

Differential regulation of *igf1* and *igf1r* mRNA levels in the two hepatic lobes following intrauterine growth restriction and its treatment with intra-amniotic insulin-like growth factor-1 in ovine fetuses

Revati A. Darp^A, Hendrina A. de Boo^A, Hui Hui Phua^A, Mark H. Oliver^A, José G. B. Derraik^A, Jane E. Harding^A and Frank H. Bloomfield^{A,B}

^ALiggins Institute, University of Auckland, Private Bag 92019, Auckland 1142, New Zealand.

^BCorresponding author. Email: f.bloomfield@auckland.ac.nz

Abstract. Intrauterine growth restriction (IUGR) has life-long health implications, yet there is no effective prenatal treatment. Daily intra-amniotic administration of insulin-like growth factor (IGF)-1 to IUGR fetal sheep improves fetal gut maturation but suppresses hepatic *igf1* gene expression. Fetal hepatic blood supply is regulated, in part, by shunting of oxygen- and nutrient-rich umbilical venous blood through the ductus venosus, with the left hepatic lobe predominantly supplied by umbilical venous blood and the right hepatic lobe predominantly supplied by the portal circulation. We hypothesised that: (1) once-weekly intra-amniotic IGF-1 treatment of IUGR would be effective in promoting gut maturation; and (2) IUGR and its treatment with intra-amniotic IGF-1 would differentially affect *igf1* and *igf1r* mRNA expression in the two hepatic lobes. IUGR fetuses received 360 µg IGF-1 or saline intra-amniotically once weekly from 110 until 131 days gestation. Treatment of IUGR fetuses with IGF-1 reversed impaired gut growth. In unembolised, untreated control fetuses, *igf1* mRNA levels were 19% lower in the right hepatic lobe than in the left; in IUGR fetuses, *igf1* and *igf1r* mRNA levels were sixfold higher in the right lobe. IGF-1 treatment reduced *igf1* and *igf1r* mRNA levels in both lobes compared with IUGR fetuses. Thus, weekly intra-amniotic IGF-1 treatment, a clinically feasible approach, reverses the impaired gut development seen in IUGR. Furthermore, *igf1* and *igf1r* mRNA levels are differentially expressed in the two hepatic lobes and relative expression in the two lobes is altered by both IUGR and intra-amniotic IGF-1 treatment.

Additional keywords: amniotic fluid, fetal therapy, gastrointestinal tract, somatotrophic axis.

Introduction

Intrauterine growth restriction (IUGR) is the failure of a fetus to achieve its intrinsic growth potential (Resnik 2002) and is a major cause of perinatal morbidity and mortality (Piper *et al.* 1996). Babies of low birthweight carry an increased risk of developing obesity, high blood pressure, coronary heart disease, Type 2 diabetes, insulin resistance and impaired cholesterol metabolism in adult life (Godfrey and Barker 2000). The increased risk of such diseases may be a consequence of permanent resetting of metabolic and homeostatic mechanisms resulting from an adverse intrauterine nutritional environment (Barker 1998), an hypothesis supported by experimental data (Langley-Evans *et al.* 1996; McMillen and Robinson 2005). Rapid postnatal growth (with upward crossing of centiles) of babies born IUGR occurs in most within the first 2 years of life (Saenger *et al.* 2007) and potentially may increase the risks of long-term disease (Ibáñez *et al.* 2006; Ong and Loos 2006; Leunissen *et al.* 2008; Rotteveel *et al.* 2008). In 10–20% of babies born IUGR there is a lack of catch-up growth with reduced final height; this is associated with impaired cognitive neurodevelopment (Fattal-Valevski *et al.* 2009). Thus, an

intervention during intrauterine life to prevent the effects of IUGR and postnatal growth perturbations may be the optimal strategy.

Clinical attempts to improve the growth of the fetus by maternal supplementation with protein (Kramer and Kakuma 2003) or oxygen (Battaglia *et al.* 1992) have not been successful. This may be because, in developed countries, placental insufficiency is one of the primary causes of IUGR, limiting delivery of nutrients and growth factors to the fetus (Robinson *et al.* 2000; Henriksen and Clausen 2002; Hendrix and Berghella 2008). Experimental attempts to increase placental blood flow with sildenafil citrate in ovine pregnancies with growth-restricted fetuses resulted in adverse outcome (Miller *et al.* 2009) and supplementation with L-arginine (a precursor of nitric oxide) in pregnant women carrying severely IUGR fetuses had no effect on fetal growth (Winer *et al.* 2009). Therefore, a treatment that bypasses the placenta may provide the most promising approach.

Previously, we investigated such an approach in IUGR ovine fetuses, namely the administration of insulin-like growth factor (IGF)-1, one of the major growth factors for late-gestation fetal

growth, into amniotic fluid (Bloomfield *et al.* 2002a; Eremia *et al.* 2007). Daily administration of a low dose (20 µg) of IGF-1 reversed the delayed maturation of the gastrointestinal tract (GIT) seen in vehicle-treated IUGR fetuses (Bloomfield *et al.* 2002a). We showed that IGF-1 administered into the amniotic fluid is taken up intact across the GIT and into the portal vein (Bloomfield *et al.* 2002b) and is thus delivered to the fetal liver. Gut *igf1* mRNA levels were not altered by intra-amniotic IGF-1 treatment, although *igf1r* mRNA levels in the jejunum were increased (Shaikh *et al.* 2005). In contrast, hepatic *igf1* mRNA levels were reduced in the right lobe of the liver, consistent with downregulation by IGF-1 taken up across the gut and delivered to the liver via the portal vein (Shaikh *et al.* 2005). A subsequent study, administering 120 µg IGF-1 into the amniotic fluid thrice weekly over a 4-week period, provided the first evidence of a treatment increasing fetal growth rate in IUGR fetuses *in utero* (Eremia *et al.* 2007). However, for a treatment to potentially be useful clinically, the frequency of intra-amniotic injections would need to be significantly less than thrice weekly, with once-weekly injections perhaps being feasible.

Blood supply to the fetal liver is complex, with approximately 70–75% of the blood coming from the nutrient- and oxygen-rich umbilical venous (UV) blood, approximately 20% from the nutrient- and oxygen-poor portal venous (PV) blood, and the remainder from the hepatic artery (HA; Edelstone *et al.* 1978; Rudolph 1983). The anatomy of the blood supply means that there is differential blood flow to the left and right hepatic lobes. In sheep, the left lobe receives over 90% of its blood from the UV, with approximately 6% and 1% from the HA and PV, respectively. In contrast, the right lobe receives only approximately 50% of its blood supply from the UV and the remainder from the PV (Edelstone *et al.* 1978). Thus, the left lobe receives a considerably greater proportion of nutrient- and oxygen-rich blood from the placenta compared with the right lobe. Because IGF-1 is regulated by nutrient supply during fetal development (Oliver *et al.* 1993, 1996), we hypothesised that *igf1* gene expression may differ in the two hepatic lobes.

Furthermore, in IUGR pregnancies there is a redistribution of blood supply from the periphery to 'essential' organs, such as the heart and brain, due to fetal hypoxia (Thornburg and Morton 1994). Shunting UV blood through the ductus venosus (DV), thereby bypassing the liver and delivering nutrient-rich blood to the systemic circulation, is one mechanism of redistributing cardiac output, which may contribute to 'brain sparing' seen in IUGR fetuses. Studies in ovine fetuses have demonstrated an increased ductal shunt during acute hypoxia induced by either reducing inspired maternal oxygen concentrations or by umbilical arterial occlusion (Edelstone *et al.* 1980; Tchirikov *et al.* 1998; Kiserud *et al.* 2000a). Studies in human IUGR fetuses suggest that a similar phenomenon is seen in these fetuses, which are typically hypoxaemic and hypoglycaemic (Weiner *et al.* 1996; Bellotti *et al.* 2006; Kiserud *et al.* 2006). Moreover, fetal growth may be partly regulated by hepatic blood perfusion (Tchirikov *et al.* 2002) and a reduction in hepatic perfusion may have lasting consequences on the metabolic adaptation of the liver, potentially affecting health in later life (Kiserud *et al.* 2006). We therefore hypothesised that if IUGR alters the proportion of nutrient-rich UV blood being shunted through

the DV, it may affect *igf1* gene expression in the two hepatic lobes differently. Finally, administration of exogenous IGF-1 into the amniotic fluid, and thereby into the PV, may also affect gene expression differently in the hepatic lobes via direct downregulation or indirectly via effects on DV shunting as a result of the vasoactive effects of IGF-1 (Haylor *et al.* 1991; Wu *et al.* 1994).

Therefore, the aims of the present study were, first, to determine whether a more clinically applicable regimen for intra-amniotic IGF-1 treatment of IUGR (reducing the treatment frequency from thrice to once weekly) would still promote the growth and development of fetal GIT morphology and affect *igf1* and *igf1r* gene expression in the fetal gut. Second, we wanted to determine the effects of IUGR and intra-amniotic IGF-1 treatment on mRNA levels of *igf1* and *igf1r* in the left and right hepatic lobes of ovine fetuses.

Materials and methods

Animals

Twenty-seven pregnant Romney ewes carrying Dorset cross-breed singleton fetuses were housed in individual cages with free access to water and pelleted food. All animal experiments were approved by the University of Auckland Animal Ethics Committee and were conducted in accordance with the National Animal Ethics Advisory Committee guidelines (National Animal Ethics Advisory Committee 2002).

Surgery

After acclimatisation to laboratory conditions, animals underwent surgery under halothane anaesthesia at 98 days of gestation (dGA; term = 147 dGA). Streptopen (250 mg procaine penicillin and 250 mg dihydrostreptomycin sulfate; Pittman Moore, Upper Hutt, New Zealand) was administered intramuscularly before surgery. Chronic indwelling catheters were inserted into a tarsal artery and vein, the amniotic sac (two catheters), a maternal femoral artery and vein, and a maternal carotid artery and jugular vein. In ewes randomised to induction of IUGR, additional catheters were inserted into both maternal uterine arteries (UA). Growth catheters were inserted subcutaneously around the fetal chest using established techniques (Harding *et al.* 1997; Eremia *et al.* 2007).

Experimental design

Sheep were randomly assigned to one of three experimental groups, namely control fetuses ($n = 11$), which were unembolised and untreated, and two IUGR groups, treated with either saline (IUGR; $n = 9$) or IGF-1 (IGF-1; $n = 7$). In the two embolised groups, UA embolisation to induce IUGR, performed as described previously by Bloomfield *et al.* (2002c) and Eremia *et al.* (2007), commenced at 103 dGA and continued for 7 days. Briefly, 20–50 µm polystyrene microspheres (Superose 12 diluted 1:100 to give approximately $1-2 \times 10^5$ beads mL⁻¹; Pharmacia Biotech, Uppsala, Sweden) were injected once or twice daily into the UA catheters, with the dose adjusted according to the fetal blood gases and daily girth increment (Bloomfield *et al.* 2002c; Eremia *et al.* 2007). Embolisation was

omitted if the fetal arterial P_{aO_2} was <16 mmHg, the fetal lactate concentration was >2.0 mM or if growth catheters had not shown an increase for 2 consecutive days. Following completion of embolisation, IUGR fetuses received intra-amniotic injections of either $360 \mu\text{g}$ ($100 \mu\text{g mL}^{-1}$) IGF-1 (Genentech, San Francisco, CA, USA; IGF-1 group) or an equivalent volume of sterile saline (IUGR group) on 110, 117 and 124 dGA.

Post mortem

Ewes were killed with an overdose of sodium pentobarbitone on 131 dGA. The uterus was removed, incised and the umbilical cord clamped. The fetus was removed, dried, weighed and measured. The fetal liver, GIT and brain were rapidly removed and weighed. The GIT was then divided into the stomach and small and large intestines and each part was weighed and measured separately. Sections of the stomach (half way along the length of the abomasum), duodenum (first 10 cm of the small intestine), jejunum (10 cm distal to the duodenojejunal junction) and ileum (10 cm proximal to the ileocaecal valve) were opened longitudinally and the bowel contents removed. Samples were rapidly frozen in liquid nitrogen and stored in sterile vials at -80°C for molecular analysis or pinned onto a cork mat with the villi facing upwards and immersed in 10% buffered formalin for later histological analysis. Liver tissue was collected from the right (1 cm to the right of the gall-bladder) and left (half way between the DV and the left margin of the liver) lobes and rapidly frozen.

Real-time polymerase chain reaction to determine igf1 and igf1r mRNA levels

Total RNA was extracted by the Trizol method (Invitrogen, Carlsbad, CA, USA). Each RNA sample was diluted 10-fold in 10 mM Tris (pH 7.0, RNase free). The concentration of each RNA sample was measured using a NanoDrop spectrophotometer equipped with 3.0.1 NanoDrop software (ND-1000 Spectrophotometer; BioLab, Auckland, New Zealand). The ratio of absorbance at 260 : 280 nm was used to assess RNA purity. First-strand cDNA was synthesised using the Superscript III first strand synthesis system (Invitrogen) according to the manufacturer's instructions. RNA samples were treated with RNase-free DNase I (Invitrogen) before real-time polymerase chain reaction (PCR) to eliminate any potential genomic DNA. Transcript abundance was determined by real-time PCR using TaqMan Gene Expression Assays (Applied Biosystems, Foster City, CA, USA). The PCR amplifications of the *igf1* and *igf1r* gene transcripts were performed in triplicate on an ABI Prism 7900HT Sequence Detector (Applied Biosystems) using standard cycling conditions recommended by the manufacturer (50°C for 2 min, 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min). Singleplex amplification was performed in 384-well plates with a total reaction volume of $20 \mu\text{L}$, containing $10 \mu\text{L}$ TaqMan Universal PCR Master Mix (Applied Biosystems, Branchbury, NY, USA), $1 \mu\text{L}$ cDNA template, 200 nM probe, 900 nM forward and reverse primers and $6 \mu\text{L}$ diethylpyrocarbonate (DEPC)-treated water. Standard curves of the target gene and 18S were included in each plate, consisting of 10-fold serial dilutions of cDNA synthesised from

a randomly chosen fetal liver sample with good RNA quality. Real-time PCR efficiencies were calculated from the slopes of the standard curves for each target gene using the equation: $E = 10^{(-1/\text{slope})}$ (Pfaffl 2001). Comparisons of mRNA levels between groups was performed according to Pfaffl (2001), expressing one group relative to another and as a ratio to relative 18S rRNA levels, used as a housekeeping gene, with 99% confidence intervals (CI) generated around the ratio to account for multiple testing (Bloomfield *et al.* 2006). Probe and primer sequences were as published previously (Bloomfield *et al.* 2006). Primers sequences for *igf1* cover the end of exon 3 and those for *igf1r* are specific for the β -subunit.

Western blotting for determination of IGF-1 and IGF-1 receptor protein levels

Protein was extracted from tissues, quantified, separated electrophoretically, transferred to nitrocellulose membranes and non-specific binding blocked as described previously (Shaikh *et al.* 2005). Blots were then incubated with anti-IGF-1 and anti-IGF-1 receptor (IGF-1R) antibodies, as described previously (Bloomfield *et al.* 2006), except that the anti-IGF-1 antibody was used at a dilution of 1 : 500. Secondary antibodies were as described previously (Bloomfield *et al.* 2006). In addition, specific protein bands were detected and captured as described previously (Shaikh *et al.* 2005). Densitometric analysis of the immunoblots that were visualised on autoradiographic film was performed on a GS800 densitometer (Bio-Rad, Hercules, CA, USA) using Quantity One software (Bio-Rad). Membranes were then stripped and processed for the detection of β -actin as described previously (Bloomfield *et al.* 2006), but using a 1 : 50 000 dilution of the primary antibody. All signals are expressed as optical densities relative to the signal for β -actin.

Histology

To confirm whether weekly treatment with IGF-1 had a similar effect on mucosal growth in the small intestine as daily treatment (Bloomfield *et al.* 2002a), we measured villus height, crypt depth, submucosal thickness and muscularis thickness in the jejunum, the region of the GIT that we have reported previously was most responsive to intra-amniotic IGF-1 treatment (Bloomfield *et al.* 2002a). Longitudinal sections of the jejunum were embedded in paraffin and $5 \mu\text{m}$ sections were cut, mounted onto poly-L-lysine-coated glass slides (Biolab Scientific, Auckland, New Zealand) and stained with haematoxylin and eosin. Image analysis was performed on a light microscope (Carl Zeiss Microimaging, Thornwood, NY, USA) using AxioVision software (version 3.1.1.1) and an AxioCam HRc camera (Carl Zeiss Microimaging). For each fetus, 12 measurements were made from each of two sections of jejunum for each of the parameters listed above, with the mean of the 24 measurements used in subsequent analyses.

Statistics

Data for real-time PCR are expressed as relative expression ratios to the control group with 99% confidence intervals to account for multiple comparisons between groups. Non-PCR

Table 1. Fetal size and organ weights

Unless indicated otherwise, data are given as the mean \pm s.d. * $P < 0.05$ compared with control. There were no significant differences between the intrauterine growth-restricted (IUGR) and insulin-like growth factor (IGF)-1-treated IUGR groups

	Control	IUGR	IGF-1
Body and organs			
<i>n</i>	11	9	7
Bodyweight (kg)	3.90 \pm 0.68	3.09 \pm 0.84*	3.22 \pm 0.69
Crown-rump length (cm)	43.9 \pm 3.4	40.6 \pm 3.0	41.5 \pm 3.1
Chest girth (cm)	33.5 \pm 2.0	31.1 \pm 2.7	31.6 \pm 2.0
Brain (g)	44.4 \pm 5.7	41.3 \pm 6.1	42.3 \pm 5.5
Brain : bodyweight ratio ($\times 10^3$)	11.6 \pm 1.6	13.8 \pm 2.2*	13.3 \pm 2.1
Liver (g)	145.2 \pm 46.8	123.9 \pm 49.2	112.5 \pm 28.8
Liver : bodyweight ratio ($\times 10^3$)	38.0 \pm 13.7	40.4 \pm 14.4	35.5 \pm 8.1
Brain : liver ratio	0.34 \pm 0.10	0.38 \pm 0.15	0.37 \pm 0.09
Gastrointestinal tract			
<i>n</i>	8	7	7
Stomach (g)	31.4 \pm 7.1	27.5 \pm 11.7	27.1 \pm 5.6
Small intestine			
Weight (g)	58.6 \pm 8.9	51.3 \pm 21.6	58.4 \pm 15.2
Length (m)	7.28 \pm 0.59	7.04 \pm 0.69	7.33 \pm 1.05
Large intestine			
Weight (g)	13.3 \pm 2.3	11.3 \pm 3.4	13.0 \pm 4.7
Length (m)	1.34 \pm 0.18	1.22 \pm 0.16	1.49 \pm 0.36
Total intestine			
Weight (g)	77.5 \pm 23.9	62.3 \pm 24.8	71.4 \pm 17.5
Length (m)	8.62 \pm 0.69	8.23 \pm 0.76	8.82 \pm 0.95

Table 2. Effects of embolisation and insulin-like growth factor (IGF)-1 treatment on the morphometry of the jejunal mucosa

Unless indicated otherwise, data are given as the mean \pm s.d. * $P < 0.05$, ** $P < 0.01$ compared with control; †† $P < 0.01$, ††† $P < 0.001$ compared with the intrauterine growth-restricted (IUGR) group

	Control (<i>n</i> = 10)	IUGR (<i>n</i> = 7)	IGF-1 (<i>n</i> = 7)
Villus height (μm)	353.1 \pm 59.0	457.1 \pm 62.9**	298.5 \pm 44.3†††
Crypt depth (μm)	88.5 \pm 11.3	77.9 \pm 10.1	93.5 \pm 15.2
Submucosal thickness (μm)	54.1 \pm 8.4	44.5 \pm 5.7*	57.7 \pm 8.6††
Muscularis externa thickness (μm)	95.3 \pm 36.5	70.9 \pm 8.5**	88.4 \pm 10.4††
Villus height : crypt depth ratio	4.1 \pm 0.9	5.8 \pm 0.9**	3.3 \pm 0.6†††

data were compared by analysis of variance (ANOVA) in Minitab (version 15; University Park, Pennsylvania State University, State College, PA, USA), with data transformed by the Box-Cox method (Box and Cox 1964) as required. Pairwise comparisons between the groups were performed using Tukey's simultaneous tests. The significance level for these analyses was set at the 5% level.

Results

Fetal size and organ weights

Consistent with previous studies (Bloomfield *et al.* 2002a; Eremia *et al.* 2007), embolised fetuses were hypoxic, polycythaemic and hypoglycaemic (data not shown). Embolisation reduced fetal bodyweight by 21% ($P < 0.05$; Table 1) and

increased the brain:bodyweight ratio by 17% ($P < 0.05$; Table 1), consistent with a brain-sparing effect. Other organ weights were not significantly different between groups (Table 1). Histological analysis of the jejunum showed that embolisation decreased the thickness of the submucosa and muscularis externa and increased villus height, thus also increasing the villus height : crypt depth ratio (Table 2).

Treatment of IUGR fetuses did not alter fetal bodyweight or size (Table 1) and had no significant effect on organ weight (Table 1). However, IGF-1 treatment significantly decreased villus height and the villus height : crypt depth ratio, and significantly increased the thickness of the submucosa and muscularis externa compared with IUGR fetuses such that these values did not differ significantly from those found in control fetuses (Table 2).

Gene and protein expression in the GIT

Embolisation reduced *igf1* mRNA levels in the stomach by nearly half ($P = 0.01$), but there were no differences in *igf1* expression in other GIT regions (Table 3). IGF-1 treatment resulted in increased *igf1* mRNA levels in the stomach compared with IUGR fetuses, although levels remained significantly lower than in control fetuses (Table 3). In the duodenum, IGF-1 treatment resulted in significantly lower *igf1* mRNA levels compared with IUGR fetuses (Table 3). In contrast, in the jejunum, *igf1* mRNA levels were increased by 4.0- and 3.6-fold compared with control and IUGR fetuses, respectively (Table 3).

Embolisation had no effect on *igf1r* mRNA levels in the GIT (Table 3). However, intra-amniotic IGF-1 treatment increased

igf1r mRNA levels in the ileum compared with control fetuses (Table 3). IGF-1 treatment had no effect on *igf1r* mRNA levels in the jejunum.

Embolisation had no significant effects on IGF-1 or IGF-1R protein levels in the GIT (Table 4). IGF-1 treatment significantly increased IGF-1 levels in the ileum compared with controls (Table 4).

Gene and protein expression in the two hepatic lobes

In control fetuses, *igf1* mRNA levels in the right hepatic lobe were 19% lower than in the left lobe, but *igf1r* mRNA levels were similar (Table 5). In IUGR fetuses, *igf1* and *igf1r* mRNA levels in the right lobe were 6.3-fold higher than in the left lobe

Table 3. *igf1* and *igf1r* mRNA levels in liver and gastrointestinal tract

Data are given as fold changes with 99% confidence intervals in parentheses in the intrauterine growth-restricted (IUGR) group ($n = 9$) relative to the control group ($n = 11$) or in the insulin-like growth factor (IGF)-1-treated IUGR group ($n = 7$) relative to either the control or IUGR groups

	mRNA levels expressed as a ratio to 18s rRNA		
	IUGR group relative to control group	IGF-1 group relative to control group	IGF-1 group relative to IUGR group
Liver			
Right lobe			
<i>igf1</i>	16.3 (11.0–24.1)	2.45 (1.02–5.87)	0.15 (0.12–0.18)
<i>igf1r</i>	9.58 (6.37–14.40)	1.28 (0.77–2.11)	0.13 (0.13–0.14)
Left lobe			
<i>igf1</i>	2.07 (1.59–2.69)	0.79 (0.66–0.94)	0.38 (0.28–0.52)
<i>igf1r</i>	1.74 (1.16–2.61)	0.86 (0.61–1.22)	0.49 (0.32–0.75)
Gastrointestinal tract			
Stomach			
<i>igf1</i>	0.55 (0.30–1.00)	0.76 (0.62–0.93)	1.38 (1.12–1.70)
<i>igf1r</i>	0.95 (0.69–1.21)	0.85 (0.52–1.24)	0.90 (0.75–1.08)
Duodenum			
<i>igf1</i>	1.02 (0.58–1.92)	0.80 (0.39–1.64)	0.76 (0.69–0.86)
<i>igf1r</i>	1.46 (0.44–2.95)	0.83 (0.57–1.21)	0.56 (0.45–1.19)
Jejunum			
<i>igf1</i>	1.10 (0.18–7.36)	3.97 (2.02–7.20)	3.57 (1.71–6.38)
<i>igf1r</i>	1.00 (0.76–1.32)	1.04 (0.51–1.95)	1.04 (0.20–1.35)
Ileum			
<i>igf1</i>	1.03 (0.53–2.03)	1.02 (0.89–1.16)	0.98 (0.63–1.54)
<i>igf1r</i>	1.03 (0.41–2.59)	1.37 (1.26–1.50)	1.33 (0.92–1.94)

Table 4. Protein levels of insulin-like growth factor (IGF)-1 and IGF-1 receptor (IGF-1R) in the gut

Data were analysed densitometrically and expressed relative to the density of the β -actin band. Data are log-transformed and expressed as the mean \pm s.d. * $P < 0.05$ compared with control. IUGR, intrauterine growth restriction

	Control group ($n = 11$)	IUGR group ($n = 9$)	IGF-1-treated IUGR group ($n = 7$)
Log(IGF-1 : β-actin) (relative densitometric units)			
Stomach	-3.0 ± 0.9	-2.6 ± 0.9	-2.2 ± 1.1
Duodenum	-4.1 ± 1.8	-2.7 ± 1.5	-3.7 ± 1.7
Jejunum	-1.0 ± 0.6	-1.8 ± 1.25	-1.0 ± 0.2
Ileum	-2.6 ± 1.3	-1.4 ± 1.0	$-1.1 \pm 0.8^*$
Log(IGF-1R : β-actin) (relative densitometric units)			
Stomach	-2.9 ± 0.9	-3.2 ± 0.9	-4.1 ± 0.5
Duodenum	0.03 ± 0.02	0.05 ± 0.10	0.04 ± 0.07
Jejunum	-0.8 ± 0.8	-2.5 ± 2.5	-2.2 ± 1.4
ileum	-2.8 ± 1.3	-2.7 ± 1.2	-1.4 ± 1.4

(Table 5). Thus, in IUGR fetuses, *igf1* and *igf1r* mRNA levels were significantly higher in both the right and left hepatic lobes than in control fetuses (Table 3).

Treatment of IUGR fetuses with IGF-1 significantly reduced *igf1* and *igf1r* mRNA levels in both the right and left hepatic lobes compared with saline-treated embolised fetuses (Table 3). The *igf1* and *igf1r* mRNA levels in the right hepatic lobe of IGF-1-treated fetuses were 2.5- and 1.7-fold higher, respectively, than in the left lobe (Table 5). However, although *igf1* mRNA levels in the right hepatic lobe of IGF-1 fetuses were 2.5-fold higher than those in the control group, *igf1* mRNA levels in the left hepatic lobe were comparatively reduced (Table 3).

Overall, IGF-1 protein levels were higher in the left hepatic lobe than the right lobe, and IGF-1R protein levels were higher

in the right lobe than the left (Fig. 1). In the left lobe, IGF-1 protein levels were significantly higher in the IGF-1-treated group than in controls (Fig. 1). The IGF-1 protein levels in the right lobe and IGF-1R protein levels in either lobe did not differ significantly between the groups (Fig. 1).

Discussion

We have shown previously that daily administration of 20 µg IGF-1 into the amniotic fluid for 10 days in late gestation results in a marked increase in growth of the GIT mucosa (Bloomfield *et al.* 2002a). In a subsequent study, we reported that extending the duration of treatment to 4 weeks, with thrice-weekly intra-amniotic administration of 120 µg IGF, improved fetal growth rates (Eremia *et al.* 2007). In the present study, we have extended the treatment interval to once weekly while still giving the same total weekly dose as used previously (Eremia *et al.* 2007) and have demonstrated that this treatment regimen still results in reversal of the adverse effects on GIT mucosal growth and development seen in IUGR. Interestingly, this reversal of the effects of IUGR on the fetal GIT occurs in the absence of any significant change in fetal weight. It is possible that other effects of IUGR may have also been altered in the absence of a change in fetal weight. For example, we have demonstrated previously that fetuses of ewes undernourished in the periconceptual period have reduced fetal growth trajectory in late gestation

Table 5. *igf1* and *igf1r* mRNA levels in the right hepatic lobe compared to the left hepatic lobe within each treatment group

Data are given as fold changes, with 99% confidence intervals in parentheses. IUGR, intrauterine growth restriction; IGF-1, insulin-like growth factor-1

	Control group (n = 11)	IUGR group (n = 9)	IGF-1-treated IUGR group (n = 7)
<i>igf1</i>	0.81 (0.74–0.88)	6.34 (4.13–9.74)	2.51 (2.26–2.78)
<i>igf1r</i>	1.16 (0.67–1.99)	6.37 (4.82–8.42)	1.72 (1.34–2.21)

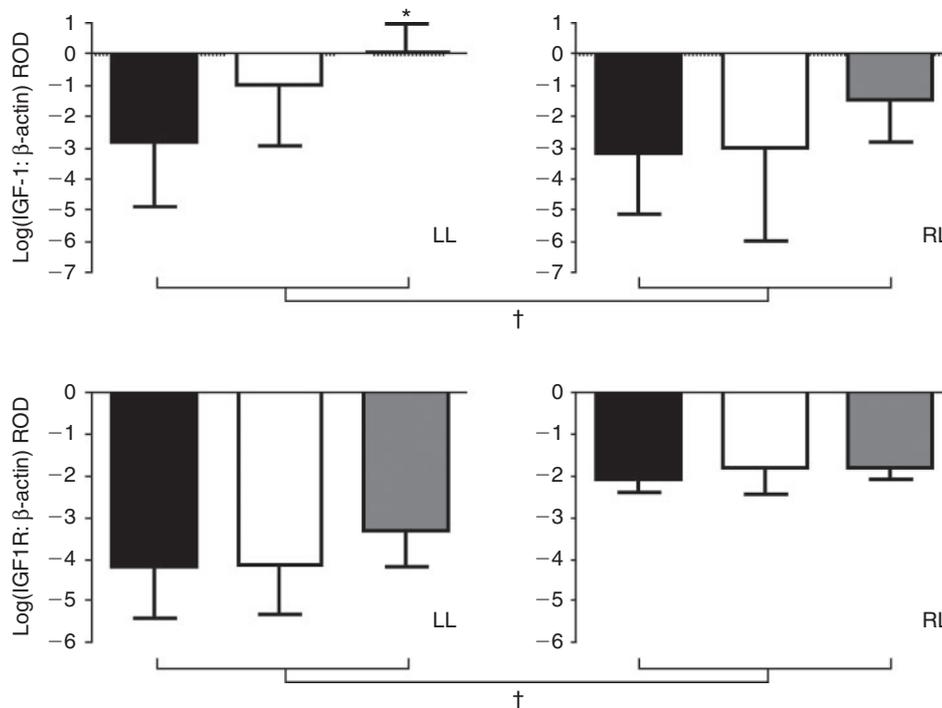


Fig. 1. Insulin-like growth factor (IGF)-1 (top panels) and IGF-1 receptor (IGF-1R; bottom panels) protein levels in the left (LL) and right (RL) hepatic lobes. Bars represent control (black), intrauterine growth-restricted (IUGR; white) and IGF-1-treated IUGR (grey) groups. Data are expressed as logarithmic transformations of the ratio of relative optical densities of IGF-1 and IGF-1R to β-actin protein levels and are given as the mean ± s.d. **P* < 0.05 compared with control; †*P* < 0.05 for comparisons between the two hepatic lobes.

without altered size at birth compared with fetuses of ewes that were well fed throughout (Oliver *et al.* 2005). However, fetuses with reduced growth trajectory go on to have impaired glucose tolerance postnatally (Todd *et al.* 2009). Thus, increasing fetal growth trajectory in IUGR fetuses may have beneficial long-term consequences, both on GIT development, as shown here, and potentially on metabolism, without a change in size at birth. The long-term consequences of intrauterine treatment of IUGR now need to be determined. Should this approach be shown to be safe and with beneficial long-term effects, a once-weekly treatment would be a feasible clinical approach.

Gut

Embolisation had little effect on *igf1* and *igf1r* mRNA and protein levels in the fetal GIT, with only *igf1* mRNA levels in the stomach being decreased. IGF-1 treatment suppressed *igf1* mRNA levels in the stomach, but increased *igf1* mRNA levels in the jejunum and IGF-1 protein levels in the ileum, and increased *igf1r* mRNA levels in the stomach. These findings differ from those of our previous study, in which IUGR resulted in decreased *igf1* and *igf1r* mRNA levels in the jejunum and IGF-1 increased *igf1r* mRNA levels throughout the GIT (Shaikh *et al.* 2005). Differences between the two studies are likely the result of variations in experimental protocols. Previously, fetuses were treated with 20 µg IGF-1 injected into the amniotic fluid on a daily basis for 10 days, commencing at 110 dGA, with tissues being collected 24 h after the last dose (Shaikh *et al.* 2005). In the present study, fetuses were treated with once weekly doses of 360 µg IGF-1 from 120 dGA, with tissues collected 7 days after the last dose. The half-life of IGF-1 in amniotic fluid is 24 h and significant amounts are still present for several days after dosing (Bloomfield *et al.* 2002b). Because the total weekly dose in the present study is more than twice that used previously, downregulation of *igf1* and *igf1r* in the proximal GIT is not unexpected. Changes in the distal small intestine may reflect systemic or local effects of IGF-1 treatment.

There was no significant effect of IGF-1 treatment on GIT weight and length. However, the effects on intestinal morphology were similar to those we reported previously (Bloomfield *et al.* 2002a). Impairment of GIT development in IUGR fetuses may lead to severe health outcomes, such as delays in establishing enteral feeding, postnatal growth failure and, particularly in developing countries, an increased risk of infection from bacterial translocation across the GIT epithelium (Lunn *et al.* 1991; Prindull and Ahmad 1993). Gastrointestinal permeability may also be permanently altered in infants born IUGR (Lunn *et al.* 1991). Supplementation of formula with IGF-1 (10 µg per 100 mL) in preterm babies, some of whom were IUGR, did not increase postnatal growth, but gut permeability was decreased (Corpeleijn *et al.* 2008). These studies emphasise that the postnatal period is not the optimal window for treatment of impaired GIT maturation. In contrast, we have demonstrated that antenatal supplementation of IGF-1 in IUGR ovine fetuses reverses the delayed maturation and impaired morphology of the GIT seen in IUGR fetuses. If the effect on morphology is translated into function, this treatment could mitigate common postnatal morbidities in IUGR infants.

Liver

In the present study, we report for the first time that mRNA and protein levels of *igf1* differ in the two hepatic lobes of ovine fetuses. This is consistent with the differential blood supply to the two lobes and with a previous report demonstrating differential regulation of numerous genes in the hepatic lobes of baboons in mid-gestation (Cox *et al.* 2006). Further, we showed that both IUGR induced by placental embolisation and its treatment with intra-amniotic IGF-1 differentially affect the expression of *igf1* and IGF-1R in the two hepatic lobes. These data suggest that the amniotic fluid is a potential route for interventions targeting both GIT growth and hepatic development.

The higher *igf1* and *igf1r* mRNA levels in the left hepatic lobe compared with the right in control fetuses is consistent with blood supply to the left lobe being almost exclusively oxygen- and nutrient-rich UV blood, whereas the right lobe receives mostly oxygen- and nutrient-depleted blood (Edelstone *et al.* 1978). This difference is secondary to shunting of UV blood through the DV, thereby bypassing the right hepatic lobe, which therefore receives a significant proportion of its blood supply from PV drainage. In fetal lambs 38–64% of the umbilical blood is shunted through the DV (Paulick *et al.* 1991; Rudolph *et al.* 1991); in human fetuses, similar shunting has been estimated at 45% at 20 weeks and 15% at term (Pennati *et al.* 1998; Kiserud *et al.* 2000b; Bellotti *et al.* 2004).

The liver is likely to have an important role in fetal growth, being the first organ to receive nutrient-rich oxygenated blood from the placenta (Tchirikov *et al.* 2002). It has a role in the synthesis and metabolism of macronutrients (Seifter and England 1994), as well as in the synthesis of IGF-1 (Sjögren *et al.* 1999; Ohlsson *et al.* 2009). When DV shunting was increased experimentally (and thus hepatic perfusion decreased) by implantation of a stent into the DV, cell proliferation in the liver, heart and skeletal muscle of ovine fetuses was reduced (Tchirikov *et al.* 2002). In contrast, increased hepatic perfusion led to greater *igf1* and *igf2* mRNA expression in the liver (Tchirikov *et al.* 2002). These data suggest a potential role of hepatic perfusion in fetal growth. Blood flow through the DV is increased during acute hypoxia in both human and ovine fetuses (Edelstone *et al.* 1980; Reuss and Rudolph 1980; Rudolph *et al.* 1991; Tchirikov *et al.* 1998; Kiserud *et al.* 2000a). In severely IUGR fetuses, greater shunting of UV blood flow through the DV provides a relatively constant flow to the heart and brain at the expense of fetal hepatic perfusion from the DV (Bellotti *et al.* 2004; Kiserud *et al.* 2006). This shunting may be mediated, at least in part, via fetal responses to intrauterine stress (Cohen *et al.* 1982; Jensen and Berger 1993; Bellotti *et al.* 2004, 2006).

It would be expected that this increased shunting of UV blood through the DV would result in decreased hepatic expression of nutrient-responsive genes, such as *igf1*. Decreased hepatic expression (the hepatic lobe sampled is not stated) of *igf1* has been reported previously in studies in which IUGR was induced by prepregnancy carunclectomy in the ewe, resulting in restricted placental size (Gentili *et al.* 2009). This results in chronic hypoxia and fetuses of varying size. Interestingly, decreased hepatic *igf1* expression was only

seen in fetuses that also had reduced liver growth. In IUGR fetuses with preserved liver growth, *igf1* mRNA levels did not differ from that in controls (Gentili *et al.* 2009). In contrast, in the present study, *igf1* mRNA levels in both hepatic lobes increased among the saline-treated IUGR fetuses compared with the controls. The much greater increase in the right hepatic lobe compared with the left (eightfold) suggests that these differences may be related to differential blood flow to the two hepatic lobes. In severe IUGR, the increased shunting of blood via the DV may lead to a reversal of blood flow in the left portal vein, with PV blood then contributing a much greater proportion of the blood supply to the left hepatic lobe than is usually the case (Kilavuz *et al.* 2003; Kessler *et al.* 2007, 2009). Furthermore, the decreased hepatic blood supply from the UV may be compensated for by an increased contribution from the hepatic arteries, which would supply both hepatic lobes (Kilavuz and Vetter 1999).

Finally, it is known that the fetal GIT can supply a significant proportion of the fetal nitrogen requirement in late gestation (Gitlin *et al.* 1972) and that this uptake increases with addition of supplementary amino nitrogen into the fetal GIT (Charlton and Reis 1981). Therefore, it is possible that, in the face of impaired placental supply, IUGR fetuses increase GIT utilisation of amino acids, including from the amniotic fluid. Our preliminary data suggest that this may indeed be the case in IUGR induced by placental embolisation (Bloomfield *et al.* 2002c). Thus, in IUGR there may be an increase in the proportion of blood supply to both hepatic lobes from hepatic arterial blood, an adjustment of the relative proportions that each hepatic lobe receives from PV blood and potentially altered nutrient supply from the GIT. Together, these changes could account for the increased *igf1* mRNA levels in both hepatic lobes reported here.

Another possible explanation for the upregulation of hepatic *igf1* and *igf1r* mRNA levels following IUGR is that in this paradigm, which reflects relatively acute placental insufficiency, there may be an initial hepatic sparing at the expense of brain sparing (Haugen *et al.* 2005). However, our previous finding of reduced hepatic weight following a similar protocol (Bloomfield *et al.* 2002a) and the tendency to reduced hepatic weight in the present study, makes this explanation unlikely.

The reduction in *igf1* mRNA levels with IGF-1 treatment is consistent with our previous study (Shaikh *et al.* 2005). In the present study, *igf1* mRNA levels decreased in both hepatic lobes, consistent with a systemic effect or downregulation in response to IGF-1 delivery via the portal system, which, as outlined above, may supply both hepatic lobes in IUGR. The magnitude of the effect is different in the two hepatic lobes, perhaps more in keeping with an effect mediated via the portal circulation. This could be either a direct effect of absorbed IGF-1, supported by increased IGF-1 protein levels because the antibody detects both human and ovine IGF-1, or via a GIT-derived mediator, such as ghrelin, or an incretin, such as glucagon-like peptide 1.

Conclusion

Treatment of IUGR fetuses with IGF-1, given as a weekly intra-amniotic injection, results in reversal of the impaired GIT

development seen with IUGR, along with altered expression of *igf1* and *igf1r*. The potential postnatal effects of this treatment warrant further investigation to determine whether intra-amniotic IGF-1 can translate into an effective clinical treatment for severely growth-restricted fetuses. Such studies should address both the immediate perinatal morbidities, including GIT function, and longer-term outcomes. In addition, both IUGR and its once-weekly treatment with intra-amniotic IGF-1 have differential effects on the expression of *igf1* and *igf1r* mRNA in the two hepatic lobes of ovine fetuses. The patterns of expression, together with existing literature, suggest that differences in the development of the two hepatic lobes during fetal life likely relate to the complex blood supply to the fetal liver and its regulation in IUGR. These data are relevant for the study of hepatic gene expression in the fetus and potentially for understanding the long-term effects of IUGR on later hepatic function.

Acknowledgements

The authors thank members of the Fetal and Neonatal Physiology Group at the Liggins Institute (University of Auckland) for their assistance with this work. The authors gratefully acknowledge financial support from the Health Research Council of New Zealand.

References

- Barker, D. J. (1998). *In utero* programming of chronic disease. *Clin. Sci.* **95**, 115–128. doi:10.1042/CS19980019
- Battaglia, C., Artini, P. G., D'Ambrogio, G., Galli, P. A., Segre, A., and Genazzani, A. R. (1992). Maternal hyperoxygenation in the treatment of intrauterine growth retardation. *Am. J. Obstet. Gynecol.* **167**, 430–435.
- Bellotti, M., Pennati, G., Gasperi, C. D., Bozzo, M., Battaglia, F. C., and Ferrazzi, E. (2004). Simultaneous measurements of umbilical venous, fetal hepatic, and ductus venosus blood flow in growth-restricted human fetuses. *Am. J. Obstet. Gynecol.* **190**, 1347–1358. doi:10.1016/J.AJOG.2003.11.018
- Bellotti, M., Gasperi, C. D., Bee, V., Zucca, I., and Rognoni, G. (2006). Cardiocirculatory changes in IUGR fetuses: longitudinal quantitative Doppler analysis. *Ultrasound Obstet. Gynecol.* **28**, 391. [Abstract] doi:10.1002/UOG.2971
- Bloomfield, F. H., Bauer, M. K., van Zijl, P. L., Gluckman, P. D., and Harding, J. E. (2002a). Amniotic IGF-I supplements improve gut growth but reduce circulating IGF-I in growth-restricted fetal sheep. *Am. J. Physiol. Endocrinol. Metab.* **282**, E259–E269.
- Bloomfield, F. H., Breier, B. H., and Harding, J. E. (2002b). Fate of (125) I-IGF-I administered into the amniotic fluid of late-gestation fetal sheep. *Pediatr. Res.* **51**, 361–369. doi:10.1203/00006450-200203000-00016
- Bloomfield, F. H., van Zijl, P. L., Bauer, M. K., and Harding, J. E. (2002c). Effects of intrauterine growth restriction and intraamniotic insulin-like growth factor-I treatment on blood and amniotic fluid concentrations and on fetal gut uptake of amino acids in late-gestation ovine fetuses. *J. Pediatr. Gastroenterol. Nutr.* **35**, 287–297. doi:10.1097/00005176-200209000-00010
- Bloomfield, F. H., van Zijl, P. L., Bauer, M. K., Phua, H. H., and Harding, J. E. (2006). Effect of pulsatile growth hormone administration to the growth-restricted fetal sheep on somatotrophic axis gene expression in fetal and placental tissues. *Am. J. Physiol. Endocrinol. Metab.* **291**, E333–E339. doi:10.1152/AJPENDO.00045.2006
- Box, G. E. P., and Cox, D. R. (1964). An analysis of transformations. *J. R. Stat. Soc. Series B Stat. Methodol.* **26**, 211–252.
- Charlton, V. E., and Reis, B. L. (1981). Effects of gastric nutritional supplementation on fetal umbilical uptake of nutrients. *Am. J. Physiol. Endocrinol. Metab.* **241**, E178–E185.

- Cohen, W. R., Piasecki, G. J., and Jackson, B. T. (1982). Plasma catecholamines during hypoxemia in fetal lamb. *Am. J. Physiol. Regul. Integr. Comp. Physiol.* **243**, R520–R525.
- Corpeleijn, W. E., van Vliet, I., de Gast-Bakker, D.-A. H., van der Schoor, S. R. D., Alles, M. S., Hoijer, M., Tibboel, D., and van Goudoever, J. B. (2008). Effect of enteral IGF-1 supplementation on feeding tolerance, growth, and gut permeability in enterally fed premature neonates. *J. Pediatr. Gastroenterol. Nutr.* **46**, 184–190. doi:10.1097/MPG.0B013E31815AFFEC
- Cox, L. A., Schlambritz-Loutsevitch, N., Hubbard, G. B., Nijland, M. J., McDonald, T. J., and Nathanielsz, P. W. (2006). Gene expression profile differences in left and right liver lobes from mid-gestation fetal baboons: a cautionary tale. *J. Physiol.* **572**, 59–66.
- Edelstone, D. I., Rudolph, A. M., and Heymann, M. A. (1978). Liver and ductus venosus blood flows in fetal lambs *in utero*. *Circ. Res.* **42**, 426–433.
- Edelstone, D. I., Rudolph, A. M., and Heymann, M. A. (1980). Effects of hypoxemia and decreasing umbilical flow liver and ductus venosus blood flows in fetal lambs. *Am. J. Physiol.* **238**, H656–H663.
- Eremia, S. C., de Boo, H. A., Bloomfield, F. H., Oliver, M. H., and Harding, J. E. (2007). Fetal and amniotic insulin-like growth factor-I supplements improve growth rate in intrauterine growth restriction fetal sheep. *Endocrinology* **148**, 2963–2972. doi:10.1210/EN.2006-1701
- Fattal-Valevski, A., Toledano-Alhadeif, H., Leitner, Y., Geva, R., Eshel, R., and Harel, S. (2009). Growth patterns in children with intrauterine growth retardation and their correlation to neurocognitive development. *J. Child. Neurol.* **24**, 846–851. doi:10.1177/0883073808331082
- Gentili, S., Morrison, J. L., and McMillen, I. C. (2009). Intrauterine growth restriction and differential patterns of hepatic growth and expression of IGF1, PCK2, and HSDL1 mRNA in the sheep fetus in late gestation. *Biol. Reprod.* **80**, 1121–1127. doi:10.1095/BIOLREPROD.108.073569
- Gitlin, D., Kumate, J., Morales, C., Noriega, L., and Arévalo, N. (1972). The turnover of amniotic fluid protein in the human conceptus. *Am. J. Obstet. Gynecol.* **113**, 632–645.
- Godfrey, K. M., and Barker, D. J. (2000). Fetal nutrition and adult disease. *Am. J. Clin. Nutr.* **71**, 1344–1352.
- Harding, J. E., Evans, P. C., and Gluckman, P. D. (1997). Maternal growth hormone treatment increases placental diffusion capacity but not fetal or placental growth in sheep. *Endocrinology* **138**, 5352–5358. doi:10.1210/EN.138.12.5352
- Haugen, G., Hanson, M., Kiserud, T., Crozier, S., Inskip, H., and Godfrey, K. M. (2005). Fetal liver-sparing cardiovascular adaptations linked to mother's slimness and diet. *Circ. Res.* **96**, 12–14. doi:10.1161/01.RES.0000152391.45273.A2
- Haylor, J., Singh, I., and Nahas, A. M. E. (1991). Nitric oxide synthesis inhibitor prevents vasodilation by insulin-like growth factor I. *Kidney Int.* **39**, 333–335. doi:10.1038/KI.1991.42
- Hendrix, N., and Berghella, V. (2008). Non-placental causes of intrauterine growth restriction. *Semin. Perinatol.* **32**, 161–165. doi:10.1053/J.SEMPERI.2008.02.004
- Henriksen, T., and Clausen, T. (2002). The fetal origins hypothesis: placental insufficiency and inheritance versus maternal malnutrition in well-nourished populations. *Acta Obstet. Gynecol. Scand.* **81**, 112–114.
- Ibáñez, L., Ong, K., Dunger, D. B., and de Zegher, F. (2006). Early development of adiposity and insulin resistance after catch-up weight gain in small-for-gestational-age children. *J. Clin. Endocrinol. Metab.* **91**, 2153–2158. doi:10.1210/JC.2005-2778
- Jensen, A., and Berger, R. (1993). Regional distribution of cardiac output. In 'Fetus and Neonate: The Circulation'. (Eds M. A. Hanson, J. Spencer and C. H. Rodeck.) pp. 23–74. (Cambridge University Press: Cambridge.)
- Kessler, J., Rasmussen, S., and Kiserud, T. (2007). The left portal vein as an indicator of watershed in the fetal circulation: development during the second half of pregnancy and a suggested method of evaluation. *Ultrasound Obstet. Gynecol.* **30**, 757–764. doi:10.1002/UOG.5146
- Kessler, J., Rasmussen, S., Godfrey, K., Hanson, M., and Kiserud, T. (2009). Fetal growth-restriction is associated with prioritization of umbilical blood flow to the left hepatic lobe, at the expense of the right lobe. *Pediatr. Res.* **66**, 113–117. doi:10.1203/PDR.0B013E3181A29077
- Kilavuz, Ö., and Vetter, K. (1999). Is the liver of the fetus the 4th preferential organ for arterial blood supply besides brain, heart, and adrenal glands? *J. Perinat. Med.* **27**, 103–106. doi:10.1515/JPM.1999.012
- Kilavuz, O., Vetter, K., Kiserud, T., and Vetter, P. (2003). The left portal vein is the watershed of the fetal venous system. *J. Perinat. Med.* **31**, 184. doi:10.1515/JPM.2003.025
- Kiserud, T., Ozaki, T., Nishina, H., Rodeck, C., and Hanson, M. A. (2000a). Effect of NO, phenylephrine, and hypoxemia on ductus venosus diameter in fetal sheep. *Am. J. Physiol. Heart Circ. Physiol.* **279**, 1166–1171.
- Kiserud, T., Rasmussen, S., and Skulstad, S. (2000b). Blood flow and the degree of shunting through the ductus venosus in the human fetus. *Am. J. Obstet. Gynecol.* **182**, 147–153. doi:10.1016/S0002-9378(00)70504-7
- Kiserud, T., Kessler, J., Ebbing, C., and Rasmussen, S. (2006). Ductus venosus shunting in growth-restricted fetuses and the effect of umbilical circulatory compromise. *Ultrasound Obstet. Gynecol.* **28**, 143–149. doi:10.1002/UOG.2784
- Kramer, M. S., and Kakuma, R. (2003). Energy and protein intake in pregnancy. *Cochrane Database Syst. Rev.* **4**, CD000032.
- Langley-Evans, S. C., Gardner, D. S., and Jackson, A. A. (1996). Maternal protein restriction influences the programming of the rat hypothalamic–pituitary–adrenal axis. *J. Nutr.* **126**, 1578–1585.
- Leunissen, R. W. J., Oosterbeek, P., Hol, L. K. M., Hellingman, A. A., Stijnen, T., and Hokken-Koelega, A. C. S. (2008). Fat mass accumulation during childhood determines insulin sensitivity in early adulthood. *J. Clin. Endocrinol. Metab.* **93**, 445–451. doi:10.1210/JC.2007-1543
- Lunn, P. G., Northrop-Clewes, C. A., and Downes, N. M. (1991). Intestinal permeability, mucosal injury, and growth faltering in Gambian infants. *Lancet* **338**, 907–910. doi:10.1016/0140-6736(91)91772-M
- McMillen, I. C., and Robinson, J. S. (2005). Developmental origins of the metabolic syndrome: prediction, plasticity, and programming. *Physiol. Rev.* **85**, 571–633. doi:10.1152/PHYSREV.00053.2003
- Miller, S. L., Loose, J. M., Jenkin, G., and Wallace, E. M. (2009). The effects of sildenafil citrate (Viagra) on uterine blood flow and well being in the intrauterine growth-restricted fetus. *Am. J. Obstet. Gynecol.* **200**, 102.e1–102.e7. doi:10.1016/J.AJOG.2008.08.029
- National Animal Ethics Advisory Committee (2002). 'Good Practice Guide for the Use of Animals in Research, Testing and Teaching.' (Ministry of Agriculture and Forestry: Wellington.)
- Ohlsson, C., Mohan, S., Sjogren, K., Tivesten, A., Isgaard, J., Isaksson, O., Jansson, J.-O., and Svensson, J. (2009). The role of liver-derived insulin-like growth factor-I. *Endocr. Rev.* **30**, 494–535. doi:10.1210/ER.2009-0010
- Oliver, M. H., Harding, J. E., Breier, B. H., Evans, P. C., and Gluckman, P. D. (1993). Glucose but not a mixed amino acid infusion regulates plasma insulin-like growth factor-I concentrations in fetal sheep. *Pediatr. Res.* **34**, 62–65. doi:10.1203/00006450-199307000-00015
- Oliver, M. H., Harding, J. E., Breier, B. H., and Gluckman, P. D. (1996). Fetal insulin-like growth factor (IGF)-I and IGF-II are regulated differentially by glucose or insulin in the sheep fetus. *Reprod. Fertil. Dev.* **8**, 167–172. doi:10.1071/RD9960167
- Oliver, M. H., Hawkins, P., and Harding, J. E. (2005). Periconceptual undernutrition alters growth trajectory, endocrine and metabolic responses to fasting in late gestation fetal sheep. *Pediatr. Res.* **57**, 591–598. doi:10.1203/01.PDR.0000155942.18096.9C

- Ong, K. K., and Loos, R. J. F. (2006). Rapid infancy weight gain and subsequent obesity: systematic reviews and hopeful suggestions. *Acta Paediatr.* **95**, 904–908. doi:10.1080/08035250600719754
- Paulick, R. P., Meyers, R. L., Rudolph, C. D., and Rudolph, A. M. (1991). Umbilical and hepatic venous responses to circulating vasoconstrictive hormones in fetal lamb. *Am. J. Physiol.* **260**, H1205–H1213.
- Pennati, G., Bellotti, M., Ferrazzi, E., Bozzo, M., Pardi, G., and Fumero, R. (1998). Blood flow through the ductus venosus in human fetus: calculation using Doppler velocimetry and computational findings. *Ultrasound Med. Biol.* **24**, 477–487. doi:10.1016/S0301-5629(98)00011-8
- Pfaffl, M. W. (2001). A new mathematical model for relative quantification in real-time RT-PCR. *Nucleic Acids Res.* **29**, e45. doi:10.1093/NAR/29.9.E45
- Piper, J. M., Xenakis, E. M.-J., McFarland, M., Elliott, B. D., Berkus, M. D., and Langer, O. (1996). Do growth-retarded premature infants have different rates of perinatal morbidity and mortality than appropriately grown premature infants? *Obstet. Gynecol.* **87**, 169–174. doi:10.1016/0029-7844(95)00400-9
- Prindull, G., and Ahmad, M. (1993). The ontogeny of the gut mucosal immune system and the susceptibility to infections in infants of developing countries. *Eur. J. Pediatr.* **152**, 786–792. doi:10.1007/BF02073371
- Resnik, R. (2002). Intrauterine growth restriction. *Obstet. Gynecol.* **99**, 490–496. doi:10.1016/S0029-7844(01)01780-X
- Reuss, M. L., and Rudolph, A. M. (1980). Distribution and recirculation of umbilical and systemic venous blood flow in fetal lambs during hypoxia. *J. Dev. Physiol.* **2**, 71–84.
- Robinson, J. S., Moore, V. M., Owens, J. A., and McMillen, I. C. (2000). Origins of fetal growth restriction. *Eur. J. Obstet. Gynecol. Reprod. Biol.* **92**, 13–19. doi:10.1016/S0301-2115(00)00421-8
- Rotteveel, J., van Weissenbruch, M. M., Twisk, J. W. R., and Delemarre-Van de Waal, H. A. (2008). Infant and childhood growth patterns, insulin sensitivity, and blood pressure in prematurely born young adults. *Pediatrics* **122**, 313–321. doi:10.1542/PEDS.2007-2012
- Rudolph, A. M. (1983). Hepatic and ductus venosus blood flows during fetal life. *Hepatology* **3**, 254–258.
- Rudolph, C. D., Meyers, R. L., Paulick, R. P., and Rudolph, A. M. (1991). Effects of ductus venosus obstruction on liver and regional blood flows in the fetal lamb. *Pediatr. Res.* **29**, 347. doi:10.1203/00006450-199104000-00004
- Saenger, P., Czernichow, P., Hughes, I., and Reiter, E. O. (2007). Small for gestational age: short stature and beyond. *Endocr. Rev.* **28**, 219–251. doi:10.1210/ER.2006-0039
- Seifter, S., and Englard, S. (1994). Energy metabolism. In 'The Liver: Biology and Pathobiology'. (Eds I. M. Arias, J. L. Boyer, N. Fausto, W. B. Jakoby, D. Schachter and D. A. Shafritz.) pp. 323–364. (Raven Press: New York.)
- Shaikh, S., Bloomfield, F. H., Bauer, M. K., Phua, H. H., Gilmour, R. S., and Harding, J. E. (2005). Amniotic IGF-I supplementation of growth-restricted fetal sheep alters IGF-I and IGF receptor type 1 mRNA and protein levels in placental and fetal tissues. *J. Endocrinol.* **186**, 145–155. doi:10.1677/JOE.1.06113
- Sjögren, K., Liu, J.-L., Blad, K., Skrtic, S., Vidal, O., et al. (1999). Liver-derived insulin-like growth factor I (IGF-I) is the principal source of IGF-I in blood but is not required for postnatal body growth in mice. *Proc. Natl Acad. Sci. USA* **96**, 7088–7092. doi:10.1073/PNAS.96.12.7088
- Tchirikov, M., Eisermann, K., Rybakowski, C., and Schröder, H. J. (1998). Doppler ultrasound evaluation of ductus venosus blood flow during acute hypoxemia in fetal lambs. *Ultrasound Obstet. Gynecol.* **11**, 426–431. doi:10.1046/J.1469-0705.1998.11060426.X
- Tchirikov, M., Kertschanska, S., Sturenberg, H. J., and Schroder, H. J. (2002). Liver blood perfusion as a possible instrument for fetal growth regulation. *Placenta* **23**(Suppl. A), S153–S158. doi:10.1053/PLAC.2002.0810
- Thornburg, K., and Morton, M. (1994). Development of the cardiovascular system. In 'Textbook of Fetal Physiology'. (Eds G. Thornburn and R. Harding.) pp. 95–130. (Oxford University Press: Oxford.)
- Todd, S. E., Oliver, M. H., Jaquier, A. L., Bloomfield, F. H., and Harding, J. E. (2009). Periconceptional undernutrition of ewes impairs glucose tolerance in their adult offspring. *Pediatr. Res.* **65**, 409–413. doi:10.1203/PDR.0B013E3181975EFA
- Weiner, Z., Divon, M. Y., and Itskovitz, J. (1996). Distribution of the umbilical venous return in normal and growth retarded human fetuses. *J. Soc. Gynecol. Invest.* **3**, 242. doi:10.1016/1071-5576(96)82815-2
- Winer, N., Branger, B., Azria, E., Tsatsaris, V., Philippe, H.-J., Rozé, J. C., Descamps, P., Boog, G., Cynober, L., and Darmaun, D. (2009). L-Arginine treatment for severe vascular fetal intrauterine growth restriction: a randomized double-blind controlled trial. *Clin. Nutr.* **28**, 243–248. doi:10.1016/J.CLNU.2009.03.007
- Wu, H. Y., Jeng, Y. Y., Yue, C. J., Chyu, K. Y., Hsueh, W. A., and Chan, T. M. (1994). Endothelial-dependent vascular effects of insulin and insulin-like growth factor I in the perfused rat mesenteric artery and aortic ring. *Diabetes* **43**, 1027–1032. doi:10.2337/DIABETES.43.8.1027

Manuscript received 1 December 2009, accepted 11 May 2010