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Effects of early gestation GH administration on placental and fetal development in sheep

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Abstract

Ovine GH (oGH) is synthesized in placental tissue during maximal placental growth and development. Our objectives were to localize oGH mRNA in the placenta, and study the impact of exogenous GH on twin pregnancies during the normal window (35–55 days of gestational age; dGA) of placental expression. *In situ* hybridization localized oGH mRNA in uterine luminal epithelium but not in tissues of fetal origin. While maternal GH and IGF-I concentrations were increased ($P < 0.001$) approximately tenfold, uterine, uterine fluid, placental, and fetal weights were unaffected by treatment at either 55 or 135 dGA. Fetal length, liver weight, and liver weight per kg of body weight were unaffected by maternal GH treatment. However, in the cotyledon, IGF-binding protein (BP)-1 and IGFBP-4 mRNA concentrations were increased

($P < 0.05$), while IGFBP-2 mRNA was decreased ($P < 0.05$). The concentration of mRNA for IGFBP-3 was unaffected by treatment. Within the caruncle, IGFBP-1 mRNA was decreased ($P < 0.05$), while IGFBP-3 and IGFBP-4 mRNA were increased ($P < 0.05$), and IGFBP-2 mRNA was unchanged due to GH treatment. While our data indicate that elevated maternal GH and IGF-I concentrations during early and mid-gestation do not enhance placental and fetal growth in twin pregnancies, localization of GH mRNA in uterine luminal epithelium could explain GHs transitory expression from 35 to 55 dGA, since by the end of this period the majority of the uterine luminal epithelium has fused with chorionic binucleate cells forming the placental syncytium.

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Introduction

Sheep have two growth hormone (GH) genes (ovine GH-1 (oGH-1) and oGH-2), and oGH-2 has multiple alleles (oGH-2N and oGH-2Z; Valinsky *et al.* 1990, Gootwine *et al.* 1993). Expression of the oGH-2 gene has been detected in sheep placenta (Lacroix *et al.* 1996, 1999) and it was inferred that both the N- and Z-alleles of the gene are expressed (Ofir & Gootwine 1997). oGH mRNA and protein have been detected in the placenta during early to mid-gestation (Lacroix *et al.* 1996, 1999), with the predominant window of oGH expression occurring between 35 and 55 days of gestational age (dGA). Immunolocalization of oGH within maternal syncytial and stromal cells, as well as the fetal trophoblast, has been reported for 40–45 dGA placentomes (Lacroix *et al.* 1996). This group also reported *in situ* hybridization of oGH mRNA in the syncytium and trophoblast layers of the placenta at 40 dGA (Lacroix *et al.* 1999). The oGH receptor (oGHR) mRNA was initially identified in the placenta at 51, 95, and 120 dGA (Klempt *et al.* 1993) and was localized to what was identified as uterine epithelium and uterine glands at 120 dGA. Lacroix *et al.*

(1999) identified oGHR mRNA in the uterine endometrium from 8 to 120 dGA and identified the primary site of oGHR mRNA being the trophoctoderm at 40 dGA. Collectively, these data suggest that a functional interaction of oGH and its receptor may exist during early to mid-gestation within the sheep uteroplacental unit.

Administration of recombinant porcine GH (rpGH) to gilts during early to mid-gestation increases fetal and placental weight (Sterle *et al.* 1995), and increases in fetal weight and length have also been reported for underfed gilts given rpGH during mid-gestation (Gatford *et al.* 2000). However, studies in sheep to elucidate the impact of GH administration during pregnancy have provided variable results. Administration of GH during conceptus elongation and early placentation has no effect on placental development (Spencer *et al.* 1999). Only endometrial gland proliferation was stimulated and this effect was seen with concurrent interferon- τ administration (Spencer *et al.* 1999). Wallace *et al.* (2004) reported an increase in uteroplacental weight due to GH infusion from 35–80 dGA; however, this is due likely to increased fetal fluid weight resulting from polyhydramnios, as total and average placentome weights and fetal weights were not statistically

different as a result of GH treatment. Furthermore, the increase in uteroplacental weight (Wallace *et al.* 2004) was only seen in adolescent ewes maintained on a high dietary intake, a feeding regimen used in adolescent ewes to generate fetal growth restriction (Wallace *et al.* 1997, Anthony *et al.* 2003). More recently, treatment of ewes from 35–65 dGA with GH failed to alter fetal or placental development (Wallace *et al.* 2006), as measured some 65 days later, and the GH was only administered to the high intake adolescent ewes. Interestingly, in over-nourished adolescent ewes, serum GH is decreased while serum insulin-like growth factor-I (IGF-I) is increased (Wallace *et al.* 1997), indicating a disconnect of the normal endocrine relationship between GH and IGF-I.

Administration of GH during mid-pregnancy (70–83 dGA) in normal sheep pregnancies results in increased maternal serum non-esterified fatty acids, IGF-I and glucose, but no change in fetal growth (Jenkinson *et al.* 1999). Fetal weight was also increased 10% by GH infusion at 98–111 dGA (Jenkinson *et al.* 1999); however, several reports saw no effect on fetal or placental weight with similar temporal administration of GH (Stelwagen *et al.* 1994, Currie *et al.* 1996, Harding *et al.* 1997). Interestingly, all of the studies discussed above did not administer exogenous GH concurrent with placental GH expression (~35–55 dGA) within the placenta (Lacroix *et al.* 1996, 1999). It remains to be seen what the effects of GH administration, concurrent with the normal placental expression window, will have in sheep. Therefore, it was our objective to further define the location of oGH expression in the placenta and to determine the effects of exogenous recombinant bovine GH (rbGH) administration during the normal window of placental oGH expression in twin sheep pregnancies. We chose to study twin pregnancies, as multiple fetuses place increased demand on the maternal system and placenta to provide adequate nutrition to the fetus.

Materials and Methods

cDNA generation

In order to verify the origin of oGH expression within the placenta of sheep, placentomes were collected from 45 dGA pregnancies following killing according to Colorado State University IACUC approved protocol 02-304A. Intact

placentomes were excised, trimmed to 5 mm cross-sections, and fixed overnight in 4% paraformaldehyde, before being embedded in paraffin. cDNA for oGH, vimentin (marker of fibroblast cell lineage), cytokeratin 18 (marker of epithelial cell lineage), and CD 18 (marker of leukocytes) were generated by RT PCR. The cDNA used for *in situ* hybridization of ovine placental lactogen (oPL) has previously been reported (Kappes *et al.* 1992). For the reverse transcriptase reaction, the SuperScript First Strand Synthesis kit (Invitrogen) was used following the manufacturer's protocol. The PCR products were generated by 40 cycles of 95 °C 1 min to denature, 1 min to anneal (°C in Table 1), and 72 °C 1 min for extension. The PCR amplicons were inserted into PCR-Script Amp SK (Stratagene, Cedar Creek, TX, USA) for transformation, sequence validation, and subsequent propagation. Plasmid DNA was harvested by alkaline lysis, and purification by centrifugation through a CsCl gradient (Liang *et al.* 1999, Limesand & Anthony 2001). The plasmid preparations were sequenced by Macromolecular Resources (Colorado State University) to verify sequence authenticity.

In situ hybridization

To generate template for *in situ* hybridization, oGH (406 bp; *EcoRI*), oPL (533 bp; *BamHI* and *NdeI*), oVimentin (369 bp; *EcoRI* and *NotI*), oCytokeratin 18 (1307 bp; *XhoI*), and oCD 18 (679 bp; *EcoRI* and *SacI*) were digested from their respective plasmids and gel purified on 1% agarose gels. For a negative control, ~400 bp of non-coding sequence from PCR-Script Amp were digested out of intact plasmid with *NspI* and *SacII*. Radiolabeling of cDNA for *in situ* hybridization was performed using a 50–100 ng template with the DECAprime II random prime labeling kit (Ambion, Austin, TX, USA) and radiolabeled cytosine tri-phosphate (³³P-α-dCTP) per the manufacturer's instructions. Only probes with specific activities exceeding 5 × 10⁸ c.p.m./μg DNA were used for *in situ* analysis. *In situ* hybridization was performed as described previously (Kappes *et al.* 1992).

Exogenous GH administration

Twenty-three whiteface crossbred ewes were naturally mated to a single sire, generating twin pregnancies. All procedures

Table 1 PCR primers, annealing temperatures, and product sizes for cDNA used in real-time PCR analysis

	Forward primer	Reverse primer	Anneal (°C)	Product (bp)
cDNA				
IGF-I	tcgcatctctcttctatctggccctgt	acagtacatctccagcctctcaga	62	238
IGF-II	gaccgcggcttctactctcag	aagaacttgcccacggggtat	62	202
IGFBP-1	tgatgaccgagtccagtgtgag	gtccagcgaagtctcacac	62	248
IGFBP-2	caatggcaggagcactctg	tggggatgtgtaggaatag	55	330
IGFBP-3	ctcagagcacagacacca	ggcatattgagctccac	54	335
IGFBP-4	tgtgcgtgtgtttgatg	gagggagccaagatgagt	62	229
RPS15	atcattctgcccgagatgggtg	tgctttacgggctttaggtg	60	134

were approved under Colorado State University IACUC protocol 02-304A. At 35 dGA, the ewes were assigned to treatment groups for s.c. injections of GH (GH, $n=10$) or buffer control (CON, $n=13$). The GH group received 0.2 mg/kg body weight (BW) of rbGH (Monsanto, St Louis, MO, USA) per day reconstituted in 100 mM NH_4HCO_3 buffer. The rbGH was administered by twice daily injections of 3 ml 12 h apart. The CON group received two injections of 3 ml of 100 mM NH_4HCO_3 buffer. The injection regimen lasted for 20 days (35–55 dGA) to coincide with the normal expression window of oGH within the placenta (Lacroix *et al.* 1996, 1999). Prior to, and every 5 days during rbGH administration, blood samples were collected by jugular venipuncture to compare serum hormone concentrations of CON and GH groups.

Between 54–56 dGA, 7 CON, and 5 GH ewes were killed (90 mg/kg sodium pentobarbital) and uterine, placental, and fetal tissues were collected for analysis. Ewe weight, gravid uterine weight, placental weight, and fetal parameters (sex, weight, crown rump length (CRL), and liver weight) were all recorded. The seven CON pregnancies yielded nine male and five female fetuses, whereas the five GH pregnancies yielded five male and five female fetuses. Using CRL and fetal weight, the ponderal index (PI) for each fetus was calculated ($\text{PI} = \text{weight in grams}/\text{length in cm}^3$). Cotyledon and caruncle tissue from each placenta were manually separated and snap frozen in liquid nitrogen for RNA analysis later. The remaining ewes (CON, $n=6$; GH, $n=5$) were gestated until 135 dGA to evaluate the effects of early gestation rbGH administration near term. Ewes were killed between 133 and 138 dGA, and the same data and tissue samples were collected as the 55 dGA groups. The 6 CON pregnancies yielded 2 male and 10 female fetuses, whereas the 5 GH pregnancies yielded 4 male and 6 female fetuses. Additionally, empty uterine weight was measured for use in calculating fluid weight within the uterus. To calculate fluid weight, fetal, placental, and empty uterine weights were subtracted from gravid uterine weight.

Serum hormone analyses

A validated GH RIA was used to determine serum GH levels (Hoefer & Hallford 1987) for duplicate samples. Using NIDDK anti-oGH and NIDDK oGH, the intra-assay coefficient of variation was 7.2% and recovery was 97% for 15 ng. A two-site IRMA (Diagnostic Systems Laboratories, Webster, TX, USA) was used to assay serum IGF-I as directed by the manufacturer. Intra- and interassay coefficients of variation were 3.43 and 3.39% for the 50 ng/ml control respectively, and 2.29 and 2.27% for the 200 ng/ml control.

Northern hybridization analysis

Radiolabeling of cDNA for northern hybridization was performed using 50–100 ng template in the DECAprime II random prime labeling kit (Ambion) and using $^{32}\text{P}\alpha\text{-dCTP}$

per the manufacturer's instructions. Only cDNAs with specific activities exceeding 5×10^8 c.p.m./ μg DNA were used for analysis. Caruncle total cellular RNA (tcRNA) was hybridized with oGH cDNA, while cotyledon tissue was hybridized with oPL cDNA as described previously (Kappes *et al.* 1992). The membranes were exposed to a Phosphor Screen (Molecular Dynamics, Sunnyvale, CA, USA) overnight for oPL and 48 h for oGH. The screens were scanned using a Storm Imager (Molecular Dynamics) to visualize hybridization. The membranes were then stripped with repeated washes of 200 ml boiling stripping buffer ($0.05 \times \text{SSC}$, 10 mM EDTA and 0.1% SDS), blocked with pre-hybridization buffer, and hybridized with ribosomal protein S15 (RPS15) cDNA (2×10^6 c.p.m./ml) to serve as a normalization control. For northern hybridization analysis, the expression of RPS15 (Rozance *et al.* 2007) was used for normalization. The concentration of RPS15 was used for normalization due to changes by treatment in other house-keeping genes tested (actin and glyceraldehydes-3-phosphate dehydrogenase). Following hybridization, the membranes were exposed to a Phosphor screen overnight and scanned with the Storm Imager. Densitometric analysis of the Storm images for all northern hybridizations was completed with ImageQuant 5.0 (Molecular Dynamics).

Quantitative reverse transcriptase PCR

tcRNA was isolated as described previously (Warren *et al.* 1990, Kappes *et al.* 1992). An aliquot of the stock tcRNA samples was diluted to a concentration of $1 \mu\text{g}/\mu\text{l} \pm 0.2 \mu\text{g}/\mu\text{l}$ for use as a working solution. The concentration of the working solutions was verified using the Ribogreen (Molecular Probes, Eugene, OR, USA) RNA quantification system. Measurements of fluorescence were taken using a Mithras LB 940 (Bertold Technologies, Oak Ridge, TN, USA). The samples were excited at 485 nm and emission was measured at 535 nm. The counting time was 0.1 s and the lamp energy was set at 10 000. The unknowns were compared against an RNA standard curve.

For the reverse transcriptase reaction, 2 μg tcRNA was used in the SuperScript First Strand Synthesis kit (Invitrogen). All samples of tcRNA for both caruncle and cotyledon were incubated at the same time with the same kit to avoid sample variation due to technician and/or kit variation. The yield of cDNA from the RT reactions was quantified with the Oligreen (Molecular Probes) ssDNA quantification kit. Samples were excited and measured with the Mithras LB 940 with the same settings as for the Ribogreen analysis. Following quantification, all samples were set to a cDNA concentration of 12.5 ng/ μl to be used in quantitative PCR (qPCR). The qPCR mix was 12.5 μl iQ SYBR Green Supermix (Bio-Rad), 200 nM primer (forward and reverse), 2 μl cDNA (25 ng), and balanced to 25 μl total volume with sterile water with a neutral pH. For all qPCRs, the first denaturing step was 95 °C for 5 min and 45 s thereafter, the annealing time was 1 min with gene-specific temperature

(Table 1), and 1 min of extension at 72 °C. The reactions were run for 40 cycles, with a fluorescence measurement after each cycle, followed by a melting curve analysis initiated at 55 °C and measuring fluorescence every 0.5 °C to 95 °C. For the qPCR analysis, cDNA for ovine IGF-I, IGF-II, and IGF-binding proteins (BP) 1, 2, 3, and 4 (de Vrijer *et al.* 2006) and RPS15 (Rozance *et al.* 2007) were used as standard templates. The standard curve template concentrations spanned 100 to 1×10^{-5} pg/ μ l at tenfold increments. For each standard, 1 μ l template was supplied to the reaction mix with the same primer concentration. Table 1 lists the primer sequences, annealing temperatures, and product sizes generated by PCR. Primers were designed to span at least one intron of the mRNA sequence for each gene of interest and produce 130–350 bp of product. For each gene of interest, the qPCRs were optimized as described previously (Erickson-Hagen *et al.* 2005) to obtain amplification efficiencies of 90–100%, and were validated by electrophoresis of the PCR products to verify correct product size prior to analysis. The standard reactions were run in duplicate and the unknown reactions were run in triplicate. The means are presented as gene of interest cycle threshold divided by RPS15 cycle threshold (C_t/C_c).

Statistical analysis

For the maternal and fetal data collected at necropsy, two-way ANOVA (treatment and fetal sex as dependent variables) was conducted on the data obtained at 55 (7 CON and 5 GH twin pregnancies) and 135 (6 CON and 5 GH twin pregnancies) dGA separately. Since there were no significant sex effects or treatment–sex interactions, fetal sex was removed from the statistical model, making student's *t*-test appropriate for comparing treatment effects (CON versus GH), at each gestational age (55 or 135 dGA). Serum hormone data means (CON and GH) for each sample day also were separated by student's *t*-test, as were normalized means for northern and qPCR analyses for the CON and GH.

Results

Localization of oGH mRNA

In situ hybridization analysis of placentome sections revealed a distinct population of cells at the maternal–fetal interface that hybridized with the oGH cDNA (Fig. 1A and B). oGH hybridization did not appear to reside within the fetal trophoblast layer, which contain chorionic binucleate cells (BNCs) as identified by oPL hybridization (Fig. 1C and D). To determine what cell-type expresses oGH, oCytokeratin 18, and oVimentin radiolabeled cDNAs were hybridized to day 45 placentome sections as well. Figure 1E–H represent light and dark phase images of oCytokeratin 18 and oVimentin hybridization respectively. Ovine cytochrome 18 hybridized to the maternal epithelium (Fig. 1E and F),

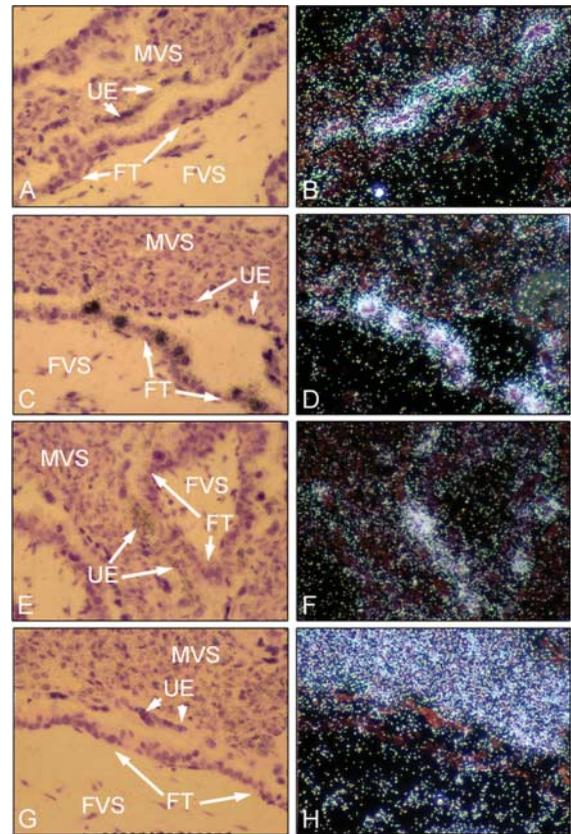


Figure 1 (A and B) *In situ* hybridization with oGH cDNA. (A) A light field image of day 45 placentome. (B) The dark field of the same location. Positive hybridization is detected in UE. (C and D) *In situ* hybridization with oPL cDNA. (C) A light field image of day 45 placentome. (D) The dark field of the same location. Positive hybridization detected in FT binucleate cells. (E and F) *In situ* hybridization with cytokeratin 18 cDNA. (E) A light field image of day 45 placentome. (F) The dark field of the same location. Positive hybridization is detected in UE. (G and H) *In situ* hybridization with vimentin cDNA. (G) A light field image of day 45 placentome. (H) The dark field of the same location. Positive vimentin hybridization is detected in MVS. FT, fetal trophoblast; FVS, fetal villous stroma; MVS, maternal villous stroma; UE, uterine epithelium; final magnification=400 \times .

whereas vimentin hybridization was apparent in the maternal villous stroma and to a lesser extent in the fetal villous stroma (Fig. 1G and H). The pattern of hybridization for oGH and oCytokeratin 18 was similarly located at the maternal–fetal interface, and was not consistently present throughout the boundary of the maternal tissue (Fig. 1A and E). The oGH-positive cells were in random clusters at outer boundaries of maternal tissue, not in the maternal stroma or fetal trophoblast, and appear to be the remnants of the maternal uterine epithelium. To verify that these cells were not of leukocyte origin, sections were hybridized with our oCD18 cDNA. The CD18-positive cells were deep in the endometrium and myometrium, far from the oGH-positive cells within the maternal epithelium (data not shown).

Effect of rbGH administration on maternal and fetal characteristics

Exogenous rbGH was administered twice daily to pregnant ewes from days 35 to 55 of gestation, resulting in greater ($P < 0.001$) concentrations of serum GH by day 5 of treatment (Fig. 2), which remained approximately tenfold higher than the controls throughout the treatment period. The exogenous rbGH was biologically active in sheep as the treated ewes exhibited increases ($P < 0.001$) in serum IGF-I concentrations, as evidenced in Fig. 2.

The physical data, for the ewes, fetuses and placenta collected at necropsy are summarized in Tables 2 and 3. For the 55 dGA groups, no differences were seen in uterine weight, placental weight, fetal weight, CRL, PI, liver weight, or liver weight expressed per kg of BW between GH and CON pregnancies. Similar results were obtained in the near term (135 dGA) ewes. No significant differences were seen between uterine, placental, calculated fluid, and fetal weights. Additionally, no significant differences were found in CRL, PI, liver weight, or liver weight/kg BW between treatments in the 135 dGA groups. There was no effect of fetal sex on the fetal parameters measured at days 55 and 135.

Effect of rbGH administration on placental gene expression

To assess placental tissue response to exogenous GH, cotyledon and caruncle tissues were collected from each 55 dGA pregnancy, and northern hybridization analysis was used to detect changes in cotyledon and caruncle concentrations of oPL and oGH mRNA (Fig. 3). Robust signal was obtained

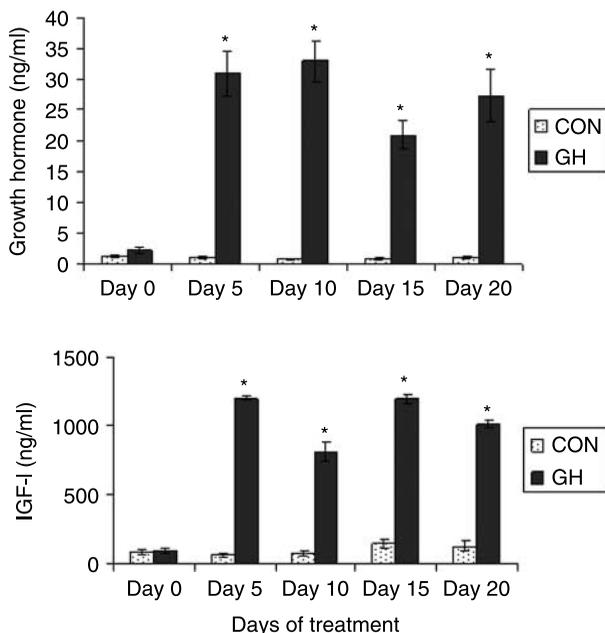


Figure 2 Serum GH and IGF-I concentrations. Concentrations for control (CON) and GH-treated (GH) ewes throughout the gestation treatment period (days 35–55) are presented as mean \pm S.E.M. with significant differences marked with an asterisk.

Table 2 Day 55 maternal and fetal physical data. Data for control (CON) and GH treated (GH) ewes are presented as mean \pm S.E.M.

	CON	GH	P value
Treatment			
Pregnancies (n)	7	5	
Days post coitus	55.14 \pm 0.26	55 \pm 0.32	0.734
Ewe weight (kg)	85.29 \pm 2.31	82.82 \pm 5.38	0.610
Gravid uterine weight (g)	1773.57 \pm 73.72	1934 \pm 59.32	0.336
Placental weight (g)	693.26 \pm 59.58	709.06 \pm 90.75	0.882
Fetal weight (g)	32.23 \pm 1.18	32.03 \pm 1.28	0.910
Crown rump length (cm)	8.26 \pm 0.16	8.48 \pm 0.15	0.346
Ponderal index (g/cm ³)	0.057 \pm 0.002	0.053 \pm 0.001	0.075
Liver weight (g)	2.64 \pm 0.15	2.48 \pm 0.11	0.426
Liver weight/kg BW	81.62 \pm 2.09	77.56 \pm 1.98	0.190

for oGH in maternal caruncles, and for oPL in the fetal cotyledons, further supporting that oGH mRNA resides in the maternal placental component. However, there was no effect of GH treatment on cotyledon oPL mRNA concentrations or maternal caruncle oGH mRNA concentrations (Fig. 3).

Cotyledon mRNA concentration for IGF-I, IGF-II, or IGFBP-3 was not impacted by GH treatment (Fig. 4). However, the concentration of mRNA encoding IGFBP-1 and -4 was increased ($P < 0.05$), while IGFBP-2 mRNA concentration was decreased ($P < 0.05$) in the GH-treated fetal placenta. Figure 5 presents the comparison of relative expression of the transcripts within the caruncle. No significant differences were detected between treatments for caruncle IGF-I, IGF-II, and IGFBP-2 mRNA concentrations. The concentration of caruncular IGFBP-1 mRNA

Table 3 Day 135 maternal and fetal physical data. Data for control (CON) and GH treated (GH) ewes are presented as mean \pm S.E.M.

	CON	GH	P value
Treatment			
Pregnancies (n)	6	5	
Days post coitus	136.00 \pm 0.97	136.20 \pm 0.80	0.880
Ewe weight (kg)	91.29 \pm 4.78	97.27 \pm 5.36	0.425
Gravid uterine weight (kg)	13.80 \pm 0.81	14.06 \pm 0.66	0.816
Placental weight (kg)	1.45 \pm 0.09	1.50 \pm 0.11	0.748
Fluid weight (kg)	2.26 \pm 0.39	2.21 \pm 0.21	0.915
Fetal weight (kg)	4.45 \pm 0.17	4.54 \pm 0.13	0.655
Crown rump length (cm)	50.36 \pm 0.79	51.70 \pm 0.82	0.332
Ponderal index (g/cm ³)	0.035 \pm 0.001	0.033 \pm 0.001	0.325
Liver weight (g)	117.43 \pm 6.36	125.20 \pm 8.39	0.462
Liver weight/kg BW	26.33 \pm 0.77	27.40 \pm 1.31	0.473

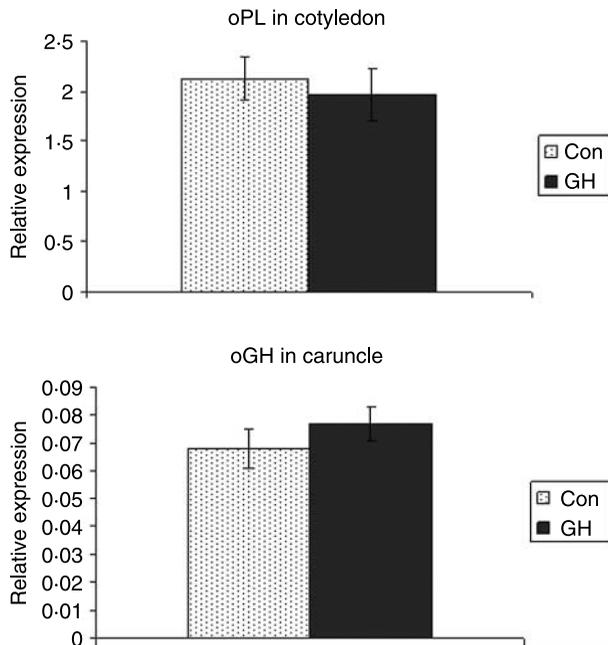


Figure 3 Relative expression of oPL in the fetal cotyledon and oGH in the maternal caruncle at 55 dGA. Densitometric values for the samples were normalized by densitometric values for RPS 15. Ten micrograms tcRNA were loaded for northern hybridization.

was significantly ($P < 0.05$) decreased, while the concentrations of IGFBP-3 and -4 mRNA were both significantly ($P < 0.05$) increased by GH treatment.

Discussion

GH has been previously detected in the ovine placenta from days 35 to 55 of gestation; however, the cellular source of GH described (Lacroix *et al.* 1996, 1999) did not coincide with our preliminary studies (TRH Regnault & RV Anthony unpublished observations). Therefore, the first objective of this

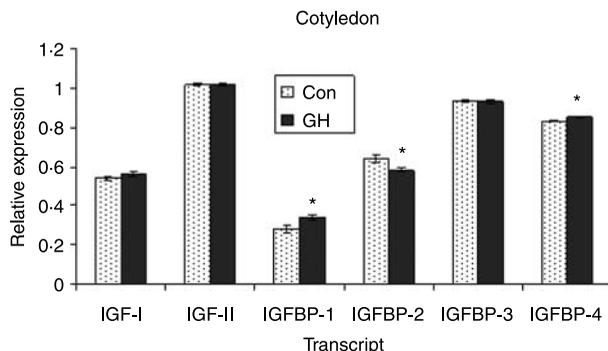


Figure 4 Relative expression of IGF-I, IGF-I, IGFBP-1, -2, -3, and -4 mRNA in cotyledon tissue. All transcripts were corrected (C_i/C_j) by RPS 15 mRNA. Data are expressed as mean \pm S.E.M. and significant differences ($P < 0.05$) are indicated by an asterisk.

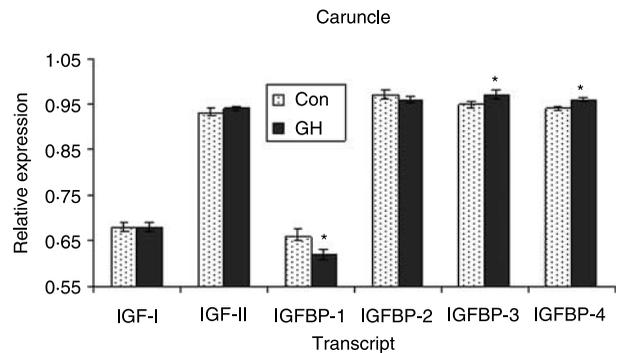


Figure 5 Relative expression of IGF-I, IGF-II, IGFBP-1, -2, -3, and -4 mRNA in caruncle tissue. All transcripts were corrected (C_i/C_j) with RPS 15 mRNA. Data are expressed as mean \pm S.E.M. and significant differences ($P < 0.05$) are indicated by an asterisk.

research was to definitively localize the expression of oGH in day 45 placentome tissue. The results of our *in situ* hybridization contrast with those reported by Lacroix *et al.* who reportedly detected oGH in maternal syncytium and stromal cells, and fetal trophoblast. The use of *in situ* hybridization and visualization at high magnification reported here specifically detected hybridization of oGH at the maternal–fetal interface (Fig. 1A and B). These cells were not chorionic BNC derived, as marked by oPL (Fig. 1C and D). The cells that hybridized to the oGH cDNA also showed positive hybridization for cytokeratin 18 (Fig. 1E and F) a marker of epithelial cells and not vimentin, a marker of fibroblast-derived cells. Additionally, the oGH-positive cells were negative for CD 18 hybridization precluding them from being leukocyte in origin (data not shown).

The results of the *in situ* hybridization analysis indicate that oGH is expressed in maternal uterine epithelium and is not detected in maternal villous stroma nor fetal trophoblast or villous stroma. Our detection of oGH in day 45 placental tissue coincides with the reported maximal expression of oGH (Lacroix *et al.* 1999). The pattern of oGH expression beginning at day 35, peaking at day 45, and decreasing to day 55 reported by Lacroix *et al.* is further supported by a much less robust detection of oGH in day 55 placentomes (TRH Regnault & RV Anthony unpublished observations). While we do not understand the initiation of oGH in the placenta at ~ 35 dGA, the mechanism by which oGH expression is terminated may be the loss of the cells producing oGH. The loss of maternal epithelium within the caruncle due to syncytium formation is well documented, and by 55–60 dGA the epithelium is not detectable (Davies & Wimsatt 1966, Stegeman 1974). Therefore, the cessation of oGH expression is likely not a change in transcriptional regulation, but the loss of oGH producing cells.

Maternal GH infusion late in gestation (days 98–111) has been shown to increase fetal growth in singleton pregnancies (Jenkinson *et al.* 1999); however, these results are not consistent within the same breed receiving the same oGH treatment (Currie *et al.* 1996). Wallace *et al.* initiated GH administration to adolescent ewes at the appropriate time (day

35); however, the GH treatment continued to day 80 (Wallace *et al.* 2004), well past the reported cessation of oGH expression in the placenta (Lacroix *et al.* 1996, 1999). Although Wallace *et al.* reported an increase in placental weight in the over-nourished adolescent ewes with singletons; these ewes are predisposed to placental and fetal growth restriction due to the high plane of nutrition. The over-nourished adolescent ewes exhibit an interesting change in GH/IGF-I endocrinology. These ewes have decreased maternal serum GH while at the same time have increased serum IGF-I (Wallace *et al.* 1997). This indicates a disconnection of the normal positive endocrinology of GH and IGF-I. Therefore, caution should be used when interpreting the results of maternal oGH infusion in this model. Maternal infusion of GH maternally may have resulted in increases in placental weight; however, this may represent a resetting of the normal GH/IGF-I system thus providing a repartitioning of nutrients toward the pregnancy to normalize placental and fetal growth.

In our research, maternal GH treatment increased ($P < 0.001$) maternal serum concentrations of GH and IGF-I tenfold higher than CON ewes, by the fifth day of treatment, and maintained the increase throughout treatment. The peak GH concentration was similar to that reported by Jenkinson *et al.* (1999) while the peak IGF-I concentration was similar to Stelwagen *et al.* (1994) and Jenkinson *et al.* (1999). Although the ewes responded to GH treatment with increased maternal serum IGF-I, exogenous GH treatment had no effect on uterine, placental, and fetal weight or fetal organ weights measured at either day 55 or 135 of gestation. While the model systems are different, our results none the less agree with the recent reports of GH administration during early gestation (Wallace *et al.* 2006). The study by Wallace *et al.* (2006) did not administer GH to the moderate intake control fed adolescent ewes, and the GH-treated high intake adolescent ewes carrying singletons, confounding the comparison between their study and ours. However, taken together, these results support the conclusion that elevated maternal serum GH and IGF-I concentrations during the normal expression window of oGH have little impact on placental and fetal growth. We specifically chose twin pregnancies to evaluate, as the multiple fetuses put increased demand on the maternal system and placenta to provide sufficient nutrition to the fetus. The only report of GH administration to ewes carrying twins was that of Jenkinson *et al.* (1999), in which ewes were treated from 98 to 111 dGA. A significant increase in fetal weight was reported (Jenkinson *et al.* 1999), but GH treatment had similar effects on the growth of both singles and twins. The response seen by Jenkinson *et al.* may well be due to partitioning of maternal nutrients toward the pregnancy late in gestation versus an alteration of placental development.

In an effort to gain insight into the potential effects of GH on gene expression within the placenta, we used northern hybridization and qPCR to quantify GH, oPL, IGF-I, IGF-II, and IGFBP-1, -2, -3, and -4 mRNA concentrations in day 55 caruncle and cotyledon tissue. Maternal GH

treatment had no effect on GH expression in the caruncle tissue, nor did GH impact oPL expression in cotyledon tissue. In placental growth, IGF-II plays a crucial role as evidenced by growth restriction in knockout and targeted disruption studies (DeChiara *et al.* 1990, Constancia *et al.* 2002). Had the exogenous GH significantly changed the IGF-II levels in the placenta, it would be expected that a change in placental growth would be evident. The role of IGF-I may be as a less potent stimulator of proliferation that has stimulatory effects on trophoblast differentiation and migration (Maruo *et al.* 1995, Lacey *et al.* 2002). With no gross changes in placental weight and fetal weight evident at necropsy, the lack of effect on IGF mRNA concentrations is not surprising.

By contrast, within the cotyledon, maternal GH treatment increased expression of IGFBP-1 and -4, while IGFBP-2 expression was decreased and IGFBP-3 was unchanged. Within the caruncle exogenous GH increased expression of IGFBP-3 and -4, while decreasing the expression of IGFBP-1. In the caruncle, IGFBP-2 was unaffected by maternal GH treatment. Previously, it was believed that IGFBP-1 was not expressed beyond 21 dGA (Wathes *et al.* 1998, de Vrijer *et al.* 2006); however, here we could detect changes in IGFBP-1 by treatment at 55 dGA. We do not discount the previous results, as our detection of IGFBP-1 is most likely due to the increased sensitivity of qPCR versus hybridization technologies in detecting transcripts. The decrease in caruncle expression of IGFBP-1 can be attributed to two possible mechanisms. First, IGFBP-1 is inversely regulated by insulin (Suikkari *et al.* 1989), and it has been shown previously that GH administration during gestation leads to hyperinsulinemia (Wallace *et al.* 2004). Given that we achieved significant stimulation of IGF-I similar to that reported by Wallace *et al.* one could reason that we generated a similar insulin response that reduced caruncle expression of IGFBP-1. Second, it has been shown that IGF-II can regulate IGFBP-1 expression at the maternal interface to control trophoblast invasion (Hamilton *et al.* 1998, Irwin *et al.* 2001). Although we did not measure maternal serum IGF-II concentrations, an increase of serum IGF-II due to GH treatment could also reduce the IGFBP-1 expression in the caruncle. The increase in caruncular IGFBP-3 agrees with reports that both GH and IGF-I are stimulatory to IGFBP-3 expression as reviewed by Clemmons (1997). The increased expression of IGFBP-4 is not surprising, as it has been reported to be a negative regulator of IGF-I action in other tissues (Clemmons 1997). Within the caruncle, IGFBP-4 may be upregulated to mitigate the effects of increased serum IGF-I.

Within the cotyledon, it is more difficult to interpret the changes in IGFBP-1, -2, and -4. Given the low amount of blood in a day 55 fetus, it is not feasible to obtain serum analysis of GH, IGF-I, IGF-II, and insulin in fetal circulation. These hormones, as discussed above, regulate the expression of IGFBPs and thus determination of their concentration would be necessary to give accurate interpretation of the transcriptional changes within the placenta. This is the first evidence of the transcriptional changes caused by GH administration during the normal placental oGH

expression window within the placenta. While we did not alter the local expression of oGH in the placenta, this may be indicative of alternative regulation of oGH in the placenta versus the pituitary. Our evidence suggests that increased serum GH does not cause the normal negative feedback on GH expression in the placenta that is seen in the pituitary. Cloning and analysis of the complete promoter sequence for oGH-1 and oGH-2 may lead to discovery of differential regulation of the promoters. Heterologous studies with the human GH genes revealed that hGH-V lacked a proximal pituitary-specific protein (Pit-1) site that is one of two present in the hGH-N promoter (Nickel *et al.* 1991). Furthermore, we could not detect expression of Pit-1 in maternal- or fetal-derived placental tissues (data not presented). This indicates a tissue-specific transcriptional regulation for human GH in the placenta when compared with the pituitary. With the lack of placental response to maternal GH treatment, further research to elucidate the role of placental oGH will likely necessitate a knockdown of oGH expression to gain insight into oGH function within the placenta.

Declaration of Interest

The authors declare that there is no conflict of interest that would prejudice the impartiality of this scientific work.

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