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Chorionic somatomammotropin RNA interference alters fetal liver glucose utilization

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Abstract

Chorionic somatomammotropin (CSH) is a placenta-specific hormone associated with fetal growth, and fetal and maternal metabolism in both humans and sheep. We hypothesized that CSH deficiency could impact sheep fetal liver glucose utilization. To generate CSH-deficient pregnancies, day 9 hatched blastocysts were infected with lentiviral particles expressing CSH-specific shRNA (RNAi) or scramble control shRNA (SC) and transferred to synchronized recipients. CSH RNAi generated two distinct phenotypes at 135 days of gestational age (dGA); pregnancies with IUGR (RNAi-IUGR) or with normal fetal weight (RNAi-NW). Fetal body, fetal liver and placental weights were reduced ($P < 0.05$) only in RNAi-IUGR pregnancies compared to SC. Umbilical artery plasma insulin and insulin-like growth factor 1 (IGF1) concentrations were decreased, whereas insulin receptor beta (INSR) concentration in fetal liver was increased ($P < 0.05$) in both RNAi phenotypes. The mRNA concentrations of *IGF1*, *IGF2*, IGF binding protein 2 (*IGFBP2*) and *IGFBP3* were decreased ($P < 0.05$) in fetal livers from both RNAi phenotypes. Fetal liver glycogen concentration and glycogen synthase 1 (GYS1) concentration were increased ($P < 0.05$), whereas fetal liver phosphorylated-GYS (inactive GYS) concentration was reduced ($P < 0.05$) in both RNAi phenotypes. Lactate dehydrogenase B (LDHB) concentration was increased ($P < 0.05$) and IGF2 concentration was decreased ($P < 0.05$) in RNAi-IUGR fetal livers only. Our findings suggest that fetal liver glucose utilization is impacted by CSH RNAi, independent of IUGR, and is likely tied to enhanced fetal liver insulin sensitivity in both RNAi phenotypes. Determining the physiological ramifications of both phenotypes, may help to differentiate direct effect of CSH deficiency or its indirect effect through IUGR.

Key Words

- ▶ chorionic somatomammotropin
- ▶ glycogen
- ▶ insulin receptor
- ▶ glycogen synthase

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Introduction

Chorionic somatomammotropin (CSH), also known as placental lactogen (PL), is a peptide hormone produced by the placenta and secreted into both fetal and maternal circulation. The source of CSH is the syncytiotrophoblast in humans, trophoblast giant cells in mice and chorionic binucleate cells in sheep (Gootwine 2004, Sibiak *et al.* 2020). CSH binding has been identified in several maternal and

fetal sheep tissues, including skeletal muscle, pancreas and liver (Chan *et al.* 1978). CSH has high affinity for prolactin (PRL) receptors as well as growth hormone (GH) receptors (Handwerger & Freemark 2000), although a distinct CSH receptor may exist in fetal liver (Freemark *et al.* 1987, Hill *et al.* 1988, Pratt *et al.* 1995). While CSH is produced by the placenta, it is found in high concentrations in both

maternal and fetal blood (Handwerger & Freemark 2000), indicating that it might have widespread roles in different physiological processes in the mother and fetus.

Ovine CSH has long been thought to play an important role in fetal growth (Hurley *et al.* 1977, Min *et al.* 1996), and reduced CSH in maternal circulation has been associated with intrauterine growth restriction (IUGR) in both human and sheep pregnancies (Spellacy *et al.* 1976, Lea *et al.* 2007). There are conflicting reports about the role of human CSH in fetal growth. Some studies have demonstrated that CSH deficiency or deletion of CSH locus in humans results in severe intrauterine growth restriction (Trapp *et al.* 1987, Rygaard *et al.* 1998); however, other studies show that CSH deficiency or gene deletion does not affect fetal growth and results in otherwise uneventful pregnancies (Wurzel *et al.* 1982, Giampietro *et al.* 1984, Barbieri *et al.* 1986, Simon *et al.* 1986). Other than its effect on fetal growth, none of these studies investigated the possible physiological ramification of CSH deficiency. We recently reported (Baker *et al.* 2016) that CSH deficiency generates two distinct phenotypes; pregnancies affected by IUGR (CSH RNAi-IUGR) and pregnancies with normal fetal weight (CSH RNAi-NW). However, Baker *et al.* (2016) reported little on the characteristics of the CSH RNAi-NW ('nonresponders') pregnancies. While correlations between CSH concentrations and fetal growth have been examined, the effect of CSH on glucose utilization in the fetal liver is not clear.

Glucose is the principal source of energy for the fetus, and optimal glucose utilization is crucial for normal fetal growth (Hay 2006). Due to high insulin sensitivity, glucose utilization in the fetal liver is primarily regulated by insulin (Hay 2006). In humans, the fetal liver plays a vital role in the maintenance of glucose metabolism and has all the necessary enzymes for gluconeogenesis and glycogen synthesis by as early as 8 weeks of gestation (Grijalva & Vakili 2013). IUGR pregnancies often exhibit an increase in fetal blood glucagon and fetal liver gluconeogenic enzymes, suggesting an early initiation of gluconeogenesis (Thorn *et al.* 2009, Culpepper *et al.* 2016, Wesolowski & Hay 2016). One such gluconeogenic enzyme is lactate dehydrogenase (LDHB) which converts lactate to pyruvate, the first substrate for gluconeogenesis (Garrett & Grisham 2001). Similarly, phosphoenolpyruvate carboxykinase 2 (PCK2) is also a key gluconeogenic enzyme which converts oxaloacetate to phosphoenolpyruvate (Yang *et al.* 2009).

CSH is believed to regulate glycogen synthesis in fetal tissues, as it stimulates glycogen synthesis and storage in fetal rat hepatocytes (Freemark & Handwerger 1984a,

1985), and infusion of CSH increased fetal sheep liver glycogen content (Schoknecht *et al.* 1996). The glycogenic action of CSH in fetal liver is thought to be regulated through insulin signaling (Freemark & Handwerger 1984b). CSH is also believed to support fetal growth by stimulating insulin-like growth factor production and by mobilizing maternal glucose and amino acids for transfer from the mother to the fetus (Handwerger & Freemark 2000). The fetal liver abundantly expresses insulin-like growth factor 1 and 2 (IGF1 and IGF2) and insulin receptor beta (IR β) (Liang *et al.* 2010). IGF2 regulates several biological functions including glycogen synthesis through its interaction with the type 1 IGF receptor and insulin receptor, and IGF2 and insulin receptor are main regulators of glycogen synthesis in fetal liver (Liang *et al.* 2010). Prenatal glycogen synthesis and storage are crucial to meet the requirements of glucose during the immediate perinatal period. Glycogen synthase (GYS), as the key enzyme involved in glycogen synthesis, is regulated by GYS kinase-3 (GSK3), which phosphorylates and inactivates GYS to p-GYS. Through activation of the insulin receptor signaling pathway, protein kinase B (AKT) phosphorylates GSK3, thereby inactivating GSK3 and hence reducing the proportion of inactivated p-GYS (Fang *et al.* 2000).

Previously, our laboratory reported a novel model of CSH-deficient pregnancies, using lentiviral-mediated trophoblast-specific RNA interference (RNAi) (Baker *et al.* 2016). Using these pregnancies, we investigated the effect of CSH RNAi on fetal liver glucose utilization, to test the hypothesis that CSH regulates fetal liver glucose utilization by regulating insulin signaling.

Materials and methods

All procedures involving use of animals or 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).

Generation of CSH-deficient pregnancies

We have previously reported the detailed procedures for the generation of these CSH-deficient pregnancies (Baker *et al.* 2016). Briefly, lentiviral particles were generated using a packaging plasmid (psPAX2), envelope plasmid (pMD2.G) and the shRNA vector (with SC or CSH specific

tg6 shRNA), and the resulting lentiviral titer was calculated by infecting human embryonic kidney cells (HEK293) and counting green fluorescent protein (GFP)-positive cells or by TCID50 assay. Day 9 hatched, and fully expanded blastocysts were collected from naturally mated/non-superovulated donor ewes, and infected with 100,000 transducing units in a 100 μ L drop of CDM-2 medium for 4–6 h. After infecting with lentiviral particles, blastocysts were surgically transferred to estrus synchronized ewes. One blastocyst was transferred in each recipient ewe. Terminal surgeries were conducted at 135 dGA and blood samples were collected from umbilical artery, umbilical vein, uterine artery and uterine vein from 8 SC and 16 CSH RNAi pregnancies. Fetal liver tissue and fetal cotyledons were collected and snap frozen in liquid nitrogen. The tissue was later pulverized using a mortar and pestle and stored in at -80°C until further use.

RNA isolation and quantitative real-time PCR

Total RNA was extracted from 135 dGA pulverized fetal cotyledons and fetal liver tissue using RNeasy Mini Kit (Qiagen) following the manufacturer's protocol. The detailed procedure and qRT-PCR primers have been previously described (Baker *et al.* 2016).

Protein extraction and Western blotting

For protein extraction, pulverized fetal liver tissue was homogenized in RIPA buffer (20 mM Tris, 137 mM NaCl, 10% glycerol, 1% nonidet P-40, 3.5 mM SDS, 1.2 mM sodium deoxycholate, 1.6 mM EDTA, pH 8) containing 1 $\times$ protease/phosphate inhibitor cocktail, and incubated for 5 min on ice. Homogenized samples were sonicated using a Bioruptor Sonication System (VWR Scientific) for five cycles of 30 s 'ON' and 30 s 'OFF'. The sonicated tissue lysate was centrifuged at 14,000 *g* for 5 min to remove tissue debris. Protein concentration in tissue lysates was measured using BCA protein assay kit (ThermoFisher), and 55–100 μ g of protein was electrophoresed through Mini-Protein 4–15% Tris-Glycine stain-free gels (Bio-Rad Laboratories) at 90 V for 15 min and 200 V for 30 min. The stain-free gels were activated for 1 min using the ChemiDoc XRS+ chemiluminescence system (Bio-Rad Laboratories) and then transferred to 0.2 μ m pore size nitrocellulose membrane at 100 V for 2 h. The membranes were imaged using the stain-free settings on the ChemiDoc XRS+ chemiluminescence system and then blocked in 5% non-fat dry milk solution in

TBST (50 mM Tris, 150 mM NaCl, 0.05% Tween 20, pH 7.6) for 1 h at room temperature. After blocking, the membranes were washed three times with 1 $\times$ TBST for 5 min each, and then incubated at 4°C overnight with specific primary antibody. After overnight incubation, the membranes were washed three times with 1 $\times$ TBST for 5 min each. After washing, the membranes were incubated with appropriate secondary antibody conjugated to horseradish peroxidase for 1 h at room temperature. After removing the secondary antibody, the membranes were washed following the same procedure and developed using Super Signal WestDura Extended Duration Substrate and imaged using ChemiDoc XRS+ chemiluminescence system. The images were quantified using Image-Lab software. Protein quantification data were normalized to total protein quantity in each lane transferred to the nitrocellulose membrane. The antibodies used and their dilutions are listed in Supplementary Table 1 (see section on [supplementary materials](#) given at the end of this article).

Glycogen assay

Glycogen quantity in fetal liver samples was measured using glycogen colorimetric/ fluorometric assay kit (BioVision) following the manufacturer's protocol. Briefly, 10 mg of pulverized fetal liver tissue was homogenized in 200 μ L dH₂O, boiled for 10 min to inactivate enzymes, and centrifuged to remove insoluble materials within the homogenate. Using a 96-well flat-bottom plate, 5 μ L of tissue extract was added in two replicate wells and final volume was brought up to 50 μ L using hydrolysis buffer. One well was used to measure glycogen while the second well was used to measure glucose background of each sample. A standard curve for glycogen was made by loading different glycogen quantities (0, 0.4, 0.8, 1.2, 1.6, and 2.0 μ g per well) and final volume was brought to 50 μ L using hydrolysis buffer. Two μ L of hydrolysis enzyme mix was added in all wells except the wells to be used to measure glucose background and incubated at room temperature for 30 min. Fifty μ L of development reaction mix for each well was prepared by mixing 46 μ L development buffer, 2 μ L development enzyme and 2 μ L OxiRed reagent. After incubation, 50 μ L of development reaction mix was added in each well and incubated for 30 min at room temperature. Absorbance (OD 570 nm) was measured using a BioTek Synergy 2 Microplate Reader (BioTek) and glycogen quantity in each sample was determined from the standard curve.

Radioimmunoassay

The serum concentrations of insulin and IGF1 were measured using RIA as previously reported (Baker *et al.* 2016).

Serum glucose measurement

Maternal and fetal serum glucose concentrations were measured using the YSI 2700 SELECT Biochemistry Analyzer (YSI, Yellow Springs, OH) following the manufacturer's protocol, as previously reported (Baker *et al.* 2016).

Statistical analysis

As described in Baker *et al.* (2016), the determination of 'responder' (RNAi-IUGR) pregnancies versus 'nonresponder' (RNAi-NW) was done by determining which pregnancies had placental weights that fell two standard deviations below the mean of the SC pregnancies. Subsequently, all data were subjected to ANOVA with treatment, fetal sex and sire as the dependent variables, and included all interactions. There was no treatment by fetal sex or treatment by sire interactions, therefore statistical analysis of the data presented herein, from both RNAi phenotypes, were compared with SC using one-way ANOVA followed by Tukey's HSD post-hoc test. Statistical significance was set at $P \leq 0.05$ while statistical tendency was set at $P \leq 0.10$. The data figures are presented as scatter plots, with the horizontal line representing the mean, and the capped vertical lines representing the S.E.M.

Results

Trophectoderm-specific CSH RNAi generates two distinct phenotypes

As previously reported (Baker *et al.* 2016), trophectoderm-specific CSH RNAi generated two distinct phenotypes, as assessed by determining which CSH RNAi (tg6) pregnancies resulted in placental weights that fell two s.d. below the mean of the SC pregnancies. As such, some fetuses were considered to be intrauterine growth restricted (RNAi-IUGR; $n = 8$) and some had normal weight (RNAi-NW; $n = 8$) compared to SC ($n = 8$) (Fig. 1A). A 52% reduction ($P < 0.05$) in placental weight and a 40% reduction ($P < 0.05$) in fetal liver weight were observed in RNAi-IUGR pregnancies compared to SC, whereas there was no significant change in placental weight and fetal liver weight in RNAi-NW pregnancies (Fig. 1B and C). CSH mRNA was reduced by 50% ($P < 0.05$) in RNAi-IUGR and by 47% ($P < 0.05$) in RNAi-NW fetal cotyledons compared to SC (Fig. 1D). Differences in cotyledonary CSH concentration for these pregnancies were previously reported (Baker *et al.* 2016).

CSH deficiency affects insulin signaling in fetal liver

There was a 48% reduction ($P = 0.07$) in umbilical artery insulin in RNAi-IUGR pregnancies and a 36% reduction ($P = 0.15$) in RNAi-NW pregnancies compared to SC (Table 1), but umbilical artery glucose concentrations were not impacted. Insulin and glucose concentrations in the uterine artery were not impacted in either RNAi-IUGR and RNAi-NW pregnancies (Table 1); however, there was a significant increase in the uterine artery to uterine

Figure 1

(A, B and C) Fetal body weight, liver weight and placental weight at 135 dGA in SC, RNAi-IUGR and RNAi-NW pregnancies. ($n = 8$ /treatment). SC and RNAi-IUGR data from Baker *et al.* (2016) are incorporated here to build a comparison. (D) Cotyledon CSH mRNA concentration in fetal cotyledons from SC, RNAi-IUGR and RNAi-NW pregnancies ($n = 8$ /treatment), $*P < 0.05$.

Table 1 Fetal and maternal serum measurements.

Serum measurements	SC (mean $\pm$ S.E.M.)	RNAi-IUGR (mean $\pm$ S.E.M.)	P value (SC vs RNAi-IUGR)	RNAi-NW (mean $\pm$ S.E.M.)	P value (SC vs RNAi-NW)
UtA insulin (ng/mL)	0.40 $\pm$ 0.08	0.94 $\pm$ 0.31	0.14	0.40 $\pm$ 0.10	0.99
UtA glucose (mmol/L)	3.39 $\pm$ 0.56	3.70 $\pm$ 0.54	0.69	3.05 $\pm$ 0.16	0.54
UtA insulin:glucose	0.12 $\pm$ 0.02	0.24 $\pm$ 0.06	0.08	0.13 $\pm$ 0.03	0.78
UmbA insulin (ng/mL)	0.56 $\pm$ 0.13	0.29 $\pm$ 0.06	0.07	0.36 $\pm$ 0.04	0.15
UmbA glucose (mmol/L)	0.80 $\pm$ 0.31	0.53 $\pm$ 0.21	0.48	0.61 $\pm$ 0.11	0.57
UmbA insulin:glucose	1.21 $\pm$ 0.27	1.77 $\pm$ 0.78	0.51	0.62 $\pm$ 0.11	0.08
UtV glucose (mmol/L)	3.06 $\pm$ 0.50	3.34 $\pm$ 0.56	0.71	2.64 $\pm$ 0.17	0.43
UtA-UtV glucose (mmol/L)	0.23 $\pm$ 0.04	0.36 $\pm$ 0.03	0.02	0.42 $\pm$ 0.06	0.02
UmbV glucose (mmol/L)	0.97 $\pm$ 0.33	0.77 $\pm$ 0.22	0.62	0.76 $\pm$ 0.10	0.56
UmbV-UmbA glucose (mmol/L)	0.17 $\pm$ 0.04	0.24 $\pm$ 0.07	0.42	0.15 $\pm$ 0.03	0.66

Serum concentrations are mean $\pm$ S.E.M. SC and RNAi-IUGR data from Baker *et al.* (2016) are incorporated here to build a comparison. ($n = 8/\text{treatment}$).

$P \leq 0.10$ indicates a trending difference from controls. $P \leq 0.05$ indicates a significant difference from controls.

UmbA, umbilical artery; UmbV, umbilical vein; UtA, uterine artery; UtV, uterine vein.

vein glucose gradient ($P < 0.05$) in both RNAi-IUGR and RNAi-NW phenotypes (Table 1). No significant difference was observed in IGF1 concentration in uterine arterial blood in either RNAi-IUGR and RNAi-NW pregnancies; however, umbilical artery IGF1 concentration was reduced ($P < 0.05$) in both RNAi phenotypes compared to SC (Fig. 2). The mRNA concentrations of *IGF1*, *IGF2*, *IGFBP2* and *IGFBP3* were reduced ($P < 0.05$) in fetal livers from both RNAi phenotypes compared to SC (Table 2). Moreover, mRNA concentration of *IGFBP1* did not change in RNAi-IUGR fetal livers but tended ($P = 0.08$) to be reduced in RNAi-NW fetal livers compared to SC (Table 2). Analysis of INSR concentrations revealed a 192% ($P < 0.05$) increase in

RNAi-IUGR fetal livers and 106% ($P < 0.05$) in RNAi-NW fetal livers, compared to SC (Fig. 3).

CSH deficiency leads to increased glycogen storage in fetal liver

Glycogen concentration in fetal livers increased by 80% ($P < 0.05$) in RNAi-IUGR pregnancies and by 89% ($P < 0.05$) in RNAi-NW pregnancies compared to SC (Fig. 4). Glycogen synthase 1 (GYS1), glycogen synthase 2 (GYS2) and phosphorylated glycogen synthase (p-GYS) were also assessed. GYS1 concentration in fetal liver increased 92% ($P < 0.05$) in RNAi-IUGR pregnancies and 47% ($P < 0.05$) in RNAi-NW pregnancies compared to SC (Fig. 5A). There was no statistical difference in GYS2 concentration in fetal livers in either RNAi-IUGR and RNAi-NW pregnancies as compared to SC (Fig. 5B). The inactive form of glycogen synthase, p-GYS, was reduced in fetal livers by 34% ($P < 0.05$) in RNAi-IUGR pregnancies and 30% ($P < 0.05$) in RNAi-NW pregnancies compared to SC (Fig. 5C).

CSH deficiency affects the expression of genes with known role in glucose metabolism

Analysis of LDHB concentrations in fetal liver revealed a 130% ($P < 0.05$) increase in RNAi-IUGR pregnancies, whereas no significant change was observed in fetal liver from RNAi-NW pregnancies, compared to SC (Fig. 6A). There was no significant difference in fetal liver PCK2 concentrations in fetal livers from either RNAi-IUGR and RNAi-NW pregnancies, compared to SC (Fig. 6B). However, IGF2 concentration was reduced by 23% ($P < 0.05$) in RNAi-IUGR fetal liver, whereas no change was observed in RNAi-NW fetal liver, as compared to SC (Fig. 6C).

Figure 2

IGF1 concentration in uterine artery (UtA; $n = 7$ for SC and RNAi-IUGR, and $n = 8$ for RNAi-NW) and umbilical artery (UmbA; $n = 8/\text{treatment}$) blood. SC and RNAi-IUGR data from Baker *et al.* (2016) are incorporated here to build a comparison ($n = 8/\text{treatment}$), * $P < 0.05$.

Table 2 Fetal liver insulin-like growth factor mRNA concentrations.

Gene	SC (mean $\pm$ S.E.M.)	RNAi-IUGR (mean $\pm$ S.E.M.)	P value (SC vs RNAi-IUGR)	RNAi-NW (mean $\pm$ S.E.M.)	P value (SC vs RNAi-NW)
<i>IGF1</i> (pg/pg)	0.04 $\pm$ 0.01	0.01 $\pm$ 0.002	0.0001	0.02 $\pm$ 0.003	0.0019
<i>IGF2</i> (pg/pg)	0.49 $\pm$ 0.08	0.14 $\pm$ 0.04	0.001	0.22 $\pm$ 0.04	0.01
<i>IGFBP1</i> (pg/pg)	0.72 $\pm$ 0.16	0.64 $\pm$ 0.31	0.813	0.37 $\pm$ 0.10	0.08
<i>IGFBP2</i> (pg/pg)	0.18 $\pm$ 0.04	0.05 $\pm$ 0.03	0.022	0.03 $\pm$ 0.01	0.006
<i>IGFBP3</i> (pg/pg)	0.09 $\pm$ 0.01	0.02 $\pm$ 0.006	0.0002	0.02 $\pm$ 0.003	0.0002

mRNA concentrations are mean $\pm$ S.E.M. for the starting quantity of mRNA of interest (pg) divided by geomean of starting quantity (pg) for the housekeeping mRNA. SC and RNAi-IUGR data in this table were previously presented (Baker *et al.* 2016) and are incorporated here to build a comparison. ($n = 8$ /treatment), $P \leq 0.10$ indicates a trending difference from controls. $P \leq 0.05$ indicates a significant difference from controls.

Discussion

In sheep, chorionic somatomammotropin (CSH) is produced by chorionic binucleate cells and is secreted into both fetal and maternal circulation (Gootwine 2004, Sibiak *et al.* 2020). CSH has been previously studied for its potential to modulate fetal growth (Spellacy *et al.* 1976, Freemark 2010, Baker *et al.* 2016, Jeckel *et al.* 2018). Recently, we generated CSH-deficient pregnancies by trophoctoderm-specific RNA interference (RNAi) of CSH (Baker *et al.* 2016, Jeckel *et al.* 2018). In this unique approach, hatched day 9 sheep blastocysts were incubated with shRNA-expressing lentiviral particles which infect specifically the trophoctoderm and leads to placenta specific modifications without affecting the inner cell mass (Baker *et al.* 2016). We reported that CSH deficiency in sheep resulted in two distinct phenotypes at 135 dGA; pregnancies with IUGR (RNAi-IUGR) or pregnancies with normal placental and fetal weights (RNAi-NW). CSH mRNA in fetal cotyledons was significantly reduced in both RNAi-IUGR and RNAi-NW phenotypes (Fig. 1D), whereas cotyledonary CSH was only significantly reduced in RNAi-IUGR pregnancies (Baker *et al.* 2016). Despite comparable reductions in CSH mRNA, variation existed on the impact of fetal and placental growth

between the CSH RNAi phenotypes. Jeckel *et al.* (2018) reported that trophoctoderm-specific RNAi in sheep causes fetal growth restriction at 50 dGA, in the absence of significant reductions in cotyledonary CSH mRNA and protein. Both in our study and Jeckel *et al.* (2018) study, the placental tissue samples were collected after terminating the pregnancies and CSH concentrations were determined at only one timepoint in gestation. However, the efficiency of RNAi is affected by several factors including stabilization of target mRNA by binding proteins, target mRNA turnover rate, abundance of target transcript, efficiency of lentivirus transduction, epigenetic modifications and shRNA-driving promoter efficiency (Ramezani *et al.* 2000, Holen *et al.* 2002, Hong *et al.* 2014). These factors can affect the degree of CSH RNAi at different stages of gestation starting from day of infection (day 9) till the termination of pregnancy. However, it is not possible to access the variability in RNAi in placenta across gestation, without terminating the pregnancies. These factors can vary from animal to animal, and there is possibility that, in some animals, RNAi efficiency is low at a critical time of fetal and placental development and vice versa, which will affect the phenotype of that pregnancy. Several studies in humans have also reported contrasting results regarding CSH's role in fetal growth. Some reports have shown that CSH deficiency or deletion of the CSH locus in humans causes severe fetal growth restriction

Figure 3

Representative immunoblots and densitometric analysis for INSR protein in fetal liver from SC, RNAi-IUGR and RNAi-NW pregnancies ($n = 8$ /treatment), $*P < 0.05$.

Figure 4

Glycogen concentration in fetal liver from SC, RNAi-IUGR and RNAi-NW pregnancies ($n = 8$ /treatment), $*P < 0.05$.

Figure 5

Representative immunoblots and densitometric analysis for GYS1, GYS2 and p-GYS in fetal liver from SC, RNAi-IUGR and RNAi-NW pregnancies ($n = 8/\text{treatment}$), $*P < 0.05$.

(Trapp *et al.* 1987, Rygaard *et al.* 1998); however, in some other cases it has no impact on fetal growth (Wurzel *et al.* 1982, Giampietro *et al.* 1984, Barbieri *et al.* 1986, Simon *et al.* 1986). Absence of growth restriction in CSH-deficient pregnancies suggests that there might be some compensatory mechanisms supporting fetal growth in these pregnancies (Freemark 2010), which are yet to be explored.

It has long been thought that, other than fetal growth, CSH also regulates glucose metabolism. According to Freemark and Handwerger (1984), ovine CSH has insulin-like effects and stimulates incorporation of glucose into glycogen in fetal rat hepatocytes in a dose dependent manner (Freemark & Handwerger 1984a). They also reported that, other than stimulating glycogen synthesis, ovine CSH inhibits glucagon-induced glycogen degradation in fetal rat hepatocytes (Freemark & Handwerger 1985). CSH receptors have also been identified in many maternal and fetal tissues including liver suggesting a role in regulating liver function (Freemark *et al.* 1986, 1987, 1992, Hill *et al.* 1988, Pratt *et al.* 1995). The expression of CSH receptors in maternal and fetal sheep liver is positively correlated with glucose and insulin concentrations in maternal and fetal blood

(Freemark *et al.* 1990, 1992). However, the effect of CSH deficiency on glucose utilization by sheep fetal liver has never been examined. As little was reported on the CSH RNAi-NW (nonresponders) in Baker *et al.* (2016), here we further report on both phenotypes (RNAi-IUGR and RNAi-NW) and extend the investigation into the fetal liver glucose utilization.

According to Schoknecht *et al.* (1996), i.v. infusion of 1.2 mg/day of ovine CSH into sheep fetal circulation starting at 122 dGA increases hepatic glycogen deposition without affecting fetal weight (Schoknecht *et al.* 1996). Therefore, it would be expected that reduced placental CSH would result in a decrease in liver glycogen stores. Glycogen, however, was found to be significantly increased in fetal liver from both RNAi-IUGR and RNAi-NW pregnancies, which is contradictory to former findings (Freemark & Handwerger 1984a, Schoknecht *et al.* 1996). One potential reason for this contradiction is that previous studies established a correlation between CSH and glycogen synthesis by CSH infusion into fetus (Schoknecht *et al.* 1996) or stimulating rat hepatocytes with ovine CSH (Freemark & Handwerger 1984a) whereas we investigated this correlation using a CSH deficiency model. We suggest that, compared to CSH

Figure 6
Representative immunoblots and densitometric analysis for LDHB, PCK2 and IGF2 in fetal liver from SC, RNAi-IUGR and RNAi-NW pregnancies ($n = 8/\text{treatment}$), * $P < 0.05$.

infusion or stimulation, CSH deficiency could modulate more pathways involved in glucose utilization. Another possible explanation is that the tissues in our study were collected after terminal surgeries performed under general anesthesia and after ~16 h of fasting. Although fasting might not impact the plasma concentrations of CSH (Butler *et al.* 1987, Bauer *et al.* 1995), the stress of the surgical procedure and general anesthesia can increase CSH concentrations in fetal and maternal plasma (Taylor *et al.* 1980). Although increased CSH concentration due to surgery or anesthesia can affect the glucose utilization by the fetal liver, it is possible that an increase in CSH concentration during the surgical procedure (~1 h), could drive enhanced glycogen concentration in fetal liver.

Several studies have demonstrated a correlation between CSH and insulin. Bennet *et al.*, showed that injection of human CSH in hypophysectomized rats increases pancreatic insulin secretion (Bennett *et al.* 1976). Handwerger *et al.* reported that injecting CSH in pregnant or non-pregnant ewes resulted in a decrease in insulin after 1 h of CSH injection which then increases above the baseline (Handwerger *et al.* 1976).

Treatment of human fetal pancreatic explants with human CSH in the presence of glucose causes a significant increase in insulin synthesis and release (Swenne *et al.* 1987). In our study, while there were reductions in umbilical artery insulin concentrations in both RNAi-IUGR and RNAi-NW pregnancies, neither reached statistical significance ($P < 0.05$), and there were no changes in umbilical glucose concentrations either. However, insulin receptor beta (INSR) concentration was significantly increased in fetal livers from both RNAi-IUGR and RNAi-NW pregnancies. Increased INSR concentration in the fetal liver might be a compensatory response to reduced insulin in fetal blood, thus increasing fetal liver insulin sensitivity (Beale 2013, Boucher *et al.* 2014). Increased insulin sensitivity could explain the increased glycogen concentration in fetal livers from both RNAi-IUGR and RNAi-NW pregnancies. Hypoinsulinemia and increased insulin sensitivity are a hallmark of human IUGR pregnancies (Gagnon 2003); however, the existence of enhanced insulin sensitivity in the absence of IUGR has not been reported.

CSH is thought to support fetal growth by stimulating the production of fetal insulin-like growth factors (IGF)

and insulin (Handwerger 1991, Schoknecht *et al.* 1996). Thorn *et al.* reported that placental-insufficiency-based IUGR in sheep resulted in a 60% reduction in fetal liver weight compared to control fetuses at 134 dGA (Thorn *et al.* 2009). They further reported that IUGR fetuses had significant reductions in plasma insulin and IGF1 concentrations and 80% increase in insulin receptor in fetal skeletal muscle; however, insulin receptor concentration in the fetal liver was not reported (Thorn *et al.* 2009). In our study, we saw a significant reduction in IGF1 in umbilical artery blood from both RNAi-IUGR and RNAi-NW groups. Moreover, fetal livers from both RNAi-IUGR and RNAi-NW pregnancies had a significant reduction in mRNA encoding *IGF1*, *IGF2*, IGF-binding protein 2 (*IGFBP2*) and *IGFBP3*. IGF1 and IGF2 stimulate glycogen synthesis by interacting with insulin receptors (Liang *et al.* 2010, Muhič *et al.* 2015). IGFBPs bind IGFs and reduced their bioavailability, and nearly 75% of IGFs are bound to IGFBP3 (Ding & Wu 2018). Based on these findings we suggest that, other than increased insulin sensitivity, increased bioavailability of IGFs due to reduced IGFBPs could also contribute in glycogen synthesis resulting in increased glycogen in fetal liver from CSH-deficient pregnancies in our study. We also measured tissue IGF2 concentrations in the fetal liver, which were significantly reduced in RNAi-IUGR pregnancies, with no change in fetal livers from RNAi-NW pregnancies. IGF2 is a key determinant of fetal growth and IGF2 infusion in mouse increases fetal growth and body weight (White *et al.* 2018). Therefore, reduced IGF2 quantity only in RNAi-IUGR fetal liver might be driving the growth restriction in this group.

By its interaction with INSR, insulin initiates a signaling cascade that results in enhanced glycogen synthesis (Bouskila *et al.* 2010). One major step in this cascade is the increase in the ratio of active to inactive GYS in response to interaction of insulin with INSR (Bouskila *et al.* 2010). In humans, glycogen synthase has two paralogous isozymes; GYS1 known as the muscle isozyme and GYS2 known as the liver isozyme (Browner *et al.* 1989, Westphal & Nuttall 1992). However, we found that sheep fetal liver has high expression of both GYS1 and GYS2, which are active forms of glycogen synthase. GYS1 concentration was significantly increased in fetal livers from RNAi-IUGR and RNAi-NW pregnancies, whereas there was no change in GYS2 concentrations. The quantity of p-GYS (inactive form of GYS) was significantly reduced in both RNAi-IUGR and RNAi-NW pregnancies, indicating overall increased proportion of active GYS.

Other studies have also shown that during late gestation in rats, insulin increases the proportion of active glycogen synthase leading to glycogen deposition in rat fetal liver (Eisen *et al.* 1973, Beurel *et al.* 2015). Our data suggest that increased insulin sensitivity in fetal liver in both RNAi-IUGR and RNAi-NW pregnancies lead to increased active GYS, and hence increased glycogen synthesis and storage.

Normally, gluconeogenesis does not occur *in utero* until the immediate perinatal period (Grijalva & Vakili 2013); however, some studies suggest that gluconeogenic activity is initiated earlier in IUGR pregnancies as an adaptive mechanism to meet the demand of glucose utilization within the fetus (Hay 2006, Limesand *et al.* 2007, Thorn *et al.* 2009, Brown *et al.* 2015). Lactate dehydrogenase B (LDHB) and phosphoenolpyruvate carboxykinase 2 (PCK2) are key gluconeogenic enzymes. LDHB converts lactate into pyruvate which is then converted to oxaloacetate through a series of reactions. With the aid of PCK2, the oxaloacetate is shuttled across the mitochondrial membrane to be converted into phosphoenolpyruvate, a substrate for gluconeogenesis (Garrett & Grisham 2001, Yang *et al.* 2009). The CSH-deficient RNAi-IUGR pregnancies had significantly increased in LDHB concentrations, but there was no significant change in PCK2 concentrations. Increased LDHB might initiate gluconeogenesis but unchanged PCK2 suggests otherwise. There was no significant change in LDHB and PCK2 quantity in RNAi-NW fetal liver. From these results we suggest that increased glycogen storage in RNAi-IUGR and RNAi-NW fetal liver is primality due to altered insulin signaling rather than gluconeogenesis.

As reported previously, CSH deficiency generated by lentiviral-mediated RNAi can result in significant fetal growth restriction (Baker *et al.* 2016, Jeckel *et al.* 2018), but at least near-term (Baker *et al.* 2016) there is a cohort of CSH RNAi pregnancies that do not exhibit IUGR. While there are a number of potential explanations for these two phenotypes (e.g. variability in RNAi efficiency, response to fasting and anesthesia, potential compensatory mechanisms, etc.), our results complement the existing case reports of human pregnancies in which the CSH locus is disrupted/deleted. As mentioned earlier, in some human cases severe fetal growth restriction (Trapp *et al.* 1987, Rygaard *et al.* 1998) occurred, whereas in some other cases there was no apparent impact on fetal growth (Wurzel *et al.* 1982, Giampietro *et al.* 1984, Barbieri *et al.* 1986, Simon *et al.* 1986). Based on our results, it could be argued that the offspring of a CSH-deficient pregnancy may well be impacted by CSH deficiency, even in the absence of

IUGR. The pregnancies that we studied were harvested at a single gestational age, and the samples collected reflect a single-point in time. As such, there is clearly a need to investigate both CSH-deficient phenotypes under steady-state non-anesthetized/non-stressed conditions, as these types of stressors (fasting and/or anesthesia) impact the acute physiological status of both the mother and fetus, in order to fully define the impact of CSH deficiency on fetal physiology.

Supplementary materials

This is linked to the online version of the paper at <https://doi.org/10.1530/JOE-20-0375>.

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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