dams were either maintained on control or LPD diets or were switched from LPD at 4.25 days (blastocyst stage) to control diet. All animal procedures were conducted in accordance with the UK Home Office Animals (Scientific Procedures) Act (1986) with the local ethics committee approval.

Embryo collection

Embryos at different stages of development were collected from oviducts or uteri between day 2 and 4 by

flushing with H6 medium containing BSA (4 mg/ml, Sigma; Sheth *et al.* 1997). Freshly collected embryos were rapidly washed once in H6 + BSA, three times with H6 + polyvinyl pyrrolidone (6 mg/ml, Sigma) medium (Sheth *et al.* 1997) and three times with PBS. Single embryos in minimal volume (2–5 μl) of PBS were then transferred to siliconised tubes, snap frozen in liquid nitrogen and stored at −80 °C until used. Inner cell masses (ICMs) were isolated by immunosurgery from blastocysts as described (Eckert *et al.* 2004) prior to snap frozen in liquid nitrogen and stored at −80 °C until used.

Isolation of Poly A$^+$ RNA in preimplantation embryos

Poly A$^+$ RNA was isolated from single embryos using Dynabeads Oligo $(dT)_{25}$ (Dynabeads mRNA DIRECT kit, Dynal Biotech, Wirral, UK). All reagents were provided in the Dynabeads mRNA DIRECT kit unless otherwise stated. Prior to isolation, 1 pg luciferase mRNA (1 pg/μl, Promega) was added to each tube containing single embryos to serve as an external standard. Single frozen-stored embryos were lysed by adding 150 μl lysis-binding buffer (100 mM Tris–HCl, pH 8.0, 500 mM LiCl, 10 mM EDTA, 1% (w/v) lithium dodecylsulfate (LiDS), 5 mM dithiothreitol) and incubated at room temperature for 10 min before addition of 10 μl washed Dynabeads Oligo $(dT)_{25}$ in lysis-binding buffer and incubation at room temperature for 10 min on a roller. The beads with bound poly A$^+$ RNA were separated employing a Dynal MPC-P-12 magnet (Dynal Biotech). The supernatant was kept for DNA extraction (see below) in order to determine the gender of the blastocyst. After two washes with 100 μl wash buffer A (10 mM Tris–HCl, pH 8.0, 0.15 M LiCl, 1 mM EDTA, 0.1% (w/v) LiDS) and three washes with 100 μl wash buffer B (10 mM Tris–HCl, pH 8.0, 0.15 M LiCl, 1 mM EDTA), the poly A$^+$ RNA was eluted from Oligo $(dT)_{25}$ beads in 10 μl nuclease-free water (Anachem, Luton, UK) by incubating at 65 °C for 2 min. RT was carried out using 80% of the eluted poly A$^+$ RNA in 20 μl reaction comprising 1× RT buffer, Sensiscript Reverse Transcriptase (Qiagen), 20 U RNase inhibitor (Roche), 0.19 μM random hexamers (Promega) and 500 μM of each dNTP (Invitrogen) according to the manufacturer's instructions. To ensure the absence of DNA contamination, the remaining 20% of eluted poly A$^+$ RNA was used in the same reaction except that Sensiscript enzyme was omitted.

Embryo gene expression

Different proportions of single embryo cDNA were used for parallel PCR to detect target gene expression using specific primers (Table 1). PCR was performed in a total volume of 50 μl containing 1× PCR buffer (20 mM Tris–HCl, pH 8.4, 50 mM KCl), 2 mM $MgCl_2$ (Invitrogen), 200 μM of each dNTP (Invitrogen) and 40 pmol of each primer. A hot start was used to ensure the specific amplification of the target gene. The PCR cycles involved one cycle of 94 °C for 3 min, 72 °C for 30 s prior to addition of 2.5 U of Taq DNA polymerase (Invitrogen). PCR was performed for varying numbers of cycles with the denaturing step at 94 °C for 30 s, different annealing temperatures (Table 1) for 30 s and extension step at 72 °C for 45 s. After the last cycle, samples were kept at 72 °C for 10 min. PCR products (10 μl) were separated on 1% agarose gel in the presence of 1 μg/ml ethidium bromide and integrated density values (IDV) generated using a digitised camera system (Alpha Imager 1220) accomplished with AlphaEase software (Alpha Innotech). After subtracting the background intensity for each band, the relative abundance of a given transcript was determined as the ratio of the IDV of that transcript to IDV value of luciferase standard (Miller *et al.* 2003).

Embryo gender determination

After embryo poly A$^+$ RNA binding to magnetic beads in Dynabead extraction, supernatant was transferred to a microfuge tube and 350 μl of nuclease-free H_2O (Anachem) added. The solution was then loaded on a Microcon YM-30 column (Millipore, Watford, UK) and centrifuged at 14 000 ***g*** for 10 min. This procedure was

Table 1 Primers and conditions for the detection of different transcripts.

Genes	Primer sequence (5′→3′)	Annealing temperature, cycle number and amount of embryo equivalent	Product size (bp)	Accession number
Igf2	CCGTGGCATCGTGGAAGAGTG GGAAGGGAAGTGGAGCAGAGA	62 °C, 36 cycles, 0.08	427	X14834.1
Igf2R	TGGCTTGTATTCCTTCTGTAG AGTTGTCTCCTTCCTCTCTGA	62 °C, 38 cycles, 0.16	455	NM_012756
H19	TGATCGGTGTCTCGGAGAGCT GACATGAGCTGGGTAGCACCA	65 °C, 36 cycles, 0.04	325	XR_000314
β-actin	TGACGATATCGCTGCGCTCG GTCCAGACGCAGGATGGCAT	70 °C, 33 cycles, 0.04	532	BC063166
Luciferase	ACTTCGAAATGTCCGTTCGG TCCGGAATGATTTGATTGCC	58 °C, 30 cycles, 0.04	535	M15077
uPA	GTCCTTCAGCAAACCTACAAT CACCTCAAACTTCATCTCTCC	56 °C, 40 cycles, 0.16	476	NM_013085

repeated two more times with 500 μl H_2O added after each spin. The solution in the column was then eluted and the eluant transferred to a new YM-30 column and centrifuged at 14 000 ***g*** for 3 min. Then, 20 μl H_2O was added to each column and resuspended for 30 s. DNA in the column was eluted and added to master mix containing 1× PCR buffer (as above), 1.5 mM $MgCl_2$, 200 μM of each dNTPs and 40 pmol of each primer (Table 2). Then, samples were heated at 94 °C for 8 min, 60 °C for 2 min for three cycles before the addition of 2.5 U Taq DNA polymerase. PCR was performed for 30 cycles consisting of 94 °C for 1 min, 60 °C for 2.5 min and 72 °C for 2.5 min. After the last cycle, the samples were kept at 72 °C for another 10 min. The first stage product (2 μl) was transferred into two tubes each containing 47.5 μl master mix (as above) either with 40 pmol of each *Hprt* and *Zfy* inner primers or with *Sry* inner primers (Table 2). After the addition of 2.5 U Taq DNA polymerase, PCR was performed at the same conditions as described above. After the second stage PCR, 15 μl product was separated in 1.8% agarose gel and DNA bands were visualised under UV illumination.

Collection of fetal and placental samples

On day 20 of gestation, conceptuses were removed from uteri and their position in the horn recorded. Each conceptus was weighed before dissecting into fetus, placenta and yolk sac and subsequently fetal organs for re-weighing. Samples were either snap frozen in liquid nitrogen or in the case of fetal liver stored in RNA later solution (Qiagen). For gene analysis in fetal liver, two fetuses were chosen randomly (prior to gender determination) from the right horn. Placental samples from these fetuses were also used for gene analysis. In addition, two placental samples were also chosen randomly (prior to gender determination) from the left horn. RNA was extracted from these samples using RNeasy Mini Kit (Qiagen). After determining the concentration by Biophotometer (Eppendorf), RNA was used in RNase protection for gene expression analysis. To determine fetal gender, DNA was extracted from liver using DNeasy Tissue kit (Qiagen) and gender determined by PCR as described above for embryos.

Preparation of biotinylated riboprobes

The PCR products of *H19*, *Igf2*, *β-actin* and *28S rRNA* from fetal liver or placenta were cloned into TOPO-2.1 vector according to manufacturer's instructions (Invitrogen). *In vitro* transcription was performed in a final volume of 20 μl containing 1 μg of linearised plasmid, 1× reaction buffer (40 mM Tris, pH 7.9, 6 mM $MgCl_2$, 2 mM spermidine and 10 mM NaCl, Promega), 0.01 M DTT, 2 mM each ATP, GTP and UTP, 0.5 mM CTP, 1 μl RNase inhibitor (10 U/μl, Promega), 0.5 mM Biotin-CTP (Amersham) and 1 μl T7 RNA polymerase (10 U/μl, Promega). Reaction mix was incubated at 37 °C for 1.5 h before treatment with RNase-free DNase I (Promega) at 37 °C for 15 min and the reaction terminated by adding 1 μl 0.5 M EDTA. *In vitro*-transcribed product was separated from unincorporated nucleotides in a 7 M urea, 6% polyacrylamide gel (Invitrogen) at 180 V for 50 min before visualising on Fluor-coated thin layer chromatography plate (Ambion, Huntingdon, UK) at 254 nm. Relevant bands were excised and eluted in probe elution buffer (Ambion).

RNase protection assay

RNase protection assay was carried out using RPA III kit (Ambion). Briefly, 5 μg total RNA from fetal liver or placenta was hybridised with biotinylated riboprobes (6 fmol for *β-actin*, *H19* and *Igf2* and 10.65 pmol for *28S rRNA*) at 42 °C overnight in hybridisation III buffer as described by the manufacturer. After hybridisation, a mixture of 2.5 U/ml RNase A and 100 U/ml RNase T1 was added and incubated at 37 °C for 30 min. Protected hybrids were purified and separated on 7 M urea, 6% polyacrylamide gel (Invitrogen) at 180 V. A 'no RNase' (undigested probes) and 'no target' (probes hybridised with yeast RNA treated with RNase enzyme mix) controls were included in every assay to check probe integrity and the completion of enzyme digestion. Then, bands were transferred to Biodyne B membrane (Pierce, Cramlington, UK) at 30 V for 2 h. Membranes were then air-dried and bands were detected using Supersignal RPA III Chemiluminescent Detection kit (Pierce) as described by the manufacturer. Band intensity was quantified using VersaDoc Imaging System and Quantity One software (Bio-Rad).

Table 2 Outer and inner primer sequences and product size for embryo and fetal gender determination.

Genes	**Primer sequence** (5′→3′)	**Product size** (bp)	**Accession number**
	Outer primers		
Hprt	GTTCTCTTCAATTGCTGGTCCA TGACAACGATTCACACTGCTGA	618	AF001282
Zfy	AAGATAAGCTTGCATAATCACATGCCTATGAAACCCTTTGCTGCACATG	611	X75172
Sry	CACAAGTTGGCTCAACAGAATC AGCTCTACTCCAGTCTTGTCCG	300	X89730
	Inner primers		
Hprt	ATGCTGGTGTTGTCTCTTCAGA ATCTGTCTGTCTCACAAGGGAA	318	
Zfy	GGAAGCATCTTTCTCATGCTGG TTTTGAGCTCTGATGGGTGACGG	207	
Sry	AGCATGCAGAATTCAGAGAT ATAGTGTGTAGGTTGTTGTCC	248	

17β-estradiol and progesterone analysis in maternal serum

Maternal blood samples were taken by cardiac puncture on day 4 of pregnancy at the same time as blastocyst collection. The serum was prepared by centrifuging the blood at 4 °C, 1000 ***g*** for 10 min. The 17β-estradiol was measured by the Estradiol Maia kit (Serono) and progesterone by the Amerlex-M progesterone kit (Amersham). Control sera gave inter-assay coefficients of variation for 17β-estradiol and progesterone of 9.9 and 9.4% respectively.

Bisulphite mutagenesis and sequencing

DNA from day 20 fetal liver was extracted using DNeasy Tissue kit (Qiagen) according to the manufacturer's instruction. DNA samples (4 μg) were bisulphite treated as described by Warnecke *et al.* (1998), and bisulphite-treated DNA was dissolved in 20 μl water and stored at −20 °C until used. PCR was performed using 8 μl bisulphite-treated DNA in a reaction volume of 50 μl containing 2.5 U Taq polymerase, 1× reaction buffer (Invitrogen), 2 mM $MgCl_2$, 200 μM of each dNTPs and 40 pmol of outer primers for H19 differentially methylated region (GenBank AF043428) for 30 cycles at 94 °C for 1 min, 50 °C for 1 min and 72 °C for 1 min. Then, 2 μl of first stage PCR product was amplified for another 30 cycles with the same conditions as the first stage reaction. Outer primers: (4207–4233) 5′ GGTTTTTATGGTTTTTG-GATTTTTAAA 3′ and (4873–4845) 5′ AAAAACCATTCCA-TAAATCCAAATACCTA 3′. Inner primers: (4237–4270) 5′ TTAGTGTGGTTTATTATTAGAAGATGTAGAAGT 3′ and (4844–4802) 5′ TAAACCTAAATAACTTAAAACTTTATCA-CAAAC 3′. PCR products were then gel purified by QIAquick Gel Extraction kit (Qiagen) and subjected to a sequencing using ABI PRISM Big-Dye v1.1 sequencing ready reaction mix (Applied Biosystems, Warrington, UK).

Statistical analysis

Data from blastocysts were not normally distributed and were normalised by $\ln(1+x)$ transformation. The gene expression data sets included either multiple embryos or at least two fetal and placental samples from the same dam. Because of the hierarchical nature of these data, a random effects regression analysis was employed (Stata program, Stata Corporation, College Station, TX, USA), which accounts for the variance between and within dams. Additional parameters such as gender, number of fetuses in the litter and their location in individual horns may also exert an effect on gene expression and were also accounted for estimating the effect of diet on gene expression (Kwong *et al.* 2004, Osmond *et al.* 2005). Thus, data presented (mean ± S.E.M.) as significantly different ($P<0.05$) are independent of these parameters. Conceptus weight data were similarly analysed by random effects regression analysis.

Results

Effect of maternal LPD on fetal and placental growth

On day 20 of gestation, the weights of placenta, fetuses and their organs were determined and the genders of these fetuses were also recorded. Using random effects regression analysis, taking into account the variables of gender, uterine position, litter size and maternal/fetal hierarchy, no significant difference in placental, fetal or organ weights or organ/body weight ratio was detected in relation to diet treatment (Table 3).

H19 expression pattern in rat embryos

The expression pattern of *H19* in mouse preimplantation embryos is controversial. While some reports suggest that it is not expressed until the postimplantation stage, others have indicated the expression at the blastocyst stage (Poirier *et al.* 1991, Szabo & Mann 1995, Doherty *et al.* 2000). Therefore, we first examined the temporal and spatial expression of *H19* and *Igf2* in rat embryos (not previously investigated) from two-cell to blastocyst stages before determining the effect of maternal LPD on embryo imprinted gene expression.

H19 transcripts were first detected at the morula stage (~16–32 cells) with increased band intensity apparent at the blastocyst stage (Fig. 1A). This finding is consistent with those reported for mouse embryos employing RT-PCR in which a major increase in *H19* mRNA was observed between eight-cell and blastocyst stages (Doherty *et al.* 2000). *Igf2* expression was first detected at the compacted eight-cell stage (Fig. 1A).

Both *in situ* hybridisation and RT-PCR data on mouse blastocysts indicate *H19* is only expressed in the trophectoderm cells (Poirier *et al.* 1991, Doherty *et al.* 2000). To determine the spatial distribution of the *H19* transcript in rat blastocysts, ICMs, immediately after immunosurgical isolation, and whole blastocysts were compared. We found *H19* only detectable in whole blastocysts but not ICM samples (Fig. 1B), indicating trophectoderm specificity. Furthermore, another reported trophectoderm-specific transcript, urokinase plasminogen activator (*uPA*) (Harvey *et al.* 1995), gave an equivalent result (Fig. 1B). In contrast, the amplification of *Igf2* transcript was detected in both isolated ICMs and whole blastocysts (Fig. 1B). In conclusion, the results indicate that *H19* and *Igf2* exhibit distinct temporal and spatial expression profiles in rat preimplantation embryos.

Gene expression analysis in blastocysts by semi-quantitative RT-PCR amplification

Although the Dynabead method for poly A^+ RNA extraction and semi-quantitation has been used in single bovine embryo analyses (Miller *et al.* 2003), this is the first report of its use in single rat embryos. This method was selected in our study because it provided a fast and

Table 3 Effect of maternal LPD on placental, fetal, organ weights and body proportion of day 20 rat fetuses.

	18% Casein (n=78, dams=14)	**9% Casein** (LPD) (n=64, dams=12)	**9–18% Casein** (switched diet) (n=76, dams=14)
(A) Male fetuses			
Fetal weight (mg)	3429±29.9	3412±36.5	3429±55.3
Placental weight (mg)	442.69±5.56	410.10±6.35	430.65±6.12
Liver weight (mg)	214.78±3.98	214.47±6.66	216.91±5.21
Heart weight (mg)	18.34±0.34	17.64±0.33	17.76±0.47
Kidney weight (mg)	27.09±0.42	28.05±0.44	28.03±0.55
Fetal/placental weight	7.84±0.12	8.43±0.14	8.03±0.13
Liver/fetal weight (%)	6.27±0.11	6.25±0.17	6.33±0.12
Heart/fetal weight (%)	0.54±0.009	0.52±0.008	0.52±0.010
Kidney/fetal weight (%)	0.79±0.011	0.82±0.009	0.82±0.008
	18% Casein (n=82, dams=14)	**9% Casein** ((LPD) n=72, dams=12)	**9–18% Casein** (switched diet) (n=70, dams=14)
(B) Female fetuses			
Fetal weight (mg)	3357±31.7	3316±29.6	3300±47.8
Placental weight (mg)	433.8±5.37	416.5±5.56	434.3±6.64
Liver weight (mg)	211.12±4.99	214.29±4.48	210.60±4.85
Heart weight (mg)	18.17±0.31	17.63±0.33	17.87±0.40
Kidney weight (mg)	27.51±0.44	27.98±0.49	27.55±0.66
Fetal/placental weight	7.82±0.11	8.04±0.10	7.69±0.13
Liver/fetal weight (%)	6.32±0.15	6.47±0.12	6.36±0.14
Heart/fetal weight (%)	0.54±0.009	0.53±0.009	0.54±0.010
Kidney/fetal weight (%)	0.82±0.011	0.84±0.014	0.83±0.013

efficient way to extract and separate poly A^+ RNA from genomic DNA so that expression and gender analysis could be performed co-ordinately.

In order to compare the relative abundance of gene expression in different samples, it is important to ensure that the PCR amplification is in the linear range. cDNA generated from each individual blastocyst was separated into appropriate amounts according to that required for each of the multiple target genes analysed. PCR was then performed for each target using different cycle numbers and also repeated in two to three different RT-PCR runs to ensure that there was no day-to-day variation. cDNA amplified for 30–38 cycles allowed the detection of individual transcripts without reaching the plateau phase of amplification. Typical semi-log plots of the amount of *H19* and luciferase PCR products in relation to cycle number are shown in Fig. 2A and B. Cycle numbers within the linear range of amplification were selected for individual transcripts. A representative gel photograph of a RT-PCR assay of the transcripts is shown in Fig. 2C.

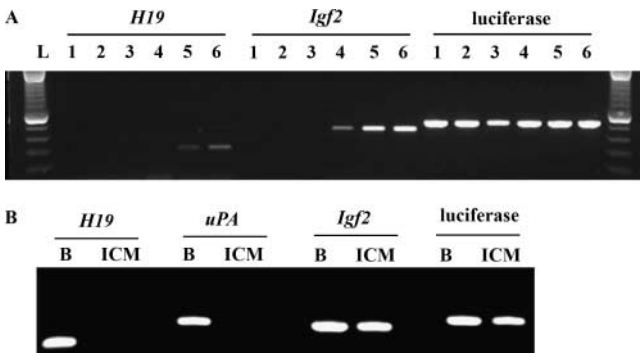

Figure 1 Temporal and spatial expression pattern of *H19* and *Igf2* during rat preimplantation embryo development. (A) *H19* was first detected in morulae whereas the expression of *Igf2* transcript was detected in eight-cell embryos. (B) *H19* expression and urokinase plasminogen activator (*uPA*) detection were confined to the trophectoderm lineage, whereas *Igf2* was present in both lineages. Lane 1, two-cell; 2, four-cell; 3, eight-cell; 4, compacted eight-cell; 5, morula and 6, blastocyst. Lane B, single blastocyst; ICM, three inner cell masses and lane L, 100 bp marker. Experiments were performed twice, in each case using two single embryos/ICM clusters (n=4 in total) for all stages and similar results were obtained.

Sex determination in single blastocysts using supernatant obtained from Dynabead extraction

Our previous observations suggested that pregnant rats fed LPD during the preimplantation period (0–4.25 days of gestation) induced a programming response in a gender-specific manner (Kwong *et al.* 2000). Therefore, embryo gender was determined co-ordinately with the gene expression pattern. Genomic DNA derived from single embryo supernatant after RNA extraction was screened with X and Y chromosome primers for *Hprt*, *Zfy* and *Sry*. In positive controls, 0.1 μg male and female liver DNA were used. PCR product from male DNA had two bands corresponding to *Hprt* and *Zfy* fragments, while female DNA only had the *Hprt* fragment (Fig. 3A). In addition, male DNA showed a band corresponding to the *Sry* fragment in a separate PCR amplification, whereas no product was found in female DNA (Fig. 3B). Hence, rat blastocysts showing *Hprt/Zfy* and *Sry* bands were assigned as male embryos (Fig. 3 lanes 3–5) and blastocysts only with a *Hprt* band were assigned as female embryos (Fig. 3 lane 6).

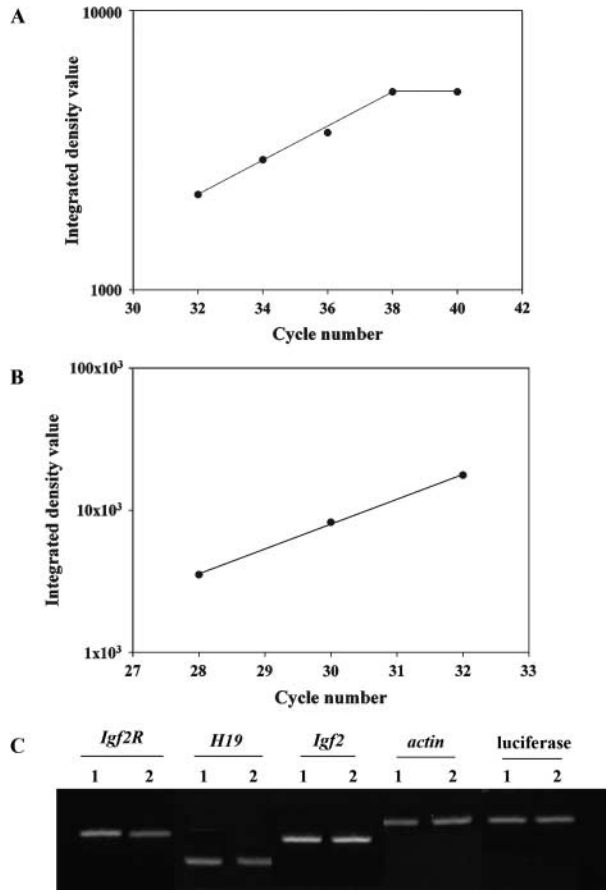

Figure 2 Validity of embryo semi-quantitative RT-PCR assay. Relationship of PCR product and cycle number for (A) *H19* and (B) luciferase transcripts. (C) Gel photograph of different transcripts amplified from two single blastocyst samples (lanes 1, 2).

Effect of maternal LPD on imprinted gene expression in sexed blastocysts

To determine the effect of LPD on gene expression, blastocysts were collected from the control or LPD dams on day 4 of gestation. The number of blastocysts recovered per dam were 9.09±0.53 (n=11) and 10.31±0.49 (n=13) for 18% casein and 9% casein dams respectively. There is no significant difference in embryos recovery number with respect to diet. After Dynabead extraction, *Igf2*, *Igf2R*, *H19*, *β-actin* and luciferase were amplified from single blastocysts (Fig. 2C). The gender of these blastocysts was also determined (Fig. 3A and B; 18%, dams=11, n=42; 9%, dams=13, n=47).

The relative abundance of *H19* transcript was reduced (30%, $P<0.05$; 18%, dams=11, n=30; 9%, dams=11, n=28) in male blastocysts derived from LPD mothers (Fig. 4A). This reduction was not observed in female blastocysts (Fig. 4B; 18%, dams=7, n=12; 9%, dams=10, n=19). In both genders, the relative expression levels of *Igf2*, *Igf2R* and *β-actin* transcripts were not altered by maternal LPD.

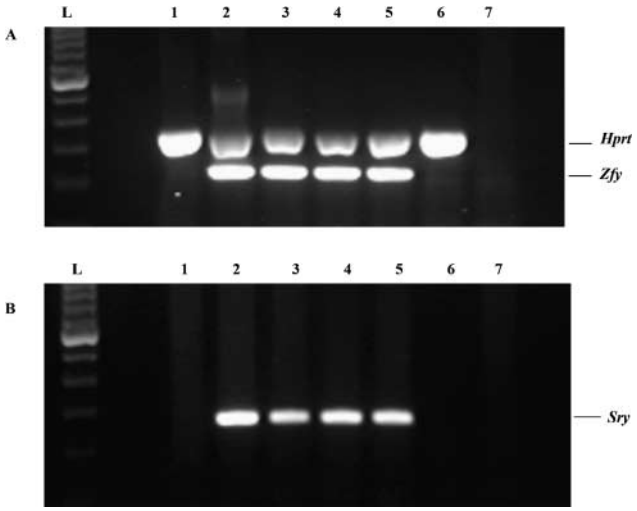

Figure 3 Sex determination in single blastocysts using supernatant obtained from Dynabead extractions. The supernatant was first purified through two YM-30 columns. Nested PCR products were electrophoresed in 1.8% agarose gel and visualised under UV illumination. (A) PCR product using *Hprt* and *Zfy* primers in nested PCR. (B) PCR product using *Sry* primer in nested PCR. Lane 1, 0.1 μg female rat liver DNA; 2, 0.1 μg male rat liver DNA. Lanes 3–6, embryo PCR product from supernatant Dynabeads extraction. Lane 7, reagent only as negative control. L, 100 bp marker.

Effect of maternal LPD on serum steroid hormones

A previous study has shown that steroid hormones alter the expression of *H19* gene in the uterus and in hormone-sensitive mammary MCF-7 cells (Adriaenssens *et al.* 1999). To investigate the possibility that reduction in *H19* expression in blastocysts may result from alteration in maternal steroid hormone concentrations, these were measured in day 4 maternal serum. Diet did not affect the level of either 17β-estradiol (control= 78.0±8.1 pM (n=18); LPD=66.7±8.0 pM (n=17)) or progesterone (control=343.8±24.2 nM (n=8); LPD= 354.9±20.5 nM (n=8)).

Effect of maternal LPD on gene expression in day 20 fetal liver and placenta

Since *H19* gene expression is reduced in male blastocysts from LPD dams, we analysed whether such changes persisted into later development. Hence, experimental dams either remained on LPD until day 20 or were switched back to control diet after preimplantation development (day 4.25). Total RNA was extracted from day 20 fetal liver and placenta, and the expression of *H19*, *Igf2*, *β-actin* and *28S rRNA* genes analysed in the same samples by multiplex RNase protection assay (Fig. 5A). The gender of these fetuses was also determined by PCR. A reduction ($P<0.05$) in relative levels of *H19* (9.4%) and *Igf2* (10.9%) transcripts in male fetal liver from the switched diet

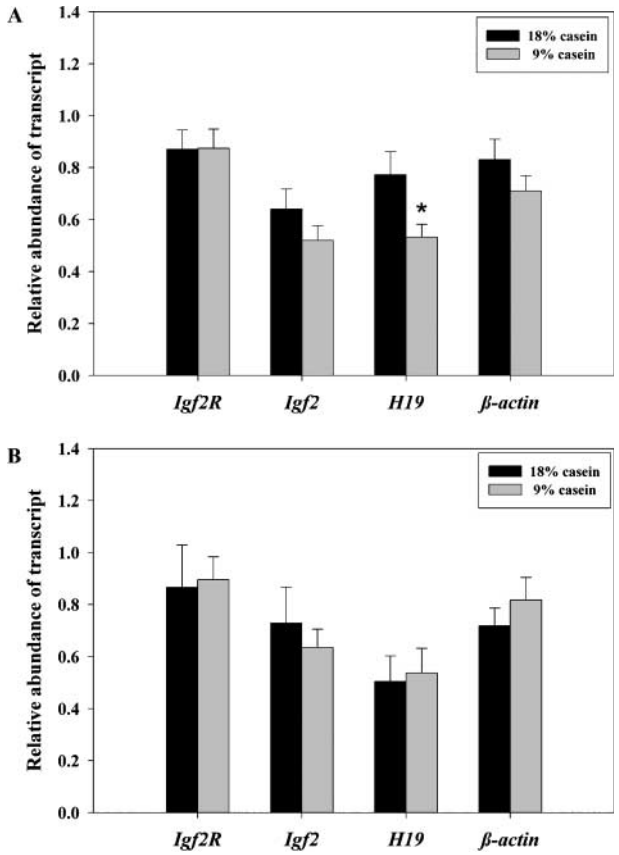

Figure 4 Relative levels of gene expression in (A) male (18% casein, $n=30$, dams$=11$; 9% casein, $n=28$, dams$=11$) and (B) female (18% casein, $n=12$, dams$=7$; 9% casein, $n=19$, dams$=10$) blastocysts. Data are shown as ratio of IDV for specific transcript to IDV for luciferase. Values are expressed as mean$\pm$S.E.M. *$P<0.05$ when compared with control.

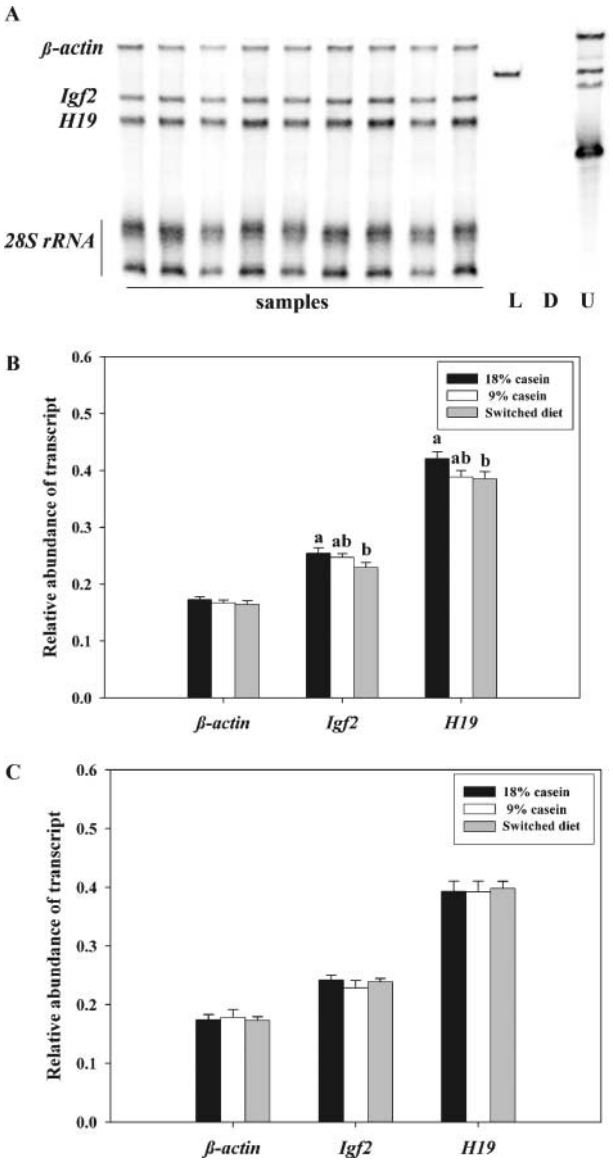

Figure 5 Relative level of gene expression in fetal liver. (A) Representative gel image of RNase protection assay. Lane D, biotinylated probe incubated with yeast RNA and treated with RNase to verify completion of digestion. Lane U, undigested probe. Lane L, *Igf2* biotinylated probe, loaded in every gel to ensure the efficiency of chemiluminescent detection. (B) The relative abundance of genes in male liver (18% casein, $n=11$, dams$=10$; 9% casein, $n=14$, dams$=10$; switched diet, $n=16$, dams$=12$) and (C) in female liver (18% casein, $n=12$, dams$=11$; 9% casein, $n=7$, dams$=6$; switched diet, $n=7$, dams$=7$). The values were calculated as ratio of IDV of specific transcript to IDV for *28S rRNA*. Data are mean$\pm$S.E.M. Different letters denote statistically significant differences, $P<0.05$.

group was observed compared with controls (Fig. 5B; 18%, dams$=10$, $n=11$; 9%, dams$=10$, $n=14$; switched diet, dams$=12$, $n=16$). In contrast, no effect on the levels of these imprinted genes was observed when LPD was maintained throughout gestation. Neither feeding regimen altered the relative expression of *β-actin* (Fig. 5B). No difference in gene expression was detected in female fetal liver in response to dietary manipulation (Fig. 5C; 18%, dams$=11$, $n=12$; 9%, dams$=6$, $n=7$; switched diet, dams$=7$, $n=7$). To investigate whether the reduction in *H19* transcript correlated with the alteration in methylation status of *H19* gene, bisulphite sequencing of differentially methylated region (DMR) (Manoharan *et al.* 2004) located upstream of the promoter was performed (Fig. 6A). By using the bisulphite treatment condition described by Warnecke *et al.* (1998), we found that the conversion of C in non-CpG to T is 100% (Fig. 6B). Preliminary experiments indicated that the maternal diet did not alter the methylation status of this region (Fig. 6B; 18%, $n=3$; 9%, $n=3$; switched diet, $n=6$).

The expression of *H19* and *Igf2* mRNA was also analysed in placenta from the same conceptuses. No differences were observed in the relative level of these genes in placenta in either sex (Fig. 7A and B; male, 18%, dams$=12$, $n=24$; 9%, dams$=11$, $n=24$; switched diet, dams$=12$, $n=26$; female, 18%,

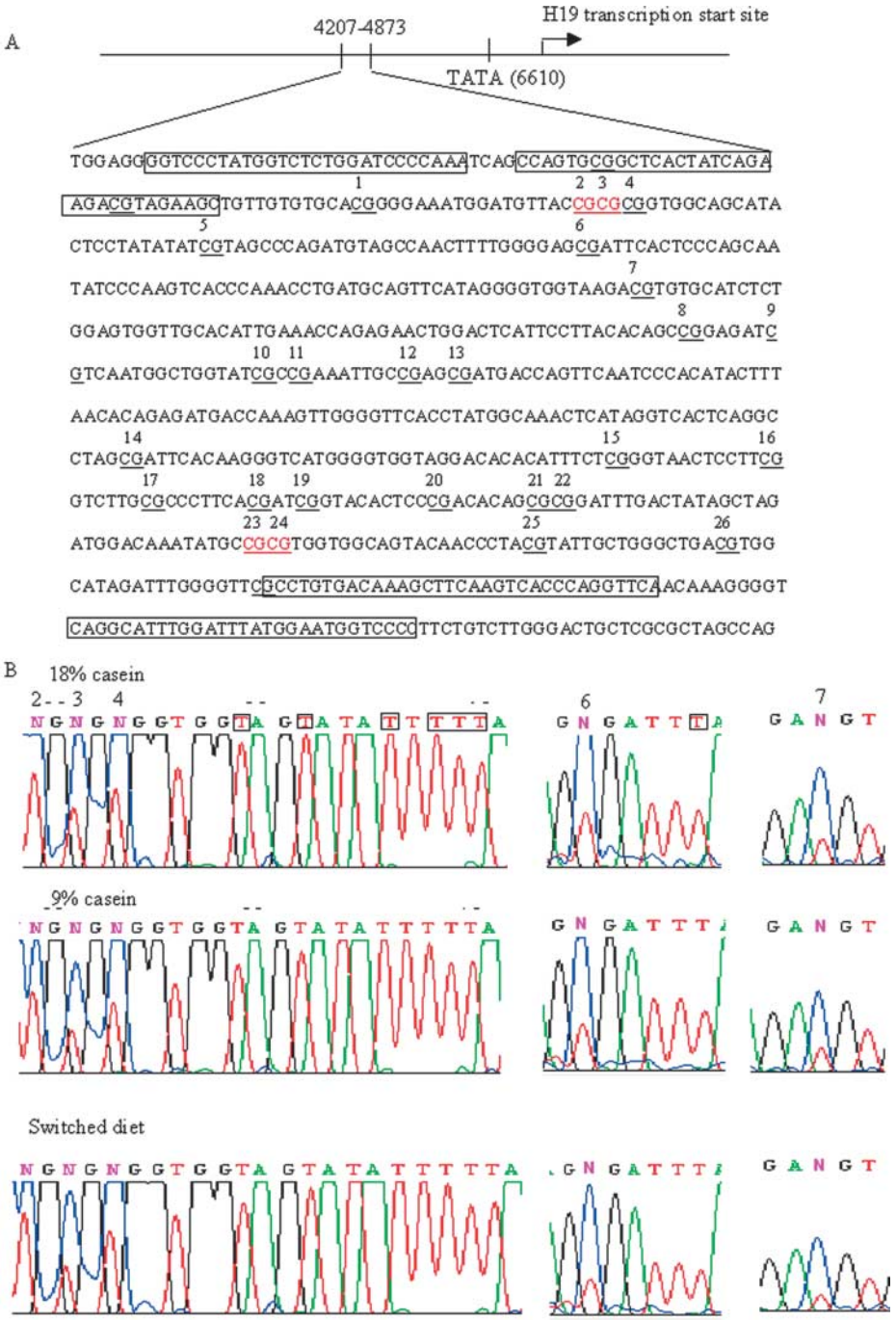

Figure 6 (A) Region of *H19* gene selected for bisulphite sequencing. CpG dinucleotides are numbered and underlined. Sequences within boxes are outer and inner PCR primers. CTCF sites are shown in red. (B) Representative bisulphite sequencing chromatograms of fetal liver samples. N on chromatogram indicates a mixture of C and T in DNA sequence and correspond to CpG sites 2, 3, 4, 6 and 7 (18% casein, $n=3$; 9% casein, $n=3$; switched diet, $n=6$). Bases within boxes are non-CpG cytosines that have been converted to T after bisulphite treatment.

dams=11, $n=23$; 9%, dams=11, $n=18$; switched diet, dams=11, $n=19$).

Since *H19* and *Igf2* genes contribute to growth regulation, the reduced levels of expression in response to maternal LPD may affect *individual* fetal growth despite the absence of effect on *total* fetal weight (Table 3). To test this possibility, a correlation analysis was performed on male fetal weight and the relative levels of *H19* and *Igf2* in the switched diet group. The analysis showed that there was no correlation between *H19* or *Igf2* gene expression and fetal weight (*H19*, $P=0.56$; *Igf2*, $P=0.92$) or combined *H19/Igf2* and fetal weight ($P=0.60$).

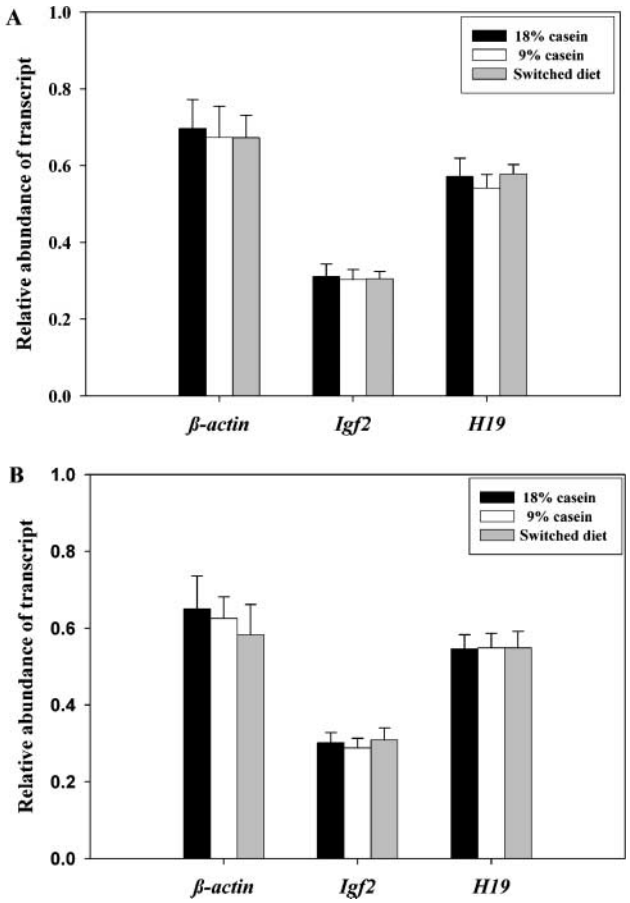

Figure 7 Relative level of gene expression in placenta. (A) male (18% casein, $n=24$, dams$=12$; 9% casein, $n=24$, dams$=11$; switched diet, $n=26$, dams$=12$) and (B) female (18% casein, $n=23$, dams$=11$; 9% casein, $n=18$, dams$=11$; switched diet, $n=19$, dams$=11$). Values are calculated as ratio of IDV of particular transcript to IDV of *28S rRNA*. Data are mean$\pm$S.E.M.

Discussion

Our data show that maternal LPD fed during just the preimplantation period of rat development leads to a decrease in *H19* imprinted gene expression in male blastocysts. At day 20 of gestation, a reduction in *H19* and *Igf2* gene expression was also observed in male fetal liver from the switched diet group (LPD only for the first 4.25 days of gestation), but not the maintained LPD group. There was no effect of any of these feeding regimens on female blastocyst or fetal liver imprinted gene expression. Similarly, the expression of these genes was not affected in placenta of either male or female conceptuses.

H19 belongs to a subgroup of imprinted genes that code for untranslated RNA (Brannan *et al.* 1990). In rat embryos, *H19* transcript is first detected at the morula stage and only in trophectoderm cells of blastocysts. The reduction in *H19* transcript level in male blastocysts likely reflects a specific response to maternal dietary treatment rather than a global effect on blastomere proliferation rate. Thus, rat blastocysts used in this study were collected on day 4.0 for gene expression analysis, and we have shown previously that the number of trophectoderm cells was unaffected by dietary treatment at this stage. In addition, the relative expression of *β-actin*, *Igf2* and *Igf2R* transcripts did not show any changes in the same blastocyst. The more pronounced effect of maternal LPD on *H19* compared with other genes suggests that it is more susceptible to environmental conditions. This relative sensitivity is in agreement with other reports (Doherty *et al.* 2000, Lane & Gardner 2003). As *H19* detection is present in trophectoderm (progenitor of most extraembryonic tissues) but not the ICM (progenitor of embryo proper) at this early stage of development, it is proposed that it plays an important role in extraembryonic tissue proliferation to support nutrient transfer and the survival of the fetus after implantation (Rossant 1986).

The analysis of *H19* and *Igf2* expression at day 20 of gestation revealed that the level of *H19* in placenta is unaffected by dietary treatment, coinciding with no effect of maternal diet on placental weight. However, we cannot exclude the possibility that the transport function of placenta is affected at this stage or at other stages of development. Interestingly, reduction in both *H19* and *Igf2* transcript levels was detected in fetal liver from the switched diet group. Although it is unclear when the reduced level of these genes is initiated, it does indicate that even a brief exposure to LPD, i.e. only during the preimplantation period, may have a long-term impact on fetal phenotype, particular on *H19* since its expression in the embryonic tissues is not initiated until day 8.5 of gestation (Poirier *et al.* 1991). The effect of maternal LPD during the preimplantation period appeared to be gender specific, with both male blastocysts and fetal livers being affected. Such gender-related programming effects have been observed in several studies (Kwong *et al.* 2000, McMullen & Langley-Evans 2005). Although the precise mechanism is unknown, male sensitivity may reflect a faster rate of male embryo development, thus making them more vulnerable to suboptimal environmental conditions (Erickson 1997).

Several lines of evidence have suggested that DNA methylation status can be altered by maternal diet. For example, pregnant mice fed with methyl-supplemented diet containing methionine, betaine, folic acid and vitamin B_{12} at conception increased DNA methylation in the intra-cisternal A particle in the A^{vy} allele of agouti mice offspring (Cooney *et al.* 2002). In another study, Rees *et al.* (2000) have shown that feeding pregnant rats with LPD, which contained excess methionine relative to other amino acids, led to hypermethylation of DNA in fetal liver. However, our results in the present study indicate that DNA methylation in this particular H19 DMR was not altered by maternal diet. Thus, the aberrant expression of *H19* in fetal livers may result from other regulatory mechanisms such as histone acetylation status

rather than alteration in DNA methylation in this region. However, we cannot exclude the possibility that the methylation status of other regions in *H19* gene may be affected by diet.

Expression of *H19* and *Igf2* transcripts is also subjected to regulation by hormones or amino acids. In mouse uterus, it has been shown that 17β-estradiol enhances *H19* expression, whereas progesterone represses it through their effect on the *H19* promoter (Adriaenssens *et al.* 1999). In the present study, however, neither 17β-estradiol nor progesterone levels in serum from pregnant dams was affected by LPD. It is possible that other estrogenic compounds or their receptors may play a role in *H19* expression in blastocysts. Glucocortiocoid has also been shown to suppress *H19* and *Igf2* transcripts levels in rat neonatal liver (Senior *et al.* 1996) and *IGF2* in fetal sheep (Li *et al.* 1993). Given that maternal undernutrition can lead to alteration in steroid hormone levels (Gonzalez *et al.* 1997, Fernandez-Twinn *et al.* 2003), this may in turn affect *H19* and/or *Igf2* gene expression. Amino acids have also been shown to affect the expression of genes such as *Igf2*, *Igf2R* and *Igf1* in preimplantation embryos (Ho *et al.* 1995). Moreover, alteration in maternal serum amino acid concentrations in response to LPD (Kwong *et al.* 2000, Petrie *et al.* 2002) may influence the expression of *H19* (Doherty *et al.* 2000) and *Igf2* expression (Straus & Takemoto 1988).

H19 and *Igf2* genes are thought to be regulated co-ordinately and reciprocally as described by the 'enhancer competition model' (Bartolomei *et al.* 1993). Based on this model, the reduction in *H19* in both male blastocysts and fetal livers may be expected to associate with an increase in *Igf2* expression. Unexpectedly, a reduction in both *H19* and *Igf2* transcript levels in blastocysts and livers was observed. Such a non-reciprocal expression of *H19* and *Igf2* has also been reported in other studies. Fetuses from mouse embryos cultured in the presence of serum had 31 and 15% reduction in *H19* and *Igf2* respectively compared with *in vivo* counterparts (Khosla *et al.* 2001*b*). In addition, fetal liver from pregnant ewes fasted for 48 h showed a 61% reduction in *H19* without any alteration in *IGF2* expression (Naimeh *et al.* 2001). It is possible that the expression of *H19* and *Igf2* are regulated independently under certain circumstances. In fact, the transcription of *Igf2* can be modulated by the degree of methylation in DMR within the *Igf2* gene (Feil *et al.* 1994). In addition, several studies have shown that *H19* and *Igf2* transcripts can be regulated posttranscriptionally (Straus & Takemoto 1988, Jouvenot *et al.* 1999).

H19 encodes an untranslated RNA and its function remains unsolved. Some experiments suggest that it plays a growth control role (possibly via controlling IGF2 expression) during embryogenesis. Thus, deleting maternal H19 allele in mice led to 27% increased in birth weight compared with their wild type littermates. These animals also showed an elevated *Igf2* transcript level in their tissues compared with wild type (Leighton *et al.* 1995*a*). In the present study, reduction in hepatic *H19* and *Igf2* gene expression in the switched diet group did not show any observed effect on fetal and organ weights nor did it influence the organ-to-body weight ratio. Statistical analysis also showed that there was no correlation between the decrease in expression of these genes and individual fetal growth. This may suggest that the level of reduction is insufficient to impede fetal growth. Alternatively, alteration in the availability of insulin-like growth factor binding proteins may account for the lack of effect on fetal growth (El-Khattabi *et al.* 2003). However, the reduction in *Igf2* (and possibly protein level) and *H19* may have a bigger impact on growth during the last 2 days of gestation. During this time, the weights of fetuses were nearly doubled (Langley-Evans *et al.* 1996). It is possible that the reduction in expression of these genes may lead to reduce weight gain or altered liver development during this rapid phase of growth. Such a possibility may explain the reduced birth weight, altered postnatal growth trajectory and reduced male offspring liver growth observed in the switched diet treatment, we have reported previously (Kwong *et al.* 2000). In relation to this speculation, mice with targeted disruption of *Igf2* gene in liver, kidney and gut exhibit a 20% reduction in birth weight (Leighton *et al.* 1995*b*).

The effect of maternal LPD on imprinted gene expression was only seen in male fetal liver from the switched diet group but not in those fed with LPD for 20 day of gestation. The present findings are consistent with those reported by El-Khattabi *et al.* (2003) in which LPD fed to pregnant rats for 21.5 day of gestation did not alter *Igf2* mRNA in LPD fetal liver. Although the mechanism by which maternal LPD at the preimplantation embryo stage can perpetuate into later gestation or postnatal life is unclear, it may indicate that a 'predictive adaptive response' can be initiated in the preimplantation embryo (Gluckman *et al.* 2005). By this concept, embryos may be able to control the growth rate or pattern of metabolism to maximise survival opportunity during future development relative to nutrient availability. However, the mismatch between preimplantation and postimplantation diets may distort such mechanisms. Consistent with this view, we have shown that programming of male hypertensive offspring can be induced irreversibly by maternal LPD fed only at the preimplanation period of development (Kwong *et al.* 2000).

In conclusion, our data show that maternal LPD for just the first 4 days of gestation can alter the pattern of expression of growth regulating imprinted genes from the blastocyst stage onwards. This early and maintained response to maternal LPD is gender specific and may contribute to changes in growth detected previously during the postnatal life (Kwong *et al.* 2000).

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