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ORIGINAL ARTICLE

Hepatic *IGF1* DNA methylation is influenced by gender but not by intrauterine growth restriction in the young lamb

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Intrauterine growth restriction (IUGR) and postnatal catch-up growth confer an increased risk of adult-onset disease. Overnourishment of adolescent ewes generates IUGR in ∼50% of lambs, which subsequently exhibit increased fractional growth rates. We investigated putative epigenetic changes underlying this early postnatal phenotype by quantifying gene-specific methylation at cytosine:guanine (CpG) dinucleotides. Hepatic DNA/RNA was extracted from IUGR [eight male (M)/nine female (F)] and normal birth weight (12 M/9 F) lambs. Polymerase chain reaction was performed using primers targeting CpG islands in 10 genes: insulin, growth hormone, insulin-like growth factor (*IGF*)*1*, *IGF2*, *H19*, insulin receptor, growth hormone receptor, IGF receptors 1 and 2, and the glucocorticoid receptor. Using pyrosequencing, methylation status was determined by quantifying cytosine:thymine ratios at 57 CpG sites. Messenger RNA (mRNA) expression of IGF system genes and plasma IGF1/insulin were determined. DNA methylation was independent of IUGR status but sexual dimorphism in *IGF1* methylation was evident (M < F, $P = 0.008$). *IGF1* mRNA:18S and plasma IGF1 were M > F (both $P < 0.001$). *IGF1* mRNA expression correlated negatively with *IGF1* methylation ($r = -0.507$, $P = 0.002$) and positively with plasma IGF1 ($r = 0.884$, $P < 0.001$). Carcass and empty body weights were greater in males ($P = 0.002$–0.014) and this gender difference in early body conformation was mirrored by sexual dimorphism in hepatic *IGF1* DNA methylation, mRNA expression and plasma IGF1 concentrations.

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Introduction

Intrauterine growth restriction (IUGR) remains a leading cause of perinatal mortality and neonatal morbidity worldwide. Survivors of IUGR are at increased risk of cardiovascular and metabolic disease in later life.[1] In particular, a mismatch between the prenatal and postnatal environments predisposes towards the development of an adverse metabolic profile.[2] Individuals born small who demonstrate 'catch-up' growth are at highest risk of adult-onset diseases (reviewed by Ong[3]), whereas the absence of catch-up growth appears to be protective.[4] It appears that early-life nutrition can influence gene expression, which can alter baseline metabolic function and thus determine vulnerability to later life exposures. Although the mechanisms by which the early-life environment mediates these gene expression changes is not completely understood, it is believed to be due at least in part to epigenetic modifications that alter gene expression in the absence of any change in the genomic DNA sequence.

The most widely studied epigenetic mechanism to date is DNA methylation, which occurs at specific palindromic sequences where a cytosine (C) residue is directly followed by a guanine (G), termed CpG dinucleotides. CpG dinucleotides occur throughout the genome but predominate in short stretches of G:C-rich and CpG-dense regions termed CpG islands, which are often (but not always) found in or near the promoter regions of genes. The C residues within CpG dinucleotides exhibit variable degrees of methylation that differ from cell to cell and tissue to tissue. The presence of a methyl group inhibits the attachment of transcription factors.[5] Accordingly, hypermethylation (particularly of the promoter region of a gene) is often associated with reduced gene expression or gene silencing, whereas relative hypomethylation is associated with enhanced gene expression. By contrast, intragenic DNA methylation may be associated with active transcription. Genome-wide DNA methylation studies comparing monozygotic with dizygotic human twin pregnancies have highlighted the important contribution that the intrauterine environment makes to the neonatal epigenome, even amongst genetically identical individuals.[6] Various rat models of IUGR are characterized by hypermethylation of genes including insulin-like growth factor (*IGF*)*1* and the glucocorticoid receptor (*NRC31*), and hypomethylation of the insulin receptor (*INSR*) in the offspring between 3 weeks and 18 months of life.[7–10]

The overnourished adolescent sheep model is characterized by placental and fetal growth restriction and premature delivery relative to the normally developing pregnancies of adolescents receiving a control dietary intake.[11] The prenatal insult leads to a 42% reduction in uteroplacental blood flow,[12] which limits nutrient supply to the fetus, resulting in hypoglycaemia and

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hypoinsulinaemia by late pregnancy.[13,14] Historically, the degree of uteroplacental compromise is such that ~50% of overnourished pregnancies result in significant IUGR (defined as a birth weight >2 S.D. below the mean birth weight of normally grown control fetuses), whereas the remaining 50% exhibit relatively 'normal' birth weight, despite the fact that they receive the same maternal nutritional manipulation and are similarly born, on average, 2–3 days preterm.[15]

Relative to the 'normal' birth weight lambs of overnourished adolescent dams, IUGR lambs demonstrate increased fractional growth rate (FGR) to weaning for anthropometric parameters including weight, height and abdominal girth, although *absolute* catch-up growth is not seen.[16] Consequently, IUGR lambs still demonstrate lower carcass weight at weaning. Nevertheless, evidence of an adverse metabolic profile is evident from 7 weeks of age, when fasting glucose is increased and insulin secretion in response to glucose challenge is decreased.[16]

We hypothesized that the placentally mediated fetal growth constraint that results in reduced birth weight and the aforementioned altered postnatal phenotype is, at least in part, epigenetically mediated. Therefore, the aim of the present study was to examine putative epigenetic changes underlying the observed differences in fractional growth between IUGR and normal birth weight lambs during early postnatal life. Given that nutrition *per se* may influence epigenetic status,[17,18] we chose to compare IUGR *v.* normal birth weight offspring of exclusively overnourished adolescent ewes, as opposed to normal birth weight lambs of control-fed adolescent ewes, as this presents a unique opportunity to examine the effects of prenatal growth status independently of maternal nutrition during pregnancy and lactation and variations in gestation length. We chose a panel of candidate genes with potential relevance to postnatal growth and metabolism and for which ovine genomic sequences were already published. We hypothesized that *IGF1* methylation, in particular, would be affected by prenatal growth restriction as differences in circulating IGF1 levels are present in the offspring of overnourished relative to control-intake adolescent ewes in fetal (0.9 gestation)[19] and neonatal life: 13.8 *v.* 10.2 ng/ml, respectively, $P < 0.05$, $n = 14$ each (Wallace JM, unpublished data).

Materials and methods

Experimental animals

Animal procedures were approved by the UK Home Office under the Animals (Scientific Procedures) Act 1986 and by local ethics committee review. Ewes were housed in individual pens under natural lighting conditions at the Rowett Institute of Nutrition and Health (57°N, 2°W). Embryos were harvested on day 4 post-oestrus from 12 superovulated donor ewes (Scottish Blackface × Border Leicester, ~2.5 years old) that had been inseminated by a single sire (Dorset Horn) on day 0. Embryos were transferred in singleton into the uteri of 65 oestrus-synchronized adolescent recipient ewes (Dorset Horn × Mule, ~8.5 months old) to establish exclusively singleton pregnancies of precisely known gestational age and maximum genetic homogeneity, as previously described.[20] Pregnancy rate was determined by transabdominal ultrasound at 45 days gestation, at which stage viable pregnancies were confirmed in 44 ewes (68%). Three ewes went on to miscarry before 90 days' gestation (term = 145 days). The remaining 41 pregnancies continued uninterrupted and were allowed to result in spontaneous vaginal delivery (see below).

Nutritional management

Following an initial 3-day re-alimentation period after embryo transfer, steadily increasing amounts of a complete diet were offered gradually over the next 2 weeks until the level of daily food refusal was ~15% of the total offered (equivalent to *ad libitum* intakes). The level of food offered was reviewed three times per week and individually adjusted on the basis of daily food refusal rates throughout pregnancy. The complete diet provided 12 MJ of metabolizable energy and 140 g of crude protein/kg and was offered in two equal rations at 8 am and 4 pm (see Wallace *et al.*[21] for details of diet composition and analyses). Following parturition, all ewes continued to be fed to appetite to maximize milk yield. Lambs had access to their mothers' feed throughout the 11-week lactation and males remained gonad intact.

Parturition management

From 135 days gestation (the earliest point commensurate with live birth in the overnourished adolescent paradigm), ewes were supervised 24 h a day and allowed to deliver spontaneously. A standardized proactive regime of neonatal care applied to all lambs (including resuscitation at birth and prophylactic antibiotic coverage) was used in view of the otherwise potentially very high rates of neonatal mortality in IUGR lambs (up to 62%) owing to prematurity and impaired passive immunity and/or low nutrient intake secondary to inadequate colostrum supply.[22] Lambs were dried and weighed at the time of birth. After delivery, 10 IU intravenous oxytocin (Intervet UK Ltd) was administered to ewes in order to induce milk let down. The udder was stripped by hand and the total volume of colostrum was determined before being fed back to the lamb by bottle or feeding tube. Lambs require at least 50 ml colostrum/kg birth weight to acquire sufficient antibody protection.[23] If maternal colostrum yield fell below this minimum requirement then the difference was provided to the lamb in the form of pooled donor colostrum that had been collected and frozen previously. Lambs were weighed at regular intervals during the neonatal period to determine whether any further supplementary feeds were required to ensure lamb survival.

Blood sampling and analysis

Venous blood was sampled at the time of birth and on the day of necropsy (see below) and immediately centrifuged at 2000 ***g***

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for 20 min at 4°C. IGF1 and insulin levels were quantified in duplicate by radioimmunoassay, as previously described.[16]

Necropsy and tissue sampling

Three lambs (two IUGR and one normal) died or were euthanized for welfare reasons during the neonatal period, which left 38 surviving lambs available for further study. Lambs were weighed at 5-day intervals until weaning at 11 weeks of age in order to determine absolute and FGRs. This time point was chosen, as the adverse postnatal phenotype that is characteristic of this IUGR model is established by this stage, including altered growth trajectory, body conformation and glucose metabolism.[16] Overall, FGR (%/day) was calculated by expressing the live weight gain between birth and necropsy at 77.5 ± 0.4 days gestation as a proportion of lamb birth weight and dividing by the time interval between birth and necropsy. Thereafter, all lambs were humanely killed by intravenous injection of pentobarbital sodium (20 ml) and underwent complete postmortem examination. All major internal organs were examined macroscopically and weighed. Samples of hepatic tissue from the same position and same lobe in each animal were snap frozen in isopentane chilled with liquid nitrogen and stored at −80°C, pending DNA and RNA extraction.

DNA extraction and pyrosequencing

From each lamb, 25 mg of hepatic tissue was lysed and homogenized, and DNA was extracted using the DNEasy Blood & Tissue Kit (Qiagen, Uppsala, Sweden). The DNA concentration of each sample was determined using a ND-1000 spectrophotometer (NanoDrop Technologies Inc., Wilmington, DE, USA) and samples were run on an Agilent 2100 Bioanalyser to exclude RNA contamination. In order to validate the pyrosequencing reactions, a fully methylated control was prepared by treating ovine genomic DNA with CpG methylase, M.*Sss*I (EC 2.1.1.37; Zymo Research Corporation, Irvine, CA, USA) and a fully unmethylated control was prepared using a modified genome-wide amplification approach with the GenomiPhi™ DNA Amplification Kit (GE Healthcare, Little Chalfont, Bucks, UK) and DNA polymerase derived from the bacteriophage φ29, as described.[24] From each test and control sample, 400 ng DNA was modified overnight with sodium bisulphite (which converts unmethylated C bases to uracil whilst leaving unmethylated ones intact) using the EZ DNA Methylation-Gold™ Kit (Zymo Research Corporation). All samples were bisulphite-converted in a single batch in order to avoid conversion bias.

Genomic DNA sequences for ovine genes of the somatotrophic axis were identified via the NCBI Nucleotide database. Principal genes associated with growth were searched for amongst all published genomic DNA sequences for *Ovis aries* (28,512 at time of writing). If an ovine sequence was available for a particular gene of interest, this was analysed using Methyl Primer Express® v1.0 (Applied Biosystems, Foster City, CA, USA) to determine the presence or absence of one or more C:G islands according to the following criteria: length >200 bp; GC content >55%; observed to expected C:G ratio >0.65. A total of 24 CpG islands were identified in the following 10 genes: insulin (*INS*), *IGF*1 and 2, *H19*, growth hormone (*GH*), *INSR*, insulin-like growth factor 1 receptor (*IGF1R*), insulin-like growth factor 2 receptor (*IGF2R*), growth hormone receptor (*GHR*) and glucocorticoid receptor (*NRC31*), detailed in Table 1. *H19* was included because, although it does not itself code for a protein, DNA methylation of this gene is inversely correlated with *IGF2* expression.[25] Sequences were subsequently modified to account for the widespread degradation of C to T (outwith CpG dinucleotides) that occurs during bisulphite conversion and highlight CpGs as sites of interrogation. Primers for polymerase chain reaction (PCR) and pyrosequencing were designed specifically on the bisulphite-converted sequences using PyroMark Assay Design software v2.0.1.15 (Qiagen). Care was taken to avoid the inclusion of CpG dinucleotides within the PCR primer sequences in order to prevent amplification bias, while optimizing the number of CpGs within the sequencing primer to maximize the number of sites at which DNA methylation could be quantified (see supplementary data file). Forward/reverse primers need to be 9–40 bases in length (optimum 20), have a melting temperature of 58–60°C and a GC content of 30–60%, avoid runs of identical nucleotides (<3 sequential Gs) and have no >2 Gs and/or Cs within the final five nucleotides at the 3′ end. Sequencing primers needed to have a melting temperature of 68–70°C, avoid any Gs at the 5′ end and be located as close as possible to the other two primers without overlapping. A total of 14 assays were designed covering a total of 57 CpG sites across the 10 genes of interest. Subsequently, PCR reactions were set up using biotinylated primers (Table 2) and the AmpliTaq Gold® DNA Polymerase Low DNA Kit (Applied Biosystems) and run on a PTC-225 Peltier Thermal Cycler (MJ Research, Watertown, MA, USA). After verifying that an amplicon of the expected length had been generated using gel electrophoresis, PCR products were sequenced in a PSQ 96MA pyrosequencer using Pyromark Gold Q96 Reagents (Qiagen) and custom-designed sequencing primers (Table 3). All 38 samples were processed on a single plate for each gene of interest. At each of the 57 individual CpG sites examined, percentage DNA methylation was calculated as the ratio of C to T, reflecting the proportion of methylated *v.* unmethylated DNA in the original sample.

RNA extraction and real-time reverse transcription PCR (RT-PCR)

Messenger RNA (mRNA) levels for four of the above 10 genes (*IGF1*, *IGF2*, *IGF1R* and *IGF2R*) were determined by quantitative RT-PCR. For each lamb, ~25 mg of hepatic tissue was lysed and homogenized and RNA was extracted using the

Table 1. *Details of 24 CpG islands identified within 10 somatotrophic axis genes*

CpG island number	Gene	Accession number	Length of gene (bases)	Length of CpG island (bases)	CG (%)
1	*INS* exons 1 to 3	U00659	1520	980	71.4
2	*IGF2* exon 1	U00659	873	180	71.4
3	*IGF2* exon 4	U00664	1760	1323	68.8
4	*IGF2* exon 5	U00664	800	800	68.8
5	*IGF2* exon 6	U00664	1932	1520	68.8
6	*IGF2* exon 7	U00665	547	262	68.3
7	*IGF2* exon 8	U00666	889	440	61.6
8	*IGF2* exon 9	U00667	611	496	69.0
9	*IGF2* exon 10	U00668	720	622	59.8
10	*IGF1* exon 2	X51357	1808	515	55.7
11	*IGF1* exon 3	X51358	560	496	55.4
12	*IGF1* exon 3	X69473	573	379	56.2
13	*IGF1* exon 4	X69474	526	469	55.7
14	*H19*	AJ566210	8767	1160	64.0
15	*H19*	AJ566210	8767	818	64.9
16	*H19*	AJ566210	8767	1502	67.8
17	*H19*	AJ566210	8767	1939	67.2
18	*H19*	AJ566210	8767	628	69.4
19	*GH*	X12546.1	2162	320	55.9
20	*GHR* exon 1B	S78252	1379	215	55.8
21	*INSR*	[a]	264	226	56.2
22	*NRC31*	HM204706	4791	2984	65.6
23	*IGF1R* exon 3	EF669473	346	343	69.4
24	*IGF2R* intron 2 DMR	AY182033	3048	2619	72.3

C, cytosine; G, guanine; *INS*, insulin; *IGF2*, insulin-like growth factor 2; *IGF1*, insulin-like growth factor 1; *GH*, growth hormone; *GHR*, growth hormone receptor; *INSR*, insulin receptor; *IGF1R*, insulin-like growth factor 1 receptor; *IGF2R*, insulin-like growth factor 2 receptor; DMR, differentially methylated region.

[a]No accession number available for *INSR* – sequence taken from McGrattan *et al.*[70]

RNeasy Mini Kit (D-40724; Qiagen), quantified and quality checked via capillary electrophoresis using the Agilent 2100 Bioanalyser. Next, ~30 ng total RNA from each sample was reverse transcribed in triplicate to complementary DNA using TaqMan reverse transcription reagents and MultiScribe reverse transcriptase (Applied Biosystems, Warrington, Cheshire, UK). Ovine-specific probes and primer sets were designed using Primer Express® and are detailed in Table 4. Polymerization and amplification reactions for each RT were performed in duplicate using a 7500 Fast Real-Time PCR system (Applied Biosystems) at 60°C for 40 cycles. Samples were randomized to ensure that each prenatal growth category and both genders were equally represented in each of four 96-well PCR plates. In addition, a quality control sample generated from a RNA pool was run on each plate and used to calculate the inter- and intra-assay coefficient of variation (cov) for each gene of interest. Intra-plate cov varied from 3.4 to 5.9% for individual genes (overall mean ± S.E.M. = 4.3 ± 0.57%), whereas inter-plate cov varied from 2.03 to 11.9% (overall mean ± S.E.M. = 6.2 ± 2.08%). The individual sample mRNA expression for each gene of interest was expressed relative to the sample's own internal 18S RNA content using 18S PDAR kit reagents (Applied Biosystems).

Data analysis

There was a continuous distribution of birth weights and lambs were classified as IUGR based on a 2 S.D. cut-off below the mean birth weight of normally grown lambs born to control-fed adolescent ewes in earlier studies using this model.[15,22] Accordingly, the threshold for IUGR was <4000 g and the remaining lambs were classified as non-IUGR. In view of anticipated gender differences, data are additionally presented by sex. Although we had no direct control over the gender of the embryos transferred, the eventual male to female ratio amongst the lambs was well balanced and supported this approach. After confirming normality and equality of variance using Q-Q plots and Levene's test, respectively, the four groups (normal male, normal female, IUGR male and IUGR female) were compared using general linear model (GLM) in order to assess the effects of IUGR status, gender and their potential interactions. In circumstances where GLM indicated strong effects of either prenatal growth status or gender, data were additionally combined, irrespective of gender (i.e. all IUGR *v.* all normal birth weight lambs) and IUGR status (all males *v.* all females) and compared using Student's *t*-test. Percentage DNA methylation was compared between groups at each individual

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Table 2. *PCR primers for 14 assays interrogating CpG sites in 10 genes of interest*

Assays	Target	Primer	Sequence
1	*INS*	Forward	5′-[biotin]AGTTATGAAGATTTTTAAGGGGGTTTTAT-3′
		Reverse	5′-AAACCCTCCACCCCTAAATTAACCT-3′
2	*IGF2* exon 1	Forward	5′-[biotin]ACCTTAATACAACCAAATCACC-3′
		Reverse	5′-ATAGGTATTTGTTTAGGTTATTAT-3′
3	*IGF2* exon 4	Forward	5′-TTTGGAATTTTTTAAGTTTTATATTGAGGA-3′
		Reverse	5′-[biotin]ACCCAAACATATAAATCAACAAACTC-3′
4	*IGF2* exon 6	Forward	5′-GGGTTTTTAAATATTTTAGAATAGTGATT-3′
		Reverse	5′-[biotin]AACTCCAACCAAAAAAAAACCAAACTTA-3′
5	*IGF1* exon 2	Forward	5′-GGGAGTGTGTGAAGAGTTGAAT-3′
		Reverse	5′-[biotin]ACTAAAAAAAAAAATCCATCCAAAATCTAC-3′
6	*IGF1* exon 3	Forward	5′-TGAATGATAGTTTGTGGTTGGTAGTTA-3′
		Reverse	5′-[biotin]AAAAAAAACTCCATCCAAAATCTACA-3′
7	*IGF1* exon 4	Forward	5′-GATATGTTTAAGGTTTAGAAGGTAAGTT-3′
		Reverse	5′-[biotin]TACTACTAAATTACTACAACCACATAACTC-3′
8	*H19*	Forward	5′-ATTAGTTTTGGAAGGTGTTGG-3′
		Reverse	5′-[biotin]ATAAATCTTCCCTTCCTTTAAATCAACCT-3′
9	*GH*	Forward	5′-GGGAGGTTAGTTGAGTTTTTTAGTTGTTAG-3′
		Reverse	5′-[biotin]TACCTAACCCCACCCCCTAA-3′
10	*IGF1R*	Forward	5′-GTGTTGGGAGGGTAGTTGG-3′
		Reverse	5′-[biotin]CACCCCCAATACCTAAACAACTACAAC-3′
11	*IGF2R*	Forward	5′-AGAGTAGAAATTTTTTTTGGAGTGTT-3′
		Reverse	5′-[biotin]AACACCTTACTCAAAACCTACCA-3′
12	*GHR*	Forward	5′-GTTTTGTTTTTTTTTTGGGAATAGG-3′
		Reverse	5′-[biotin]CCTCCTAAAAAAAAATATTAATAATTACAA-3′
13	*NRC31*	Forward	5′-GAGTTATATAAATGGTAGTATGTGT-3′
		Reverse	5′-[biotin]CAACCCCCTTCCCAAACT-3′
14	*INSR*	Forward	5′-GTTTTTTAGAAGGTTTGGGGATGAAAATT-3′
		Reverse	5′-[biotin]AACCCACTCTCAAATCCTCAAAAAACTAA-3′

PCR, polymerase chain reaction; *INS*, insulin; *IGF2*, insulin-like growth factor 2; *IGF1*, insulin-like growth factor 1; *GH*, growth hormone; *IGF1R*, insulin-like growth factor 1 receptor; *IGF2R*, insulin-like growth factor 2 receptor; *GHR*, growth hormone receptor; *INSR*, insulin receptor.

CpG site and as an average for each individual assay and gene of interest. Correlations were assessed using Pearson's product moment test. All data are presented as mean ± S.E.M., unless otherwise stated. Formal statistical significance was considered to have been reached where $P < 0.05$.

Results

Anthropometric and biochemical parameters at birth and necropsy

Table 5 details anthropometric and biochemical parameters at the time of birth and at necropsy at ~77 days of age, presented by gender and dichotomous IUGR status. There were no significant differences in the requirements for or indeed the duration of supplementary feeding between lambs classified as IUGR or 'normal' birth weight ($P > 0.05$, data not shown). IUGR lambs were, on average, 32% lighter at birth than their normally grown counterparts (3.26 ± 0.14 *v.* 4.80 ± 0.12 kg, $P < 0.001$, respectively, for males and females combined). The average weight of the entire cohort was 4.11 kg. In spite of increased FGR relative to normal lambs (11.0 ± 0.38 *v.* 8.5 ± 0.22%/day, $P < 0.001$), absolute catch-up growth was not observed within the first 11 weeks of life and prenatally growth-restricted lambs remained ~13% lighter at the time of necropsy (live weight: 31.1 ± 1.02 *v.* 35.8 ± 0.77 kg, $P < 0.001$). Although there were no significant gender differences in birth weight, by 11 weeks of age males were significantly heavier than females (35.7 ± 0.98 *v.* 31.5 ± 0.81 kg, $P = 0.002$ for IUGR and normal birth weight combined). The reduced liver weight attributable to both IUGR status and female gender was largely proportionate and was no longer significantly different from normal birth weight and/or male lambs when expressed in relative (i.e. empty body weight specific) terms ($P > 0.05$). At birth, plasma concentrations of both IGF1 and insulin were reduced ($P < 0.02$) in IUGR lambs (in keeping with reduced fetal nutrient supply) and were independent of gender. However, at necropsy the influence of IUGR status on IGF1 levels was reversed, with modestly but significantly higher plasma concentrations in the prenatally growth-restricted lambs ($P < 0.05$). Furthermore, irrespective of growth category, male lambs had markedly higher circulating

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Table 3. *Sequencing primers for 14 assays interrogating CpG sites in 10 genes of interest*

Assays	Target	PCR primer score	PCR product size	Sequencing primer
1	*INS*	66	132	5′-ACCCCTAAATTAACCTC-3′
2	*IGF2* exon 1	95	110	5′-ATAGGTATTTGTTTAGGTTATTAT-3′
3	*IGF2* exon 4	82	114	5′-AGTTTTATATTGAGGATTTTGT-3′
4	*IGF2* exon 6	65	154	5′-TTTTAGAATAGTGATTTTAGATGTT-3′
5	*IGF1* exon 2	80	145	5′-GTGTTGGTAGTTATTTTTAGTT-3′
6	*IGF1* exon 3	83	119	5′-AGTTTTAGAGATTTTTTATTTTAAT-3′
7	*IGF1* exon 4	93	100	5′-GGGGAGGAGGTGAGGG-3′
8	*H19*	88	117	5′-TTGGAAGGTGTTGGT-3′
9	*GH*	59	174	5′-TTGTTAGTTATTTGTTGTTATTTTT-3′
10	*IGF1R*	70	110	5′-GGGAGGGTAGTTGGG-3′
11	*IGF2R*	85	231	5′-AAAGGTGAGGTAGGA-3′
12	*GHR*	76	80	5′-TTTTTTTTTGGGAATAGGG-3′
13	*NRC31*	70	97	5′-AGTATGTGTAGTTTAAGGTAGG-3′
14	*INSR*	89	104	5′-GATGAAAATTAAGTTGTGTAGGTA-3′

PCR, polymerase chain reaction; *INS*, insulin; *IGF2*, insulin-like growth factor 2; *IGF1*, insulin-like growth factor 1; *GH*, growth hormone; *IGF1R*, insulin-like growth factor 1 receptor; *IGF2R*, insulin-like growth factor 2 receptor; *GHR*, growth hormone receptor; *INSR*, insulin receptor.

Table 4. *RT-PCR primers and probes for four somatotrophic axis genes of interest*

Target	Oligonucleotide	Nucleotide sequence	Accession number[a]
IGF1	Forward	5′-ACATCCTCCTCGCATCTCTTCT-3′	NM001009774
	Reverse	5′-CGTGGCAGAGCTGCTGAA-3′	
	Probe	5′(6FAM)-CTGGCCCTGTGCTTGCTCGC-(TAMRA)3′	
IGF2	Forward	5′-GCTTCCGGACGACTTCACA-3′	NM001009311
	Reverse	5′-GGACTGCTTCCAGGTGTCAGA-3′	
	Probe	5′(6FAM)-CATACCCCGTGGGCAAGTTCTTCCA-(TAMRA)3′	
IGF1R	Forward	5′-GCCTTTTACTCTGTACCGAATCG-3′	AY162434
	Reverse	5′-GCGCTGCAGCCAAGCT-3′	
	Probe	5′(6FAM)-TCCACAGCTGTAACCACGAGGCTGAG-(TAMRA)3′	
IGF2R	Forward	5′-TGTCCAGCCTCTCCAAGAACA-3′	AF327649
	Reverse	5′-TGCACACCCCCACACTGTAG-3′	
	Probe	5′(6FAM)-CTTCAAGGTGACCCGAGGCCCG-(TAMRA)3′	

RT-PCR, reverse transcription polymerase chain reaction; *IGF1*, insulin-like growth factor 1; *IGF2*, insulin-like growth factor 2; *IGF1R*, insulin-like growth factor 1 receptor; *IGF2R*, insulin-like growth factor 2 receptor.

[a]Nucleotide sequences for ovine-specific genes were obtained from the National Center for Biotechnology Information (NCBI) database.

IGF1 levels than female lambs (597 ± 30 *v.* 380 ± 18 ng/ml, $P < 0.001$ from GLM and by *t*-test). Peripheral insulin concentrations at necropsy were also higher in male *v.* female lambs ($P < 0.05$) and were independent of prenatal growth status. Moderate variability was noted in plasma insulin levels, which likely reflects the fact that these were not fasted samples, given that lambs were kept together with their mothers until point of necropsy to prevent separation anxiety.

Hepatic DNA methylation

Table 6 shows the mean percentage methylation for each of the 14 assays investigating a total of 10 different genes of interest in relation to postnatal growth and metabolism, whereas Table 7 details the methylation status at each individual CpG site. There were no statistically significant differences between IUGR and normal birth weight lambs for any of the genes examined, although *IGF2R* methylation tended to be greater in IUGR *v.* normal birth weight lambs (54.3 ± 0.47 *v.* $53.2 \pm 0.41\%$, $P = 0.087$), independent of gender. In contrast, independent of IUGR status, *IGF1* exons 2 and 3 methylation was increased in female *v.* male lambs (75.5 ± 0.14 *v.* $74.6 \pm 0.26\%$, $P = 0.01$; and 90.1 ± 0.19 *v.* $89.3 \pm 0.29\%$, $P < 0.05$, respectively, from GLM and by *t*-test) and consequently overall *IGF1* gene methylation was significantly greater in females (86.2 ± 0.14 *v.* $85.3 \pm 0.22\%$, $P = 0.008$), independent of IUGR status. Further, overall hepatic *IGF1* methylation was negatively correlated with IGF1 plasma levels at necropsy ($r = -0.455$, $n = 38$, $P = 0.005$), irrespective of gender.

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Table 5. *Selected anthropometric and biochemical parameters at the time of birth and necropsy at 77.5 ± 0.5 days postnatal age*

	Prenatal growth status and gender				*P*-value		
Parameters	Normal female ($n = 9$)	IUGR female ($n = 9$)	Normal male ($n = 12$)	IUGR male ($n = 8$)	Prenatal growth status	Gender	Interaction
Birth weight (kg)	4.60 ± 0.13	3.13 ± 0.22	4.95 ± 0.19	3.40 ± 0.17	**<0.001**	0.103	0.830
Live weight at necropsy (kg)	33.4 ± 0.5	29.5 ± 1.2	37.6 ± 1.0	32.8 ± 1.5	**<0.001**	**0.002**	0.650
FGR between birth and necropsy (%/day)	8.51 ± 0.23	11.31 ± 0.70	8.56 ± 0.35	10.83 ± 0.22	**<0.001**	0.620	0.535
Carcass weight at necropsy (kg)	21.7 ± 0.4	18.9 ± 0.8	23.8 ± 0.8	20.9 ± 1.0	**<0.001**	**0.011**	0.914
Empty body weight at necropsy (kg)	27.2 ± 0.4	24.2 ± 1.0	39.0 ± 0.9	26.3 ± 1.2	**<0.001**	**0.014**	0.718
Liver weight at necropsy (g)	556 ± 19	530 ± 22	659 ± 16	565 ± 28	**0.006**	**0.002**	0.113
Relative liver weight (g/kg empty body weight)	20.4 ± 0.69	21.9 ± 0.45	22.1 ± 0.50	21.5 ± 0.374	0.386	0.267	0.061
Plasma IGF1 at birth (ng/ml)	254 ± 19	139 ± 18	216 ± 15	169 ± 24	**<0.001**	0.783	0.105
Plasma IGF1 at necropsy (ng/ml)	359 ± 16	405 ± 31	555 ± 38	672 ± 39	**0.022**	**<0.001**	0.308
Plasma insulin at birth (ng/ml)	0.84 ± 0.20	0.64 ± 0.10	0.96 ± 0.10	0.53 ± 0.05	**0.019**	0.976	0.378
Plasma insulin at necropsy (ng/ml)	2.52 ± 0.18	2.78 ± 0.42	3.36 ± 0.43	3.70 ± 0.41	0.463	**<0.032**	0.920

IUGR, intrauterine growth restriction; FGR, fractional growth rate; IGF1, insulin-like growth factor 1.
Significant *P* values (<0.05) are highlighted in bold.

Table 6. *Mean DNA methylation of 10 somatotrophic axis genes (presented by exon where applicable) in hepatic tissue at 77.5 ± 0.5 days postnatal age*

	Prenatal growth status and gender				*P*-value		
Genes	Normal female ($n = 9$)	IUGR female ($n = 9$)	Normal male ($n = 12$)	IUGR male ($n = 8$)	Prenatal growth status	Gender	Interaction
INS	89.7 ± 0.38	89.6 ± 0.38	89.0 ± 0.33	89.4 ± 0.30	0.511	0.673	0.060
IGF1 exon 2	75.4 ± 0.18	75.6 ± 0.22	74.7 ± 0.36	74.5 ± 0.39	0.974	**0.010**	0.519
IGF1 exon 3	90.3 ± 0.24	89.9 ± 0.30	89.4 ± 0.42	89.1 ± 0.39	0.358	**0.026**	0.793
IGF1 exon 4	92.6 ± 0.40	93.0 ± 0.45	92.2 ± 0.33	92.7 ± 0.35	0.271	0.332	0.981
IGF1 mean	86.1 ± 0.12	86.2 ± 0.28	85.4 ± 0.31	85.3 ± 0.28	0.898	**0.008**	0.673
IGF2 exon 1	34.3 ± 0.41	34.9 ± 0.98	34.6 ± 0.57	34.5 ± 0.40	0.703	0.933	0.536
IGF2 exon 4	27.9 ± 1.16	31.0 ± 1.34	31.0 ± 1.05	30.1 ± 1.70	0.395	0.386	0.137
IGF2 exon 6	6.8 ± 0.84	6.4 ± 0.48	6.5 ± 0.48	6.8 ± 0.59	0.953	0.938	0.578
IGF2 mean	23.0 ± 0.63	24.1 ± 0.76	24.0 ± 0.52	23.5 ± 0.65	0.655	0.727	0.214
H19	47.8 ± 0.51	47.2 ± 0.95	49.5 ± 0.90	48.2 ± 0.65	0.278	0.106	0.674
GH	69.4 ± 2.13	68.9 ± 1.93	62.7 ± 4.23	67.9 ± 1.48	0.150	0.383	0.288
INSR	77.7 ± 0.68	77.5 ± 0.54	76.6 ± 1.47	77.1 ± 0.50	0.920	0.463	0.726
IGF1R	78.9 ± 1.09	77.8 ± 0.86	77.7 ± 0.62	79.7 ± 0.46	0.577	0.716	0.058
IGF2R	53.0 ± 0.47	54.4 ± 0.52	53.4 ± 0.62	54.3 ± 0.86	0.087	0.771	0.685
GHR	1.6 ± 0.12	1.6 ± 0.19	1.8 ± 0.10	1.8 ± 0.14	0.926	0.344	0.930
NRC31	1.9 ± 0.25	1.8 ± 0.07	2.2 ± 0.22	2.2 ± 0.23	0.766	0.133	0.593

IUGR, intrauterine growth restriction; *INS*, insulin; *IGF1*, insulin-like growth factor 1; *IGF2*, insulin-like growth factor 2; *GH*, growth hormone; *INSR*, insulin receptor; *IGF1R*, insulin-like growth factor 1 receptor; *IGF2R*, insulin-like growth factor 2 receptor; *GHR*, growth hormone receptor.
Significant *P* values (<0.05) are highlighted in bold.

Hepatic mRNA expression

Table 8 shows the relative mRNA expression of *IGF1*, *IGF2*, *IGF1R* and *IGF2R* in hepatic tissues obtained at necropsy. In keeping with the plasma IGF1 results detailed above, *IGF1* mRNA expression was marginally greater in IUGR relative to normal birth weight lambs ($P < 0.05$), and was markedly higher in males *v.* females (23.9 ± 1.48 *v.* 11.4 ± 0.49, $P < 0.001$ from GLM and by *t*-test), independent of prenatal growth status. Irrespective of gender or growth status, mRNA expression of *IGF1* was positively correlated with plasma IGF1 levels at necropsy ($r = 0.884$, $n = 38$, $P < 0.001$) and

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Table 7. *DNA methylation of 50 individual CpG sites across 10 somatotrophic axis genes in hepatic tissue at 77.5 ± 0.5 days postnatal age*

		Prenatal growth status and gender				*P*-value		
Genes	CpG position	Normal female (n = 9)	IUGR female (n = 9)	Normal male (n = 12)	IUGR male (n = 8)	Prenatal growth status	Gender	Interaction
INS	1	83.5 ± 0.51	83.8 ± 0.83	81.8 ± 0.98	82.7 ± 0.37	0.921	0.969	0.182
INS	2	93.0 ± 0.22	92.8 ± 0.21	92.3 ± 0.28	92.9 ± 0.23	0.320	0.335	0.270
INS	3	87.5 ± 0.55	88.6 ± 0.98	86.6 ± 0.46	87.6 ± 0.63	0.080	0.239	0.994
INS	4	94.0 ± 1.03	92.6 ± 0.61	93.2 ± 0.86	93.6 ± 0.99	0.585	0.865	0.324
INS	5	90.9 ± 0.52	90.2 ± 0.79	90.1 ± 0.48	90.0 ± 0.67	0.560	0.441	0.637
IGF1 exon 2	1	87.9 ± 1.01	89.5 ± 0.94	85.9 ± 0.91	86.6 ± 2.03	0.360	**0.050**	0.752
IGF1 exon 2	2	4.2 ± 0.18	4.5 ± 0.16	4.6 ± 0.27	4.5 ± 0.18	0.824	0.364	0.416
IGF1 exon 2	3	90.6 ± 0.46	89.7 ± 0.91	89.7 ± 0.56	89.8 ± 0.46	0.533	0.606	0.450
IGF1 exon 2	4	93.5 ± 0.17	92.3 ± 1.39	92.4 ± 0.64	93.6 ± 0.93	0.985	0.915	0.185
IGF1 exon 2	5	91.1 ± 0.67	92.2 ± 0.55	92.7 ± 0.84	91.0 ± 0.99	0.727	0.793	0.076
IGF1 exon 2	6	85.0 ± 0.46	87.3 ± 1.98	82.9 ± 0.68	82.9 ± 0.68	0.291	**0.005**	0.314
IGF1 exon 3	1	88.6 ± 0.28	88.7 ± 0.33	87.7 ± 0.43	87.8 ± 0.49	0.837	**0.028**	0.985
IGF1 exon 3	2	92.3 ± 0.27	91.8 ± 0.18	91.6 ± 0.26	91.8 ± 0.39	0.561	0.188	0.167
IGF1 exon 3	3	92.1 ± 0.44	91.6 ± 0.59	91.8 ± 0.60	91.3 ± 0.64	0.406	0.601	0.972
IGF1 exon 3	4	88.3 ± 0.46	87.5 ± 0.40	86.5 ± 0.65	85.7 ± 0.47	0.158	**0.003**	0.962
IGF1 exon 4	1	97.7 ± 0.74	96.3 ± 1.22	96.9 ± 0.81	96.3 ± 1.19	0.341	0.688	0.724
IGF1 exon 4	2	98.8 ± 0.61	99.8 ± 0.15	99.7 ± 0.15	100 ± 0.00	0.057	0.100	0.305
IGF1 exon 4	3	81.3 ± 0.39	83.0 ± 0.55	80.0 ± 0.70	81.7 ± 0.29	**0.007**	**0.032**	1.000
IGF2 exon 1	1	37.0 ± 0.48	36.9 ± 1.02	37.1 ± 0.76	35.7 ± 0.66	0.341	0.473	0.431
IGF2 exon 1	2	39.8 ± 0.53	41.7 ± 1.17	41.6 ± 1.02	40.1 ± 0.51	0.849	0.919	0.087
IGF2 exon 1	3	25.9 ± 0.65	26.2 ± 1.08	25.2 ± 0.49	27.5 ± 0.56	0.075	0.673	0.161
IGF2 exon 4	1	20.3 ± 1.03	20.9 ± 1.02	21.8 ± 0.95	20.9 ± 1.67	0.929	0.534	0.539
IGF2 exon 4	2	35.4 ± 2.08	41.0 ± 1.94	40.2 ± 1.46	39.4 ± 2.07	0.218	0.411	0.092
IGF2 exon 6	1	5.2 ± 0.47	4.9 ± 0.35	5.7 ± 0.56	5.5 ± 0.24	0.698	0.280	0.986
IGF2 exon 6	2	7.6 ± 0.84	6.9 ± 0.64	7.4 ± 0.65	8.2 ± 1.16	0.980	0.531	0.356
H19	1	48.2 ± 0.51	47.6 ± 0.77	48.9 ± 1.03	48.7 ± 0.81	0.600	0.304	0.860
H19	2	50.0 ± 0.58	49.9 ± 1.27	51.9 ± 0.95	51.2 ± 0.98	0.685	0.123	0.761
H19	3	47.3 ± 0.77	46.9 ± 1.18	50.3 ± 0.82	48.4 ± 0.63	0.216	**0.016**	0.391
H19	4	38.5 ± 0.93	37.7 ± 1.04	39.2 ± 0.81	38.2 ± 0.48	0.297	0.478	0.907
H19	5	48.4 ± 0.56	47.5 ± 0.85	50.0 ± 1.22	48.8 ± 0.65	0.296	0.164	0.879
H19	6	54.1 ± 0.64	53.6 ± 1.12	56.5 ± 0.96	54.1 ± 1.08	0.143	0.158	0.359
GH	1	69.5 ± 3.19	71.4 ± 2.59	66.9 ± 5.00	71.5 ± 2.77	0.732	0.164	0.879
GH	2	69.4 ± 2.61	66.4 ± 2.13	58.9 ± 3.68	64.2 ± 0.71	**0.019**	0.158	0.359
INSR	1	76.2 ± 0.84	76.1 ± 0.56	76.5 ± 0.59	74.3 ± 0.67	0.087	0.284	0.125
INSR	2	79.3 ± 0.8	78.9 ± 0.96	76.6 ± 3.02	79.8 ± 0.70	0.500	0.688	0.386
IGFR1	1	73.9 ± 0.67	72.5 ± 0.88	72.7 ± 0.58	74.7 ± 0.39	0.639	0.485	**0.013**
IGFR1	2	88.4 ± 0.93	86.9 ± 0.81	87.1 ± 0.48	88.5 ± 0.63	0.921	0.8113	0.051
IGFR1	3	74.5 ± 1.97	74.1 ± 1.11	73.2 ± 1.01	75.8 ± 0.74	0.397	0.853	0.248
IGFR2	1	54.5 ± 0.41	55.0 ± 0.44	54.1 ± 0.44	53.8 ± 0.52	0.896	0.082	0.386
IGFR2	2	54.2 ± 0.47	55.0 ± 0.53	55.3 ± 0.79	54.8 ± 0.31	0.841	0.501	0.294
IGFR2	3	49.9 ± 0.49	51.7 ± 0.59	52.6 ± 1.25	51.4 ± 0.41	0.793	0.198	0.120
IGFR2	4	53.6 ± 1.48	51.7 ± 0.91	53.8 ± 1.74	53.3 ± 0.94	0.435	0.547	0.661
IGFR2	5	44.3 ± 0.38	45.5 ± 0.36	42.1 ± 2.72	49.8 ± 5.01	0.114	0.705	0.245
IGFR2	6	56.5 ± 0.93	57.1 ± 1.00	58.1 ± 0.81	59.9 ± 2.48	0.384	0.105	0.695
IGFR2	7	48.3 ± 0.30	49.4 ± 1.37	48.2 ± 0.87	49.2 ± 0.69	0.268	0.878	0.923
IGFR2	8	59.7 ± 1.07	59.9 ± 0.61	58.7 ± 1.34	63.1 ± 5.29	0.360	0.650	0.391
IGFR2	9	34.2 ± 1.05	35.5 ± 1.12	35.7 ± 1.09	38.9 ± 1.56	0.071	0.052	0.476
IGFR2	10	73.8 ± 1.74	77.5 ± 3.52	76.6 ± 4.00	66.2 ± 5.53	0.410	0.293	0.083
IGFR2	11	54.0 ± 2.64	59.7 ± 1.19	52.3 ± 2.64	59.4 ± 4.64	0.032	0.734	0.813
GHR	1	1.6 ± 0.12	1.6 ± 0.19	1.8 ± 0.10	1.8 ± 0.14	0.926	0.344	0.930

IUGR, intrauterine growth restriction; *INS*, insulin; *IGF1*, insulin-like growth factor 1; *IGF2*, insulin-like growth factor 2; *GH*, growth hormone; *INSR*, insulin receptor; *IGF1R*, insulin-like growth factor 1 receptor; *IGF2R*, insulin-like growth factor 2 receptor; *GHR*, growth hormone receptor.
NRC31 methylation is not shown by individual CpG site as minimal detectable methylation at several loci prevented meaningful comparisons between groups.
Significant *P* values (<0.05) are highlighted in bold.

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Table 8. *mRNA expression of the insulin-like growth factors (IGF) and their receptors relative to 18S in hepatic tissue at 77.5 ± 0.5 days postnatal age*

	Prenatal growth status and gender				*P*-value		
Genes	Normal female ($n = 9$)	IUGR female ($n = 9$)	Normal male ($n = 12$)	IUGR male ($n = 8$)	Prenatal growth status	Gender	Interaction
IGF1:18S	10.41 ± 0.671	12.44 ± 0.571	22.06 ± 1.270	26.62 ± 3.040	**0.044**	**<0.001**	0.428
IGF2:18S	15.41 ± 0.591	15.77 ± 0.892	16.87 ± 0.757	16.13 ± 0.972	0.816	0.274	0.507
IGF1R:18S	12.78 ± 0.995	10.85 ± 1.227	12.08 ± 0.622	11.23 ± 0.503	0.123	0.861	0.546
IGF2R:18S	17.06 ± 0.647	16.56 ± 1.051	20.28 ± 1.967	17.68 ± 0.400	0.281	0.133	0.463
18S	0.035 ± 0.001	0.033 ± 0.001	0.034 ± 0.002	0.033 ± 0.001	0.358	0.886	0.838

mRNA, messenger RNA; IUGR, intrauterine growth restriction; *IGF1R*, insulin-like growth factor 1 receptor; *IGF2R*, insulin-like growth factor 2 receptor.
Significant *P* values (<0.05) are highlighted in bold.

negatively correlated with overall hepatic *IGF1* methylation ($r = -0.507$, $n = 38$, $P = 0.002$). There was no impact of prenatal growth status or gender on hepatic *IGF2*, *IGF1R* and *IGF2R* mRNA abundance.

Associations between molecular parameters and phenotype

Figure 1 summarizes the gender differences seen in three different aspects of *IGF1* gene function, namely protein levels (a), mRNA expression (b) and DNA methylation (c). In addition to the strong relationships between these three parameters in the expected and biologically meaningful direction, *IGF1* methylation was negatively correlated with carcass weight ($r = -0.460$, $n = 38$, $P = 0.005$) and plasma insulin levels ($r = -0.580$, $n = 38$, $P < 0.001$) at necropsy. By contrast, *H19* methylation was positively correlated with carcass weight at necropsy ($r = 0.494$, $n = 38$, $P = 0.002$). These relationships were independent of lamb gender. In addition, *IGFR1* and *GH* methylation were inversely correlated with lamb birth weight ($r = -0.524$, $n = 38$, $P = 0.018$) and plasma IGF1 levels at necropsy ($r = -0.535$, $n = 38$, $P = 0015$), respectively, in male lambs only, whereas IGF2 methylation was positively correlated with plasma insulin levels at necropsy ($r = 0.519$, $n = 38$, $P = 0.027$) in female lambs only, irrespective of IUGR status.

Discussion

Effects of IUGR

In the present study, IUGR lambs were noted to have significantly higher *IGF1* mRNA expression and higher circulating IGF1 protein levels relative to normal birth weight lambs. This represented a switch from the pattern observed at the time of birth, when IGF1 concentrations were lower in IUGR lambs, most likely reflecting reduced fetal nutrient supply. This reversal of the IGF1 differential by 11 weeks postnatal life and markedly altered absolute and fractional growth velocity during the early neonatal period are in keeping with the phenomenon of neonatal catch-up growth, which occurs in the event of a mismatch between the pre- and postnatal environments[26] and is often attributed to putative epigenetic changes. However, in the present study, despite a major differential in birth weight and markedly altered rates of growth, prenatal growth restriction had no significant impact on the methylation status of 10 genes variously involved in somatic growth and metabolism. A statistical trend towards higher *IGF2R* methylation was noted in IUGR lambs ($P = 0.087$) relative to normally grown lambs, which could well represent a chance observation, especially as it was not accompanied by any measurable change in *IGF2R* mRNA expression. Alternatively, however, it may reflect an antecedent effect of IUGR during intrauterine life. Unlike IGF1R, which mediates the mitogenic effects of IGF1 and IGF2, IGF2R is a clearance receptor that antagonizes IGF2 action, and disruption of the *IGF2R* gene results in increased serum and tissue levels of IGF2 and fetal overgrowth.[27] Relative hypermethylation of the *IGF2R* gene at the particular site examined, which is an imprinted differentially methylated region, would hypothetically be associated with reduced gene expression, which would serve to maximize IGF2 availability. The fact that IGF2 is predominant during fetal but not neonatal life might potentially explain the failure to demonstrate differences in *IGF2R* expression at 11 weeks of age, at which time persistent differences in DNA methylation may simply represent stigmata of earlier events in the life course. *IGF2R* hypermethylation has been reported previously in children referred to geneticists for short stature following IUGR.[28]

It was interesting to observe striking yet consistent differences in the overall degree of methylation between genes (e.g. ~90% for *INS v.* <2% for *GHR*) and within genes (e.g. ~4% at a single locus in *IGF1* exon 2 compared with >85% for all others tested). The *GHR* had the lowest overall methylation, yet clearly maintained the potential to become heavily methylated, as a 94% methylation status was achieved in the control sample treated with M.SssI. The region examined was just proximal to exon 1B, which is expressed in multiple tissues and contains a putative promoter.[29] In addition, *NRC31* was noted to have no measurable methylation at many loci, and minimal degrees of methylation were detectable. The degree of *NRC31*

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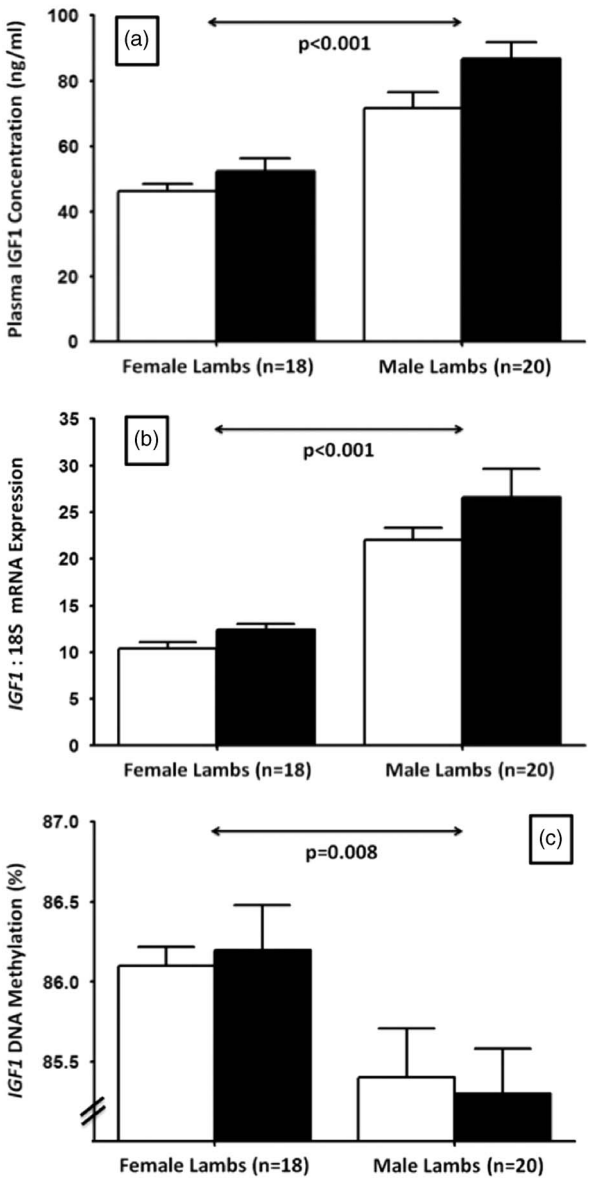

Fig. 1. Plasma insulin-like growth factor 1 (*IGF1*) levels (a) plus hepatic mRNA expression (b) and DNA methylation of the *IGF1* gene presented by gender at 77.5 ± 0.5 days postnatal age in 38 lambs born to overnourished adolescent ewes and characterized as normal birth weight ($n = 21$, open bars) or IUGR ($n = 17$, closed bars).

methylation observed herein is consistent with that reported in the hypothalamus of prenatally undernourished sheep, which ranges from 0.3 to 0.6% during fetal life[17] to ~1% at 5 years of age.[30]

In general, the apparent lack of a measurable epigenetic effect of IUGR following overnourishment of the pregnant adolescent ewe suggests that no permanent alterations in somatotrophic gene function are associated with this prenatal insult, which is reassuring. The lack of apparent epigenetic changes may relate to the timing of the insult in our model, given that the onset of IUGR is relatively late, being confined to the final third of gestation.[31] Although the impact of IUGR is arguably greatest in late gestation, when energy demand is maximal, there is accumulating evidence that the epigenome is more susceptible at an earlier window of development. Initial studies on individuals prenatally exposed to the Dutch famine of 1944–1945 reported reduced methylation of *IGF2*[32] and increased methylation of interleukin 10 (*IL10*), ATP-binding cassette subfamily A member 1 (*ABCA1*), maternally expressed 3 (non-protein coding) (*MEG3*), leptin (*LEP*) and GNAS antisense RNA (*GNASAS*)[33] only when the exposure occurred around the time of conception and not during late gestation. The same group subsequently tested for differences at the same loci in a separate cohort of adults born preterm after IUGR and found no differences in DNA methylation of *IGF2*, *GNASAS*, *INSIGF* and *LEP* relative to appropriate birth weight controls.[34] The latter results suggest that not all long-term morbidity of IUGR resulting from programming *in utero* is mediated by changes in DNA methylation and may involve other epigenetic mechanisms such as histone modifications[35] or microRNAs (miRNAs).[36] For example, IUGR induced by bilateral uterine artery ligation in the rat has been demonstrated to modify the histone code of the *IGF1*, *PPAR-γ* and *NRC31* genes,[7,37,38] and levels of miR132.[39] Alternatively, non-epigenetic insults on tissue structure and function may be responsible for long-term adverse effects on health. For example, IUGR in the rat is characterized by decreased pancreatic β-cell mass and islet vascularity leading to impaired insulin secretion,[40] whereas in the human low birth weight is associated with reduced nephron numbers[41] and exaggerated sympathetic nerve activity during adulthood,[42] both of which are implicated in the prenatal programming of hypertension. Given that there was a measurable impact of IUGR on *IGF1* mRNA expression and circulating IGF1 in the present study, both of which were increased relative to normal birth weight lambs, it remains possible that alternative epigenetic or indeed non-epigenetic mechanisms may be responsible.

Effects of gender

In the present study, irrespective of prenatal growth status, male lambs had significantly higher *IGF1* mRNA expression and higher circulating IGF1 protein levels compared with female lambs. No gender differences were present at the time of birth, suggesting that this sexual dimorphism in IGF1 emerges during the neonatal period, reflected by serial IGF1 samples over the first 11 weeks of life.[16] Hepatic IGF1 mRNA expression and IGF1 protein levels were each inversely correlated with hepatic *IGF1* DNA methylation, which was significantly greater in female *v.* male lambs at 11 weeks postnatal age. Relative *IGF1* hypomethylation in males is consistent with increased transcription and hence greater IGF1 protein levels. However, *absolute* differences between male and female lambs were ultimately very small at <1%, which raises questions about their biological significance. Notably, however, these gender differences were highly statistically significant, were

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present at five separate CpG sites and occurred in the expected direction of effect relative to the clear differences in protein and mRNA expression. The chances that differences in three separate aspects of *IGF1* gene function were detectable by chance are infinitely small; however, it is accepted that DNA methylation changes alone are unlikely to explain the total variation in *IGF1* mRNA and protein expression observed in this cohort of lambs. Notably, the small differential between groups (<1%) is similar to previous reports of DNA methylation in humans across a wide range of genes, including male *v.* female[43] and type 2 diabetic *v.* non-diabetic comparisons.[44] These studies have also revealed similar variance with respect to DNA methylation (<1%) among human subjects, despite the arguably much greater degree of genetic heterogeneity when compared with our highly controlled sheep model. Furthermore, in the aforementioned studies of hypothalamic *NRC31* DNA methylation, a small decrease of only 0.6–0.3% was associated with a five-fold increase in mRNA expression.[17,30]

We found that the differences in *IGF1* methylation were confined to exons 2 and 3. The lack of similar changes in exon 4 may reflect the fact that its mRNA is not expressed at all in sheep.[45] In support of this assumption, methylation proximal to exon 4 was greater than at any region studied, suggesting minimal gene expression or gene silencing. Interestingly, the putative promoter site for *IGF1* is located just proximal to exon 1 but is reportedly not GC rich;[46] therefore, it is unsurprising that no conventional CpG island was found at this location in the published ovine sequence. Given that the aims of the present study were to examine DNA methylation in CpG islands, we did not measure methylation of this putative promoter, which is a potential limitation of our approach. Moreover, as the regions of *IGF1* investigated were all intragenic, it is possible that differential methylation here may have effects that are different to promoter methylation.[47] To our knowledge, gender differences in *IGF1* methylation have not previously been reported in any species, although other genes have been shown to be differentially methylated in males *v.* females. For example, the aforementioned studies on survivors of the Dutch Hunger Winter found that effects on *INS*, *IGF* and *LEP* were restricted to men and that changes in *GNASAS* were more pronounced in women. Moreover, *IGFR2* methylation in normal (unexposed) controls was 2.6% higher in men *v.* women in adult life.[33] *IGFR1* methylation was higher in male db/db mice (homozygous for a point mutation in the leptin receptor) compared with female db/db mice and both male and female controls.[48] In general, human epigenetic studies have shown that CpG sites generally show *greater* methylation in males, with the exception of imprinted genes, in which DNA methylation appears to be more equal between sexes.[49] Consequently, the relative hypomethylation of *IGF1* seen here in males is novel but certainly in keeping with recognized gender differences in circulating IGF1 in the young lamb.[16] Studies in other species have similarly demonstrated elevated IGF1 levels in males *v.* females at various stages of the life course. For example, peripheral IGF1 concentrations are higher in male mice during early puberty,[50] whereas in the rat no measurable differences occur until well into adult life, at around 12 weeks of age.[51] In humans, IGF1 levels tend to be higher in boys than girls between 6 and 18 years of age;[52] however, no significant differences in IGF1 are apparent in early adulthood[53] or old age,[54] despite persistent gender differences in body composition and glucose metabolism in both humans and our sheep model of IUGR.[16,22,55] In females, there were clear associations between lower circulating IGF1 levels, body weight and radial/ulnar growth rates, and higher adiposity, leptin levels and thoracic growth rates; however, to what extent this sexual dimorphism is governed by differences in DNA methylation remains unclear. As discussed above in relation to IUGR status, it remains possible that alternative epigenetic mechanisms are responsible for the observed differences at the mRNA/protein level. For example, gender differences have been reported in histone acetylation[56] and miRNA regulation,[57] some of which may be mediated by differences in sex steroids. Although circulating levels of oestrogen and testosterone are likely to have been low at the point of necropsy several weeks before puberty, these steroid hormones could nevertheless have impacted pituitary GH secretion resulting in altered hepatic IGF1 action. Clinical studies have demonstrated associations between hypogonadism and low GH and precocious puberty and high GH,[58] and exposure to sex steroids during neonatal life can impact the GH secretory pattern later in life by modulating the number of GH releasing hormone neurones in the hypothalamus.[59] In addition, exposure to xenoestrogens during neonatal life alters GH-dependent liver proteins[60] and oestrogen replacement has been associated with reduced circulating IGF1 levels in adult women.[61] Accordingly, a direct or indirect effect of reproductive steroids on the IGF1 system cannot be ruled out and may interact with or indeed eclipse the relatively small epigenetic changes observed herein.

Strengths and weaknesses

Bisulphite sequencing methods provide very high-resolution assessment of methylation at specific loci, but are limited by the fact that only a small number can be examined in any one reaction. Furthermore, the specific sites are limited to those around which functional primer sets can be designed, which are largely dependent upon the neighbouring gene sequences. Finding primers that are specific enough and do not form secondary (e.g. hairpin or dimer) structures when working with bisulphite-converted DNA is a major challenge, as the standard four-base genetic code is massively simplified to one comprising just three bases. In addition, the highly stringent primer specifications (detailed in the Materials and methods section) greatly limited the number of viable sets for each (relatively short) gene sequence, greatly restricting the sites within the genomic DNA that could be examined. Clearly, it remains possible that changes outwith the 57 CpG sites studied could have been missed. Array-based technologies have been developed

for the human and mouse that can simultaneously examine up to 450,000 CpG sites in a single assay; however, no such commercial kits are currently available for sheep. More recently, the role of 5-hydroxymethylcytosine (5hmC), which is a marker of so-called 'active demethylation' in which methylated C is oxidized by ten-eleven translocation enzymes, has been attracting increasing attention, especially in the field of cancer epigenetics.[62] Unfortunately, the methodology used herein does not currently distinguish between 5-methylcytosine and 5hmC; therefore, we are unable to comment on any potential influence of demethylation, and any future studies should take this into account. It is also a potential limitation that the methodology used cannot recognize differences in methylation between the maternal and paternal alleles, called imprinting, which is known to influence a number of different genes including *H19*. This may arguably be less important here, given the use of a single sire, which completely controls for paternal genetics.

A further limitation is that, although accepted criteria were used to identify CpG islands within the available ovine gene sequences, the biological significance of differential DNA methylation at these specific sites has largely not been assessed. Most of the ovine genes investigated herein were originally sequenced with the aim of examining their exonic arrangement rather than focussing on the 5′ untranslated regions (proximal introns) where the majority of CpG islands are known to lie.[5] As the DNA methylation assays developed herein are novel, direct comparisons with the limited number of previous sheep studies are not possible. Sinclair *et al.*[63] reported alterations in DNA methylation in sheep fetuses following periconceptual manipulation of maternal dietary vitamin B and methionine content at 4% of 1400 loci in a gender-specific manner using restriction landmark genome scanning; however, the identity of these loci was not explicitly stated. Wang *et al.*[64] examined methylation of *IGF2/H19* and *IGF2R* in the late fetal and early postnatal heart using combined bisulphite restriction analysis, but found no significant changes secondary to ovine IUGR induced by carunclectomy. Begum *et al.*[17] and Lan *et al.*[18] recently demonstrated altered methylation status of *NRC31* and the proopiomelanocortin gene, and *IGF2R* and *H19*, respectively, in a variety of ovine fetal tissues following maternal dietary manipulations (underfeeding and variable energy source, respectively), but did not report any associated impact on fetal weight or mRNA expression of the same panel of genes. Direct comparisons with other ovine studies may also be limited by the fact that the present study used donor superovulation, which is known to influence DNA methylation of imprinted genes including *H19*[65] and *IGF2*.[66] As all animals herein underwent the same assisted reproductive techniques and oocytes from 9 of 11 donors ultimately produced both IUGR and normal birth weight lambs with a balanced ratio of males to females, it is unlikely that this limits comparisons between groups. The use of a single sire also limited any variation in paternal genetics.

It should also be noted that we only examined DNA methylation in a single tissue and at a single time point. Liver was chosen for this study as most published work on the epigenetic impact of IUGR has been carried out using this tissue type, and because the liver is an important metabolic organ that plays a central role in the regulation of postnatal growth. However, ultimately epigenetic changes observed in one tissue cannot necessarily be extrapolated to others.[67] For example, adipose tissue would be a good candidate for further investigation into the influence of IUGR and gender on early-life metabolism. As we only examined animals at weaning, it is unknown if sexual dimorphism in *IGF1* methylation is present at the time of birth, or indeed whether it persists into adult life. Hence, it remains unclear whether differential *IGF1* methylation is programmed *in utero* or represents dissimilarities that emerge alongside other sex differences such as fasting metabolite levels and relative adiposity during postnatal growth and development. In support of this concept, there is evidence that epigenetic changes can occur and can be prevented during postnatal life. For example, amelioration of catch-up growth by dietary manipulation in growth-restricted rat pups appears to prevent changes in *IGF1* methylation that otherwise emerge in the first few weeks,[68] and is associated with an improved metabolic profile.[69] Consequently, it is possible that the changes seen herein simply represent gender differences in ontogeny of the somatotrophic axis, rather than a 'fetal programming' effect *per se*.

Conclusion

In summary, IUGR induced by overnourishment of pregnant adolescent ewes did not significantly impact DNA methylation of key growth axis genes, but gender differences in live weight and body conformation in early postnatal life were associated with sexual dimorphism in hepatic DNA methylation and mRNA expression of the *IGF1* gene and plasma IGF1 concentrations. Future work should focus on more targeted methylation analysis around putative promoter and regulatory regions of IGF1 and evaluate for possible changes in 5hmC.

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Conflicts of Interest

None.

Ethical Standards

The authors assert that all procedures contributing to this work comply with the ethical standards of the relevant national guides on the care and use of laboratory animals (sheep) and has been approved by the institutional committee (Rowett Institute local ethics review committee).

Supplementary material

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