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Chorionic somatomammotropin impacts early fetal growth and placental gene expression

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Abstract

Several developmental windows, including placentation, must be negotiated to establish and maintain pregnancy. Impaired placental function can lead to preeclampsia and/or intrauterine growth restriction (IUGR), resulting in increased infant mortality and morbidity. It has been hypothesized that chorionic somatomammotropin (CSH) plays a significant role in fetal development, potentially by modifying maternal and fetal metabolism. Recently, using lentiviral-mediated *in vivo* RNA interference in sheep, we demonstrated significant reductions in near-term (135 days of gestation; dGA) fetal and placental size, and altered fetal liver gene expression, resulting from CSH deficiency. We sought to examine the impact of CSH deficiency on fetal and placental size earlier in gestation (50 dGA), and to examine placental gene expression at 50 and 135 dGA. At 50 dGA, CSH-deficient pregnancies exhibited a 41% reduction ($P \leq 0.05$) in uterine vein concentrations of CSH, and significant ($P \leq 0.05$) reductions ($\approx 21\%$) in both fetal body and liver weights. Placentae harvested at 50 and 135 dGA exhibited reductions in *IGF1* and *IGF2* mRNA concentrations, along with reductions in *SLC2A1* and *SLC2A3* mRNA. By contrast, mRNA concentrations for various members of the System A, System L and System y^+ amino acid transporter families were not significantly impacted. The IUGR observed at the end of the first-third of gestation indicates that the near-term IUGR reported previously, began early in gestation, and may have in part resulted from deficits in the paracrine action of CSH within the placenta. These results provide further compelling evidence for the importance of CSH in the progression and outcome of pregnancy.

Key Words

- ▶ chorionic somatomammotropin
- ▶ placental lactogen
- ▶ sheep
- ▶ placenta
- ▶ lentivirus
- ▶ RNA interference
- ▶ *SLC2A1*
- ▶ *SLC2A3*

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Introduction

Various complications during pregnancy can impact the health and survival of a fetus, among the most significant of these being intrauterine growth restriction (IUGR). IUGR affects upwards to 8% of human pregnancies, and occurs when a fetus fails to grow to its full potential (Gagnon 2003). IUGR is a leading cause of perinatal mortality and is associated with an increased risk of adult-onset disease such as hypertension, heart disease, diabetes and stroke (Barker & Osmond 1986, Barker *et al.* 1989, 1990, 1993a,b,

Gagnon 2003). Functional placental insufficiency accounts for at least half of IUGR cases when the fetus is normally formed (Ghidini 1996). As the placenta mediates the exchange of nutrients and oxygen from the mother to the fetus (Barry & Anthony 2008), placental insufficiency results in relative fetal undernutrition, impairing normal growth and development. Furthermore, the placenta is itself a highly metabolic organ, metabolizing a majority of the glucose and oxygen delivered to it (Meschia *et al.* 1980,

Bell *et al.* 1986), as well as producing an array of hormones and growth factors that impact maternal and fetal metabolism and promote fetal growth and development. Of these, chorionic somatomammotropin (CSH, a.k.a. placental lactogen) has long been hypothesized to impact metabolism in both the mother and fetus (Handwerger 1991, Handwerger & Freemark 2000).

CSH is a member of the growth hormone/prolactin gene family that is synthesized and secreted by syncytiotrophoblasts in the human placenta and binucleate cells in the sheep placenta (Gootwine 2004). Both human and sheep IUGR pregnancies are associated with reduced concentrations of CSH in maternal circulation (Spellacy 1976, Lea *et al.* 2008), and CSH is among the most abundantly produced placental proteins, continuously secreted into maternal and fetal circulation throughout pregnancy (Walker *et al.* 1991, Wooding & Burton 2008). However, until recently (Baker *et al.* 2016), there was no direct evidence as to whether CSH deficiency had a causative, correlative or dependent relationship with IUGR. Baker *et al.* (2016) reported the generation of CSH-deficient sheep pregnancies, generated by lentiviral-mediated RNA interference *in vivo*, which resulted in significant IUGR near-term.

At 135 days of gestation (dGA), CSH-deficient pregnancies (Baker *et al.* 2016) exhibited 52% and 32% reductions in placental and fetal weights, respectively, as a result of 50% and 38% reductions in CSH mRNA and protein. The observed growth restriction was associated with significant reductions in umbilical concentrations of insulin and insulin-like growth factor 1 (IGF1), as well as fetal hepatic concentrations of *IGF1*, *IGF2*, *IGFBP2* and *IGFBP3* mRNA, supporting hypotheses about CSH actions within the fetus (Handwerger 1991, Handwerger & Freemark 2000). However, maternal concentrations of insulin and IGF1, as well as maternal hepatic concentrations of *IGF1*, *IGF2* and *IGFBP* mRNA, were not impacted by CSH deficiency, contrary to what had been previously hypothesized about the actions of CSH on maternal physiology (Handwerger 1991, Handwerger & Freemark 2000). To determine whether CSH deficiency impacted placental and fetal growth prior to late gestation, we generated additional pregnancies, which were harvested at the end of the first-third of gestation (50 dGA), and utilized the resulting tissue, as well as tissue harvested at 135 dGA (Baker *et al.* 2016), to investigate the impact of CSH deficiency on placental expression of the *IGFs*, glucose and amino acid transporters. It was our hypothesis that CSH deficiency impacts placental growth during early- to mid-gestation, setting the stage for fetal growth restriction during late gestation.

Materials and methods

All procedures conducted with animals and lentivirus were approved by the Colorado State University Institutional Animal Care and Use Committee (Protocol 14-5257A) and the Institutional Biosafety Committee (Protocols 11-034B and 13-043B), respectively.

Lentivirus generation

Generation of hLL3.7 tg6, which expresses an shRNA targeting CSH mRNA, was previously described (Baker *et al.* 2016). The scrambled control sequence used by Baker *et al.* (2016) was cloned into LL3.7, using the same procedures as described for hLL3.7 tg6, thereby generating hLL3.7 NTS (non-targeting sequence/scrambled control). The shRNA sequences for both hLL3.7 tg6 and hLL3.7 NTS are presented in Table 1. Generation and titering of virus harboring the hLL3.7 tg6 and hLL3.7 NTS constructs followed the procedures extensively described by Baker *et al.* (2016).

Blastocyst collection and transfer

Individual hatched and fully expanded blastocysts, harvested from donor ewes 9 days after breeding, were infected with 100,000 transducing units of either hLL3.7 NTS or hLL3.7 tg6, as previously described (Baker *et al.* 2016). Following ~5 h of incubation with the virus, the blastocysts were thoroughly washed in HCDM-2 media (Baker *et al.* 2016) and a single blastocyst was surgically transferred to each synchronized recipient ewe. Recipient ewes were monitored for return to standing estrus daily. The recipient ewes were group housed and fed to meet, or slightly exceed, their gestational nutrient requirements.

Tissue collection

At 50 dGA, 8 hLL3.7 NTS pregnancies (control) and 6 hLL3.7 tg6 pregnancies (CSH-deficient) were harvested at terminal surgery as previously described (Baker *et al.* 2016). Uterine vein blood was harvested prior to euthanization of the ewe and fetus (90 mg/kg sodium pentobarbital; intravenous infusion of the uterine vein and umbilical vein), and the resulting serum was stored at -80°C , before being assayed for CSH concentrations (Kappes *et al.* 1992, Lea *et al.* 2008, Baker *et al.* 2016). Fetal body weight (FBW) and crown-rump length (CRL) were recorded for each fetus. Fetal livers were harvested, recorded for weight and stored in 50-mL conical tubes snap-frozen

Table 1 Scrambled control and CSH-targeting shRNA sequences.

Oligonucleotide	Sequence (5'-3')
NTS shRNA sense	GAGTTAAAGGTTTCGGCACGAATTCAAGAGATTCGTGCCGAACCTTTAACTC
tg6 shRNA sense	AAGGCCAAAGTACTTGTAGACTTCAAGAGAGTCTACAAGTACTTTGGCCTT

in liquid nitrogen. After hysterectomies were performed, all placentomes (combination of maternal caruncle and fetal cotyledon) were excised, washed in PBS and recorded for placental weight (PW) and total placentome number. The fetal cotyledons were separated from the maternal caruncles, and the fetal cotyledons were snap-frozen in liquid nitrogen, before being pulverized using a mortar and pestle. Pulverized tissue was kept frozen at -80°C for later use. Harvest of placental tissue (fetal cotyledon) from control and CSH-deficient pregnancies at 135 dGA, as well as the characteristics of those pregnancies, has been previously reported (Baker et al. 2016).

RNA isolation

Total cellular RNA was isolated from 50 dGA fetal placenta and liver, as well as from 135 dGA fetal placenta (Baker et al. 2016) using the RNeasy Mini Kit (Qiagen) according to the manufacturer's protocol. The BioTek Synergy 2 Microplate Reader (BioTek) was used to quantify RNA concentration, and measure RNA purity using the 260/280 absorbance ratio. Samples were stored at -80°C until use.

cDNA synthesis and quantitative real-time PCR

cDNA was generated from 1 μg of total cellular RNA using iScript Reverse Transcription Supermix (Bio-Rad) according to the manufacturer's protocol. All cDNA samples were treated with 5 units of RNase H (Thermo Fisher Scientific) at 37°C for 20 min. To control for variance in efficiency of the reverse transcription reaction, cDNA was quantified using the Quant-iT OliGreen ssDNA Assay Kit (Invitrogen) according to the manufacturer's protocol. An equal mass of cDNA (20 ng) was used for each sample in the quantitative real-time PCR (qPCR) reaction.

qPCR was performed using the LightCycler 480 (Roche Applied Science) and protocol previously described (Baker et al. 2016). Forward and reverse primers for qPCR were designed using Oligo software (Molecular Biology Insights, Cascade, CO, USA) to amplify an intron-spanning product. Primer sequences and amplicon sizes are summarized in Table 2. A PCR product for each gene was generated using cDNA from 135 dGA fetal placenta as a template and cloned into the StrataClone vector (Agilent Technologies). Amplification of the correct cDNA

was verified by sequencing each PCR product (Colorado State University Proteomics and Metabolomics Facility). Standard curves were generated for each mRNA from 1×10^2 to 1×10^{-5} pg using the PCR products amplified from the sequenced plasmids and were used to measure amplification efficiency. Starting quantities (pg) were normalized by dividing the starting quantity of the mRNA of interest by the starting mRNA quantity (pg) of ovine ribosomal S15 (*RPS15*). All primers were annealed at 60°C . The starting quantity (pg) of *RPS15* mRNA was not impacted ($P \geq 0.25$) by CSH deficiency in either 50 or 135 dGA tissues.

Western immunoblotting

Cellular protein from 50 and 135 dGA fetal placenta (cotyledon) tissue was assessed using Western immunoblot analysis. To isolate total cellular protein, cotyledon tissue (100 mg) was lysed in 500 μL of Western lysis buffer (0.48 M Tris, pH 7.4; 10 mM EGTA, pH 8.6; 10 mM EDTA, pH 8; 0.1 mM PMSF; 0.1 mM AEBSF; 0.0015 mM pepstatin A; 0.0014 mM E-64; 0.004 mM bestatin; 0.002 mM leupeptin; and 0.00008 mM aprotinin) and sonicated on ice. 10–50 μg of protein from each sample was electrophoresed through a 4–12% Bis-Tris gel (Life Technologies) and transferred to a 0.45- μm pore nitrocellulose membrane. To visualize CSH (1:25,000 dilution, α -oPL-S4; Kappes et al. 1992), SLC2A1 (1:2000 dilution, product no. 07-1401; EMD Millipore) and SLC2A3 (1:500 dilution, product no. ab125465; Abcam), an anti-rabbit horseradish peroxidase-conjugated IgG (1:5000 dilution, product no. sc-2004; Santa Cruz Biotechnology) was used. As a loading control and housekeeping protein to normalize CSH, SLC2A1 and SLC2A3, a polyclonal antibody to β -actin (ACTB, 1:2500 dilution, product no. sc-47778; Santa Cruz Biotechnology) bound by a horseradish peroxidase-conjugated secondary antibody (1:5000, product no. sc-2005; Santa Cruz Biotechnology) was used. Membranes were developed using an ECL Western Blotting Detection Reagent chemiluminescent kit (Amersham) and imaged using the ChemiDoc XRS+ chemiluminescence system (Bio-Rad). Densitometry calculations were performed using Image Lab Software (Bio-Rad). Technical error between membranes was corrected by including a common sample in each Western

Table 2 Primers and product sizes for cDNA used in qPCR.

cDNA	Forward primer	Reverse primer	Product (bp)
IGF1	TCG CAT CTC TTC TAT CTG GCC CTG T	ACA GTA CAT CTC CAG CCT CCT CAG A	240
IGF2	GAC CGC GGC TTC TAC TTC AG	AAG AAC TTG CCC ACG GGG TAT	203
IGFBP1	TGA TGA CCG AGT CCA GTG AG	GTC CAG CGA AGT CTC ACA C	248
IGFBP2	CAA TGG CGA GGA GCA CTC TG	TGG GGA TGT GTA GGG AAT AG	330
IGFBP3	CTC AGA GCA CAG ACA CCC A	GGC ATA TTT GAG CTC CAC	336
RPS15	ATC ATT CTG CCC GAG ATG GTG	TGC TTG ACG GGC TTG TAG GTG	134
SLC2A1	GAC AGG GAG GAG CAA GCC AAA	TAG GGT GAA GCC AGG GAT GTG	380
SLC2A3	CTC TAC TGC TGG GCT TCA CC	ACT TGC TTC TCC TGC GAC AT	211
SLC38A1	CCC GAA GAC GAT AAC ATT AGC AA	CTC CCA GCT TTT CAT AAA CCA	382
SLC38A2	CTT GCT GCC CTC TTT GGA T	CAA CAC AGC CAA ACG GAC AA	120
SLC38A4	CTA TTC GCA CAT CAG TGA C	TGA AAA TGA TGC CAA CCA C	279
SLC7A5	GCT CGG CTT CAT CCA GAT C	AGG CAA AGA GGC CGC TGT A	122
SLC7A8	TAT TGC TCC TCA CAT GGG TCA	GAC AAG CTC CTC AGT CAC GTA	272
SLC7A1	CTT ACG GTA TCA GCC CGA GCA	GAA AGA CTG CTG CCC ACA ACT CCC	320

immunoblot. Densitometry measurements were adjusted based on the average densitometry measurement of the common sample and normalized to ACTB.

Statistical analyses

All data were subjected to analysis of variance using SAS software (SAS Institute, Cary, NC, USA) and the PROC Mixed procedure, with treatment and fetal sex as dependent variables and the treatment $\times$ fetal sex interaction. There was not a significant fetal sex effect (50 dGA), or a significant treatment by fetal sex interaction; subsequently data from control and CSH-deficient pregnancies were compared by Student's *t*-test within a gestational age. Statistical significance was set at $P \leq 0.05$, and statistical tendency was set at $P \leq 0.10$. Data are reported as the mean $\pm$ S.E.M.

Results

Day 50 fetal and placental measurements

Following viral infection and single blastocyst transfer, 8 control (50% males) and 6 CSH-deficient (33% males) pregnancies were harvested at 50 dGA. FBW, CRL, fetal liver weight (FLW) and PW are presented in Table 3. As evidenced in Table 3, both FBW and FLW

were significantly ($P \leq 0.01$) reduced in the 50 dGA CSH-deficient pregnancies, whereas CRL and PW only tended ($P \leq 0.10$) to be impacted. Placental efficiency (FBW/PW) was not impacted ($P \geq 0.10$) by CSH deficiency (Table 3). Uterine vein CSH concentrations were reduced ($P \leq 0.05$) $\approx 41\%$ in CSH-deficient pregnancies (Fig. 1), as compared to control pregnancies. CSH mRNA and protein concentrations within the placenta were both reduced $\approx 21\%$ in CSH-deficient pregnancies, but these differences were not statistically significant (Fig. 1).

Insulin-like growth factor and insulin-like growth factor binding proteins

At 50 dGA, there were no statistical differences in mRNA concentrations for *IGF1*, *IGF2*, or any of the three *IGFBPs* (Table 4) within the fetal liver. Within the placenta, *IGF1* mRNA concentrations were reduced at 50 ($P = 0.12$) and 135 ($P \leq 0.05$) dGA (Table 5), whereas *IGF2* mRNA concentration was reduced ($P \leq 0.05$) only at 135 dGA. Placental expression of *IGFBP1* was undetectable at 50 dGA (Table 5), whereas at 135 dGA, there was a tendency ($P = 0.11$) for increased *IGFBP1* mRNA concentration in CSH-deficient pregnancies relative to control pregnancies. By contrast, *IGFBP2* mRNA concentration was reduced ($P \leq 0.05$) at 50 dGA in CSH-deficient pregnancies, but there was no treatment effect at 135 dGA (Table 5).

Table 3 Fetal and placental measurements for 50 dGA pregnancies.

50 dGA	Controls	CSH-deficient	P value	% difference
Fetal weight (g)	17.17 $\pm$ 0.39	13.65 $\pm$ 0.61	0.01	20.5
CRL (cm)	9.5 $\pm$ 0.11	8.5 $\pm$ 0.20	0.10	10.4
Fetal liver (g)	1.32 $\pm$ 0.06	1.04 $\pm$ 0.08	0.01	20.9
Placenta weight (g)	105.5 $\pm$ 4.42	88.1 $\pm$ 10.31	0.11	16.5
Placental efficiency (g/g)	0.165 $\pm$ 0.008	0.165 $\pm$ 0.02	0.98	0.32

Figure 1

(A) Uterine vein CSH concentration at 50 dGA. (B) Placental CSH mRNA concentrations at 50 dGA. (C) Placental CSH concentrations at 50 dGA. * $P \leq 0.05$ when CSH-deficient pregnancies are compared with controls.

Placental *IGFBP3* mRNA concentration was not statistically impacted by treatment at either 50 or 135 dGA.

Nutrient transporters

As evidenced in Fig. 2, *SLC2A1* mRNA concentration was reduced ($P \leq 0.05$) in CSH-deficient placenta at 50 and 135 dGA, relative to controls. However, placental *SLC2A1* concentration (Fig. 2) was not statistically reduced at either 50 or 135 dGA. By contrast *SLC2A3* mRNA (Fig. 3) was reduced only by 24% ($P \geq 0.10$) at 50 dGA, but was significantly less (49%; $P \leq 0.05$) in CSH-deficient placenta at 135 dGA. Placental *SLC2A3* concentration (Fig. 3)

mirrored the changes in mRNA concentrations, with a nonsignificant ($P \geq 0.10$) 17% reduction at 50 dGA, and a significant 32% reduction ($P \leq 0.05$) at 135 dGA in CSH-deficient pregnancies. There was no effect of CSH deficiency at 50 dGA on *SLC2A8* mRNA concentration (Fig. 4), but *SLC2A8* mRNA was increased ($P \leq 0.05$) in CSH-deficient placenta at 135 dGA (Fig. 4). We were unable to identify an antiserum that allowed the specific assessment of sheep *SLC2A8* by Western immunoblot analysis.

The mRNA concentration of System A (*SLC38A1*, *SLC38A2* and *SLC38A4*), System L (*SLC7A5* and *SLC7A8*) and System y⁺ (*SLC7A1*) amino acid transporter families was assessed in our control and CSH-deficient pregnancies harvested at 50 and 135 dGA. As evidenced in Fig. 5, there were no significant changes in the mRNA concentration of any of these amino acid transporters at 50 dGA, as a result of CSH deficiency. At 135 dGA (Fig. 5), the only transporter that appeared to be impacted by CSH deficiency was *SLC38A4* (SNAT4), which tended ($P \leq 0.10$) to be reduced.

Discussion

Using lentiviral-mediated RNA interference, we recently reported the generation of CSH-deficient pregnancies in sheep (Baker et al. 2016). Near-term (135 dGA), CSH-deficient pregnancies (Baker et al. 2016) were characterized by significant fetal (32% reduction) and placental (52% reduction) growth restriction, associated with reductions in placental CSH mRNA (50%) and protein (38%). These results provided support for the long-held hypothesis that CSH promotes fetal growth (Handwerger 1991, Handwerger & Freemark 2000). The purpose of the current study was to determine whether CSH deficiency manifested changes in placental function and fetal growth at the end of the first-third of gestation (50 dGA), a time point when the placenta is fully established and continuing to grow.

At the end of the first-third of gestation, we observed significant ($P \leq 0.01$) reductions in fetal weight and FLWs, suggesting that the growth restriction observed near-term

Table 4 50 dGA fetal liver insulin-like growth factor mRNA concentrations.

	Controls	oCSH-deficient	P value	% difference
IGF1 (pg/pg)	0.0015±0.00015	0.0013±0.00013	0.24	17.0
IGF2 (pg/pg)	1.05±0.12	0.74±0.13	0.14	29.3
IGFBP1 (pg/pg)	0.68±0.12	0.73±0.09	0.77	6.9
IGFBP2 (pg/pg)	0.052±0.010	0.043±0.011	0.53	18.1
IGFBP3 (pg/pg)	0.30±0.05	0.28±0.05	0.85	4.7

Table 5 50 and 135 dGA placenta insulin-like growth factor mRNA concentrations.

	Controls	CSH-deficient	P value	% difference
50 (dGA)				
IGF1 (pg/pg)	0.000081±0.000028	0.000024±0.000008	0.12	69.6
IGF2 (pg/pg)	0.082±0.012	0.064±0.009	0.28	21.4
IGFBP1 (pg/pg)	0	0		
IGFBP2 (pg/pg)	0.0022±0.00060	0.0006±0.00025	0.04	73.1
IGFBP3 (pg/pg)	0.026±0.006	0.019±0.004	0.41	27.3
135 (dGA)				
IGF1 (pg/pg)	0.003±0.0009	0.001±0.0002	0.03	65.7
IGF2 (pg/pg)	0.36±0.07	0.17±0.05	0.04	53.3
IGFBP1 (pg/pg)	0.00021±0.00007	0.00057±0.00019	0.11	62.5
IGFBP2 (pg/pg)	0.0024±0.0006	0.0029±0.0010	0.69	16.8
IGFBP3 (pg/pg)	0.047±0.008	0.045±0.007	0.88	3.4

(Baker *et al.* 2016) in CSH-deficient pregnancies started during early gestation. Although PW only tended to be smaller at 50 dGA (Table 3), the ratio of fetal weight to PW (i.e., placental efficiency) was equivalent between control and CSH-deficient pregnancies. This would suggest that placental size/function was the driving force behind the fetal growth restriction observed at 50 dGA.

In contrast to what we observed (Baker *et al.* 2016) near-term, there was a significant ($P \leq 0.05$) reduction in uterine vein concentrations of CSH. At 50 dGA, due to the size of the fetus and amount of fetal blood, we could not harvest sufficient umbilical blood to assess CSH concentrations, as we did at 135 dGA (Baker *et al.* 2016). As discussed in Baker *et al.* (2016), blood concentrations of CSH become quite variable toward the end of gestation (Taylor *et al.* 1980, Butler *et al.* 1987, Bauer *et al.* 1995), and surgery elevates both maternal and fetal concentrations of CSH for up to 5 days (Taylor *et al.* 1980). Similar studies of CSH variability over time, during early to mid-gestation, have not been reported. However, simply comparing the overall (both control and CSH-deficient) coefficients of variation for the uterine vein values obtained at 50 dGA ($\approx 16\%$) vs 135 dGA ($\approx 1858\%$) infers that CSH secretion into maternal circulation is not nearly as variable during early to mid-gestation as it is near-term. Maternal and fetal vascular cannulation and harvest of serial samples obtained under non-stressed/non-anesthetized conditions, along with blood flow measurements, will be necessary to accurately assess the impact of CSH RNA interference on maternal and fetal concentrations of CSH. To our surprise, within the CSH-deficient placenta at 50 dGA, we could not demonstrate a statistically significant change in CSH mRNA and protein, as we had at 135 dGA. Unfortunately, we cannot fully explain discrepancies between these two studies as a result of *in vivo* RNA interference on placental CSH mRNA and protein. This could result from day-to-day variability in shRNA expression and function, but to our

knowledge, there is no approach to directly address this potential variability *in vivo*.

In contrast to the robust reduction in fetal liver mRNA concentrations for *IGF1*, *IGF2*, *IGFBP2* and *IGFBP3* observed at 135 dGA (Baker *et al.* 2016), at 50 dGA, neither *IGF1* and *IGF2* mRNA concentration were significantly changed, nor were fetal liver mRNA concentrations for all three *IGFBPs* impacted by CSH deficiency. Due to the major impact of CSH deficiency on placental size observed

Figure 2 (A) Effect of CSH deficiency on *SLC2A1* mRNA concentrations in the placenta at 50 and 135 dGA. (B) Effect of CSH deficiency on *SLC2A1* concentrations in the placenta at 50 and 135 dGA. $*P \leq 0.05$ when CSH-deficient pregnancies are compared with controls, within a gestational age.

Figure 3

(A) Effect of CSH deficiency on *SLC2A3* mRNA concentrations in the placenta at 50 and 135 dGA. (B) Effect of CSH deficiency on *SLC2A3* concentrations in the placenta at 50 and 135 dGA. * $P \leq 0.05$ when CSH-deficient pregnancies are compared with controls, within a gestational age.

at 135 dGA (Baker *et al.* 2016), we reasoned that at least part of CSH's impact may result from paracrine actions within the placenta. Accordingly, we examined the mRNA concentrations of *IGF1*, *IGF2*, *IGFBP1*, *IGFBP2* and *IGFBP3* in the placenta harvested at both 50 and 135 dGA (Baker *et al.* 2016). At 50 dGA, placental *IGF1* tended (Table 5) to be reduced, and *IGFBP2* was significantly lower, whereas *IGF2*, *IGFBP1* and *IGFBP3* were not impacted. However, near-term (135 dGA) placental concentrations of *IGF1* and *IGF2* mRNA were both significantly reduced, as they were in the near-term fetal liver (Baker *et al.* 2016). In contrast to what was observed in the near-term fetal liver

Figure 4

Effect of CSH deficiency on *SLC2A8* mRNA concentrations in the placenta at 50 and 135 dGA. * $P \leq 0.05$ when CSH-deficient pregnancies are compared with controls, within a gestational age.

(Baker *et al.* 2016), neither *IGFBP2* nor *IGFBP3* mRNA concentration was impacted by placental CSH deficiency at 135 dGA. While it is difficult to draw firm conclusions from these samples, the disparity in expression of the various IGF axis components between the placenta and fetal liver, and between the two gestational ages examined, may result from both direct effects (i.e., directly within the placenta or fetus) and indirect effects (i.e., placental mediated effects on the fetus) of CSH.

Due to the impact of CSH deficiency on placental IGF expression, and evidence that IGF can impact the expression of placental nutrient transporters (Wali *et al.* 2012, Jones *et al.* 2013, 2014, Baumann *et al.* 2014), we turned our attention to the expression of glucose and amino acid transporters within CSH-deficient placentae. Fitting with the hypothesis that CSH drives placental *IGF1* expression and *IGF1* in turn enhances *SLC2A1* expression (Baumann *et al.* 2014), we observed diminished ($P \leq 0.05$) expression of *SLC2A1* mRNA at both 50 dGA and 135 dGA,

Figure 5

(A) Effect of CSH deficiency on amino acid transporter mRNA concentrations in the placenta at 50 dGA. (B) Effect of CSH deficiency on amino acid transporter mRNA concentrations in the placenta at 135 dGA. † $P \leq 0.10$ when CSH-deficient pregnancies are compared with controls, within a gestational age.

whereas *SLC2A3* mRNA was significantly reduced ($P \leq 0.05$) only at 135 dGA. In contrast to what was observed with the reductions in *SLC2A1* mRNA, Western blot analysis of fetal placental homogenates revealed no significant change in *SLC2A1* at either 50 or 135 dGA. Janzen *et al.* (2013) reported a significant reduction in *SLC2A1* mRNA within the basal plate region of human IUGR placenta, similar to what we observed in this study, with no change in the concentration of *SLC2A1* protein. It is possible that *SLC2A1* is so highly expressed in the placenta that there is a functional 'reserve' of *SLC2A1* mRNA, making *SLC2A1* less susceptible to 'outside' influences.

In contrast to *SLC2A1*, there was a similar magnitude of change at both 50 and 135 dGA for both *SLC2A3* mRNA and protein. Similar to the human, *SLC2A1* is much more abundant than *SLC2A3* in the sheep placenta, but in sheep, *SLC2A1* is located on the trophoblast basolateral surface, whereas *SLC2A3* is located solely on the maternal-facing apical microvillous surface (Wooding *et al.* 2005). While the expression of *SLC2A3* increases significantly during the latter half of gestation (Ehrhardt & Bell 1997), due to its location (Wooding *et al.* 2005), affinity and glucose transport capacity (Simpson *et al.* 2008), similar to the human (Brown *et al.* 2011), *SLC2A3* may play a significant role during the first-half of gestation as well, by mediating the uptake of glucose into the trophoblast. Due to the greater impact of CSH deficiency on *SLC2A3* protein, lack of *SLC2A3* may have been a driving force behind the growth restriction observed.

At 50 dGA, there was no difference in *SLC2A8* mRNA, but at 135 dGA, there was a $\approx 76\%$ increase ($P \leq 0.05$) in *SLC2A8* mRNA concentration. It has been reported that *SLC2A8* placental mRNA and protein expression is decreased in the hyperthermic-model of sheep IUGR (Limesand *et al.* 2004). However, Wooding *et al.* (2005) was unable to identify significant membrane localization of *SLC2A8* in any of the ruminant placenta examined. *SLC2A8* is a class III glucose transporter (*SLC2A1* and *SLC2A3* are class I transporters; Joost *et al.* 2002), which is primarily localized to endosomes, lysosomes and endoplasmic reticulum membranes (Schmidt *et al.* 2009), and is thought to catalyze hexose transport across intracellular membranes. *SLC2A8* might be up-regulated in response to deficiencies in *SLC2A1* or *SLC2A3*. If its role is as an intracellular transporter (Schmidt *et al.* 2009), its up-regulation may infer a mechanism by which the placenta preserves its metabolic function, which would fit with the reductions in mitochondrial membrane potential and ATP generation in sperm of *Slc2a8*^{-/-} mice (Gawlik *et al.* 2008). While our data on *SLC2A8* are intriguing, since there is no evidence

for its role in glucose transport to the fetus, its role in the resulting IUGR observed in CSH-deficient pregnancies at 50 and 135 dGA is not readily apparent.

In contrast to the glucose transporters, we did not observe significant changes in mRNA concentrations for *SLC38A1*, *SLC38A2*, *SLC38A4*, *SLC7A1*, *SLC7A5* or *SLC7A8*, except for a trend ($P \leq 0.10$) for reduced *SLC38A4* (SNAT4) at 135 dGA. Placental transfer of amino acids, especially essential amino acids, is reduced in human and sheep IUGR pregnancies (Marconi *et al.* 1999, de Vrijer *et al.* 2004, Regnault *et al.* 2005). However, amino acid transporter mRNA and protein concentration is not a perfect surrogate for transporter activity in IUGR pregnancies (Mando *et al.* 2013, Chen *et al.* 2015, Dunlap *et al.* 2015, Pantham *et al.* 2016). Thorough *in vivo* assessment of placental uptake, net utilization and transfer to the fetus is required before any changes in placental nutrient transporter mRNA or protein concentrations can be interpreted completely.

Collectively, our current results, combined with the work of Baker *et al.* (2016), provide compelling evidence for the importance of CSH in the progression and outcome of pregnancy. Our initial study (Baker *et al.* 2016) supported the long-held hypotheses (Handwerger 1991, Handwerger & Freemark 2000) that CSH promoted fetal growth by impacting fetal concentrations of IGF1 and insulin. Our current results expand the possible impact of CSH to actions within the placenta itself, including the expression of *IGF1*, *IGFBP2* and glucose transporters. These paracrine actions within the placenta may have been responsible for the reduction in fetal growth that occurred during early gestation (50 dGA), but may have also set the stage for functional placental insufficiency during late gestation (135 dGA). Unfortunately, with both of our studies, it was not feasible to chronically catheterize the pregnancies, allowing steady-state investigations under non-stressed/non-anesthetized conditions that will be required to determine direct effects of CSH within the placenta vs actions directly within the fetus. Regardless of these shortcomings, our results highlight the utility of *in vivo* lentiviral-mediated RNA interference to study the function of genes expressed by the placenta of large mammals. More importantly, they provide compelling evidence that CSH plays a critical role in the progression of pregnancy and lay the groundwork for future studies that can delineate the exact mechanisms by which CSH promotes fetal growth.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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