Chorionic somatomammotropin deficiency perturbs the metabolic transcriptome in the ruminant placenta

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INTRODUCTION

Chorionic somatomammotropin (CSH; aka placental lactogen) is a member of the growth hormone/prolactin family and is secreted by the binucleate cells of the ruminant placenta. While CSH is the most abundantly produced placental hormone, the exact biological functions of CSH continue to evade elucidation. Decreases of CSH in maternal circulation have been associated with intrauterine growth restriction (IUGR) in sheep (Lea et al., 2007) but until recently, no direct evidence existed to demonstrate the necessity of CSH for adequate fetal growth. To this end, our lab developed an approach to generate CSH-deficient pregnancies in sheep using lentiviral-mediated RNA interference (RNAi) as described by Baker et al. (2016). Using this approach, we demonstrated CSH RNAi directly resulted in the development of IUGR at both 50 days of gestational age (dGA; Jeckel et al., 2018) and 135 dGA (near-term; Baker et al., 2016).

Interestingly, near-term (135 dGA) two distinct phenotypes emerge in response to CSH RNAi: 1) pregnancies with IUGR and 2) pregnancies with normal fetal and placental weights (Baker et al., 2016; Ali et al., 2020). These two phenotypes resemble those documented in the human in response to CSH loci mutations/deletions (Daikoku et al., 1979; Rygaard et al., 1998). Both CSH RNAi phenotypes displayed reduced umbilical insulin-like growth factor 1 (IGF1), as well as increased uterine artery to vein glucose gradients, an indicator of impaired glucose uptake (Ali et al., 2020). This led us to believe that metabolic perturbations as a result of CSH RNAi were occurring regardless of changes in fetal and placental weight. This was supported by in vivo physiological experiments on CSH RNAi pregnancies without IUGR which exhibited reduced uterine blood flow and increased glucose utilization by the placenta, ultimately leading to reduced glucose transfer to the fetus (Tanner et al., 2021).

Thus, to investigate transcriptomic changes in CSH RNAi placentae, we employed RNA sequencing. We hypothesized that CSH RNAi would result in transcriptional perturbations of genes involved in metabolism and cell function in both IUGR and non-IUGR phenotypes. Therefore, the objective of this study was to characterize placental transcriptional pathways that are impacted due to CSH deficiency, in order to provide insight into altered placental metabolism observed in vivo, and to aid in describing the progression of IUGR in CSH-deficient pregnancies.

MATERIALS AND METHODS

Creation of CSH RNAi Pregnancies and Tissue Collections

All procedures were approved by the Colorado State University Institutional Animal Care and
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Use Committee (#14-5257A) and the Institutional Biosafety Committee (#11-034B and 13-043B). The generation of these CSH RNAi pregnancies was extensively described by Baker et al. (2016). Briefly, fully expanded and hatched blastocysts were collected at 9 dGA. Each blastocyst was infected with 100,000 transducing units of either control RNAi (scramble control; \( n = 8 \)) or CSH RNAi (target 6/tg6; \( n = 16 \)) lentivirus. After 4 h of infection, a single washed blastocyst was surgically transferred into a synchronized recipient ewe. Placentomes were collected at terminal surgery at 135 dGA and were processed by separating the fetal portions of the placenta (cotyledons) from the maternal portion of the placenta (caruncle) which were subsequently snap-frozen in liquid \( N_2 \) and stored at \(-80^\circ C\). Selection of cotyledonary samples for transcriptomic analysis was selected based on four traits: 1) placental weight, 2) fetal weight, 3) uterine artery-vein glucose gradient, and 4) umbilical artery IGF1 concentrations. The five samples that most distinctly clustered within each treatment (CON \( n = 5 \); CSH-IUGR \( n = 5 \); CSH-NW \( n = 5 \)) based on SD from the treatment mean for the combination of aforementioned traits were selected for RNA sequencing.

**RNA Sequencing, Alignments, and Analysis**

Frozen cotyledons were pulverized, and RNA was isolated using RNeasy Mini Kit (Ref# 74104; QIAGEN, Hilden, Germany) according to manufacturer’s instructions. Samples were submitted to the University of Colorado Genomics and Microarray Core (Aurora, CO) for sequencing. RNA integrity was assessed by Agilent TapeStation (Agilent Technologies, Inc., Boulder, CO) and all samples had a minimum RNA integrity number (RIN) of \( \geq 9.0 \). NuGEN Universal mRNA library prep kit (cat # 0508; NuGEN Technologies, Inc., Redwood City, CA) was utilized according to manufacturer’s instructions. Both automated clusters generation (150 bp) and RNAseq analysis were by the Illumina NovaSEQ6000 (100,000,000 paired-end reads). Adapter sequences were trimmed with Trimmomatic (Bolger et al., 2014). Paired-end reads were mapped to the reference ovine genome (Ovis aries, Oar.ra_1.0, Baylor College of Medicine) using HISAT2 version 2.2.0 (Kim et al., 2015). Transcripts were assembled and gene counts were obtained through StringTie version 1.3.3 (Pertea et al., 2015, 2016; Kovaka et al., 2019). DESeq2 using fragment bias correction was utilized to determine differentially expressed genes (DEGs) between each CSH RNAi phenotype and controls (CON vs. CSH-IUGR and CON vs. CSH-NW). The minimum alignment count was 15. The threshold for significance after multiple testing corrections (\( Q \)-value) was set to \( Q \leq 0.10 \) (equivalent to \( P < 0.00035 \)). All genes that were false discovery rate protected (\( Q \leq 0.10 \)) were used for pathway analysis with DAVID 6.8 (Huang et al., 2009a, 2009b) and Ingenuity Pathway Analysis (IPA; QIAGEN Inc.; https://www.qiagenbioinformatics.com/products/ingenuity-pathway-analysis). The threshold for considering a pathway or ontology significant was \( P \leq 0.05 \). Real-time qPCR validation of a subset of genes identified by DEG analysis is ongoing.

**RESULTS**

A total of 346 DEGs meeting a threshold of \( Q \leq 0.10 \) were identified between CSH-IUGR vs. CON, with 162 being upregulated and 184 being downregulated in CSH-IUGR pregnancies. Analysis of CSH-NW vs. CON pregnancies revealed 96 DEGs, 40 of which were upregulated and 56 were downregulated in CSH-NW pregnancies. Thirty-six common DEGs were identified in both CSH-IUGR and CSH-NW pregnancies, indicating likely direct effects of CSH RNAi. Table 1 contains a list of DEGs impacted by CSH RNAi that could prove interesting to further investigate to test how CSH RNAi differentially impacts placental function in IUGR vs. normal weight pregnancies.

DAVID 6.8 functional annotation and ontological analysis of pathways revealed numerous categories impacted in response to CSH RNAi with IUGR. The categories presented in Table 2 describe pathways or ontologies (\( n = 127 \); \( P < 0.05 \)) that could potentially contribute to the development of IUGR including: extracellular exosomes (\( n = 46 \)), metabolic pathways (\( n = 27 \)), extracellular space (\( n = 17 \)), protein processing in the endoplasmic reticulum (\( n = 11 \)), carbon metabolism (\( n = 6 \)), cellular responses to hypoxia (\( n = 5 \)), regulation of cell proliferation (\( n = 5 \)), lysine degradation (\( n = 4 \)), cyclic adenosine monophosphate (cAMP) regulation in metabolic processes (\( n = 3 \)), and negative regulation of protein catabolism (\( n = 3 \)).

IPA revealed functional categories similarly disrupted (\( P \leq 0.01 \); Table 3) by CSH RNAi regardless of fetal weight included 1) hematological development and function, 2) cell death and survival, 3) cell-to-cell signaling, and 4) immune cell trafficking. By comparing CON vs. CSH-IUGR, four canonical pathways were identified (\( P \leq 0.01 \); Table 3) including eicosanoid signaling, 3-phosphoinositidine biosynthesis, and protein ubiquitination.
pathways. The cell functions disrupted ($P \leq 0.01$) in CSH-IUGR pregnancies included cellular movement, protein synthesis, lipid metabolism, and tissue morphology. In CON vs. CSH-NW pregnancies, the canonical pathways implicated ($P \leq 0.01$; Table 3) included the mitotic roles of polo-like kinases and signaling by Rho family GTPases. Cell functions uniquely disrupted ($P \leq 0.01$) by CSH RNAi in pregnancies with normal weights included cell functions posttranslational modification, cell signaling, and connective tissue development.

**DISCUSSION**

We hypothesized CSH RNAi would result in perturbation of placental metabolism and cell function, identified through transcriptomic alterations to genes in both IUGR and non-IUGR phenotypes. The results from the functional analyses support this hypothesis as CSH RNAi placentae possessed DEGs and functional groups that indicate disrupted metabolism and cell function. By examining common DEGs between both CSH RNAi
Table 3. IPA of canonical pathways, cell functions, and physiological systems impacted by CSH RNAi

| Contrast          | Category               | Name                                   | Molecules impacted | P-value |
|-------------------|------------------------|----------------------------------------|--------------------|---------|
| **CON vs. CSH RNAi** | Physiological systems  | Hematological development and function | 41                 | 0.013   |
|                   | Cell functions          | Cell death and survival                | 34                 | 0.014   |
|                   | Cell functions          | Cell-to-cell signaling                 | 33                 | 0.011   |
|                   | Physiological systems   | Immune cell trafficking                | 20                 | 0.011   |
| **CON vs. CSH-IUGR** | Canonical pathways     | Eicosanoid signaling                   | 3                  | 0.005   |
|                   | Canonical pathways      | 3-Phosphoinositide biosynthesis        | 6                  | 0.008   |
|                   | Canonical pathways      | Protein ubiquitination pathway         | 8                  | 0.009   |
|                   | Cell functions          | Cellular movement                      | 24                 | 0.011   |
|                   | Cell functions          | Protein synthesis                      | 50                 | 0.007   |
|                   | Cell functions          | Lipid metabolism                      | 40                 | 0.011   |
|                   | Physiological systems   | Tissue morphology                      | 17                 | 0.011   |
| **CON vs. CSH-NW** | Canonical pathways      | Mitotic roles of polo-like kinase      | 3                  | 0.001   |
|                   | Canonical pathways      | Signaling by Rho GTPases               | 4                  | 0.007   |
|                   | Cell functions          | Posttranslational modification         | 7                  | 0.012   |
|                   | Cell functions          | Cell signaling                         | 7                  | 0.010   |
|                   | Physiological systems   | Connective tissue development          | 11                 | 0.015   |

*Comparison between control (n = 5) and all CSH RNAi pregnancies (n = 10).
†Comparison between control (n = 5) and CSH RNAi pregnancies with IUGR (n = 5).
‡Comparison between control (n = 5) and CSH RNAi pregnancies with normal fetal and placental weights (n = 5).

phenotypes, it is possible to examine the direct effects of CSH RNAi on the placental transcriptome. The 36 commonly impacted DEGs included genes involved in lipid and amino acid transfer, and positive regulation of cAMP. Additionally, both CSH RNAi phenotypes saw changes with functional groups associated with hematological development and function, cell death, and cell-to-cell signaling, thus indicating direct impacts of CSH RNAi on those pathways regardless of phenotype. Taken together, these DEGs and pathways support the idea that CSH RNAi placenta do differ from normal pregnancies. This is further supported by the altered physiology of both CSH RNAi phenotypes including reduced umbilical IGF1 and increased uterine artery-vein glucose gradients (Ali et al., 2020). Furthermore, the direct impacts of CSH RNAi on hematological function are supported by reductions in uterine blood flow as well as vascular endothelial nitric oxide synthase (NOS3) in CSH RNAi pregnancies (Tanner et al., 2021).

CSH RNAi pregnancies resulting in IUGR were more transcriptionally perturbed (346 DEGs) than CSH RNAi pregnancies with normal weights (90 DEGs). Furthermore, these pregnancies had increased metabolic transcriptional disruptions as evidenced by the impacted functional groups of carbon and lipid metabolism, cAMP metabolic processes, and protein catabolism and degradation. This is supported by the in vivo studies where these pregnancies experienced 32% and 52% reductions in fetal and placental weights, respectively, as well as reduced fetal liver weights (Baker et al., 2016). Interestingly, this RNAseq analysis revealed an increase in the Na’-dependent glucose cotransporter SLC5A1 in CSH RNAi pregnancies with IUGR. In the future, this could be examined as a potential compensatory mechanism as these placentae have been demonstrated to have decreased SLC2A1 (GLUT1), SLC2A3 (GLUT3) mRNA expression (Jeckel et al., 2018). Ultimately, these unique transcriptional disruptions to nutrient transporters, metabolic pathways, and metabolic enzymes could in part explain why some CSH RNAi pregnancies experience IUGR and some do not.

**IMPLICATIONS**

CSH-deficient pregnancies can result in fetal growth restriction, and sheep that have low birth weights often experience a high neonatal death loss. Often, those that survive have reduced postnatal growth potential which can impact the economic return of producers. This study examined placental transcriptomic alterations due to CSH deficiency to 1) identify novel genes and pathways that could explain the progression of growth restriction and 2) combine transcriptomic and physiological data to outline novel functions of CSH to be tested in the future. These data will provide insights to improve neonatal survival in livestock.

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