Maternal nutrient restriction in early pregnancy programs hepatic mRNA expression of growth-related genes and liver size in adult male sheep

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Abstract

The liver is a major metabolic and endocrine organ of critical importance in the regulation of growth and metabolism. Its function is determined by a complex interaction of nutritionally regulated counter-regulatory hormones. The extent to which hepatic endocrine sensitivity can be programed *in utero* and whether the resultant adaptations persist into adulthood is unknown and was therefore the subject of this study. Young adult male sheep born to mothers that were fed either a control diet (i.e.100% of total live weight-maintenance requirements) throughout gestation or 50% of that intake (i.e. nutrient restricted (NR)) from 0 to 95 days gestation and thereafter 100% of requirements (taking into account increasing fetal mass) were entered into the study. All mothers gave birth normally at term, the singleton offspring were weaned at 16 weeks, and then reared at pasture until 3 years of age when their livers were sampled. NR offspring were of similar birth and body weights at 3 years of age when they had disproportionately smaller livers than controls. The abundance of mRNA for GH, prolactin, and IGF-II receptors, plus hepatocyte growth factor and suppressor of cytokine signaling-3 were all lower in livers of NR offspring. In contrast, the abundance of the mitochondrial protein voltage-dependent anion channel and the pro-apoptotic factor Bax were up regulated relative to controls. In conclusion, maternal nutrient restriction in early gestation results in adult offspring with smaller livers. This may be mediated by alterations in both hepatic mitogenic and apoptotic factors.

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Introduction

Fetal hepatic growth is sensitive to maternal nutrient intake, particularly, during late gestation, when a reduction in food consumption can markedly compromise liver size at birth (Bauer *et al.* 1995). This change in fetal configuration is accompanied by a range of endocrine adaptations including a decrease in plasma insulin-like growth factor (IGF)-I and IGF-II and increased growth hormone (GH) concentration (Bauer *et al.* 1995). In contrast, maternal nutrient restriction between early to mid gestation, coincident with the period of maximal placental growth, has no effect on fetal body or liver mass in singleton fetuses (Whorwood *et al.* 2001), although this has been observed when both singleton and twin pregnancies were studied (Vonnahme *et al.* 2003). However, nutrient restriction at this stage results in an age-dependent increase in hepatic mRNA abundance for IGF-I, IGF-II, and the GH receptor (GHR; Brameld *et al.* 2000). These adaptations are accompanied by enhanced hepatic glucocorticoid sensitivity as mRNA abundance for the glucocorticoid receptor (GR) is increased, whereas both enzyme activity and mRNA abundance of 11β-hydroxysteroid dehydrogenase (11βHSD) type 2, which catalyzes the inactivation of cortisol to inert cortisone, is reduced (Whorwood *et al.* 2001). These responses within the fetus may be mediated in part by changes in maternal and therefore fetal cortisol exposure although whether this is due to an increase (Edwards & McMillen 2001) or decrease (Bispham *et al.* 2003) in maternal plasma cortisol remains in doubt.

Cortisol acting through the GR has been shown to have a critical role in regulating the development of the fetal liver in preparation for life after birth. This includes the onset of hepatic gluconeogenesis (Gardner *et al.* 2001, Forhead *et al.* 2003) in conjunction with an increase in mRNA abundance of a number of genes that can potentially regulate liver function including IGF-I, IGF-II (Forhead *et al.* 2000), and

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prolactin receptors (PRLR; Hyatt *et al.* 2004). Activation of these receptors subsequently regulates a range of cell signaling pathways, including JAK/Stat (Harding *et al.* 2000), the negative feedback loop initiated from changes in abundance of suppressor of cytokine signaling (SOCS) molecules (Ram & Waxman 1999). These pathways are nutritionally sensitive, being down regulated by fasting in 6-week-old rats (Beauloye *et al.* 2002). However, it is not known whether this occurs in the adult offspring as a consequence of previous exposure to maternal nutrient restriction. Other growth factors important in regulating liver growth include the hepatocyte growth factor (HGF; Duncan *et al.* 2004) and its receptor c-met (Bladt *et al.* 1995). HGF can regulate liver mass by acting as a mitogen and survival factor as well as by inducing apoptosis (Santoni-Rugui *et al.* 1996, Conner *et al.* 1997). The rate of apoptosis is also determined by the abundance of both pro- (e.g. Bax) and anti- (e.g. Bcl-2) apoptotic genes (Nakagami *et al.* 2002). Furthermore, the outer mitochondrial protein voltage-dependent anion channel (VDAC) has been implicated in the mitochondrial control of this process (Madesh & Hajnoczky 2001, Zhang *et al.* 2001). It is currently not known whether these processes are susceptible to long-term nutritional programming.

The abundance of mRNA for growth factor genes is established to be nutritionally regulated in those fetal tissues whose mass is altered at birth. For example, up regulation of mRNA for both IGF-I receptor (R) and IGF-IIR in adipose tissue is accompanied by enhanced fat stores in the term fetus (Bispham *et al.* 2003). To date, however, there have been no studies that have examined the longer term consequences of fetal exposure to maternal nutrient restriction from the time of conception up to mid gestation on later liver mass or function. This is important as it is now established that exposure to an adverse nutritional environment at specific stages of fetal development can have differential long-term consequences with respect to later disease risk in the resulting offspring (Barker 1998). To this extent, perturbed liver growth and later function have been associated with increased plasma concentrations of low density lipoprotein (LDL) cholesterol, fibrinogen, and factor VII (Barker *et al.* 1992, Roseboom *et al.* 2000) that could potentially contribute to metabolic complications in adult life (Barker 1998, Roseboom *et al.* 2001). We have previously shown that maternal nutrient restriction commencing from the time of either conception or uterine attachment has no effect on weight at birth, postnatal growth, or later body composition but does result in pronounced cardiovascular (Gopalakrishnan *et al.* 2004), but not metabolic (Gardner *et al.* 2005), adaptations during young adulthood that are potential precursors of later disease. The aim of the present study was therefore to extend these investigations by examining whether they are accompanied by changes in liver mass, GH–IGF sensitivity, and apoptotic potential as assessed by determining liver weight and mRNA abundance for hepatic GH, IGF-I and IGF-II receptors, and expression of apoptotic genes in control and nutrient-restricted offspring.

Materials and Methods

Animals and diets

Eighteen mature Scottish Blackface sheep of similar age, live weight, and body condition score were studied. Only sheep with male offspring were included in the present study and these were taken from a larger animal cohort for which female offspring were not followed up to 3 years of age. Following mating, the mothers were randomly allocated to receive either a control (C; $n=8$) or a nutrient-restricted (NR; $n=10$) diet. Control mothers were fed 100% metabolizable energy requirements for live weight maintenance (8·0 MJ/day) as defined by the Agricultural and Food Research Council (AFRC 1993), while NR sheep were initially fed to 50% of that amount (4·0 MJ/day) until 95 days gestation. Thereafter, all animals were fed to 100% of requirements with daily rations being increased fortnightly according to the changing requirements associated with the predicted increase in conceptus weight for a singleton bearing ewe (AFRC 1993). All diets were fed according to current body weight. The basal diet consisted of an average of 250 g (fresh weight) hay per day (1 MJ/day) and 650 g (fresh weight) dried grass pellets per day (7 MJ/day; pelleted ration, North Eastern Farmers Ltd., Aberdeen, UK). The average NR diet consisted of a similar portion of hay (i.e. 250 g) but reduced pellet intake of 280 g (3 MJ/day). All diets contained an adequate amount of vitamins (vitamin A, 8121 mg/kg; D, 2005 mg/kg; and E, 50 mg/kg diet) with minerals provided as blocks ('Baby Rockies', Tithebarn Ltd, Winsford, Cheshire, UK; containing magnesium, 1 g/kg; iron 200 mg/kg; manganese, 100 mg/kg; iodine, 50 mg/kg; zinc, 120 mg/kg; cobalt, 100 mg/kg; selenium, 20 mg/kg; and sodium, 38%). All mothers remained healthy throughout pregnancy, ate normally, and showed no symptoms of pregnancy toxemia. At term, all offspring were delivered naturally with no intervention, and birth were weights recorded. The male offspring remained intact (i.e. were not castrated) and were reared with their mother until weaning at 16 weeks of age and thereafter grass-fed at the Macaulay Institute Glensaugh Research Station, Aberdeen until 3 years of age. Throughout their life, there were no differences in mean body weights between nutritional groups. The animals that were entered into this study were those whose long-term cardiovascular outcomes we have previously published (Gopalakrishnan *et al.* 2004) plus a further animal from the same cohort for which blood pressure measurements were not obtained due to catheter blockage. At 3 years of age, all animals were catheterized via the jugular vein, to enable non-invasive blood sampling, which included six post-prandial samples (i.e. taken hourly on the same day between 1 and 6 h after feeding) taken for the measurement of plasma IGF-I concentration. During this period, each animal was fed 1 kg chopped hay per day plus 500 g pelleted food. All animals were then killed and their livers plus all other major organs (for full details see Gopalakrishnan *et al.* 2004) were rapidly dissected, weighed,

and a representative portion of the liver (i.e. 20 g from the same position of the right lobe from each animal) placed in liquid nitrogen and stored at −80 °C until further analysis. All procedures were performed with the necessary institutional ethical approval as designated under the UK Animals (Scientific Procedures) Act, 1986.

Laboratory analyses

mRNA detection Total RNA was isolated from a central region of the right lobe (see above) using Tri-Reagent (Sigma). Total RNA concentration (μg/μl) was determined by measuring optical density absorbance at 260 nm with a nanodrop. Then, using this value together with the volume (μl) of water needed to dissolve the initial RNA pellet and total liver mass, total RNA content was calculated for each animal (Gnanalingham *et al.* 2005). RNA integrity was further assessed by running on a denatured agarose/formaldehyde gel electrophoresis and 28S and 18S rRNA visualized in a 2:1 ratio. In order to maximize sensitivity, a two-tube approach to reverse transcription (RT) was adopted as previously described by Bispham *et al.* (2005) and Gnanalingham *et al.* (2005). The conditions used to generate first strand cDNA RT were: 70 °C (5 min), 4 °C (5 min), 25 °C (5 min), 25 °C (10 min), 42 °C (1 h), 72 °C (10 min), and 4 °C (5 min). The RT reaction (final volume 20 μl) contained: buffer (250 mM Tris–HCl, 40 mM $MgCl_2$, 150 mM KCl, 5 mM dithioerythritol; pH 8·5), 2 mM dNTPs, 1× hexanucleotide mix, 10 units RNase inhibitor, 10 units M-MLV reverse transcriptase, and 1 μg total RNA. All these commercially available products were purchased from Roche Diagnostics Ltd. Each RT reaction yielded 2 μg/μl of cDNA for use in semi-quantitative PCR.

The expression of each gene was determined by RT-PCR, as previously described (Gnanalingham *et al.* 2005). The analysis used oligonucleotide cDNA primers to each gene under test by generating specific exon–intron spanning products (Table 1). Briefly, the PCR program consisted of an initial denaturation (95 °C (15 min)), amplification (stage I, 94 °C (30 s); stage II, annealing temperature (30 s); stage III, 72 °C (60 s) and final extension (72 °C (7 min); 8 °C 'hold'). The PCR mixture (final volume 20 μl) contained 7 μl DEPC H_2O, 10 μl Thermo-Start PCR Master Mix (50 μl contains 1·25 units Thermo-Start DNA Polymerase, 1× Thermo-Start reaction buffer, 1·5 mM $MgCl_2$ and 0·2 mM each of dATP, dCTP, dGTP, and dTTP, catalog number AB-0938-DC-15 ABgene), 1 μM forward primer, 1 μM reverse primer, and 1 μl RT (2 μg of cDNA) product. For 18S PCR reactions, 1 μl of the optimized 18S primer dilution (18S PCR primer mix (5 μM) obtained from Ambion) was serially diluted in nuclease-free water and dose-response PCRs performed to determine the optimal primer dilution needed to amplify 18S PCR product in the same linear range as the target gene transcript (Table 1). The annealing temperature and cycle numbers of all primers were optimized so as to be in the linear range (see Table 1) and the intra- and interassay coefficients of variation were 2·3–4·8% and 5·6–9·2% for all 18 animals for each gene set respectively. A representative standard curve of mRNA detection is illustrated in Fig. 1. It was not possible to make mRNA measurements of VDAC, as there is currently insufficient detail of its precise gene sequence in the ovine species.

Agarose gel electrophoresis (2·0–2·5%) and ethidium bromide staining confirmed the presence of a single amplicon for both target genes and 18S at the expected sizes. Densitometric analysis was performed on each gel by image detection using a Fujifilm LAS-1000 cooled charge-coupled device camera and mRNA abundance determined for each gene. Consistency of lane loading for each sample was verified from the measurement of 18S rRNA. All results were then expressed as a ratio of a reference sample (liver tissue cDNA from of 1-day-old lamb) included on all gels. All analyses and gels were performed in duplicate with appropriate positive (same as reference sample) and negative (no RNA (RT stage) and no RT (PCR stage)) controls and a range of molecular weight markers. The average of duplicate PCR experiments was used for statistical analysis. The coefficient of variation (intra and inter) was below 10% for all 18 animal samples for each gene set. The resultant PCR product was extracted using a commercially available kit (QIAquick gel extraction kit, catalog number 28704; Qiagen) and 20 ng of RT-PCR gel purified amplicon (quantified using a nanodrop) together with 1 pg of the corresponding primer set (forward and reverse primers) were submitted to Qiagen DNA sequencing centre, Germany, for analysis. DNA sequence results were cross-referenced against accession numbers used for primer design (Table 1) and Genebank database to determine specificity of primers. In addition, we undertook analyses for mRNA abundance of IGF-IR and Bcl-2 and, although these were highly abundant in the relevant positive control samples i.e. RNA from fetal liver and adult spleen respectively, they were both undetectable in adult liver samples from the present study.

Protein detection

Mitochondria, crude plasma membranes, and whole cell lysates were all prepared from 0·5 g of frozen liver tissue (Nevalainen *et al.* 1996, Budge *et al.* 2000). Their protein contents together with that of the initial liver homogenate were then determined (Lowry *et al.* 1951). These results were then used to calculate total liver protein. For each assay, 20 μg of each protein preparation were loaded on each 6–10% SDS-polyacrylamide gel for every sample (6%, IGF-IIR, GHR, and 10% GR). Densitometric analysis of Ponceau red staining of all membranes confirmed that equal amounts of protein were transferred before immunodetection (Mostyn *et al.* 2003). The expression of VDAC was determined using an antibody raised in rabbits to ovine VDAC (purified from the kidney) at a dilution in the ratio 1:2000 (Mostyn *et al.* 2003). Western blot analysis of plasma membrane and whole cell lysate preparations were also performed to confirm that mRNA abundance of each candidate gene was translated into protein, where appropriate antibodies

Table 1 Primer sequences and optimal PCR conditions used in the adult sheep liver

	Accession number	Position	Product size (bp)	Primer sequence	Annealing temperature (°C)	Cycle number[a]	18S dilution[a]
Primer set							
GHR	Ovine sequence M82912	943–962 bp	315	F 5′-ATG AAC CCA TCT GCA TGT GA-3′	59·7	29	1:3
		1258–1236 bp		R 5′-TTC AGT CTT CTC ATC AGG GTC A-3			
PRLR (all forms)	Ovine sequence AF041977	184–203 bp	586	F 5′-CTG ACT TAC CGC AAG GAA GG-3′	59·9	34	1:7
		769–750 bp		R 5′-CCA CTG CCC AGA CCA TAA TC-3′			
PRLR-L	Ovine sequence AF041257	693–713 bp	200	F 5′-CCA GAT ACC TAA TGA CTT CCC-3′	60	32	1:5
		893–873 bp		R 5′-TCT TCG GAC TTG CCC TTC TCC-3′			
PRLR-S	Ovine sequence AF041977	672–879 bp	229	F 5′-CCA GAT ACC TAA TGA CTT CCC-3′	60	31	1:5
		900–879 bp		R 5′-GCC CTT CTA TTA AAA CAC AGA C-3′			
SOCS-3	Gift from Professor Caroline McMillen, Adelaide, Australia		164	F 5′-GCT GGT GGT GAA CGC AGT GC-3′	58	34	1:7
				R 5′-GGG TCT TGA CGC TGA GCG TG-3′			
IGF-I	Ovine sequence M31735	46–65 bp	401	F 5′-CCC ATC TCC CTG GAT TTC TT-3′	58·1	30	1:3
		446–427 bp		R 5′-ACA TCT CCA GCC TCC TCA GA-3′			
IGF-IR	Bovine sequence X54980	278–297 bp	498	F 5′-GCC TCC AAC TTT GTC TTT GC-3′	58·1	33	1:7
		765–746 bp		R 5′-GCT GAA ATA CTC CGG GTT CA-3′			
IGF-II	Ovine sequence M89789	945–964 bp	248	F 5′-TCA CAG CAG GAA AGT CGA TG-3′	59·1	28	1:3
		1192–1173 bp		R 5′-GGC ACA GTA AGT CTC CAG CA-3′			
IGF-IIR	Ovine sequence AF327649	5–24 bp	401	F 5′-ACC GGC ACTTCA ACT ACA CC-3′	60	32	1:5
		405–386 bp		R 5′-ACT CAG AAT GAC GGC TTC GT-3′			
HGF	Ovine sequence AF213397	1–20 bp	314	F 5′-ATT TGG CCA TGA ATT TGA CC-3′	60	32	1:5
		314–296 bp		R 5′-TCG ATA ACT CTC CCC ATT GC-3′			
c-Met	Ovine sequence AF213398	220–240 bp	352	F 5′-CGG TCT TCA AGT AGC CAA GG-3′	60	30	1:3
		572–541 bp		R 5′-ACC AGT TCA GAA AAC GGA TGG-3′			
Bax	Ovine sequence AF163774	70–91 bp	164	F 5′-CAG GAT GCA TCC ACC AAG AAG C-3′	59·2	32	1:5
		233–210 bp		R 5′-TTG AAG TTG CCG TCG GAA AAC ATT-3			
GR (type 2)	Ovine sequence X70407	402–422 bp	150	F 5′-ACT GCC CCA AGT GAA AAC AGA-3′	59·7	35	1:7
		552–531 bp		R 5′-ATG AAC AGA AAT GGC AGA CAT T-3′			
11βHSD-1	Ovine sequence GI57164386	139–160 bp	160	F 5′-GTG CCA GAT CCC TGT CTG GAT-3′	60·4	29	1:3
		299–280 bp		R 5′-AGC GGG ATA CCA CCT TCT TT-3′			
11βHSD-2	Ovine sequence AH005138	764–784 bp	260	F 5′-CGC ATT GTG ACC GTA AGC-3′	58·4	32	1:5
		924–904 bp		R 5′-CAG GCA GGC AGG ATG ATG-3′			
18S			324	Ambion Classic II 18S Internal Standards Catalogue no. 1717			

Growth hormone receptor (GHR); total prolactin receptor (PRLR); prolactin receptor-long (PRLR-L) and –short (PRLR-S); suppressor of cytokine signalling-3 (SOCS-3); insulin-like growth factor-I (IGF-I) and –type I receptor (IGF-IR); insulin-like growth factor-II (IGF-II) and type –II receptor (IGF-IIR); hepatocyte growth factor (HGF); glucocorticoid receptor (GR); 11β-hydroxysteroid dehydrogenase (HSD) type 1 and 2. All primer sets underwent strict optimisation processes to determine optimal annealing temperature and cycle number needed to ensure that all PCR products are amplified in the linear range. Target gene and internal control 18S PCR reactions were performed at the same time on the same RT reactions in order to minimise potential impact on amplification efficiency.
[a]The number of cycles and 18S primer dilution used for PCR to amplify both the target gene and 18S at a linear rate.

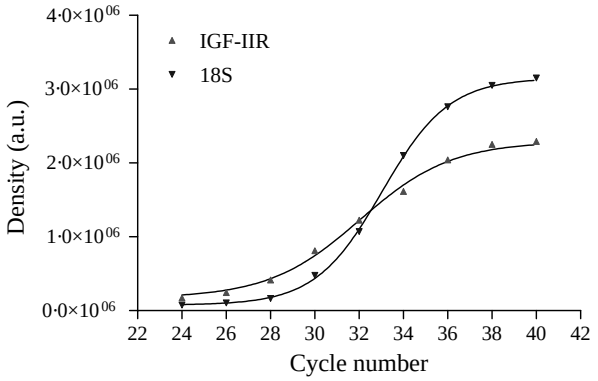

Figure 1 Representative dose response curve for the optimization of PCR cycle number for amplification of IGF-IIR and 18S using the optimal 60 °C annealing temperature. IGF-IIR: log (EC_{50}) 32 cycles, 95% confidence intervals 31·1–32·7, $R^2=0·99$; 18S: log (EC_{50}) 32·7 cycles, 95% confidence intervals 32·3–33·1, $R^2=0·99$.

for use on ovine samples were available. Antibodies were tested for GHR (two polyclonal GHR antibodies raised to the transmembrane domain of GHR were tested (anti-rabbit (RDI-GHRabm-5, Research Diagnostics Inc., Division of Fitzgorald Industries, Concord, MA, USA) and anti-mouse (gift from G Thordarson, University of California, Santa Cruz)), IGF-IIR (polyclonal IGF-IIR – a gift from P Lobel, Rutgers University, New Jersey as previously described (Young *et al.* 2001)), and GR (Santa Cruz rabbit polyclonal IgG cat no. SC 8992 raised to the internal domain of GRα). All antibodies were tested at a range of dilutions from 1:250 to 1:1000 under both mild-reducing and non-reducing conditions as recommended (Ilkbahar *et al.* 1999) using up to 80 μg of plasma membrane and/or whole cell lysate. However, none of the antibodies tested produced a consistently detectable signal in ovine samples, which was in accord with the predicted molecular mass for each product (GHR: 85 kDa, IGF-IIR: 120 kDa, and GR: 95 and 90 kDa). However, the necessary positive controls (concentrated purified GHR and GR protein) did react as expected. Our lack of success with these GHR and GR antibodies is therefore due to a lack of cross-reactivity and not due to technical failure. Owing to the previous success (Young *et al.* 2001) of the IGF-IIR in detecting ovine proteins and a lack of purified IGF-IIR)) hepatic plasma membrane prepared from 1-day-old lamb was used as a positive control. Failure of IGF-IIR antisera to detect ovine proteins was due to a technical failure of using an older antibody 'batch'. Specificity of detection was confirmed using non-immune rabbit serum. All gels were run in duplicate and included a range of molecular weight markers, and a reference sample (newborn and adult ovine liver samples) allowed comparison between gels. Densitometric analysis for VDAC detection was performed on each gel following image detection using a Fujifilm LAS-1000 cooled CCD camera (Fuji Photo Film Co. Ltd, Tokyo, Japan) and all values were expressed in densitometric units.

Liver dry weight and plasma IGF-I analyses

Liver dry weight was determined by freeze drying a representative portion. The concentration of plasma IGF-I was determined by ELISA (Heasman *et al.* 2000).

Statistical analyses

The data were first tested for normality of distribution using a Kolmorgorov–Smirov test. Data were not of normal distribution and therefore appropriate non-parametric tests were used. A Mann–Whittney *U*-test was used to statistically determine the effect of maternal nutrient restriction upon gene expression. Linear associations were described by Spearman's rank correlation coefficient. All data are expressed as means ± S.E. unless otherwise stated. Statistical comparisons were conducted using SPSS 11.1 (SPSS, Chicago, IL, USA). For all comparisons, statistical significance was accepted when $P<0·05$.

Results

Liver mass and composition

There was no effect of maternal nutrient restriction on either birth (C, 4·43 ± 0·15 (mean ± S.E.; $n=8$); NR, 4·0 ± 0·33 kg ($n=10$)) or adult live body weight (C, 75·63 ± 2·76; NR, 75·0 ± 2·6 kg). Both fresh and dry liver weights were significantly lower in NR offspring, irrespective of whether these values were expressed in absolute or relative terms (Table 2), but there were no differences in either total (or fractional (data not shown)) hepatic RNA, protein or mitochondrial protein content between groups. All other organ weights (i.e. adipose tissue, adrenal, heart, kidney, lung,

Table 2 Effect of early to mid gestational maternal nutrient restriction on the liver wet and dry weights, plus total protein, mitochondrial and RNA content as measured in the adult offspring born to mothers that consumed 50% (nutrient restricted (NR) $n=10$) or 100% (control (C) $n=8$) of their metabolisable energy requirements for maternal metabolism and fetal growth between 0 and 95 days gestation. Values are means with their standard errors

	Control	Nutrient restricted	*P* value
Liver wet weight (g)	1262 ± 38	1118 ± 36	0·017
Relative liver wet weight to body weight (g/kg)	16·28 ± 0·61	14·9 ± 0·48	0·035
Liver dry weight (g)	916 ± 30	785 ± 23	0·003
Relative liver dried weight to body weight (g/kg)	12·18 ± 0·47	10·54 ± 0·37	0·015
Protein (g)	265·1 ± 13·7	230·4 ± 6·1	NS
Mitochondria (g)	25·8 ± 3·6	23·0 ± 2·0	NS
RNA (g)	1·10 ± 0·13	1·04 ± 0·12	NS

For all analyses, statistical significance was accepted when $P<0·05$ as assessed by Mann–Whittney *U*-test. NS, not significant.

liver, pancreas, and spleen) were similar in the two nutritional groups (Gopalakrishnan *et al.* 2004). Food intake was similar in all animals during the 2–3 week period, over which *in vivo* measurements were made (data not shown) as were plasma IGF-I concentrations between groups (C, 12·8±0·8; NR, 13·7±0·6 nmol/l).

Cytokine receptors, HGF, GR, and IGF ligand mRNA abundance

The mRNA abundance for the GH, prolactin (PRL; all forms), and IGF-II receptors were all significantly lower in NR compared with control offspring (Fig. 2). These adaptations were accompanied by significantly reduced mRNA abundance for both SOCS-3 and HGF (Fig. 2). In contrast, there was no difference in mRNA abundance between groups for the long and short forms of PRLR, IGF-I and IGF-II, or the HGF receptor c-met (Table 3). Significant positive relationships between mRNA abundance for IGF-I and either the GHR or the long or short forms of the PRLR were observed in control but not NR offspring (Table 4).

Hepatic GR mRNA abundance was higher in offspring of NR mothers but this was not statistically significant (i.e. $P=0{\cdot}07$; Fig. 3). However, there was no difference in mRNA abundance for 11βHSD types 1 or 2 between groups (Table 3).

VDAC and Bax

The abundance of VDAC in liver mitochondria was significantly increased in livers of NR offspring compared with controls (Fig. 4) and was accompanied by an up regulation of Bax mRNA.

Discussion

The major finding of our study is the magnitude of long-term adaptations in endocrine and apoptotic-related genes within the livers of 3-year-old offspring born to mothers NR between 0 and 95 days gestation. Importantly, differences in the hepatic GH–IGF axis were accompanied by a 10–15% reduction in liver wet and dry mass, but not RNA or protein content. We interpret these findings as being indicative of impaired liver growth possibly as a consequence of an imbalance between mitogenic and pro-apoptotic mechanisms in conjunction with decreased hepatic IGF sensitivity.

This is the first time that a programmed reduction in liver mass has been shown in the absence of any effects on total body mass. Previous animal studies have shown that fetal growth restriction following maternal consumption of a low protein diet permanently changes the balance of two liver enzymes phosphoenolpyruvate and glucokinase, involved in the synthesis and breakdown of glucose respectively (Desai *et al.* 1995). The reduction in liver mass appeared to relate to a global reduction in mass rather than any one specific component. It is unlikely to represent a change in glycogen content, as this makes up a comparatively small proportion of liver mass even under conditions in which it is increased (Clarke *et al.* 1996). In addition, all livers were healthy and had no visible difference in appearance or any signs of lipid accumulation.

Gluconeogenesis is regulated by glucocorticoids and GH as well as catecholamines and insulin (Cherrington 1999), and there is good evidence that fetal hepatic sensitivity to both

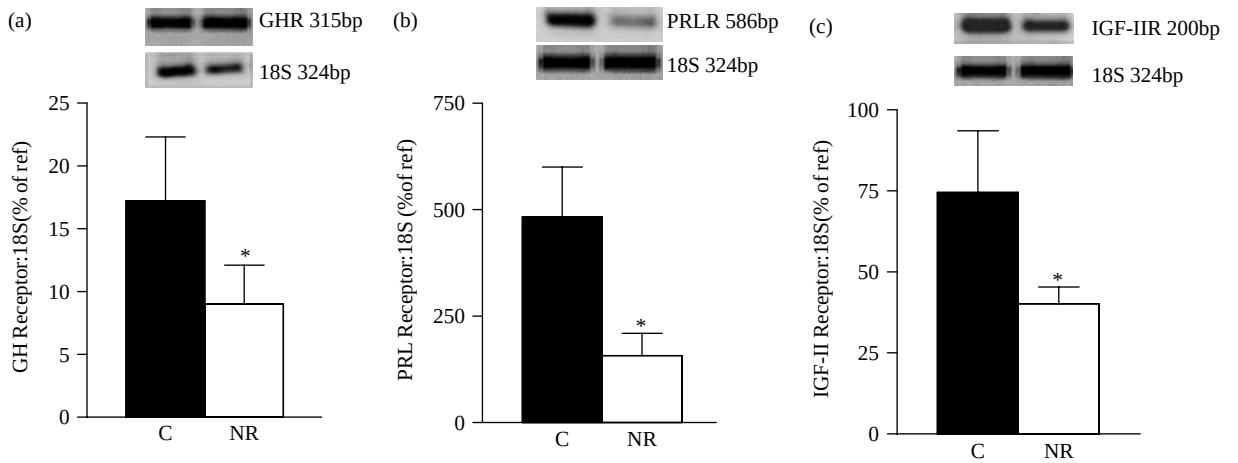

Figure 2 Effect of maternal nutrient restriction on the mRNA abundance for (a) growth hormone receptor (GHR), (b) prolactin receptor (PRLR), and (c) insulin-like growth factor-II receptor (IGF-IIR) in the livers of the adult offspring born to mothers that consumed 50% (nutrient restricted (NR) $n=10$) or 100% (control (C) $n=8$) of their metabolisable energy requirements for maternal metabolism and fetal growth between 0 and 95 days gestation. A representative RT-PCR image is provided for a C and NR animal for each target gene and corresponding 18S. Values are means±S.E.M. expressed as a percentage of a reference sample. *$P<0{\cdot}05$, mean value significantly different from control group as assessed by Mann–Whittney *U*-test.

Table 3 Effect of early to mid gestational maternal nutrient restriction on the mRNA for abundance for the long (L) and short (S) forms of the prolactin receptor (PRLR), insulin-like growth factor (IGF)-I and –II, hepatocyte growth factor receptor c-met, and 11β-hydroxysteroid dehydrogenase (11βHSD) types 1 and 2 in the liver of the adult male offspring born to mothers that consumed 50% (nutrient restricted $n=10$) or 100% (control $n=8$) of their metabolisable energy requirements for maternal metabolism and fetal growth between 0 and 95 days gestation. Values are means with their standard errors

	Control	Nutrient restricted
PRLR-L	19·52±7·91	10·63±3·12
PRLR-S	11·61±5·95	7·15±2·8
IGF-I	79·48±16·24	73·10±9·3
IGF-II	110·16±12·15	134·22±18·89
c-met	38·65±4·91	59·65±16·51
11βHSD-1	138·38±35·42	108·84±17·59
11βHSD-2	77·55±6·23	114·40±10·28

cortisol (Whorwood *et al.* 2001) and GH (Brameld *et al.* 2000, Hyatt *et al.* 2004) can be programmed *in utero*. In the present study, there was a statistical trend for NR offspring to exhibit increased GR mRNA abundance despite no difference in plasma cortisol (Gopalakrishnan *et al.* 2004) or 11βHSD types 1 and 2 mRNA, which is therefore likely to be reflected in similar hepatic enzyme activities between groups (Whorwood *et al.* 2001). The extent to which plasma concentrations of GH may be reset in NR offspring remains to be explored, but not only was mean plasma IGF-I similar between groups, also, to date, we have seen no effect of nutrient restriction on glucose, cortisol, or leptin plasma profiles in these offspring (Gopalakrishnan *et al.* 2004). However, when these offspring are reared under an environment in which their activity is restricted and they become obese, then marked health problems ensue including raised plasma cortisol and symptoms of mineral toxicity

Table 4 Effect of early to mid gestational maternal nutrient restriction on the relationship (Spearmans Rank Order Test) between hepatic mRNA abundance of insulin-like growth factor-I (IGF-I), growth hormone receptor (GHR) and long (L) and short (S) forms of the prolactin receptor (PRLR) in the adult male offspring born to mothers that consumed 50% (nutrient restricted $n=10$) or 100% (control $n=8$) of their metabolisable energy requirements for maternal metabolism and fetal growth between 0 and 95 days gestation

	Control		Nutrient restricted	
	r	*P* value	*r*	*P* value
Correlations between IGF-I mRNA and				
GHR	0·47	NS	0·55	NS
PRLR-L	0·78	$P=0·021$	0·10	NS
PRLR-S	0·78	$P=0·021$	0·73	NS

NS, not significant.

(Symonds *et al.* 2006). The findings from the present study are therefore important with regard to providing baseline information necessary to completely understand why these adverse adaptations occur with adult obesity in nutrient-restricted offspring but are not seen in animals born to control fed mothers on becoming obese.

Liver growth is usually regulated such that its size is proportional to body mass and a number of intrinsic signals, including HGF (Somerset *et al.* 2000), GH (Pennisi *et al.* 2004), PRL (Phillips *et al.* 1997), and IGF (Demori *et al.* 2000), both positively and negatively regulate liver mass until an appropriate size is reached (Kam *et al.* 1987, Van Thiel *et al.* 1987, Kawasaki *et al.* 1992). Our finding that liver weight was reduced, both in absolute and relative terms, in the adult NR offspring, when maintained under the same plane of postnatal nutrition, in conjunction with decreased mRNA abundance of a range of receptors critically involved in liver growth is novel. One possibility is that the 50% reduction in hepatic HGF mRNA directly contributes to the decrease in liver size. Mice lacking HGF fail to complete development as the mutation affects the embryonic liver which is reduced in size and shows extensive loss of parenchymal cells (Uehara *et al.* 1995, Somerset *et al.* 2000, Duncan *et al.* 2004). The extent to which HGF expression was altered *in utero* in this study remains to be examined, but one potential mechanism by which nutrient restriction produced smaller livers may be due to decreased abundance of hepatic HGF secondary to reduced nutrient availability in early gestation. Any nutritional effect on HGF was not accompanied by a reduction in the abundance of its cognate receptor c-met, and is perhaps not unexpected as c-met is a marker of hepatic stem cells (Somerset *et al.* 2000).

Although paracrine signalling of HGF potentially plays a role in the programming of liver size, HGF *in vivo* only causes a weak induction of hepatocyte proliferation (Webber *et al.* 1994, Fausto 2000). The expression of functional GHR is essential for normal postnatal growth and metabolic homeostasis (Zhou *et al.* 1997). GHR and PRLR are present in the sheep fetus from early gestation, the expression of which increases with advancing gestational age (Gluckman *et al.* 1983, Klempt *et al.* 1993, McMillen *et al.* 2001). However, the precise function of GHR and PRLR in promoting growth and development of the fetus remains to be fully defined, although they can be detected in the embryo suggesting a role in early development (Waters & Kaye 2002). GH is unlikely to have an effect on fetal liver growth and development, as its receptor becomes functional only after birth (Gluckman *et al.* 1983, McMillen *et al.* 2001). Interestingly, statistically significant relationships were present between the expression of either GHR or the long and short forms of PRLR and hepatic IGF-I mRNA in livers from the control, but not NR offspring. Both short and intermediate forms of the PRLR are derived from alternative splicing resulting in a frameshift and truncated intracytoplasmic domain that acts to inhibit the activation induced by PRL through the long form of PRLR (Trott *et al.* 2003). We also found that whilst early nutrient restriction did not alter

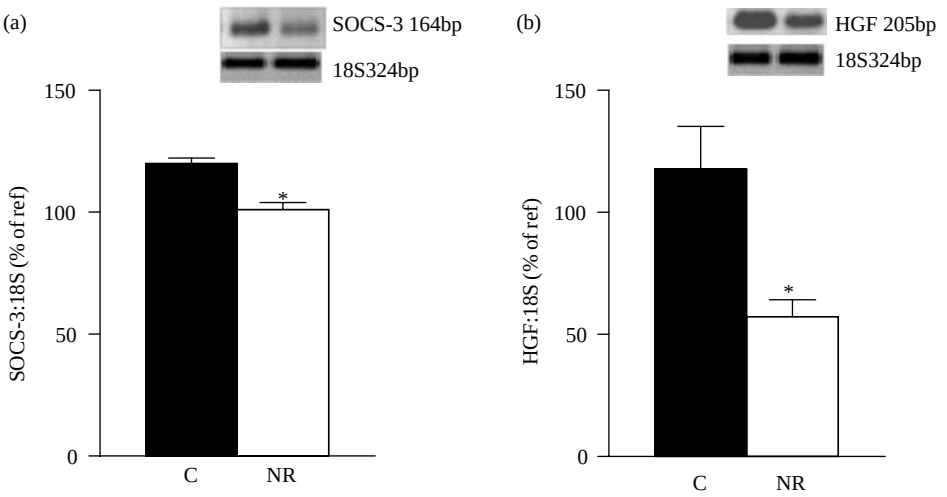

Figure 3 Effect of maternal nutrient restriction on the mRNA abundance for (a) suppressor of cytokine signaling (SOCS)-3 and (b) hepatocyte growth factor (HGF) mRNA in the livers of the adult offspring born to mothers that consumed 50% (nutrient restricted (NR) $n=10$) or 100% (control (C) $n=8$) of their metabolizable energy requirements for maternal metabolism and fetal growth between 0 and 95 days gestation. A representative RT-PCR image is provided for a C and NR animal for each target gene and corresponding 18S. Values are means ± S.E.M. expressed as a percentage of a reference sample. *$P<0{\cdot}05$, mean value significantly different from control group as assessed by Mann–Whitney *U* test.

mRNA abundance for either the long or short form of the PRLR, it reduced the mRNA abundance for the total PRLR in the adult liver. It is therefore possible that, in the sheep, early nutrient restriction alters the expression of other PRLR isoforms which have been previously identified in humans (Kline *et al.* 1999). Clearly, further work is needed to confirm the existence of a third 'intermediate' PRLR isoform in sheep. SOCS-3 expression is tightly regulated by a range of growth factors including GH, and PRL (Johnston & O'Shea 2003). It is therefore not surprising that the decreased cytokine receptor mRNA abundance in livers from NR offspring is accompanied by a down regulation of hepatic SOCS-3 mRNA expression. This response to maternal nutrient restriction may be an adaptive response to ensure that hepatic sensitivity to cytokines and other factors including IGF-I is maintained.

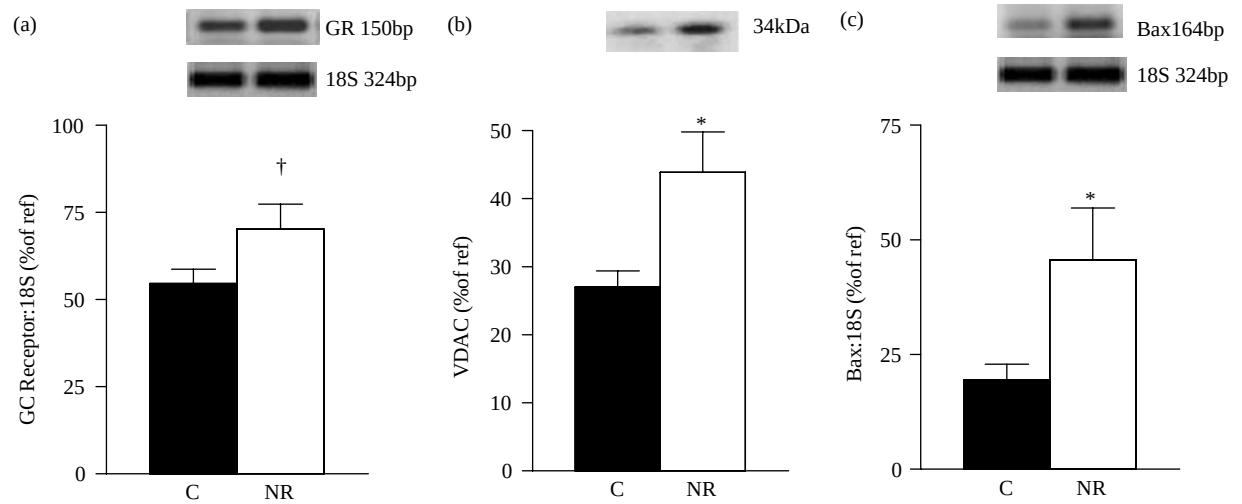

Figure 4 Effect of maternal nutrient restriction on the abundance of (a) glucocorticoid receptor (GR) mRNA, (b) voltage-dependent anion channel (VDAC) protein, and (c) Bax mRNA in the livers of the adult offspring born to mothers that consumed 50% (nutrient restricted (NR) $n=10$) or 100% (control (C) $n=8$) of their metabolizable energy requirements for maternal metabolism and fetal growth between 0 and 95 days gestation. Representative RT-PCR and western blot images are provided for a C and NR animal for each gene. Values are means ± S.E.M. expressed as a percentage of a reference sample. *$P=0{\cdot}05$, †$P<0{\cdot}05$, ‡$P<0{\cdot}07$ between nutrition groups as assessed by Mann–Whitney *U* test.

Hepatic IGF-IR mRNA levels were undetectable in adult sheep, irrespective of prenatal nutrition. Expression of IGF-IR in liver tissue normally decreases with postnatal age (Hyatt *et al.* 2004) and tissue maturation. Hepatic IGF-II expression was similarly unaffected by prenatal nutrition. The bioavailability of IGF-II depends upon a number of factors (Forhead *et al.* 1998, Oldham *et al.* 1999) including the abundance of the IGF-IIR (O'Dell & Day 1998), which normally acts as a negative regulator of its anabolic effects by sequestering plasma IGF-II into the cell for degradation (O'Dell & Day 1998). In the present study, reduced IGF-IIR mRNA was accompanied by reduced, and not enhanced, liver mass suggesting that IGF-II plays a relatively limited role in hepatic growth in postnatal life. Additionally, the inner mitochondrial protein VDAC and mRNA abundance for the pro-apoptotic factor Bax were both up regulated in the livers of the NR offspring. VDAC is proposed to have a role in apoptosis and, taken together, these adaptations may be indicative of an increased susceptibility to apoptosis (Zhang *et al.* 2001). Under the basal conditions in which these animals were studied and in conjunction with our inability to detect mRNA for the anti-apoptotic factor Bcl mRNA in these livers suggests that under conditions in which apoptosis would be raised, such as prolonged food restriction (Grasl-Kraupp *et al.* 1994) or drug exposure (Halsted *et al.* 1996), then such an adaptation is likely to be greater in NR offspring.

In conclusion, our results demonstrate that the reduction in liver size with maternal nutrient restriction is associated with alterations in both mitogenic and pro-apoptotic-related genes and proteins. These data therefore indicate that exposure to an adverse nutritional environment in early pregnancy has a long-term impact on the growth and functional development of the liver and such changes may contribute to the programing of poor adult health in later life.

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References

AFRC 1993 *Agricultural and Food Research Council. Energy and Protein requirements of Ruminants. An Advisory Manual Prepared by the AFRC Technical Committee on Responses to Nutrients*, Wallingford, Oxfordshire, UK: CAB International.

Barker DJP 1998 *Mothers, Babies and Health in Later Life*. Edinburgh: Churchill Livingstone.

Barker DJP, Meade TW, Fall CH, Lee A, Osmond C, Phipps K & Stirling Y 1992 Relation of fetal and infant growth to plasma fibrinogen and factor VII concentrations in adult life. *British Medical Journal* **304** 148–152.

Bauer MK, Breier BH, Harding J, Veldhuis JD & Gluckman PD 1995 The fetal somatotrophic axis during long term maternal undernutrition in sheep; evidence of nutritional regulation *in utero*. *Endocrinology* **136** 1250–1257.

Beauloye V, Willems B, de Coninck V, Frank SJ, Edery M & Thissen JP 2002 Impairment of liver GH receptor signaling by fasting. *Endocrinology* **143** 792–800.

Bispham J, Gopalakrishnan GS, Dandrea J, Wilson V, Budge H, Keisler DH, Broughton Pipkin F, Stephenson T & Symonds ME 2003 Maternal endocrine adaptation throughout pregnancy to nutritional manipulation: consequences for maternal plasma leptin and cortisol and the programming of fetal adipose tissue development. *Endocrinology* **144** 3575–3585.

Bispham J, Gardner DS, Gnanalingham MG, Stephenson T, Symonds ME & Budge H 2005 Maternal nutritional programming of fetal adipose tissue development: differential effects on mRNA abundance for uncoupling proteins, peroxisome proliferator activated and prolactin receptors. *Endocrinology* **146** 3943–3949.

Bladt F, Riethmacher D, Isemann S, Aguzzi A & Birchmeier C 1995 Essential role for the c-met receptor in the migration of myogenic precursor cells into the limb bud. *Nature* **376** 768–771.

Brameld JM, Mostyn A, Dandrea J, Stephenson T, Dawson J, Buttery P & Symonds ME 2000 Maternal nutrition alters the expression of insulin-like growth factors in fetal sheep liver and skeletal muscle. *Journal of Endocrinology* **167** 429–437.

Budge H, Bispham J, Dandrea J, Evans E, Heasman L, Ingleton PM, Sullivan C, Wilson V, Stephenson T & Symonds ME 2000 Effect of maternal nutrition on brown adipose tissue and its prolactin receptor status in the fetal lamb. *Pediatric Research* **47** 781–786.

Cherrington AD 1999 Banting Lecture 1997. Control of glucose uptake and release by the liver *in vivo*. *Diabetes* **48** 1198–1214.

Clarke L, Andrews DC, Lomax MA & Symonds ME 1996 Effect of maternal glucose infusion on brown adipose tissue and liver development in the neonatal lamb. *Reproduction, Fertility, and Development* **8** 1045–1054.

Conner EA, Wirth PJ, Kiss A, Santoni-Rugiu E & Thorgeirsson SS 1997 Growth inhibition and induction of apoptosis by HGF in transformed rat liver epithelial cells. *Biochemical and Biophysical Research Communications* **236** 396–401.

Demori I, Balocco S, Voci A & Fugassa E 2000 Increased insulin-like growth factor binding protein-4 expression after partial hepatectomy in the rat. *American Journal of Physiology* **278** G384–G389.

Desai M, Crowther NJ, Ozanne SE, Lucas A & Hales CN 1995 Adult glucose and lipid metabolism may be programmed during fetal life. *Biochemical Society Transactions* **23** 331–335.

Duncan BB, Schmidt MI, Pankow JS, Bang H, Couper D, Ballantyne CM, Hoogeveen RC & Heiss G 2004 Adiponectin and the development of type 2 diabetes: the atherosclerosis risk in communities study. *Diabetes* **53** 2473–2478.

Edwards LJ & McMillen IC 2001 Maternal undernutrition increases arterial blood pressure in the sheep fetus during late gestation. *Journal of Physiology* **533** 561–570.

Fausto N 2000 Liver regeneration. *Journal of Hepatology* **32** 19–31.

Forhead AJ, Li J, Gilmour RS & Fowden AL 1998 Control of hepatic insulin-like growth factor II gene expression by thyroid hormones in the fetal sheep near term. *American Journal of Physiology* **275** E149–E156.

Forhead AJ, Li J, Saunders JC, Dauncey MJ, Gilmour RS & Fowden AL 2000 Control of ovine hepatic growth hormone receptor and insulin-like growth factor I by thyroid hormones *in utero*. *American Journal of Physiology* **278** E1166–E1174.

Forhead AJ, Poore KR, Mapstone J & Fowden AL 2003 Developmental regulation of hepatic and renal gluconeogenic enzymes by thyroid hormones in fetal sheep during late gestation. *Journal of Physiology, London* **548** 941–947.

Gardner DS, Fletcher AJ, Fowden AL & Giussani DA 2001 Plasma adrenocorticotropin and cortisol concentrations during acute hypoxemia after a reversible period of adverse intrauterine conditions in the ovine fetus during late gestation. *Endocrinology* **142** 589–598.

Gardner DS, Tingey K, van Bon BWM, Ozanne SE, Wilson V, Dandrea J, Keisler DH, Stephenson T & Symonds ME 2005 Programming of glucose-insulin metabolism in adult sheep after maternal undernutrition. *American Journal of Physiology* **289** R1407–R1415.

Gluckman PD, Butler JH & Elliott TB 1983 The ontogeny of somatotrophic binding sites in ovine hepatic membranes. *Endocrinology* **112** 1607–1612.

Gnanalingham MG, Mostyn A, Gardner DS, Stephenson T & Symonds ME 2005 Developmental regulation of adipose tissue: nutritional manipulation of local glucocorticoid action and uncoupling protein 2. *Adipocytes* **1** 221–228.

Gopalakrishnan G, Gardner DS, Rhind SM, Rae MT, Kyle CE, Brooks AN, Walker RM, Ramsay MM, Keisler DH, Stephenson T *et al.* 2004 Programming of adult cardiovascular function after early maternal undernutrition in sheep. *American Journal of Physiology* **287** R12–R20.

Grasl-Kraupp B, Bursch W, Ruttkay-Nedecky B, Wagner A, Lauer B & Schulte-Hermann R 1994 Food restriction eliminates preneoplastic cells through apoptosis and antagonizes carcinogenesis in rat liver. *PNAS* **91** 9995–9999.

Halsted CH, Villanueva J, Chandler CJ, Stabler SP, Allen RH, Muskhelishvili L, James SJ & Poirier L 1996 Ethanol feeding of micropigs alters methionine metabolism and increases hepatocellular apoptosis and proliferation. *Hepatology* **23** 497–505.

Harding R, Cock ML, Louey S, Joyce BJ, Davey MG, Albuquerque CA, Hooper SB & Maritz GS 2000 The compromised intra-uterine environment: implications for future lung health. *Clinical and Experimental Pharmacology and Physiology* **27** 965–974.

Heasman L, Brameld JM, Mostyn A, Budge H, Dawson J, Buttery PJ, Stephenson T & Symonds ME 2000 Maternal nutrient restriction during early to mid gestation alters the relationship between IGF-I and body size at term in fetal sheep. *Reproduction, Fertility, and Development* **12** 345–350.

Hyatt M, Walker D, Stephenson T & Symonds ME 2004 Ontogeny and nutritional manipulation of the hepatic prolactin (PRL)-growth hormone (GH)-insulin-like growth factor (IGF) axis in the ovine fetus, neonate and juvenile sheep. *Proceedings of the Nutrition Society* **63** 127–135.

Ilkbahar YN, Thordarson G, Camarillo IG & Talamantes F 1999 Differential expression of the growth hormone receptor and growth hormone-binding protein in epithelia and stroma of the mouse mammary gland at various physiological stages. *Journal of Endocrinology* **161** 77–87.

Johnston JA & O'Shea JJ 2003 Matching SOCS with function. *Nature Immunology* **4** 507–509.

Kam I, Lynch S, Svanas G, Todd S, Polimeno L, Francavilla A, Penkrot RJ, Takaya S, Ericzon BG, Starzl TE *et al.* 1987 Evidence that host size determines liver size: studies in dog receiving orthtopic liver transplants. *Hepatology* **7** 362.

Kawasaki S, Makuuchi M, Ishizone S, Matsunami H, Terada M & Kawarazaki H 1992 Liver regeneration in recipients and donors after transplantation. *Lancet* **339** 580–581.

Klempt M, Bingham B, Breier BH, Baumbach WR & Gluckman PD 1993 Tissue distribution and ontogeny of growth hormone receptor messenger ribonucleic acid and ligand binding to hepatic tissue in midgestation sheep fetus. *Endocrinology* **132** 1071–1077.

Kline JB, Roehrs H & Clevenger CV 1999 Functional characterization of the intermediate isoform of the human prolactin receptor. *Journal of Biological Chemistry* **274** 35461–35468.

Lowry OH, Rosenbrough NJ, Farr AL & Randall RJ 1951 Protein measurement with the Folin phenol reagent. *Journal of Biological Chemistry* **193** 265–275.

Madesh M & Hajnoczky G 2001 VDAC-dependent permeabilization of the outer mitochondrial membrane by superoxide induces rapid and massive cytochrome *c* release. *Journal of Cell Biology* **155** 1003–1015.

McMillen IC, Houghton DC & Phillips ID 2001 Maturation of the cytokine receptors in preparation for birth. *Biochemical Society Transactions* **29** 63–68.

Mostyn A, Wilson V, Dandrea J, Yakubu DP, Budge H, Alves-Guerra MC, Pecqueur C, Miroux B, Symonds ME & Stephenson T 2003 Ontogeny and nutritional manipulation of mitochondrial protein abundance in adipose tissue and the lungs of postnatal sheep. *British Journal of Nutrition* **90** 323–328.

Nakagami H, Morishita R, Yamamoto K, Taniyama Y, Aoki M, Yamasaki K, Matsumoto K, Nakamura T, Kaneda Y & Ogihara T 2002 Hepatocyte growth factor prevents endothelial cell death through inhibition of bax translocation from the cytosol to mitochondrial membrane. *Diabetes* **51** 2604–2611.

Nevalainen M, Valve EM, Ingleton PM & Harkonen PL 1996 Expression and hormone regulation of prolactin receptors in rat dorsal and lateral prostate. *Endocrinology* **137** 3078–3088.

O'Dell SD & Day INM 1998 Molecules in focus: insulin-like growth factor II (IGF-II). *International Journal of Biochemistry and Cell Biology* **30** 767–771.

Oldham JM, Martyn JA, Hua KM, MacDonald NA, Hodgkinson SC & Bass JJ 1999 Nutritional regulation of IGF-II, but not IGF-I, is age dependent in sheep. *Journal of Endocrinology* **163** 395–402.

Pennisi PA, Kopchick JJ, Thorgeirsson SS, LeRoith D & Yakar S 2004 Role of growth hormone (GH) in liver regeneration. *Endocrinology* **145** 4748–4755.

Phillips ID, Anthony RV, Butler TG, Ross JT & McMillen IC 1997 Hepatic prolactin receptor gene expression increases in the sheep fetus before birth and after cortisol infusion. *Endocrinology* **138** 1351–1354.

Ram PA & Waxman DJ 1999 SOCS/CIS protein inhibition of growth hormone-stimulated STAT5 signaling by multiple mechanisms. *Journal of Biological Chemistry* **274** 35553–35561.

Roseboom TJ, van der Meulen JH, Ravelli AC, Osmond C, Barker DJP & Bleker OP 2000 Plasma fibrinogen and factor VII concentrations in adults after prenatal exposure to famine. *British Journal of Haematology* **111** 112–117.

Roseboom TJ, van der Meulen JHP, Ravelli ACJ, Osmond C, Barker DJP & Bleker OP 2001 Effects of prenatal exposure to the Dutch Famine on adult disease in later life: an overview. *Twin Research* **4** 293–298.

Santoni-Rugui E, Preisegger KH, Kiss A, Audolfsson T, Shoiota G, Schmidt EV & Thorgeirsson SS 1996 Inhibition of neoplastic development in the liver by hepatocyte growth factor in a trasgenic mouse model. *PNAS* **93** 9577–9582.

Somerset DA, Jauniaux E, Strain AJ, Afford S & Kilby MD 2000 Hepatocyte growth factor concentration in maternal and umbilical cord blood samples and expression in fetal liver. *Journal of the Society for Gynecologic Investigation* **7** 333–337.

Symonds ME, Budge H, Mostyn A, Stephenson T & Gardner DS 2006 Nutritional programming of fetal development: endocrine mediators and long-term outcomes for cardiovascular health. *Current Nutrition and Food Science* **2** 389–398.

Trott JF, Hovey RC, Koduri S & Vonderhaar BK 2003 Alternative splicing to exon 11 of human prolactin receptor gene results in multiple isoforms including a secreted prolactin-binding protein. *Journal of Molecular Endocrinology* **30** 31–47.

Uehara Y, Minowa O, Mori C, Shiota K, Kuno J, Noda T & Kitamura N 1995 Placental defect and embryonic lethality in mice lacking hepatocyte growth factor/scatter factor. *Nature* **373** 702–705.

Van Thiel DH, Gavaler JS, Kam I, Francavilla A, Polimeno L, Schade RR, Smith J, Diven W, Penkrot RJ & Starzl TE 1987 Rapid growth of an intact liver transplanted into a recipient larger than the donor. *Gastroenterology* **93** 1414–1419.

Vonnahme KA, Hess BW, Hansen TR, McCormick RJ, Rule DC, Moss GE, Murdoch WJ, Nijland MJ, Skinner DC, Nathanielsz PW *et al.* 2003 Maternal undernutrition from early- to mid-gestation leads to growth retardation, cardiac ventricular hypertrophy, and increased liver weight in the fetal sheep. *Biology of Reproduction* **69** 133–140.

Waters MJ & Kaye PL 2002 The role of growth hormone in fetal development. *Growth Hormone and IGF Research* **12** 137–146.

Webber EM, Godowski PJ & Fausto N 1994 *In vivo* response of hepatocytes to growth factors requires an initial priming stimulus. *Hepatology* **19** 489–497.

Whorwood CB, Firth KM, Budge H & Symonds ME 2001 Maternal undernutrition during early to midgestation programs tissue-specific alterations in the expression of the glucocorticoid receptor, 11β-hydroxysteroid dehydrogenase isoforms, and type 1 angiotensin II receptor in neonatal sheep. *Endocrinology* **142** 2854–2864.

Young L, Fernandes K, McEvoy TG, Butterwith SC, Gutierrez CG, Carolan C, Broadbent PJ, Robinson JJ, Wilmut I & Sinclair KD 2001 Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. *Nature Genetics* **27** 153–154.

Zhang CY, Baffy G, Perret P, Krauss S, Peroni O, Grujic D, Hagen T, Vidal-Puig AJ, Boss O, Kim YB *et al.* 2001 Uncoupling protein-2 negatively regulates insulin secretion and is a major link between obesity, beta cell dysfunction, and type 2 diabetes. *Cell* **105** 745–755.

Zhou Y, Xu BC, Maheshwari HG, He L, Reed M, Lozykowski M, Okada S, Cataldo L, Coschigamo K, Wagner TE *et al.* 1997 A mammalian model for Laron syndrome produced by targeted disruption of the mouse growth hormone receptor/binding protein gene (the Laron mouse). *PNAS* **94** 13215–13220.

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