Postnatal effects of intrauterine treatment of the growth-restricted ovine fetus with intra-amniotic insulin-like growth factor-1

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Key points

- Fetal growth restriction increases the risk of fetal and neonatal mortality and morbidity, and contributes to increased risk of chronic disease later in life.
- Intra-amniotic insulin-like growth factor-1 (IGF1) treatment of the growth-restricted ovine fetus improves fetal growth, but postnatal effects are unknown.
- Here we report that intra-amniotic IGF1 treatment of the growth-restricted ovine fetus alters size at birth and mechanisms of early postnatal growth in a sex-specific manner.
- We also show that maternal plasma C-type natriuretic peptide (CNP) products are related to fetal oxygenation and size at birth, and hence may be useful for non-invasive monitoring of fetal growth restriction.
- Intrauterine IGF1 treatment in late gestation is a potentially clinically relevant intervention that may ameliorate the postnatal complications of fetal growth restriction.

Abstract Placental insufficiency-mediated fetal growth restriction (FGR) is associated with altered postnatal growth and metabolism, which are, in turn, associated with increased risk of adult disease. Intra-amniotic insulin-like growth factor-1 (IGF1) treatment of ovine FGR increases growth rate in late gestation, but the effects on postnatal growth and metabolism are unknown. We investigated the effects of intra-amniotic IGF1 administration to ovine fetuses with uteroplacental embolisation-induced FGR on phenotypical and physiological characteristics in the 2 weeks after birth. We measured early postnatal growth velocity, amino-terminal propeptide of C-type natriuretic peptide (NTproCNP), body composition, tissue-specific mRNA expression, and milk intake in singleton lambs treated weekly with 360 μg intra-amniotic IGF1 (FGRI; $n = 13$ females, 19 males) or saline (FGRS; $n = 18$ females, 12 males) during gestation, and in controls (CON; $n = 15$ females, 22 males). There was a strong positive correlation between maternal NTproCNP and fetal oxygenation, and size at birth in FGR lambs. FGR lambs were ~20% lighter at birth and demonstrated accelerated postnatal growth velocity. IGF1 treatment did not alter perinatal mortality, partially abrogated the reduction in newborn size in females, but not males, and reduced accelerated growth in both sexes. IGF1-mediated upregulation of somatotrophic genes in males during the early postnatal period could suggest that treatment effects are associated with delayed axis maturation, whilst treatment outcomes in females may rely on the reprogramming of nutrient-dependent mechanisms of growth. These data suggest that the growth-restricted fetus is responsive to intra-amniotic intervention with IGF1, and that sex-specific somatotrophic effects persist in the early postnatal period.

Introduction

Fetal growth restriction (FGR) increases morbidity and mortality and necessitates intensive antenatal surveillance and specialist management to optimise the timing of birth (Alberry & Soothill, 2007). Although constrained fetal growth in the face of nutritional deprivation is immediately beneficial for fetal survival (Patel & Kalhan, 1992; Diderholm *et al.* 2007), compromised metabolic regulation (Chiesa *et al.* 2008; Diderholm, 2009) and growth (Modi *et al.* 2006) persist postnatally. The growth-restricted neonate is smaller and thinner than a normally-grown baby, with reduced lean (Padoan *et al.* 2004) and fat mass (Verkauskiene *et al.* 2007), and greater risk of adverse perinatal outcomes (Bernstein *et al.* 2000; Unterscheider *et al.* 2013). Although vasodilatory hormones, such as C type natriuretic peptide (CNP), have been suggested to play an important role in maintaining placental function and fetal growth in compromised pregnancy (Prickett *et al.* 2007; McNeill *et al.* 2009; Reid *et al.* 2014; Espiner *et al.* 2015), non-invasive fetal monitoring of late gestation compromised pregnancy is limited. There are no therapies that have been shown to improve fetal growth during compromised pregnancy in the clinical setting. Prenatal management focuses on regular assessment to balance the risk of intrauterine death with the adverse effects of preterm delivery (Baschat *et al.* 2001; Hecher *et al.* 2001; The GRIT Study Group, 2004).

Management of the growth-restricted neonate focuses on mitigation of morbidity in the perinatal period, such as hypoglycaemia and hypothermia, and appropriate post-natal growth. Small size at birth and thinness followed by accelerated postnatal growth velocity, termed "catch-up" growth, is associated with increased visceral adiposity (Singhal *et al.* 2010) and increased leptin concentrations in childhood (Beltrand *et al.* 2009). It has been proposed that infant nutrition should be managed to prevent postnatal compensatory growth acceleration in order to reduce the future risk of obesity (Chomtho *et al.* 2008). However, this may contribute to post-natal growth restriction and compromise developmental outcomes (Sakurai *et al.* 2008). Thus, an intra-uterine intervention that improves fetal growth may present an opportunity to improve perinatal and long-term outcomes by reducing the adverse effects associated with both FGR and abnormal postnatal growth patterns.

Previous ovine FGR research has been integral to the assessment of prenatal interventions to improve *in utero* growth (Spiroski *et al.* 2016). A potential therapy that has been studied extensively through maternal (Liu *et al.* 1994), fetal (Jensen *et al.* 1999), and amniotic routes (Bloomfield *et al.* 2002*a*, *b*; Darp *et al.* 2010) is insulin-like growth factor-1 (IGF1). IGF1 is widely expressed in the developing fetus (Han *et al.* 1988) and is implicated in the regulation of placental function and fetal growth (Owens, 1991; Gluckman, 1997). Fetal IGF1 is regulated predominantly by fetal nutritional supply (Bloomfield *et al.* 2013), whereas maturation of the somatotrophic axis and endocrine activation occur around parturition (Gluckman *et al.* 1983). Intra-amniotic IGF1 treatment of the FGR ovine fetus has been shown to increase fetal growth rate in late gestation (Eremia *et al.* 2007), even when given in physiological concentrations at weekly intervals (Wali *et al.* 2012). However, the effects of this brief prenatal intervention on phenotypic, physiologic, and molecular outcomes after birth are unknown. Thus, we investigated the effect of intra-amniotic IGF1-treatment of the growth-restricted fetus on perinatal mortality and on neonatal growth, including assessment of key somatotrophic gene expression in liver and muscle, body composition, metabolism, and also on concentrations of glucocorticoid and thyroid hormones, key to maturational changes associated with birth transition. We predicted that FGR would reduce size at birth and accelerate post-natal growth, and that intra-amniotic IGF1 infusion would abrogate these changes.

Methods

Ethical approval

All experimental procedures were approved by the University of Auckland Animal Ethics Committee (AEC/02/2008/R628 and AEC/03/2011/R874) and conducted in accordance with these approvals. Reporting of this experiment conforms with *The Journal of Physiology*'s *Principles and Standards for Reporting Animal Experiments* (Grundy, 2015) and with the ARRIVE Guidelines (Kilkenny *et al.* 2010).

Generation of experimental groups

The primary outcome for the experiment was glucose tolerance at 18 months of age (young adulthood); these data will be reported elsewhere and this manuscript reports fetal and neonatal outcomes following intra-amniotic IGF1 treatment. Based on previous studies of postnatal endocrinology in young adult sheep, we calculated that to detect a change in mean glucose area under the curve in a glucose tolerance test of 15% from 712 (SD 99) $mmol \cdot min^{-1} \cdot L^{-1}$, with 80% power and a 2-tailed significance level of 0.05, 12 successful studies at 18 months were required per experimental group. Accounting for perinatal and post-perinatal losses and exclusions due to technical difficulties, we estimated that we needed to generate 102 pregnant ewes.

Multiparous Romney ewes purchased from Landcorp Farming Limited (Wellington, New Zealand) were bred on

site with Poll Dorset rams, and scanned ∼40 and ∼70 days after mating to confirm pregnancy and fetal number. Singleton-carrying ewes were acclimatised to a feedlot with 12-hour controlled photoperiods, free access to water and a complete pelleted feed diet (Oliver *et al.* 2005). Ewes were allocated using a random number generator to control (CON) or placental embolisation-induced fetal growth restriction (FGR) initially, with a second randomisation of FGR ewes once embolization was complete to either intra-amniotic vehicle (saline, FGRS), or IGF1 (FGRI) to reduce potential bias.

Ewes randomised to FGR underwent surgery between 97 and 100 days' gestational age (dGA). After an overnight fast with free access to water, surgery was conducted under isoflurane anaesthesia (Medsource, Linyi, Shangdong, China). Both fetal femoral arteries were catheterised (Murotsuki *et al.* 1995). Maternal uterine arteries were catheterised bilaterally and the catheters advanced into the primary artery supplying each horn (Jensen *et al.* 1999) and an amniotic catheter was placed in the amniotic sac. Because CON fetuses did not undergo surgery, fetal data are presented for growth-restricted groups only. Fetal blood gas, plasma hormone and metabolite concentrations in chronically-catheterised fetal CON sheep have been published previously from similar preparations (Bloomfield *et al.* 2002*a*; de Boo *et al.* 2008; Wali *et al.* 2012).

FGR was induced by twice-daily bilateral uterine artery embolisation with 20–50 μm Superose® 12 polymerised polysaccharide microspheres (GE Healthcare, Auckland, NZ) over a 5-day period (103–107 dGA), titrated against fetal blood gas and metabolite measures as previously described (Bloomfield *et al.* 2002*b*).

FGR fetuses were randomly allocated to receive either 360 μg (100 μg·mL^{-1}) human recombinant IGF1 (FGRI; Genetech, San Francisco, California, United States) or an equivalent volume of sterile saline (FGRS) once-weekly as previously described (Wali *et al.* 2012), commencing at 107 dGA with the last treatment at 135 dGA. Following the final intra-amniotic injection, the flank wound was thoroughly cleaned, catheters occluded and tucked subcutaneously, the wound closed and the ewes given 3.0 mL intramuscular Duplocillin (Schering-Plough Animal Health Ltd., Upper Hut, NZ).

Lambs were born vaginally after spontaneous labour, and remnants of protruding catheters were removed after birth. If within 1 h of birth the lamb was unable to latch and feed, the ewe was hand-milked and the lamb fed manually, repeated as necessary for up to 2 h after birth or until the lamb displayed successful feeding behaviour.

If euthanasia was recommended by the attending veterinarian, sheep were euthanised with 100–120 mg·kg^{-1} sodium pentobarbitone (300 mg·mL^{-1}, Provet, Auckland, NZ) administered intravenously.

Blood sampling, hormone and metabolite analyses

Fetal whole blood was sampled 1–2 days post-surgery to monitor blood gases and at 102 dGA to establish basal measures. Basal partial pressures of carbon dioxide (P_{aCO_2}, mmHg) and oxygen (P_{aO_2}, mmHg), and pH were measured on an i-STAT® (Abbott Point of Care, Inc., Princeton, New Jersey, USA) with CG3+ or CG4+ i-STAT® cartridges. Glucose (mmol·L^{-1}) and lactate (mmol·L^{-1}) were measured on an YSI 2300 SELECT™ Biochemistry Analyser (Yellow Springs Instruments, Queensland, AU).

Two hours post-delivery the lamb was removed from the ewe but kept within line-of-sight and hearing of the ewe. Blood samples were collected by venepuncture, centrifuged at 3,220 *g*, 4°C for 10 min and the resultant plasma stored at −80°C until analysis. Blood sampling using the same method was repeated weekly.

Hormone and metabolite analyses

11-deoxycortisol (S), cortisol (F) and cortisone (E) concentrations were analysed with high-performance liquid chromatography-tandem mass spectrometry (HPLC-MS) as described previously (Rumball *et al.* 2008). Mean inter- and intra-assay coefficients of variation (CVs) were 5.1% and 10.7%, respectively, for 11-deoxycortisol, 5.9% and 6.5% for cortisol, and 4.5% and 7.0% for cortisone.

L-3,3′,5-triiodothyronine (T3) and thyroxine (T4) were analysed on an Elecsys 2010 (Hitachi High-Technologies Corporation, Tokyo, Japan). Mean inter- and intra-assay CVs were 1.1% and 1.3%, respectively, for T3, 3.1% and 1.7% for T4.

Metabolite concentrations were measured on a Hitachi 902 autoanalyser (Hitachi High-Technologies Corporation, Tokyo, Japan). Mean inter- and intra-assay CVs were 5.1% and 3.7%, respectively, for lactate; 3.4% and 2.4% for non-esterified fatty acids (NEFA); 4.3% and 3.8% for urea, and mean intra-assay CV for glucose was 1.6%.

Insulin was measured by radioimmunoassay (RIA) with ovine insulin (Sigma-Aldrich, St. Louis, Missouri, USA) as the standard (Oliver *et al.* 1993). The minimum level of detection was 0.03 ng·mL^{-1}. Mean inter- and intra-assay CVs were 8.6% and 11.7%, respectively. IGF1 was measured by an IGFBP-blocked RIA (Blum & Breier, 1994). The minimum level of detection was 0.03 ng·mL^{-1}. Mean inter- and intra-assay CVs were 3.6 and 11.4%, respectively. Amino-terminal propeptide of C-type natriuretic peptide (NTproCNP) was measured by RIA as described previously (Prickett *et al.* 2001, 2004) using primary rabbit antiserum (J39) raised against human NTproCNP (1-15). The inter- and intra-assay CVs were 6.5% and 5.0%, respectively at 33 pmol·L^{-1}.

Milk intake during the second week after birth was measured using the deuterium oxide (D_2O) dilution technique as described previously (Jaquiery *et al.* 2011).

Measurement of growth and body composition

Lamb weight, crown-rump length (CRL), chest and abdominal girth (ABDO), forelimb (FL), hindlimb (HL) and hock-to-toe length (HT), and biparietal diameter (BPD) were measured at birth, on the third day after birth and at days 7 and 14.

Relative weight per unit body length was expressed as body mass index (BMI, $kg \cdot CRL^{-2}$), G index (GI, $kg \cdot CRL^{-1.5}$) (Gootwine, 2013), and ponderal index (PI, $kg \cdot CRL^{-3}$) (Rohrer, 1921). Growth velocity was calculated using an exponential method (Patel *et al.* 2005):

$$GV \ (g \cdot kg^{-1} \cdot d^{-1}) = 1000 \times \left[\frac{\ln\left(\frac{W_n}{W_1}\right)}{D_n - D_1} \right]$$

Where W_1 represents WT in kg at D_1 and W_n represent weight on any given day after D_1, D_n. Growth velocity of linear measures were calculated in the same manner.

At one week of age, dual X-ray absorptiometry (DXA) scans were conducted with a Norland, XR-800 (Cooper Surgical Ltd, Fort Atkinson, WI, USA) followed by percutaneous liver and muscle biopsies under intravenous sedation induced with $5.0 \ mg \cdot kg^{-1}$ ketamine (Parnell Co. Ltd, Auckland, NZ) and $0.25 \ mg \cdot kg^{-1}$ diazepam (Ceva Animal Health Ltd., Auckland, NZ), titrated as needed to maintain a stable plane of anaesthesia.

DXA scans were undertaken at a spatial resolution of 3.0×3.0 mm. The area analysed was defined proximo-distally as the area of the trunk from the thoracic inlet along the curve of the spine through the base of the tail and around the rump through the pelvic limb just distal to the tibial plateau, along the curve of the abdomen and the anterior margin of the breast. Fat mass, lean mass and bone mineral content were calculated using Norland software (Cooper Surgical Ltd, Fort Atkinson, WI, USA) and are expressed in absolute grams and relative to total bodyweight.

Muscle and liver biopsies

Following the DXA procedure a percutaneous biopsy of the right lobe of the liver, and of the *vastus lateralis* were collected using aseptic techniques. The liver biopsy was performed from a posterior approach using a 10-gauge trocar advancing through the diaphragm and into the liver. The lamb was placed in left lateral recumbency, with the neck supported in line with the spine. The biopsy site, located at the 4th intercostal space posteriorly, was infiltrated subcutaneously with 2% lignocaine

hydrochloride (AstraZeneca, Auckland, NZ) and the biopsy needle advanced approximately 1.5 cm into the thorax, orienting ventrocaudally at a $30°$ angle from the spine (pointing towards the umbilicus) and $20°$ from the external plane of the trunk. The needle was advanced until the diaphragm was punctured, then rotated towards the point of the sternum and advanced approximately 2.0 cm into the liver at a $45–60°$ angle from the spine and $30°$ from the external plane of the trunk until a granular-textured tissue was identified. The trocar was removed and a 5.0 mL sterile syringe was fitted to the cannula. Negative pressure was developed by withdrawing the syringe approximately 2.5 mL whilst advancing the cannula 0.5–1.0 cm. The negative pressure was maintained while the apparatus was withdrawn. The incised skin was immediately closed with Superglue™ to avoid pneumothorax and sprayed with TetraVet (Bomac Laboratories Ltd., Auckland, NZ). The muscle biopsy site was infiltrated subcutaneously with 2% lignocaine hydrochloride (AstraZeneca, Auckland, NZ). *Vastus lateralis* tissue was collected and the skin was closed and treated as described previously. Samples were snap frozen and stored at $-80°C$ until analysis. The lamb was given 1.0 mL intramuscular Duplocillin and monitored for adverse events. The lamb was returned to the ewe once capable of independent ambulation and following return of feed-seeking behaviours.

Quantitative PCR

A TRIzol®-based extraction protocol was utilised to isolate mRNA from a $\sim$20 mg tissue sample (Kimura *et al.* 2004; Triant & Whitehead, 2009). We were unable to isolate protein from the same sample to a high enough quality to perform Western blot analyses. Briefly, snap-frozen tissue was ground with a liquid nitrogen pre-cooled stainless steel mortar and pestle and scooped into a 1.5 mL Eppendorf tube kept on dry ice. 1.0 mL TRIzol® (Life Technologies, Auckland, NZ) was added and the solution vortexed to mix. Samples were sonicated at 35%, 2-cycle for 15 s and stored on ice. Sonication was repeated until sample was fully solubilised. Samples were centrifuged at $4°C$, $10 \ 621 \ g$ for 10 min and fatty layers discarded.

$200 \ \mu L$ chloroform (Sigma-Aldrich, St. Louis, Missouri, USA) per mL TRIzol® was added and the solution vortexed to mix. Samples were incubated for 5 min at room temperature, and centrifuged at $4°C$, $17 \ 949 \ g$ for 15 min. The aqueous phase was aspirated and transferred to a fresh nuclease-free Eppendorf tube.

Cold isopropanol (500 μL, Sigma-Aldrich, St. Louis, Missouri, USA) was added to the aqueous phase and the solution was incubated 30–40 min at $-20°C$. The precipitate was centrifuged at $4°C$, $17 \ 949 \ g$ for 15 min and the supernatant discarded. The pellet was washed with 1.0 mL 75% ethanol (Sigma-Aldrich, St. Louis, Missouri, USA) and centrifuged at $4°C$, $17 \ 949 \ g$ for 5 min. The wash step

was repeated and the pellet semi-dried for 10 min at room temperature. The pellet was dissolved on ice in 10–20 μL nuclease-free water (Life Technologies, Auckland, NZ) and stored overnight at −80°C.

During protocol development mRNA integrity and purity were confirmed with a 1.5% agarose (Life Technologies, Auckland, NZ) RNA denaturation gel and NanoDrop ND-1000 spectrophotometer (3.1.2 NanoDrop Software, BioLab Ltd., Auckland, NZ). Due to the small tissue sample size, mRNA template isolation was limited. Thus, to ensure sufficient mRNA to complete the study in its entirety, mRNA purity was assessed by NanoDrop, and a standard DNase I gDNA elimination step was conducted on all samples prior to reverse transcription. Total mRNA concentration was quantified with a NanoDrop. Samples were of acceptable purity if 260/280 absorbance was >1.9 and 260/230 absorbance was >1.6.

mRNA template concentration for each primer/probe set was optimised for each tissue using samples from a CON male lamb. 1.5 μg mRNA was incubated with 4 U RNase-free DNase I (Life Technologies, Auckland, NZ) for 15 min at room temperature to eliminate potential gDNA contamination. To halt DNA digestion, 1.0 μL 25 mmol EDTA was added and samples were incubated at 65°C for 10 min. Complementary DNA (cDNA) was synthesised in 20 μL reaction volumes with SuperScript® VILO™ cDNA synthesis kits (Life Technology, Auckland, NZ s). 1x VILO™ reaction mix, 1x SuperScript® III enzyme mix containing SuperScript® III reverse transcriptase and RNaseOUT™ recombinant ribonuclease inhibitor were added to 1.5 μg DNase-treated mRNA. Reverse transcription PCR (RT-PCR) was run on an Eppendorf Mastercycler (Eppendorf AG, Hamburg, Germany) under the following conditions: 25°C for 10 min, 42°C for 60 min, 85°C for 5 min and cooled to 4°C. Complementary DNA (cDNA) was stored at −80°C until qPCR analysis.

Transcript abundance was determined by singleplex amplification in 384-well plates in triplicate by qPCR, including intra- and inter-plate, positive and negative controls. 10.0 μL reaction volumes with 5.0 μL TaqMan Master Mix (Applied Biosystems, Foster City, California, USA), 2.0 μL cDNA template, 900 nmol forward and reverse primers and 200 nmol probe (Applied Biosystems) were assayed on an ABI 7900HT sequence detector (Applied Biosystems) under the following conditions: 50°C for 2 min and 95°C for 10 min, 40 cycle-repeats of 95°C for 15 s and 60°C for 1 min. FAM fluorescent reporter dye was bound to all TaqMan probes except *18S* (VIC reporter) at the 3′ end, and a molecular-groove binding non-fluorescence quencher (MGBNFQ) was bound at the 5′ end.

Ovine target genes involved in the somatotrophic axis and nutrient uptake were analysed: insulin-like growth factor binding protein acid labile subunit (*IGFALS*), *IGF1*,

IGF2, IGF1 receptor (*IGF1R*), growth hormone receptor (*GHR*), IGF binding protein-1 (*IGFBP1*) and -3 (*IGFBP3*), glucose transporters *SLC2A2* and *SLC2A4*, mammalian target of rapamycin (*MTOR*) and glucocorticoid receptor (*NR3C1*), (Table 1). The stability of seven potential housekeeping genes were determined for each tissue: 18S ribosomal protein 1 (*18S*); beta actin (*ACTB*); peptidylprolyl isomerase A (*PPIA*); glyceraldehyde-3-phosphate dehydrogenase (*GAPDH*); hypoxanthine phosphoribosyltransferase 1 (*HPRT1*); ribosomal protein L 19 (*RPL19*); tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, and zeta poly-peptide (*YWHAZ*) (Table 1).

GenBank ovine sequences were analysed for target genes with Primer Express Software (Applied Biosystems). Minor groove binder primer and probe locations were determined, and sequences were BLAST searched to confirm target-specific oligonucleotide sequences. PCR amplicons were verified as previously described (Bansal *et al.* 2015). Hepatic cDNA was undiluted for *IGF1R*, and *SLC2A4*, diluted 5-fold for *MTOR*, 10-fold for *IGFBP1*, *IGFBP3*, and *NR3C1*, 25-fold for *GHR*, 100-fold for *IGFALS*, *IGF1*, *IGF2*, *SLC2A2*, *HPRT1*, and *YWHAZ*, and 1000-fold for *GAPDH*. Skeletal muscle cDNA was undiluted for *NR3C1*, diluted 5-fold for *MTOR*, 10-fold for *IGF1R*, *GHR*, *IGFBP3*, *SLC2A4*, and *HPRT1*, 100-fold for *IGF1*, *IGF2*, and *PPIA*, and 1000-fold for *ACTB*. Amplification efficiencies for all target genes and potential housekeeping genes were calculated from the slopes of the standard curves run on cDNA from the tissues for optimisation (Table 2). Housekeeping gene stability of expression across a random selection of samples was assessed mathematically by assessing the coefficient of variation and standard deviation of a panel of available genes.

The geometric mean (GEO) of the three most stable housekeeping genes across the experimental groups were calculated for each tissue (*vastus lateralis*: *ACTB*, *HRPT1* and *PPIA*; liver: *GAPDH*, *HRPT1* and *YWHAZ*) (Pfaffl *et al.* 2004). The threshold cycle (Ct) for target gene (TG) mRNA expression levels were calculated relative to the housekeeping geometric mean with a mathematical model which accounts for variation in transcript amplification efficiency (Pfaffl, 2001). The standard error of the mean (SEM) was calculated for each group and a 99% confidence interval (CI) was used for statistical analysis of qPCR results, where b is the experimental group compared with either CON or FGRS (a):

$$99\% \ \text{CI} = \frac{(E_{TG})^{\Delta Ct \ TG \ (a-b) \pm 2.58 \times SEM}}{(E_{GEO})^{\Delta Ct \ TG \ (a-b) \pm 2.58 \times SEM}}$$

Results are reported as fold-change with 99% CI relative to either Control (FGRS and FGRI) or FGRS (FGRI).

Table 1. Target genes analysed by qPCR

Gene accession ID	Primer/probe	Sequences (5′–3′)	T_m (°C)	Location (bp)	Size (bp)
IGFALS	F	GCG TCA AGG CCA ATG TCT TC	59	1178–1197	63
XM_004021110.1	R	GGT TGT GGT CCA GGT AGA G	59	1222–1240	
	P	6FAM-TCA AGC TGC CCA AGC T-MGBNFQ	70	1199–1214	
IGF1	F	CTT CCG GAG CTG TGA TCT GA	58	389–408	73
NM_001009774.3	R	TGA GCG GGC CGA CTT G	59	446–461	
	P	6FAM-CTG TGC GCC TCT CAA G-MGBNFQ	68	425–440	
IGF2	F	CGA GGC ATC CAG CGA TTA G	58	623–641	62
NM_001009311.1	R	TAG ATG GTG TCA CTT GGC AGA ATT	59	661–684	
	P	6FAM-AGT GAG CCA AAG TGT C-MGBNFQ	69	643–658	
IGF1R	F	TCT AAC TTT GTC TTT GCA AGA ACC A	58	73–97	64
AY162434.1	R	TCA CTG GCC CAG GAA TGT C	58	118–136	
	P	6FAM-CCT GCA GAA GGA GCA G-MGBNFQ	69	100–115	
GHR	F	CAT AAA GCC TGG AGG AAA CCA T	58	119–140	59
NM_001009323.2	R	CCG GCA GAC AAT CCT GAA A	58	159–177	
	P	6FAM-CGA AAA TCC AGC CTC T-MGBNFQ	68	142–157	
IGFBP1	F	GCC AAA CTG CAA CAA GAA TGG	59	210–221	52
NM_001145177.1	R	TCC GTC AGC CGA AGT CTC A	59	242–261	
	P	6FAM-TTC TAT CAC AGC AAA CAG T-MGBNFQ	69	223–241	
IGFBP3	F	GCC AGC GCT ACA AGG TTG AC	59	59–78	68
EU616622.1	R	CTT GGA CTC GGA GGA GAA GTT CT	59	104–126	
	P	6FAM-ACG AGT CTC AGA GCA C-MGBNFQ	69	80–95	
SLC2A2	F	GAA GAA TCA AAG CCC TGT TGG T	60	258–279	70
AJ318925.1	R	TTC GAA AAC CCC ATC AAG AGA	58	307–327	
	P	6FAM-CAA ACA TTC TTT CAT TAG TTG GA-MGBNFQ	69	282–304	
SLC2A4	F	AAC CCA GCA CAG AAC TGG AGT AC	58	71–93	60
AB005283.1	R	CCT GTG TGG ACC CTC AGT CA	58	110–130	
	P	6FAM-AGG GCC GGA TGA GA-MGBNFQ	68	96–109	
MTOR	F	CTG CAC GTC AGC ACC ATC A	59	3772–3790	80
NM_001145455.1	R	AGC CAT CCA ACC AAA TCA TCT T	58	3830–3851	
	P	6FAM-CCT CCA AAA GGC C-MGBNFQ	69	3792–3804	
NR3C1	F	GGG CCA ACA TAA TTG GCA ATA A	60	509–531	84
NM_001114186.1	R	CCC AGA GGT ACT CAC ACC ATG A	59	570–592	
	P	6FAM-ATG TCT GCC ATT TCT-MGBNFQ	69	532–547	
RNA18S		Eukaryotic 18S rRNA 6VIC/MGBNFQ			
ACTB	F	ACC AGT TCG CCA TGG ATG ATG	61	76–96	53
NM_001009784.1	R	CCG GAG CCG TTG TCA AC	58	113–128	
	P	6FAM-ACG AGC GCA GCA ATA T-MGBNFQ		97–112	
GAPDH	F	GGG CTG CTT TTA ATA CTG GCA AA	60	91–114	81
NM_001190390.1	R	CAT GTA GAC CAT GTA GTG AAG GTC AA	61	146–171	
	P	6FAM-CAT CGT TGC CAT CAA TG-MGBNFQ		120–136	
HPRT1	F	AGG TGT TTA TTC CTC ATG GAC TAA TTA TGG	61	1–30	75
JN811683.1	R	CAC CCA TCT CCT TCA TCA CAT CTC	61	52–75	
	P	6FAM-ACA GGA CCG AAC GAC TG-MGBNFQ		31–47	
PPIA	F	GTA CTG GTG GCA AGT CCA TCT	60	105–125	72
JX534530.1	R	CAG GAC CTG TAT GCT TCA GAA TGA	60	153–176	
	P	6FAM-ATG GCG AGA AAT TTG-MGBNFQ		126–140	
RPL19	F	CAA AAA CAA GCG GAT CTC ATG	58	340–361	65
AY158223.1	R	GCT TCT GCG AGC CTG TCT	58	385–404	
	P	6FAM-AAC ATA TCC ACA AGC TGA A-MGBNFQ		363–381	
YWHAZ	F	GAG GGT CGT CTC AGT ATT GA G	60	25–46	67
NM_001267887.1	R	TTC TCG AGC CAT CTG CTG TTT T	61	70–91	
	P	6FAM-CAG CAC CTT CCG TCT TT-MGBNFQ		49–65	

Sequences for forward (F) and reverse (R) primers; fluorescent reporter dye (FAM, VIC) bound TaqMan probes with molecular-groove binding non-fluorescence quencher (MGBNFQ). Melting temperature (T_m) and base pairs (bp) noted.

Table 2. qPCR validation

Gene	Standard curve dilution (fold)	Regression equation	r^2	Efficiency
Vastus lateralis				
GHR	10	$y = -3.3289x + 24.37$	0.9877	2.00
IGF1	10	$y = -3.4452x + 19.718$	0.9979	1.95
IGF2	10	$y = -3.4162x + 17.996$	0.9951	1.96
IGF1R	10	$y = -3.1689x + 24.118$	0.9968	2.03
IGFBP3	10	$y = -3.7169x + 23.946$	0.9967	1.86
SLC2A4	10	$y = -3.126 \times 22.529$	0.9987	2.05
MTOR	5	$y = -3.5147 + 23.11$	0.9997	1.93
NR3C1	10	$y = -3.1675x + 24.181$	0.9927	2.07
ACTB	10	$y = -3.6882x + 18.27$	0.9968	1.87
HRPT1	10	$y = -3.4986x + 23.159$	0.9978	1.93
PPIA	10	$y = -3.0689x + 20.22$	0.9889	2.12
Liver				
GHR	5	$y = -3.3357x + 19.696$	0.9907	1.99
IGF1	10	$y = -3.2571 + 20.527$	0.9995	2.03
IGF2	10	$y = -3.1495x + 19.883$	0.9987	2.08
IGF1R	10	$y = -3.2151x + 26.03$	0.9931	2.05
IGFBP1	10	$y = -3.0762x + 25.996$	0.9802	2.11
IGFBP3	10	$y = -3.4713x + 21.13$	0.987	1.94
IGFALS	10	$y = -3.135x + 22.746$	0.9988	2.08
SLC2A2	10	$y = -3.0713x + 21.828$	0.9983	2.12
MTOR	5	$y = -3.1686x + 24.506$	0.9973	2.07
NR3C1	10	$y = -3.0665x + 24.463$	0.9967	2.12
HRPT1	10	$y = -2.9729x + 21.684$	0.9817	2.17
GAPDH	10	$y = -3.454x + 19.719$	0.9846	1.99
YWHAZ	10	$y = -3.1492x + 22.65$	0.9996	2.08

Statistics

Data were analysed in JMP 12 (SAS Institute Inc., Cary, North Carolina, USA). Distribution was verified with the Shapiro-Wilk test. Non-parametric data were log transformed to approximate a normal distribution where necessary. The effects of FGR and intra-amniotic treatment of FGR with IGF1 were analysed by t test, factorial or repeated measures analysis of variance (ANOVA and RM ANOVA, respectively) by sex with breeding year as a random effect. RM ANOVA was used to examine longitudinal changes over time. Breeding year was included in the analyses to adjust for the effect on outcomes. Significance was set at $P < 0.05$ unless otherwise stated. Tukey's *post hoc* testing was conducted where appropriate. Data are presented as the mean $\pm$ SEM.

Results

One hundred and ten singleton sheep were generated for the purposes of this experiment over two consecutive breeding years (Fig. 1).

Fetal metabolic status and survival

Fetal hock-to-toe at the time of surgery was not different between FGRS and FGRI groups (both 9.9 ± 0.1 cm). Placental embolisation increased the partial pressure of carbon dioxide (P_{aCO_2}) ($P = 0.02$, Fig. 2A) and decreased the partial pressure of oxygen (P_{aO_2}, $P < 0.0001$, Fig. 2B), pH ($P < 0.0001$, Fig. 2C) and arterial blood glucose concentration ($P = 0.05$, Fig. 2D), but did not alter arterial blood lactate concentrations ($P = 0.1$, Fig. 2E). Fetal blood gas and metabolite concentrations did not differ between FGR groups. Fifty-seven FGR fetuses died prior to delivery. Fetal mortality ($P = 0.2$) and gestational age at death, defined as the date of abortion, did not differ between FGRS and FGRI fetuses ($P = 0.7$) nor between sexes ($P = 0.9$). Postnatal mortality also was not different between FGR groups (FGRS, $n = 3$; FGRI, $n = 3$), and did not occur in control lambs (Table 3).

Plasma NTproCNP concentration

Maternal mean NTproCNP was not different ($P = 0.9$) during the embolisation period (102–106 dGA) between ewes subsequently allocated FGRS and FGRI groups (105.6 ± 27.8 *vs.* 104.3 ± 27.8 pmol·L^{-1}). There was a

significant positive correlation between maternal mean NTproCNP and fetal P_{aO_2} during the embolisation period ($R^2 = 0.43$, $P = 0.006$, Fig. 2F). There was a positive correlation between mean maternal plasma NTproCNP concentration during the embolisation period and birthweight ($R^2 = 0.64$, $P = 0.0005$), CRL ($R^2 = 0.52$, $P = 0.004$) and HT length ($R^2 = 0.57$, $P = 0.002$) in growth-restricted lambs (Fig. 3). There were no differences amongst groups in plasma NTproCNP in

lambs at birth (CON, 96.5 ± 5.2; FGRS, 95.4 ± 6.7; FGRI, 86.8 ± 4.8 pmol·L^{-1}), day 7 (CON, 68.6 ± 4.7; FGRS, 78.3 ± 5.2; FGRI, 70.2 ± 4.3 pmol·L^{-1}), or day 14 (CON, 63.5 ± 2.9; FGRS, 69.1 ± 3.4; FGRI, 67.6 ± 2.9 pmol·L^{-1}).

Size at birth and neonatal growth

Birth characteristics. Gestational age at birth in surviving fetuses (Table 4) was not different amongst groups
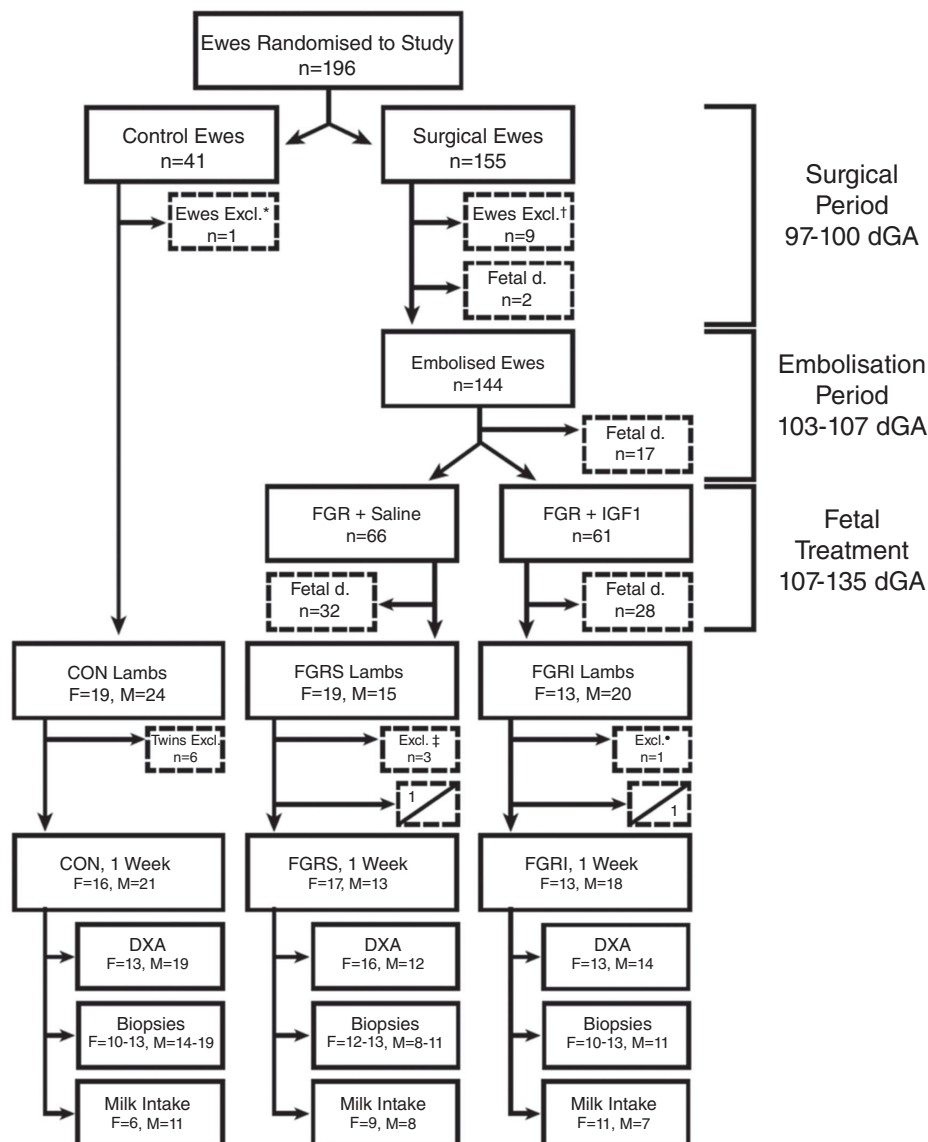

Figure 1. Characteristics of experimental groups and animal use
Animals utilised during the project according to experimental group and sex. Dashed boxes report exclusions, deaths and killing. Split boxes reporting postnatal deaths indicate the number of females (top left) and males (bottom right) for each experimental group: control (CON), saline-treated fetal growth restricted (FGRS), and insulin-like growth factor 1-treated fetal growth restricted (FGRI). Dual X-ray absorptiometry (DXA), biopsies, and milk intake were conducted in all groups. Circumstances constituting exclusion from the project and/or killing were: *pregnancy toxaemia, †mammary abscess $n = 5$, aspiration of rumen contents prior to intubation $n = 1$, twin pregnancy $n = 2$ and intrauterine infection $n = 1$; ‡twin lambs $n = 2$, intestinal atresia $n = 1$; °preterm (<136 dGA) birth $n = 1$.

Table 3. Animal losses during experimental procedures

	CON	FGR	FGRS	FGRI
Prenatal losses				
Experimentally induced	—	12	26	21
Catheter tangles	—	0	3	0
Congenital defect	—	3•	2•	3•
Intrauterine infection	—	2	0	2
Stillbirth	—	0	1	2
Early postnatal losses				
Experimental complications	0	—	1‡	1‡, 1¥
Illness	0	—	1†	1†
Congenital defect	0	—	1*	0

Congenital defects were observed during the prenatal (•, brachycephaly) and postnatal (*, intestinal atresia) experimental period in fetal growth restricted (FGR) lambs prior to randomisation to experimental groups, saline-treated fetal growth restricted (FGRS), and insulin-like growth factor 1-treated fetal growth restricted (FGRI) lambs. Control (CON) lambs did not undergo instrumentation. Early postnatal losses include respiratory distress and/or congenital heart failure secondary to pulmonary aneurysm (†), and experimental complications (‡, liver biopsy; ¥, anaesthesia).

($P = 0.2$, Female; $P = 0.4$, Male). FGRS, but not FGRI, female lambs were smaller than CON at birth with reduced birthweight, birthweight Z-score, abdominal circumference, BMI and GI (all, $P < 0.05$, Table 4); both FGRS and FGRI female lambs had shorter hindlimb length than CON. Males in both growth-restricted groups were lighter than CON ($P = 0.005$) with FGRS lambs having shorter forelimb length than CON ($P = 0.03$) and FGRI lambs having reduced GI ($P = 0.02$) and lesser chest circumference and hindlimb length than CON ($P = 0.01$ and $P = 0.001$, respectively) (Table 4).

Neonatal growth velocity. In female FGRS, but not FGRI, lambs, growth velocity of weight ($P = 0.009$) and of CRL ($P = 0.002$) in week 2 were greater than in CON lambs (Table 5). Despite this, female FGRS lambs remained lighter with smaller CRL, hindlimb and hock-to-toe length than CON from birth to one week whereas CON and FGRI females were not different in size (Fig. 4). In males, GV of weight was greater in FGRS than in CON in the first week after birth ($P = 0.04$), but was greater in FGRI than CON in the second week ($P = 0.02$) (Table 5). In contrast to females, male FGRI lambs were lighter with reduced
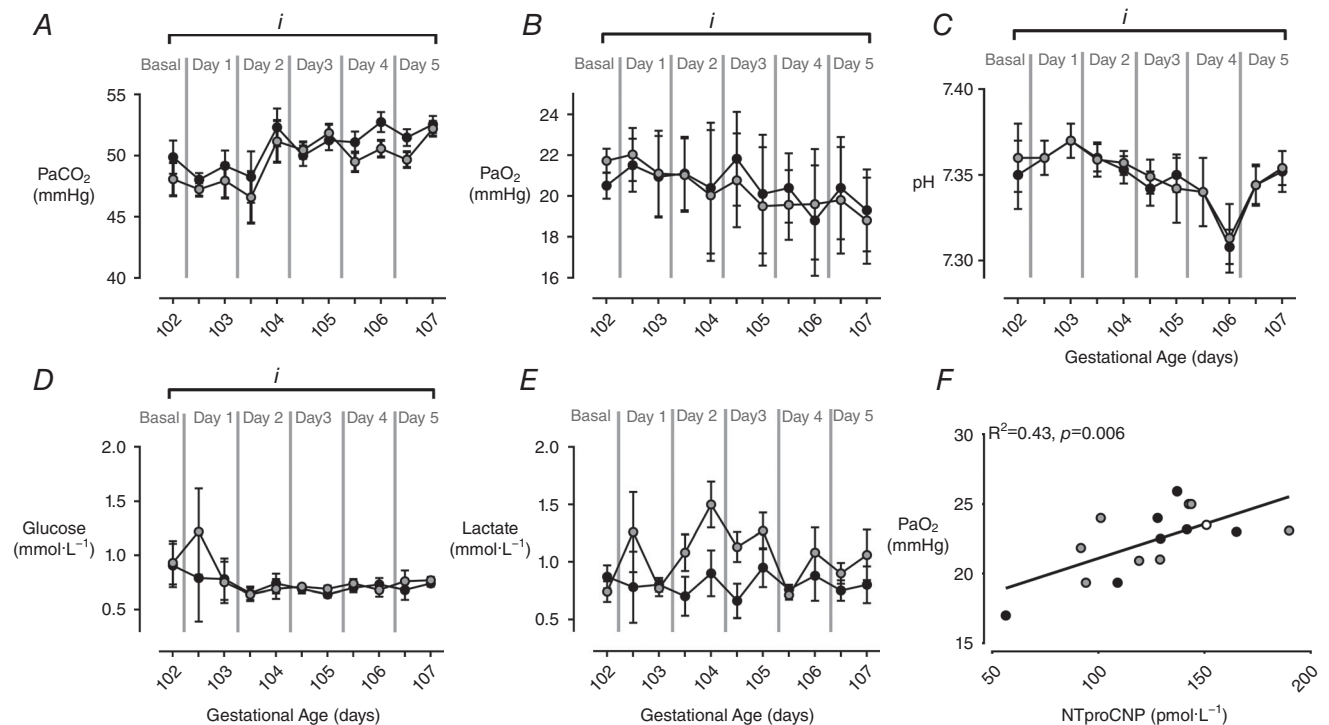

Figure 2. Fetal whole blood measures and maternal mean plasma N-terminal pro-C-type natriuretic peptide
Data are means ± SEM. Whole blood measures of partial pressure of carbon dioxide (P_{aCO_2}, mmHg; *A*); partial pressure of oxygen (P_{aO_2}, mmHg; *B*); pH (*C*); glucose (mmol·L^{-1}; *D*); and lactate (mmol·L^{-1}; *E*) in FGRS (grey, $n = 20$–34) and FGRI (black, $n = 20$–32) fetuses; and the correlation between mean maternal N-terminal pro-C-type natriuretic peptide (NTproCNP, pmol·L^{-1}) during the embolisation period (103–107 dGA) and fetal P_{aO_2} in growth-restricted FGR (white, $n = 2$), FGRS (grey, $n = 7$), and FGRI (black, $n = 7$) fetuses (*F*). Symbols denote significant differences over time (RM ANOVA: i, $P < 0.05$).

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Table 4. Characteristics of experimental groups at birth

	Female			Male			P value for treatment effect	
	CON n = 15	FGRS n = 18	FGRI n = 13	CON n = 22	FGRS n = 12	FGRI n = 19	Female	Male
GA at birth (days)	147 ± 1	147 ± 1	149 ± 1	148 ± 1	148 ± 1	147 ± 1	ns	ns
BW (kg)	5.7 ± 0.3	4.6 ± 0.3	5.1 ± 0.3	6.4 ± 0.2	5.4 ± 0.2	5.4 ± 0.2	0.02*	0.005*†
BW Z-Score	0.3 ± 0.2	−0.4 ± 0.2	−0.1 ± 0.2	0.8 ± 0.1	0.2 ± 0.2	0.1 ± 0.2	0.02*	0.005*†
CRL (cm)	51.0 ± 0.8	48.5 ± 0.7	49.8 ± 0.7	52.3 ± 0.6	50.0 ± 0.7	49.0 ± 1.0	ns	ns
Chest (cm)	39.5 ± 0.6	37.4 ± 0.5	38.2 ± 0.6	41.2 ± 0.4	39.2 ± 0.6	38.3 ± 0.6	ns	0.01†
Abdomen (cm)	40.7 ± 1.0	36.9 ± 0.9	39.0 ± 1.2	41.8 ± 0.6	40.3 ± 0.8	39.2 ± 0.8	0.01*	ns
Hock–toe (cm)	21.1 ± 0.3	19.9 ± 0.3	20.2 ± 0.3	21.7 ± 0.2	20.8 ± 0.3	21.0 ± 03	ns	0.06
Hindlimb (cm)	39.5 ± 0.6	37.4 ± 0.5	37.3 ± 0.9	41.4 ± 0.3	39.3 ± 0.5	38.5 ± 0.2	0.03*†	0.001†
Forelimb (cm)	34.1 ± 0.5	32.5 ± 0.5	33.1 ± 0.5	35.5 ± 0.4	33.7 ± 0.5	33.9 ± 0.5	ns	0.03*
BPD (cm)	6.4 ± 0.1	6.2 ± 0.1	6.2 ± 0.1	6.6 ± 0.04	6.5 ± 0.06	6.4 ± 0.03	ns	ns
BMI (kg CRL^{-2})	22.0 ± 0.9	19.5 ± 0.9	20.7 ± 1.0	23.6 ± 0.8	21.6 ± 0.9	22.01 ± 0.9	0.02*	ns
GI (kg CRL$^{-1.5}$)	15.7 ± 0.7	13.6 ± 0.6	14.6 ± 0.7	17.0 ± 0.5	15.5 ± 0.6	15.3 ± 0.6	0.01*	0.02†
PI (kg CRL^{-3})	43.6 ± 1.9	40.1 ± 1.8	41.9 ± 2.0	45.6 ± 1.8	43.1 ± 2.2	44.6 ± 2.0	ns	ns

Data are means ± SEM. Gestational age (GA), birthweight (BW), crown–rump length (CRL), chest circumference (Chest), abdominal circumference (Abdomen), hock–toe length, hindlimb length, forelimb length, biparietal diameter (BPD), body mass index (BMI), g index (GI), and ponderal index (PI) at birth. Symbols denote significant differences between experimental groups on *post hoc* testing: *CON *vs.* FGRS; †CON *vs.* FGRI; ns, not significant.

crown-rump and hindlimb length, chest circumference and biparietal diameter than CON, whilst there was no difference between FGRS and CON males (Fig. 5).

Early postnatal body composition. FGRS and FGRI females had reduced bone mineral density ($P < 0.01$ for both) compared with CON; FGRS lambs also had reduced bone mineral content compared with CON ($P = 0.04$) (Table 6). There were no significant differences in body composition measures between groups in males. Fat mass at one week of age was below the limit of detection in lambs from all groups (Table 6).

Neonatal plasma hormone and metabolite concentrations

FGRS, but not FGRI, females had lower plasma IGF1 concentrations at birth than CON; there were no differences amongst groups in males (Fig. 6). There was a positive correlation between plasma IGF1 concentration and birthweight in FGRS and FGRI lambs (females: $P = 0.007$ and $P = 0.001$; males $P = 0.002$ and $P = 0.06$ respectively). This relationship was not present in CON lambs of either sex (Fig. 6). The correlation between plasma IGF1 concentration and birthweight was not different between male and female lambs ($P = 0.055$).
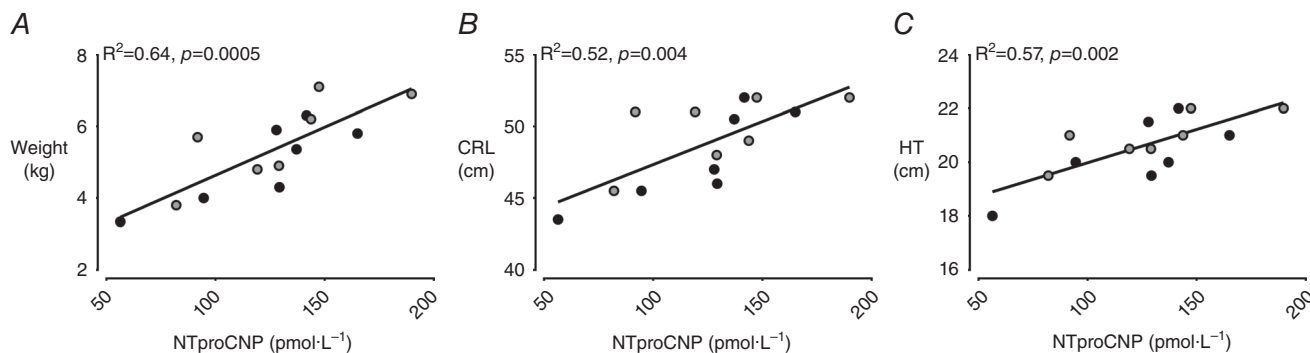

Figure 3. Maternal mean plasma N-terminal pro-C-type natriuretic peptide and offspring size at birth
Data are means ± SEM. The correlation between mean maternal N-terminal pro-C-type natriuretic peptide (NTproCNP) during the embolisation period (103–107 dGA) and birthweight (A), crown–rump length (B), and hock–toe length (C) in FGRS (grey, n = 7), and FGRI (black, n = 7) lambs.

Table 5. Growth velocity in the first 2 weeks after birth

	Female			Male			P value for treatment effect	
	CON $n = 15$	FGRS $n = 16$	FGRI $n = 13$	CON $n = 22$	FGRS $n = 12$	FGRI $n = 17$	Female	Male
GV_{0-1} WT	53.6 ± 3.4	62.1 ± 3.2	55.6 ± 4.0	52.6 ± 2.0	62.4 ± 2.4	58.3 ± 3.1	0.08	0.04*
GV_{0-1} CRL	2.0 ± 0.2	1.6 ± 0.2	1.9 ± 0.2	1.7 ± 0.2	1.6 ± 0.2	1.7 ± 0.2	ns	ns
GV_{1-2} WT	41.2 ± 3.4	49.3 ± 2.2	44.2 ± 2.5	40.4 ± 1.4	41.7 ± 1.8	45.6 ± 1.7	0.009*	0.02†
GV_{1-2} CRL	1.2 ± 0.2	1.9 ± 0.2	1.5 ± 0.2	1.6 ± 0.1	1.6 ± 0.1	1.6 ± 0.1	0.002*	ns

Growth velocity (GV) in the first (GV_{0-1}) and second (GV_{1-2}) week after birth for body weight (WT; g kg^{-1} day^{-1}) and crown–rump length (CRL; cm m^{-1} day^{-1}). Data are means $\pm$ SEM. Symbols denote significant differences amongst experimental groups on *post hoc* testing: *CON *vs.* FGRS; †CON *vs.* FGRI; ns, not significant.

Plasma concentrations of cortisol, cortisone and the 11-deoxycortisol, the cortisol to cortisone ratio, the cortisol:11-deoxycortisol ratio, and the cortisol to weight ratio were not different amongst groups in either sex (Table 7).

Plasma T4 concentrations were lower at birth in both FGRS and FGRI females compared with CON females ($P < 0.01$) and remained lower at one week after birth in FGRI females compared with both CON and FGRS lambs ($P < 0.0001$). Plasma T3 concentrations were lower in male FGRI lambs compared with CON at birth, but not different at one week. There were no differences amongst groups in either sex in the T3:T4 ratio (Table 7).

Plasma glucose and insulin concentrations, and the glucose: insulin ratio over the first two weeks after birth were not different amongst groups in either sex (Figs 7 and 8). Female, but not male, FGRS lambs had a greater increase in plasma insulin from birth to day seven compared with CON ($P = 0.04$) (Fig. 7).

Female FGRS lambs had greater plasma lactate concentrations at birth than both CON and FGRI groups ($P = 0.009$ and $P = 0.04$, respectively, Fig. 7), but these were comparable amongst groups by day seven (time*experimental group interaction, $P < 0.05$). Plasma lactate concentrations were not significantly different amongst groups at any time point in males, although there was a time*experimental group interaction ($P < 0.05$) reflecting a greater drop in concentration from birth to day 7 in the FGR groups than in CON (Fig. 8). Plasma NEFA and urea concentrations were not different amongst groups (Figs 7 and 8).
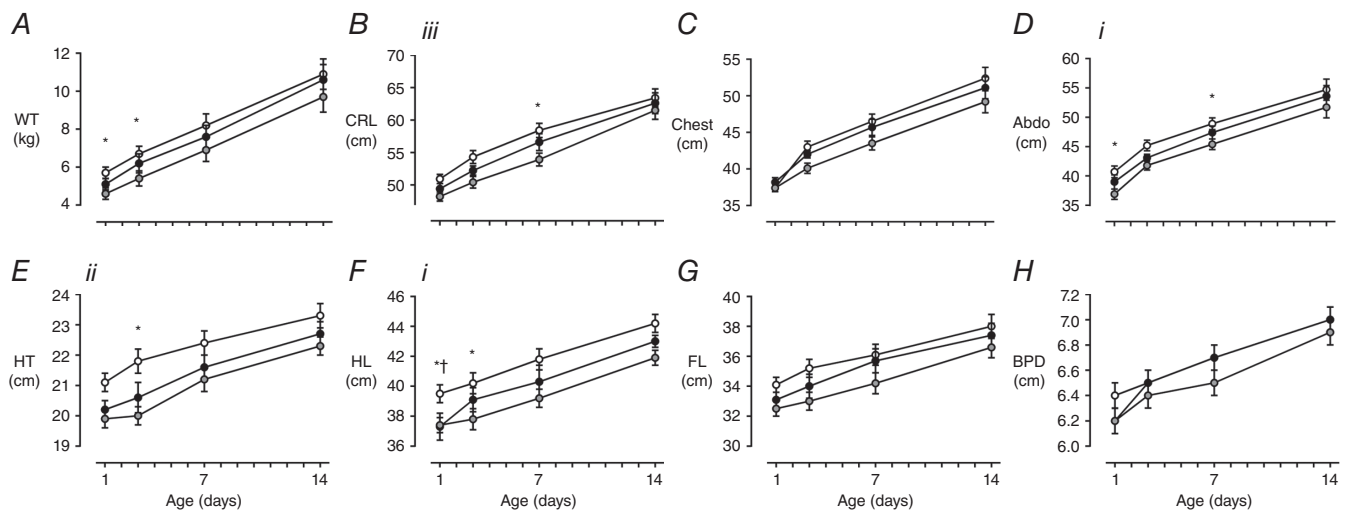

Figure 4. Early postnatal growth in females
Data are means $\pm$ SEM. *A*, bodyweight (WT); *B*, crown–rump length (CRL); *C*, chest circumference; *D*, abdominal circumference (Abdo); *E*, hock–toe length (HT); *F*, hindlimb length (HL); *G*, forelimb length (FL); *H*, biparietal diameter (BPD). CON (white, $n = 15$), FGRS (grey, $n = 16$–18), FGRI (black, $n = 13$). Roman numerals denote the significant difference between experimental groups (RM ANOVA: $^i P < 0.05$; $^{ii} P < 0.01$; ^{iii}time × experimental group interaction $P < 0.05$), symbols refer to *post hoc* P values for differences between experimental groups at each time point (ANOVA: CON *vs.* FGRS: **P < 0.05; CON *vs.* FGRI: †$P < 0.05$).

Table 6. Body composition at one week of age

	Female			Male			P value for treatment effect	
	CON $n = 12$	FGRS $n = 15$	FGRI $n = 13$	CON $n = 20$	FGRS $n = 11$	FGRI $n = 14$	Female	Male
BMC (g)	96 ± 5	78 ± 4	83 ± 4	104 ± 4	92 ± 6	91 ± 5	0.04*	ns
BMD (g cm⁻²)	0.53 ± 0.02	0.45 ± 0.01	0.46 ± 0.01	0.53 ± 0.01	0.49 ± 0.02	0.50 ± 0.02	<0.01*†	ns
LM (kg)	6.3 ± 0.3	5.5 ± 0.2	5.7 ± 0.3	7.0 ± 0.3	6.1 ± 0.4	6.2 ± 0.3	0.08	0.06

Bone mineral content (BMC), bone mineral density (BMD) and lean mass (LM) measured by dual X-ray absorptiometry. Data are means ± SEM. Symbols denote significant differences amongst experimental groups on *post hoc* testing: *CON *vs.* FGRS; †CON *vs.* FGRI; ns, not significant.

Neonatal milk intake

Milk intake in the second week after birth was approximately 20% greater in FGRI females compared with CON ($P = 0.03$) and FGRS ($P = 0.1$) (172 ± 7, 142 ± 9 and 147 ± 9 ml·kg⁻¹·d⁻¹, respectively). There was no difference in milk intake amongst groups in males (CON, 148 ± 5; FGRS, 148 ± 5; FGRI, 151 ± 5 ml·kg⁻¹·d⁻¹).

Skeletal muscle mRNA expression

In female FGRS, but not FGRI lambs, skeletal muscle mRNA levels were increased for *GHR* and *SLC2A4* and decreased for *IGF2* and *IGF1R* compared to CON. mRNA levels in FGRI compared with FGRS lambs were increased for *IGF1R* and decreased for *GHR*, *IGF1*, and *MTOR* (Table 8).

In male FGRS lambs, skeletal muscle mRNA levels were increased for *IGF1* and *IGFBP3* compared with CON. mRNA levels in both FGRS and FGRI lambs were decreased for *IGF1R* and *SLC2A4* compared with CON. In FGRI lambs mRNA levels were increased for *GHR* and decreased for *IGF1R*, *MTOR*, and *SLC2A4* compared to both CON and FGRS and also decreased for *NR3C1* compared with FGRS (Table 8).

Hepatic mRNA expression

In females, hepatic mRNA levels of *IGF1*, *IGFBP3*, and *SLC2A2* were increased in both FGRS and FGRI compared with CON lambs. FGRI lambs had decreased mRNA levels of *IGF1* but increased mRNA levels of *IGF1R*, *IGF2*, *IGFALS*, *IGFBP3*, *MTOR*, *NR3C1*, and *SLC2A2* compared with FGRS (Table 8).
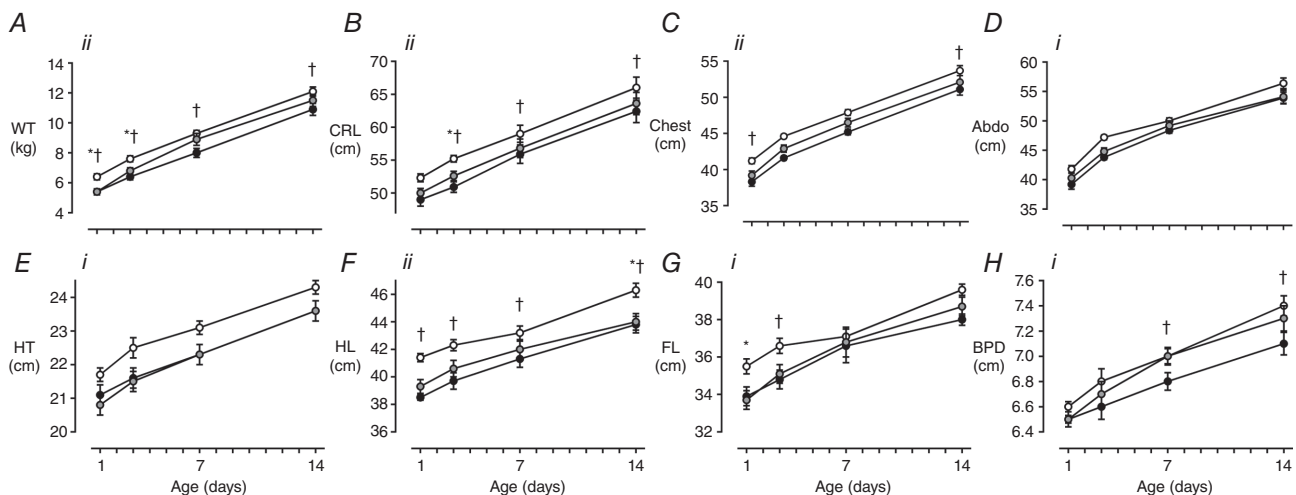
Figure 5. Early postnatal growth in males
Data are means ± SEM. *A*, bodyweight (WT); *B*, crown–rump length (CRL); *C*, chest circumference; *D*, abdominal circumference (Abdo); *E*, hock—toe length (HT); *F*, hindlimb length (HL); *G*, forelimb length (FL); *H*, biparietal diameter (BPD). CON (white, $n = 21–22$), FGRS (grey, $n = 13$), FGRI (black, $n = 18$-19). Symbols denote significant difference between experimental groups (RM ANOVA: ⁱ$P < 0.05$; ⁱⁱ$P < 0.01$), and refer to *post hoc P* values for differences between experimental groups at each time point (ANOVA: CON *vs.* FGRS: *$P < 0.05$; CON *vs.* FGRI: †$P < 0.05$).

In males, hepatic mRNA levels of *IGFBP1*, and *IGFBP3* were increased in both FGRS and FGRI compared with CON. FGRS lambs also had increased *IGF1*, and decreased *IGFALS* and *NR3C1* mRNA levels compared with CON. FGRI lambs had increased *IGF1R*, *IGF2*, *IGFALS*, and *SLC2A2* mRNA levels compared with both CON and FGRS (Table 8).

Discussion

This study demonstrates that intra-amniotic IGF1 treatment of fetuses with FGR induced by placental embolisation, which we previously have reported increases late gestation fetal growth (Wali *et al.* 2012), does not alter perinatal morbidity or mortality. Although, in contrast to previous studies (Wali *et al.* 2012), fetal lactate concentrations during embolisation were not statistically increased, there was a trend towards higher concentrations in embolised fetuses. As the lambs in this study were to be born naturally and followed postnatally, the extent of embolisation had to be judged both to ensure longer fetal survival to term (compared with termination in late gestation in previous studies) and survival of labour. Thus, it is likely that the more modest trend in elevated lactate concentrations reflects less severe embolisation.

Similarly, we did not measure daily intrauterine growth as in previous studies as live birth with growth catheters *in situ* was not deemed feasible. Birthweight of FGR lambs treated with vehicle was significantly less than controls in both males and females; however, only in females did IGF1-treatment increase birthweight to be similar to controls. We have not found this sexually dimorphic effect in previous studies, although this experiment was intentionally powered to detect effects by sex, in contrast to the earlier fetal studies.

Consistent with our hypothesis and the effect of IGF1 treatment on intra-uterine growth in females, postnatal growth velocity over the first two weeks after birth was similar in female FGRI lambs and controls, in contrast to the accelerated postnatal growth seen in FGRS female lambs. The pattern of postnatal growth in male lambs is intriguing, with accelerated growth velocity in the first week in FGRS but not FGRI, but the reverse pattern in week 2, despite similar birthweights. Accelerated postnatal growth in the first 2–3 weeks after birth has been reported in studies of ovine FGR induced by carunclectomy (De Blasio *et al.* 2007; Duffield *et al.* 2009). Although the patterns of growth in those studies were not reported to be different between males and females (although males grew faster), of note is the fact that growth rate in week one in
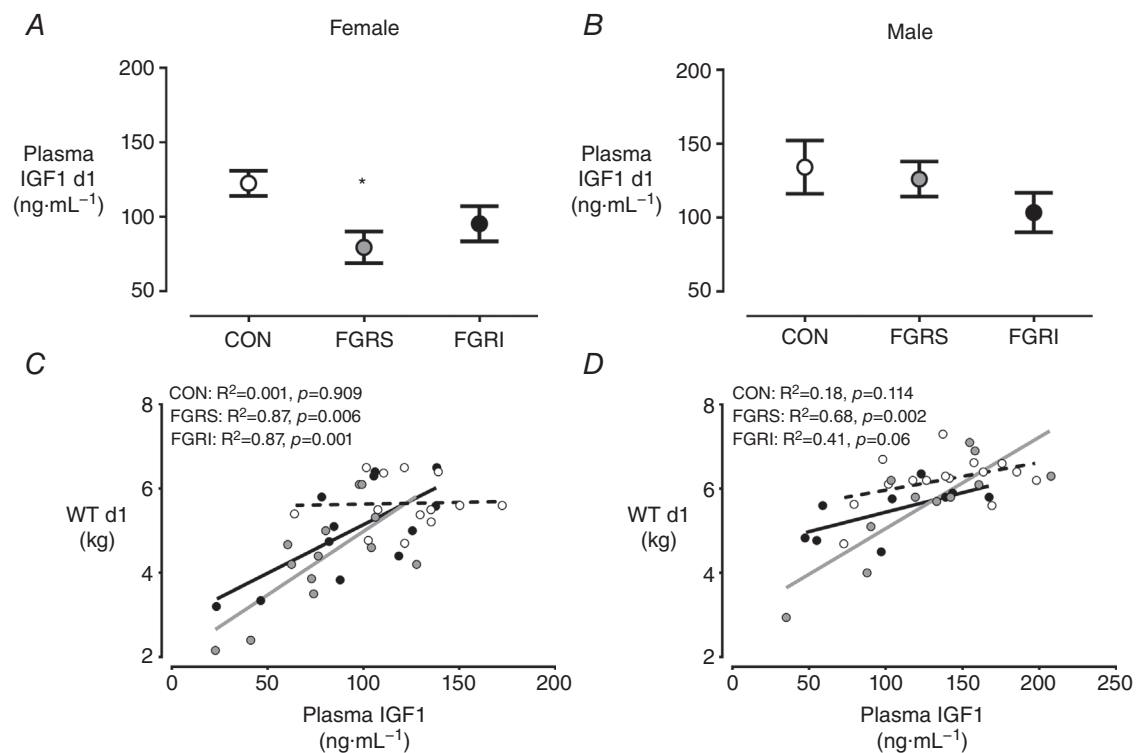

Figure 6. Plasma IGF1 characteristics at birth
Plasma concentrations of IGF1 in females (*A*) and males (*B*), and their correlation with weight at birth (*C* and *D*, respectively). Female CON (white) $n = 13$, FGRS (grey) $n = 13$, FGRI (black) $n = 12$, and male CON (white) $n = 15$, FGRS (grey) $n = 11$ and FGRI (black) $n = 9$ lambs. Data are means ± SEM. Symbols refer to *post hoc* P values for differences between experimental groups (ANOVA: CON *vs.* FGRS: *$P < 0.05$).

Table 7. Plasma corticosteroids and thyroid hormones in the first week after birth

	Female			Male			P values for treatment effect	
	CON $n = 11–15$	FGRS $n = 10–13$	FGRI $n = 10–12$	CON $n = 12—16$	FGRS $n = 10–11$	FGRI $n = 9–10$	Female	Male
Day 1								
Cortisol (ng mL^{-1})	70.1 ± 9.9	59.5 ± 9.5	52.5 ± 9.5	72.0 ± 25.8	64.3 ± 22.8	80.5 ± 24.2	ns	ns
Cortisone (ng mL^{-1})	20.0 ± 5.6	22.2 ± 5.8	15.8 ± 5.8	20.1 ± 7.0	16.8 ± 6.8	21.7 ± 7.0	ns	ns
ln 11-deoxycortisol	-0.8 ± 0.2	-1.2 ± 0.2	-1.1 ± 0.2	-0.8 ± 0.6	-1.0 ± 0.3	-0.6 ± 0.4	ns	ns
ln cortisol:cortisone ratio	1.4 ± 0.2	1.5 ± 0.2	1.4 ± 0.2	1.4 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	ns	ns
Cortisol:11-deoxycortisol ratio	155 ± 42	183 ± 45	123 ± 45	187 ± 16	144 ± 29	170 ± 33	ns	ns
T3 (pg mL^{-1})	17.5 ± 1.7	14.0 ± 1.9	14.7 ± 2.2	18.0 ± 2.9	13.3 ± 2.9	11.9 ± 3.0	ns	0.04[†]
T4 (pmol mL^{-1})	73.0 ± 4.5	49.7 ± 5.8	44.4 ± 7.5	59.3 ± 1.06	56.1 ± 10.4	49.8 ± 10.6	<0.01*[†]	ns
ln T3:T4 tatio	-1.4 ± 0.1	-1.3 ± 0.1	-1.2 ± 0.1	-1.2 ± 0.1	-1.4 ± 0.1	-1.5 ± 0.1	ns	ns
Day 7								
Cortisol (ng mL^{-1})	31.2 ± 8.8	39.2 ± 9.9	22.4 ± 9.8	25.5 ± 4.6	48.4 ± 8.3	31.7 ± 9.4	ns	ns
Cortisone (ng mL^{-1})	8.3 ± 1.3	8.1 ± 1.5	5.5 ± 1.5	8.2 ± 0.7	10.8 ± 1.3	7.4 ± 1.5	ns	ns
ln 11-deoxycortisol	-1.3 ± 0.5	-0.9 ± 1.2	-1.6 ± 1.3	-1.3 ± 0.4	-0.7 ± 0.4	-0.8 ± 0.5	ns	ns
ln cortisol:cortisone ratio	1.6 ± 0.2	1.8 ± 0.2	1.9 ± 0.1	1.5 ± 0.1	1.5 ± 0.1	1.9 ± 0.1	ns	ns
Cortisol:11-deoxycortisol ratio	98.5 ± 16.9	76.0 ± 19.4	80.6 ± 19.2	73.4 ± 15.7	76.7 ± 14.0	65.1 ± 15.4	ns	ns
T3 (pg mL^{-1})	16.0 ± 0.6	16.7 ± 0.7	16.1 ± 0.7	15.7 ± 1.3	15.6 ± 1.2	14.5 ± 1.3	ns	ns
T4 (pmol mL^{-1})	45.2 ± 1.1	44.0 ± 1.3	36.8 ± 1.2	41.2 ± 2.6	39.5 ± 2.4	36.3 ± 2.6	<0.0001*[‡]	ns
ln T3:T4 ratio	-1.5 ± 0.1	-1.1 ± 0.1	-1.1 ± 0.1	-1.2 ± 0.1	-1.2 ± 0.1	-1.2 ± 0.1	ns	ns

Absolute plasma corticosteroids and thyroid hormones, natural log transformation (ln) of plasma corticosteroids and thyroid hormones and ratios at birth (Day 1) and 7 days after birth. L-3,3′,5-triiodothyronine (T3), thyroxine (T4). Data are means ± SEM. Symbols denote significant differences amongst experimental groups upon *post hoc* testing: *CON *vs.* FGRS; [†]CON *vs.* FGRI; [‡]FGRS *vs.* FGRI; ns, not significant.

males was associated with concentrations of non-esterified fatty acids, whereas no such relationship existed in females (Duffield *et al.* 2009) further supporting the concept that growth is regulated differently in males and females. Body composition analysis by DXA demonstrated decreased lean mass in female FGRS, but not FGRI, lambs and decreased bone mineral density in both groups of FGR female lambs. Birthweight in male and female FGR lambs in both IGF1 and vehicle treated groups was moderately to strongly correlated with plasma IGF1 concentrations shortly after birth, consistent with both the nutritional regulation of fetal IGF1 concentrations and the key role IGF1 plays in fetal growth.

The pattern of mRNA expression in key genes in the somatotrophic axis may help provide further insight into these findings. In the FGRS females, mRNA levels of *GHR* in muscle and of *IGF1* and *IGFBP3* in liver were significantly greater than in controls and FGRI lambs, which also had lower levels of *IGF1* mRNA in muscle than FGRS. In contrast, *IGF1R* mRNA levels in FGRI were greater than in FGRS. In the fetal liver, expression levels of somatotrophic and metabolic genes are altered by changes in nutrition and oxygenation (Gentili *et al.* 2009; Thorn *et al.* 2012), to the extent that expression can vary in the two hepatic lobes which receive differing levels of nutrition and oxygen from the umbilical and portal circulations

(Darp *et al.* 2010). We speculate that intra-uterine administration of physiological doses of IGF1 impacted upon the fetal somatotrophic response to FGR, possibly due to a tissue-specific delay in somatotrophic maturation, which persisted beyond the period of birth transition into the postnatal period.

In females, both FGR groups had reduced plasma T4, which persisted at postnatal day 7. Although this is consistent with other studies of FGR lambs utilising placental restriction (De Blasio *et al.* 2006), FGR females did not have reduced plasma T3 concentrations, which could suggest a growth restriction-mediated reduction in capacity for thyroid hormone secretion that is not resolved by IGF1 treatment. Appropriate thyroid function is necessary to mediate somatotrophic axis maturation around parturition in the liver and skeletal muscle (Forhead *et al.* 2000, 2002). Thus, reduced plasma T3 at birth in FGRI males could delay the somatotrophic switch from fetal to postnatal GH-dependent growth. In addition to reduced plasma T3 at birth, FGRI males grew faster in the second week after birth. Upregulation of hepatic somatotrophic (*IGF2, IGF1R, IGFBP1, IGFBP3,* and *IGFALS*) and metabolic (*SLC2A2*) mRNA expression in FGRI compared with CON males, with similar changes compared with FGRS at postnatal day 7, could indicate that T3 is the predominant factor affecting hepatic

somatotrophic maturation in males. As FGRI females and males had similar changes in hepatic mRNA expression compared to FGRS without disparity in plasma T3 concentration, this could suggest that somatotrophic maturation and nutrient sequestration in females in the early postnatal period is reliant on other mechanisms.

Independent of alterations in plasma corticosteroids, FGRI males had a significant reduction in plasma T3 compared with CON lambs at birth. The pre-partum rise in plasma T3 is associated with hepatic upregulation of gluconeogenic gene expression (Forhead *et al.* 2003) and increased hepatic glyconeogenesis (Forhead *et al.* 2009).

Thus, reduced plasma T3 in FGRI males could suggest compromised gluco- and glyco-neogenic capacity at birth. This is supported by plasma glucose concentrations within the first 2 h after birth in FGRI males that were 2.0 mmol·L^{-1} lower than in both CON and FGRS groups, although this finding was not statistically significant. Interestingly, FGRS females had a significantly greater plasma lactate concentration at birth than both control and IGF1-treated females, which could suggest reduced metabolic flexibility. Assessment of hepatic molecular gluco- and glyco-neogenic gene expression was not feasible due to the small quantity of tissues collected.
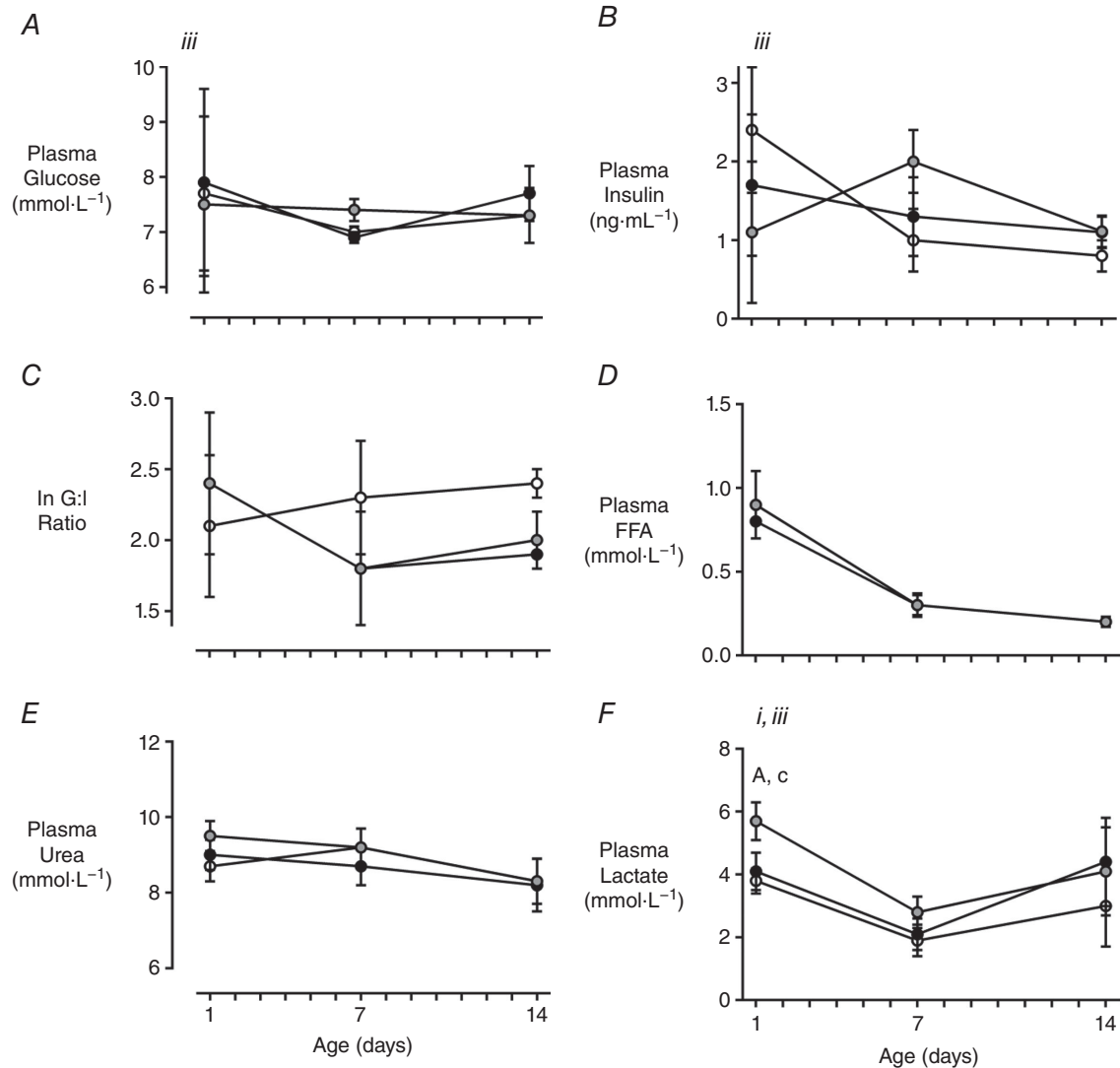

Figure 7. Plasma hormone and metabolite concentrations over the first 2 weeks after birth in females
Plasma concentrations of glucose (*A*) insulin (*B*), non-esterified fatty acids (NEFA; *F*), urea (*G*) and lactate (*H*), and the change in insulin over the first (*C*) and second (*D*) weeks, and the natural log transformation (ln) of the glucose to insulin (G:I) ratio in the first 2 weeks after birth (*E*). CON (white) *n* = 14, FGRS (grey) *n* = 11–14 and FGRI (black) *n* = 13 lambs. Data are means ± SEM. Roman numerals denote the significant difference between experimental groups (RM ANOVA: [i]*P* < 0.05, [iii]time × experimental group interaction *P* < 0.05). Symbols denote *post hoc P* values for differences between experimental groups at each time point (ANOVA: CON *vs.* FGRS: **P* < 0.05; CON *vs.* FGRI: [†]*P* < 0.05; [‡]*P* < 0.05 FGRS *vs.* FGRI).

Despite accelerated postnatal growth velocity in female FGRS lambs compared with CON in the first two weeks after birth, and increased mRNA levels of hepatic *IGF1* and *SLC2A2* and skeletal muscle *GHR SLC2A4*, lean mass remained reduced at postnatal day 7. During this post-natal period of rapid weight gain, fat deposition is greatest (Ricordeau *et al.* 1961), and is due to increased peripheral insulin sensitivity (Gardner *et al.* 2005) and activation of adipogenic and lipogenic pathways (Graugnard *et al.* 2009; Isganaitis *et al.* 2009). Interestingly, FGRI females had greater milk intake than controls, but this was not associated with differential skeletal muscle mRNA expression or accelerated growth velocity. As FGRI females

did not display a greater glucose to insulin ratio, a proxy measure for basal insulin sensitivity (Green *et al.* 2011), which could suggest greater reliance on non-insulin dependent nutrient uptake, and altered mechanisms of neonatal growth compared with saline-treated lambs.

The novel finding of a relationship between maternal NTproCNP, which derives from the placenta in sheep (McNeill *et al.* 2011), and fetal oxygenation and size at birth reported in this study is significant. Due to rapid degradation of CNP at source, plasma NTproCNP, which is biologically inactive and physiologically stable, is the best marker for CNP secretion. In sheep, the CNP signalling pathway is greatly upregulated in the
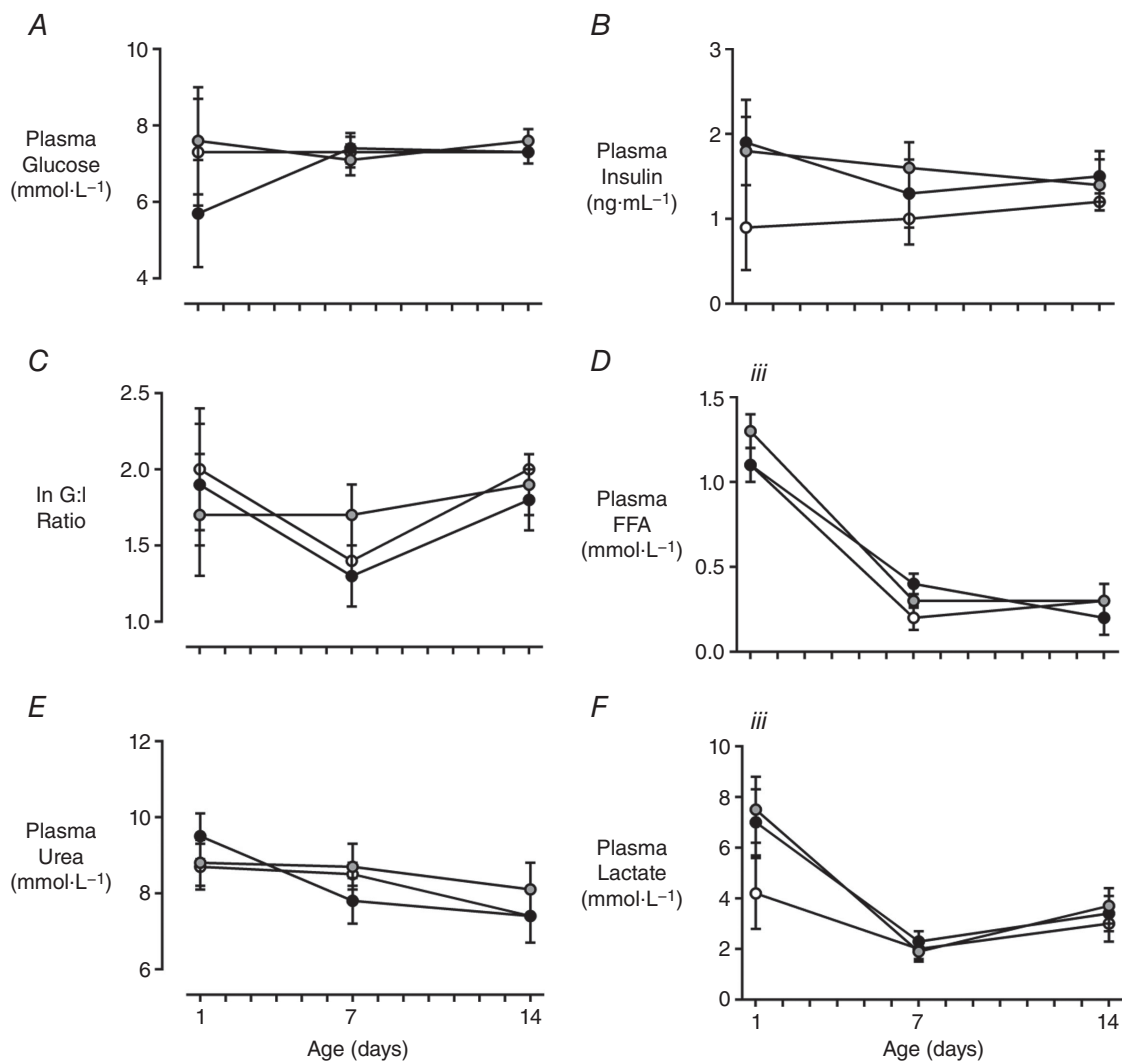

Figure 8. Plasma hormone and metabolite concentrations over the first 2 weeks after birth in males
Plasma concentrations of glucose (*A*), insulin (*B*), non-esterified fatty acids (NEFA; *F*), urea (*G*) and lactate (*H*), and the change in insulin over the first (*C*) and second (*D*) weeks, and the natural log transformation (ln) of the glucose to insulin (G:I) ratio in the first 2 weeks after birth (*E*). CON (white) $n = 16$, FGRS (grey) $n = 11$ and FGRI (black) $n = 10–11$ lambs. Data are means ± SEM. Roman numerals denote significant difference between experimental groups (RM ANOVA: [iii]time × experimental group interaction $P < 0.05$). Symbols denote *post hoc* P values for differences between experimental groups at each time point (ANOVA: CON *vs.* FGRS: *$P < 0.05$; CON *vs.* FGRI: [†]$P < 0.05$).

Table 8. Skeletal muscle and hepatic mRNA expression 1 week after birth

	Female			Male		
	FGRS *vs.* CON	FGRI *vs.* CON	FGRI *vs.* FGRS	FGRS *vs.* CON	FGRI *vs.* CON	FGRI *vs.* FGRS
Vastus lateralis						
Somatotrophic axis						
GHR	1.39 (1.17–1.64)*	0.82 (0.26–2.57)	0.59 (0.46–0.75)*	0.99 (0.89–1.10)	1.37 (1.20–1.57)*	1.38 (1.13–1.69)*
IGF1	1.20 (0.92–1.57)	0.54 (0.16–1.81)	0.45 (0.42–0.48)*	1.14 (1.01–1.30)*	0.91 (0.67–1.24)	0.80 (0.55–1.14)
IGF2	0.79 (0.73–0.86)*	0.74 (0.19–2.87)	0.93 (0.61–1.41)	0.71 (0.47–1.06)	0.66 (0.41–1.01)	0.93 (0.58–1.47)
IGF1R	0.88 (0.87–0.90)*	1.02 (0.28–3.69)	1.16 (1.09–1.23)*	0.86 (0.85–0.87)*	0.68 (0.66–0.70)*	0.79 (0.78–0.81)*
IGFBP3	1.15 (0.91–1.45)	0.74 (0.18–3.02)	0.64 (0.05–8.00)	3.37 (1.64–6.93)*	1.20 (0.83–1.74)	0.36 (0.14–0.88)*
Nutrient uptake						
SLC2A4	1.23 (1.05–1.44)*	0.93 (0.38–2.23)	0.75 (0.56–1.01)	0.68 (0.57–0.81)*	0.25 (0.17–0.37)*	0.37 (0.29–0.46)*
MTOR	1.50 (0.93–2.40)	0.64 (0.31–1.32)	0.43 (0.24–0.78)*	1.18 (0.69–2.00)	0.31 (0.18–0.51)*	0.26 (0.15–0.44)*
Glucocorticoid receptor						
NR3C1	0.95 (0.82–1.11)	0.96 (0.30–3.04)	1.01 (0.51–1.97)	0.81 (0.50–1.32)	0.47 (0.25–0.88)*	0.58 (0.30–1.12)
Liver						
Somatotrophic axis						
GHR	1.28 (0.93–1.74)	1.27 (0.99–1.62)	0.99 (0.88–1.13)	0.96 (0.74–1.23)	0.55 (0.28–1.10)	0.58 (0.30–1.10)
IGF1	1.84 (1.45–2.34)*	1.55 (1.33–1.80)*	0.84 (0.77–0.91)*	1.24 (1.08–1.42)*	0.67 (0.48–0.94)*	0.55 (0.37–0.81)*
IGF2	1.00 (0.94–1.07)	1.08 (0.97–1.21)	1.08 (1.05–1.11)*	0.97 (0.89–1.05)	1.61 (1.34–1.93)*	1.67 (1.31–2.12)*
IGF1R	0.35 (0.32–0.37)*	0.51 (0.33–0.80)*	1.48 (1.22–1.80)*	0.94 (0.89–1.00)	1.21 (1.19–1.24)*	1.29 (1.28–1.30)*
IGFBP1	0.71 (0.44–1.17)	0.48 (0.27–0.86)*	0.67 (0.34–1.32)	4.42 (1.90–10.28)*	2.84 (1.50–5.39)*	0.64 (0.41–1.00)
IGFBP3	1.30 (1.15–1.47)*	1.56 (1.12–2.16)*	1.20 (1.19–1.20)*	1.98 (1.27–3.10)*	1.97 (1.46–2.66)*	0.99 (0.91–1.08)
IGFALS	0.95 (0.90–0.99)*	0.99 (0.66–1.50)	1.05 (1.04–1.06)*	0.68 (0.56–0.83)*	1.25 (1.18–1.31)*	1.83 (1.75–1.91)*
Nutrient uptake						
SLC2A2	2.43 (1.43–4.13)*	2.50 (2.23–2.80)*	1.03 (1.03–1.03)*	0.97 (0.83–1.12)	1.47 (1.32–1.65)*	1.53 (1.42–1.64)*
MTOR	0.58 (0.46–0.68)*	0.76 (0.63–0.92)*	1.31 (1.28–1.34)*	0.98 (0.79–1.21)	0.95 (0.83–1.09)	0.98 (0.79–1.21)
Glucocorticoid receptor						
NR3C1	0.48 (0.39–0.59)*	0.63 (0.38–1.03)	1.30 (1.10–1.53)*	0.71 (0.62–0.82)*	0.93 (0.83–1.04)	1.31 (1.31–1.31)*

Skeletal muscle (Female: CON, *n* = 7; FGRS, *n* = 6; FGRI *n* = 4; Male: CON, *n* = 8; FGRS, *n* = 5–7; FGRI *n* = 5-8) and liver (Female: CON, *n* = 7; FGRS, *n* = 8; FGRI *n* = 8; Male: CON, *n* = 8; FGRS, *n* = 8; FGRI *n* = 8) mRNA expression in lambs 1 week after birth. Data are normalised to the geometric mean of 3 HK genes, and are reported as fold change (99% CI). Where 99% CIs do not include 1.0 (*), mRNA expression is statistically different from CON (FGRS and FGRI) or FGRS (FGRI) at the 1% level.

uterine arterial endothelium where it is thought to mediate, in part, the massive increase in uterine blood flow in late gestation (Itoh *et al.* 1998). Previous experimental studies have shown that both acute and chronic fetal nutrient restriction, and increasing litter size, increase maternal plasma CNP and NTproCNP (Prickett *et al.* 2007; McNeill *et al.* 2009; Madhavan *et al.* 2017) indicating fetal-placental regulation of CNP concentrations in the maternal circulation that reflect fetal status. These findings are supported by studies in women that report higher CNP and NTproCNP concentrations in maternal plasma from mid-pregnancy in women who later develop complications associated with a precarious fetal nutrient supply, such as pre-eclampsia, hypertension and growth restriction (Reid *et al.* 2014; Espiner *et al.* 2015). The current work extends these findings in suggesting that the maintenance of higher fetal P_{aO_2} during isolated placental insufficiency induced through placental embolisation could be mediated, at least in part, by a CNP-evoked adaptive vasodilator response. Although

this study was not designed to elucidate the underlying mechanisms linking fetal P_{aO_2} with maternal CNP, the potential clinical utility of maternal plasma NTproCNP in non-invasive fetal testing for the monitoring or prediction of late-gestation placental insufficiency warrants investigation. Conceivably, positive findings could open up the possibility of therapeutic use of exogenous CNP, which has already been approved for use in humans (ClinicalTrials.gov.NCT055157), in settings of maternal vascular complications.

This is the first study to describe the postnatal effects of an intra-amniotic intervention on endocrine, metabolic and somatotrophic maturation associated with alterations in growth velocity and body composition in the early postnatal period. These physiological and molecular adaptations during the early postnatal period following IGF1 treatment suggest differential mechanisms for the abrogation of early postnatal catch-up growth in a sex-specific manner. In FGRS males, catch-up growth occurred in the first week following birth, and in females

in the second week; IGF1 delayed accelerated growth velocity in males only. Together, these data suggest that, rather than relying on postnatal interventions initiated long after phenotypic and physiologic adaptation to fetal deprivation, intrauterine intervention provides an opportunity to alter fetal growth potential, influence post-natal growth, and potentially abrogate FGR-associated disease development prior to the development of post-natal complications. Whether the observed changes in mRNA expression in IGF1-treated lambs are advantageous in abrogating growth restriction-mediated alterations in somatotrophic sensitivity remains unclear. However, we acknowledge that discrete and limited tissue-specific measurements should be interpreted with care. Future time-course studies could help determine the timing and magnitude of tissue-specific alterations in the somatotrophic axis in response to intra-amniotic IGF1 treatment, and identify the appropriate sex-specific timing of intervention.

Perspectives and significance

This is the first study to show that a brief intra-amniotic intervention alters the postnatal growth of the growth-restricted fetus and may, therefore, have clinical utility. Discordance in treatment effects between the sexes could be associated with compensatory mechanisms in response to treatment, or differential mechanisms associated with physiological maturation and, thus, capacity for postnatal growth. Elucidation of longitudinal changes would provide insight into the postnatal implications of this brief prenatal intervention.

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Additional information

Competing interests

None declared.

Author contributions

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Translational perspectives

Fetal growth restriction (FGR) is a significant pregnancy complication for which there currently is no treatment. Babies with FGR frequently are born early and small, with increased perinatal mortality and morbidity. They then commonly show accelerated growth soon after birth, and this is associated with increased risks of later metabolic disease. Previous animal studies have shown that physiological doses of insulin-like growth factor (IGF) 1, a key fetal growth factor, administered into amniotic fluid are swallowed by the fetus and increase fetal growth. However, it is not known whether this treatment alters perinatal mortality or postnatal growth and metabolism. We hypothesised that intra-amniotic IGF1 given to the FGR ovine fetus would mitigate the accelerated postnatal growth without adverse effects on perinatal mortality and morbidity. We found that this approach appears safe, with no increase in perinatal mortality or morbidity. Furthermore, there were sex-specific effects on postnatal growth that were not related to milk intake but were associated with changes in expression of key genes in the somatotrophic axis. We also report that maternal concentrations of N-terminal pro-C-type natriuretic peptide (NTproCNP), a placental hormone, correlate with fetal arterial partial pressures of oxygen, suggesting that this may have potential as a novel non-invasive maternal test of fetal well-being. These findings suggest that intrauterine treatment of FGR may be feasible. However, data on long-term effects on growth, body composition, metabolic profile are needed before clinical application. The potential utility of NTproCNP as a marker of fetal oxygenation merits further investigation.