THESIS

IMPACT OF CHORIONIC SOMATOMAMMOTROPIN *IN VIVO* RNA INTERFERENCE PHENOTYPE ON UTEROPLACENTAL EXPRESSION OF THE IGF AXIS

Submitted by

Taylor Hord

Department of Biomedical Sciences

In partial fulfillment of the requirements

For the Degree of Master of Science

Colorado State University

Fort Collins, Colorado

Summer 2023

Master's Committee:

Advisor: Russell V. Anthony
Co-Advisor: Quinton A. Winger

Jason Bruemmer
Rebekah Kading

ABSTRACT

IMPACT OF CHORIONIC SOMATOMAMMOTROPIN *IN VIVO* RNA INTERFERENCE PHENOTYPE ON UTEROPLACENTAL EXPRESSION OF THE IGF AXIS

While fetal growth is dependent on many factors, optimal placental function is a prerequisite for a normal pregnancy outcome. The majority of fetal growth restricted (FGR) pregnancies result from placental insufficiency (PI). The insulin-like growth factors (IGF1 and IGF2) not only stimulate fetal growth, but also placental development and function. Previously, we demonstrated that *in vivo* RNA interference (RNAi) of the placental hormone, chorionic somatomammotropin (CSH), resulted in two phenotypes. One phenotype exhibits significant placental and fetal growth restriction (PI-FGR), impaired placental nutrient transport, and significant reductions in umbilical insulin and IGF1. The other phenotype does not exhibit statistically significant changes in placental or fetal growth (non-FGR). It was our objective to further characterize these two phenotypes by determining the impact of CSH RNAi on placental (maternal caruncle and fetal cotyledon) expression of the IGF axis. The trophectoderm of hatched blastocysts (9 days of gestation, dGA) were infected with a lentivirus expressing either a non-targeting sequence (NTS RNAi) control or CSH-specific shRNA (CSH RNAi) prior to embryo transfer into synchronized recipient ewes. At ≈125 dGA, pregnancies were fitted with vascular catheters to undergo steady-state metabolic studies. Nutrient uptakes were determined and tissues were harvested at necropsy. In both CSH RNAi non-FGR and PI-FGR pregnancies, uterine blood flow was significantly reduced ($P \leq 0.05$), while umbilical

blood flow (P≤0.01), both uterine and umbilical glucose and oxygen uptakes (P≤0.05), and umbilical concentrations of insulin and IGF1 (P≤0.05) were reduced in CSH RNAi PI-FGR pregnancies. Fetal cotyledon *IGF1* mRNA concentration was reduced (P≤0.05) in CSH RNAi PI-FGR pregnancies, whereas neither *IGF1* nor *IGF2* mRNA concentrations were impacted in the maternal caruncles, and either placental tissue in the non-FGR pregnancies. Fetal cotyledon *IGF1R* and *IGF2R* mRNA concentrations were not impacted for either phenotype, yet *IGF2R* was increased (P≤0.01) in the maternal caruncles of CSH RNAi PI-FGR pregnancies. For the IGF binding proteins (IGFBP1, IGFBP2, IGFBP3), only *IGFBP2* mRNA concentrations were impacted, with elevated *IGFBP2* mRNA in both the fetal cotyledon (P≤0.01) and maternal caruncle (P=0.08) of CSH RNAi non-FGR pregnancies. These data support the importance of IGF1 in placental growth and function, but may also implicate IGFBP2 in salvaging placental growth in non-FGR pregnancies.

ACKNOWLEDGMENTS

I want to extend my gratitude to the people who took the time and resources to guide and support me throughout my time as a graduate student. It may not take an army for a graduate student to successfully reach the finish line, but from my experience it made the journey much more feasible. I would not be the person and scientist I have grown to be without the many individuals I had the opportunity to learn from.

Thank you, Dr. Russ Anthony, for taking me under your wings and welcoming me into your lab as the efforts and resources you provided allowed me this opportunity to receive my master's degree. That will always mean so much to me. Thank you for challenging me to be better and for trusting me to independently figure out aspects of my projects. This allowed my confidence to develop as a researcher. It showed me what I am be capable of, where my limitations are, and to know when to ask for help. Thank you for your patience, understanding, and encouragement when I needed it. Thank you for the hands-on learning opportunities provided during surgery days. From standing across from you as your surgical assistant, to sedating and intubating sheep, to taking care of the embryos, it allowed me to enjoy my time as a student even more and I always had so much fun. Thank you for the laughs, stories of your unique life experiences, being able to share experiences from Mizzou, and teaching me that an exhausting workday calls for opening a bottle of scotch.

I also want to thank my other co-advisor, Dr. Quint Winger. Thank you for always having my back and encouraging me throughout my time as a graduate student. There is no doubt that your guidance and support were a major contribution to my success and

ability to graduate. Everything you have done for me to get me to where I am today will always mean so much to me. Thank you for always being available to provide valuable advice and feedback whenever I have been in need. Your input provided new and helpful perspectives and it always gave me peace of mind knowing I could count on you as my mentor. Thank you for always providing a positive outlook and for believing in me. I would leave our meetings feeling hopeful and confident due to your steadfast optimism and reassurance. I enjoy the memories made from the many hours spent searching for embryos and performing embryo transfers together. I am immensely grateful for your constant encouragement as my mentor.

I want to thank my committee members, Dr. Jason Bruemmer and Dr. Rebekah Kading. Thank you for your continuous support throughout my time as a graduate student. I am grateful to have a committee dedicated to my progress and success. Thank you for always being available when needed and for your suggestions and helpful input during my degree. I appreciate the time and effort it took for the both of you to serve on my committee.

I also want to thank my lab mates, Dr. Amelia Tanner, Tori Kennedy, Cameron Lynch, and Anna Donovan. I could always count on each of you and while there were many days that required hard work and many hours, I couldn't have asked for better teammates to endure through those moments. Each of you could make some of the most mundane tasks into something silly and fun. Thank you for teaching me many skills and for providing support and encouragement. Thank you for allowing me to share life with you all as a graduate student. Without having lab mates like you guys, my time as a

student would have been quite lonely. Thank you for giving me the opportunity to leave CSU with not only academic family, but as friends I can cherish.

Thank you to all of the hard-working undergraduate students in the lab, Bailyn Furrow, Serhat Tasdelen, Malini Desai, Blake Gash, Natalia Miller-Forest and Anna Trunzo as well as the farm crew Rick, Greg, and Brandon. Your help and hard work made the rigorous sheep seasons possible. Thank you all for being such wonderful students and colleagues to work alongside. I would like to thank my family, fiancé, and friends that supported me throughout my degree. Thank you for wholeheartedly believing in me and encouraging me. Your support got me to where I am today. Lastly, I would like to thank my dog, Hailey, for some days being the only reason I would go outside and see the sunlight and for keeping me sane.

TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

CHAPTER I: LITERATURE REVIEW

PLACENTATION

The placenta is unlike any other organ in a multitude of ways, and it doesn't get credit for how truly fascinating its capabilities are. It is the only internal organ that isn't programmed to develop within the body during organogenesis. It can only develop once a blastocyst interacts with the uterine wall and the trophectoderm cellular layer can invade into the endometrium to establish pregnancy. It presents in various morphologies dependent on the species and develops specific structures for maternal and fetal blood flow exchange within its assigned species. Nutrient and gas exchange needed for healthy fetal growth would not be possible without the interface the placenta provides. Not only does the placenta assist maternal-fetal communication, it's also a metabolic organ with high nutrient demands and acts as an endocrine organ with a variety of cell types that produce hormones and other factors that are needed to maintain a healthy pregnancy. In instances where the placenta does not develop properly during pregnancy, placental insufficiency, the degeneration in placental function and reduced placental transfer of oxygen and nutrients to the fetus (Gagnon, 2003), can be an etiology associated with fetal growth restriction (FGR) or preeclampsia.

FGR is determined when the fetus' expected growth potential is below the 10th percentile (Bruin et al., 2021). FGR can be diagnosed with serum biochemical markers that reflect placental dysfunction, like placental growth factor, and use of ultrasound to monitor fetal weight, disproportionate asymmetric growth, reduced amniotic fluid volume, and aberrant Doppler waveforms in the umbilical artery, ductus venosus, and middle

cerebral artery (Crocker, 2011; Bruin et al., 2021). FGR is the second-leading cause of perinatal death and is a pregnancy complication in 4-6% of human pregnancies worldwide (Gagnon, 2003). FGR fetuses have a five to six times higher risk of perinatal death and a three-fold greater risk of spontaneous preterm delivery (Gagnon, 2003). The use of Sildenafil as a potential therapeutic treatment has been administered in an attempt to improve vasodilation of uterine spiral arteries in a state of placental insufficiency for early-onset FGR. However, this therapy has not provided sustained evidence that it improves FGR pathophysiology throughout pregnancy. The therapeutic use of arginine and nitrogen carbamoyl glutamate supplementation has suggested evidence to improve fetal growth in complicated pregnancies, but the efficacy in treating human FGR needs further investigation. There are currently no treatments available that are past clinical trials to improve FGR outcomes (Bruin et al., 2021). Not only does FGR impact the fetus' development during gestation, but these individuals end up living with health complications throughout life. The surviving growth restricted neonates experience many health complications from their developmental ages through adulthood such as cognitive deficits, heart disease, diabetes, and hypertension (Gagnon, 2003).

It's understandable that there has not been a successful treatment developed for humans with FGR pregnancies to combat placental insufficiency. FGR is not a simple pregnancy complication as there is a conglomerate of factors feeding into the onset of FGR and various etiologies of FGR make it difficult to pinpoint the exact pathophysiology occurring with placental insufficiency that leads to FGR. Therefore, developing animal models with placental insufficiency that induce FGR is ideal with the hope it could lead to a better understanding of how to develop a reliable therapy for FGR in human

pregnancies. Animal models have been shown to mimic similar presentations of FGR during pregnancy as humans do, such as reduced placental and fetal mass, fetal hypoxia and hypoglycemia, reduced umbilical vein blood flow, reduced umbilical vein oxygen, glucose, and amino acid uptake, increased fetal blood pressure, and increased umbilical artery pulsatility index, which makes experimentation widely available without the need for human subjects (Anthony et al., 2003).

Human placenta

The human placenta is characterized by a disc shaped fetal chorionic plate where the fetal chorionic villi branch out from and invade into maternal endometrial spiral arteries allowing the maternal blood supply to be in direct contact with the fetal chorion. This characterization is defined as a discoid placenta, which is found in higher primates and rodents. The placenta develops from the trophectoderm (TE) of the blastocyst about five days post fertilization (dpf) (Turco et al., 2019). The blastocyst is composed of the inner cell mass (ICM), which develops into the fetus, and the TE. The polar side of the TE, the area that lies adjacent to the ICM, adheres to the epithelial surface of the uterine endometrium where implantation will occur (Turco et al., 2019). Following adhesion to the endometrium, the TE differentiates into cytotrophoblasts, trophoblast cells beneath the syncytium, and invades into the endometrial cells. During invasion the syncytiotrophoblast develops around seven dpf, which is a tissue type developed from the division and fusion of cytotrophoblasts to allow the growth of the syncytiotrophoblast to invade deeper into the uterus to reach the maternal blood supply. 8 dpf is the lacunar stage where fluid filled spaces called lacuna form within the syncytiotrophoblast, which provide space for the

maternal blood supply. The syncytium will also invade decidual glands to allow the syncytium to bathe in glandular secretions (Turco et al., 2019). By 14 dpf, the blastocyst will be completely embedded in the decidua. The decidua is the underlying endometrium that morphs into a specialized tissue in response to progesterone released from the corpus luteum, so it develops six days prior to implantation of the embryo during the secretory phase of the menstrual cycle. During this time from 12 to 15 dpf the villous stage of development occurs where primary chorionic villi form by proliferation of cytotrophoblasts on the outside of the embryo that push through the syncytium. These primary chorionic villi are composed of a cytotrophoblast core with a syncytiotrophoblast outer layer and will become the primary site of nutrient exchange between maternal and fetal blood flow (Turco et al., 2019). By 18 dpf the secondary chorionic villi form and are characterized by extraembryonic mesenchymal cells invading the villi. The mesenchyme are mesodermal embryonic cells that develop into connective tissue, blood vessels and lymphatic tissue. Fetal capillaries start to appear within the villous core, therefore characterizing the development of tertiary chorionic villi (Turco et al., 2019). At this point mature chorionic villi are surrounding the embryo and develop within a thin layer of syncytiotrophoblast containing prominent microvilli that face the maternal blood supply, a cytotrophoblast layer in contact with the decidua (the maternal-fetal interface), individual cytotrophoblast cells migrate from the cytotrophoblast shell to invade into the decidua as extravillous trophoblasts (EVT), and lastly a layer of mesenchyme that contains fetal blood vessels. EVT's completely remodel uterine spiral arteries by migrating from the cytotrophoblast shell into the spiral artery and replace the endothelial cells lining the uterine spiral arteries. This allows the fetus to take over the spiral arteries blood supply,

which dumps into the intervillous space on the fetal side to be picked up by the chorionic villi, and keeps the vessels dilated no matter what the maternal side is signaling. Without spiral artery remodeling, the fetus would not get the adequate blood supply it needs. At this point, the lacuna are fused together to create an intercommunicating intervillous space for the maternal blood supply to dump into. This marks the end of the first trimester with the programmed design of the placenta established.

A mature discoid shape hemochorial placenta is composed of the basal plate attached to the uterus for the maternal blood supply to enter and the chorionic plate, which contains the placenta that faces the fetus and the amniotic cavity that encapsules the fetus. Maternal oxygenated blood will enter into the intervillous space via uterine spiral arteries, bathing the chorionic villi (Gude et al., 2004). Since maternal and fetal blood supplies never mix, the chorionic villi pick up oxygen via rapid diffusion from the maternal blood supply surrounding the chorionic villi. Since fetal hemoglobin has a higher affinity for oxygen and a lower affinity for carbon dioxide compared to maternal hemoglobin, this favors the transfer of oxygen to fetal blood and carbon dioxide to maternal blood (Gude et al., 2004). Oxygen is carried to fetal circulation through the umbilical vein. After circulating through the fetal body, the now deoxygenated blood from the fetal aorta leaves the body through the umbilical artery and goes back to the placenta. The umbilical artery with deoxygenated blood flows into the chorionic villi and carbon dioxide is diffused into uterine maternal veins through the intervillous space (Gude et al., 2004). Nutrients have different methods of transportation from maternal to fetal circulation. Glucose, the primary source of energy for the fetus, is transported via protein-mediated facilitated diffusion and involvement of glucose transporters. Amino acids, required by the fetus for protein

synthesis, use active transport via amino acid transporters to get from maternal to fetal circulation. Lipids, which include free fatty acids, triacyclglycerols, phospholipids, glycolipids, and more, are bound to proteins within plasma. Free fatty acids and glycerol can readily cross from maternal to fetal circulation by simple diffusion or use of membrane-bound and cytosolic fatty acid binding proteins. The placenta is also able to readily transport long chain polyunsaturated fatty acids into fetal circulation. Water is passively transferred across the chorionic villi with the use of hydrostatic and osmotic pressure and ions use either active or passive transport across the placenta. Vitamins and minerals are also readily transferred from maternal to fetal circulation (Gude et al., 2004).

Ruminant placenta

The ruminant placenta is characterized as a cotyledonary, epitheliochorial placenta with specialized attachment sites called placentomes throughout the placenta. Placentomes are sites of interdigitation between maternal and fetal tissues identified as caruncles derived from the uterine endometrium and cotyledons derived from the trophectoderm containing chorionic villi, respectively (Regnault et al., 2002). Caruncles are projections that are always present within the uterus of sheep and are non-glandular, yet well vascularized. The developing chorion (fetal cotyledon) that creates a tight association over the caruncles establishes the foundation for placental development (Regnault et al., 2002). Fetal derived vascularization of the placenta develops from the allantois. This process occurs 15 dpf after the allantois has expanded from the fetal hind-gut, allowing for fusion with the chorion, the vascularized allantois provides placental

vascularization into the caruncles (Barry & Anthony, 2008). Unlike the human conceptus that completely invades into the endometrium, the sheep conceptus is not invasive. At four weeks of gestation, the fetal and maternal placental tissues start to interdigitate with each other (Barry & Anthony, 2008). On the surface area where the maternal caruncles and fetal cotyledons are closely associated, ridges and grooves form between the two tissues that will give rise to the fetal villi (Regnault et al., 2002). As the maternal caruncles grow, they develop crypts where the cotyledonary villi elongate and branch, forming a network of fetal villi within the maternal caruncular crypts (Regnault et al., 2002). The development of caruncular crypts allowing the branching of cotyledonary villi maximizes the surface area for blood flow and the surface area is regulated by the size of the whole placentome (Regnault et al., 2002). The structural interrelationship of the vessels of caruncular crypts and the fetal cotyledonary villi is closed, without any formation of lacunas (Leiser et al., 1997). Blood flows in a strictly determined way, whereas in the human, maternal blood flow circulates through the lacuna intervillous space in an open fashion (Leiser et al., 1997). Even though the sheep placenta harbors numerous attachment sites to allow for the exchange between maternal and fetal blood flow, the fetal villi structure is similar to the fetal villi in the human placenta. While human fetal villous trees differ in form and size, both species villous trees are made up of the stem, intermediate villi, and terminal villi (Leiser et al., 1997). Although, sheep chorionic villi extend into the maternal caruncle whereas human chorionic villi exist in the intervillous space where they are bathed in maternal blood. Another similarity shared between these species is the fetal vessels within the villi are comprised of stem arteries and veins, intermediate arterioles and venules, and terminal capillaries that are analogous to the

human (Barry & Anthony, 2008). While there is no perfect comparison to the human placenta, sheep and humans share important similarities in functional structures in their placenta. As previously mentioned, the similarity in fetal derived vascular structures as well as the maturity of the fetus at birth and the ability to obtain maternal and fetal blood samples during gestation makes the sheep an ideal research model to study placental development (Barry & Anthony, 2008). Specifically, sheep can be utilized to induce FGR to further understand the pathophysiology of FGR in human fetal development without the need of human experimental subjects.

SHEEP MODELS OF IUGR

While FGR has many etiologies associated and investigating the exact singular cause of FGR may not be a realistic goal, we can still address the mechanisms that are involved in the development of FGR. Many methods have been developed to induce FGR pregnancies in experimental sheep models. FGR can be induced in sheep pregnancies by nutrient restriction, overfeeding, administration of glucocorticoids, utero-placental embolism, limitation of placental implantation sites (carunclectomy), umbilical artery ligation, and exposure to heat stress (Regnault et al., 2002; Anthony et al., 2003).

Maternal nutrient restriction

In 1948, Wallace established the ability to restrict fetal growth by maternal undernutrition in sheep throughout most of pregnancy and this experimental model has further been utilized over the years (Wallace, 1948). Understandably so, the fetus is reliant on maternal nutrient delivery via maternal blood flow to the fetus for optimal growth

and development. When nutrients are significantly altered, we could assume it would directly impact the fetus and possibly placental growth. Maternal nutrient restriction at any time during gestation has shown to have long-term ramifications on the health of the offspring, yet fetal growth restriction only occurred when nutrient restriction was during mid- or late gestation (Roseboom et al., 2001). Nutrient restriction from early to mid-gestation (approximately day 30-80) in sheep does not result in fetal growth restriction or reduced placental weight near term (Steyn et al., 2001). Yet as mentioned previously, nutrient restriction from early to late gestation results in significant reductions in fetal and placental weight (Mellor and Murray, 1982; Wallace, 1948). Furthermore, nutrient restriction during late gestation results in both fetal growth restriction and no fetal growth restriction, which these varying results may have been dependent on maternal nutrient reserves at the onset of restriction (Mellor, 1983; Chandler et al., 1985). It has also been shown that maternal nutrient restriction results in reduced vascular endothelial growth factor (VEGF), a placental endothelial proliferation promotor during placental development, protein by 45 dGA in caruncle tissue and 90 dGA in cotyledonary tissue of the placenta (Reed et al., 2022). Altogether, these findings indicate that nutrient restriction affects the fetus directly more so during mid- to late gestation. During maximal placental development from early to mid-gestation, nutrient restriction does not result in placental insufficiency (Anthony et al., 2003). Since the degree of which maternal nutrient restriction is only effective at inducing FGR from mid- to late gestation, it does not truly model human FGR. Nevertheless, these studies have been fundamental in developing our understanding of fetal programming (Barker, 1997). Not only does undernutrition impact the growth of the fetus during development, but it can lead to long-term health

ramifications for the offspring. When sheep underwent nutrient restriction from 28-79 dGA, the male offspring demonstrated increased body weight and fat deposition up to 280 days of age as well as dysregulated glucose uptake (Ford et al., 2007). The pregnant ewes were fed the control diet after the period of nutrient restriction during early to mid-gestation. These lambs were not growth restricted at birth, so this could suggest fetal growth is able to recover with proper nutrition after a period of nutrient restriction.

Maternal overnutrition

While we could expect over-nourished pregnant ewes to develop healthy lambs, or even large for gestational age lambs, to term with excess to give to the fetus, excess maternal nutrition on singleton-bearing adolescent ewes throughout gestation results in FGR with significant reductions in fetal and placental weight (Wallace et al., 1996, 1997, 1999a). The maintenance of maternal growth seems to take priority in response to overnutrition to promote maternal tissue synthesis at the expense of the nutrient requirements of the gravid uterus (Wallace et al., 1997, 1999a). Evidence to further support this conclusion appeared when the carcasses of ewes on the over-nourished diet displayed increased body fat deposition (Wallace et al., 1999b). Similar to recovering maternal nutrition in the undernutrition models by mid-gestation, reducing the dietary intake from overnutrition to moderate nutrition at 50 dGA recovers placental and fetal growth, suggesting maternal overnutrition effects on fetal and placental growth can be reversible in early gestation. (Wallace et al., 1999b). On the other hand, shifting the maternal moderate diet to a high intake diet at 50 dGA results in reduced fetal and placental growth (Wallace et al., 1999b). The overnutrition model has been beneficial in

understanding human FGR pregnancies in that the fetus displays similar physiological responses when the ewe is over-nourished throughout gestation. The similarities include fetal hypoxia and hypoglycemia by late gestation, which is thought to be due to reduced uterine arterial blood flow, reduced umbilical venous blood flow, and reduced umbilical venous oxygen and glucose uptake accompanied with reduced placental and fetal weight (Wallace et al., 2002). The changes observed in uterine-umbilical blood flow and umbilical nutrient uptake could be a result of reduced placental and fetal size, rather than altered placental vascularity or resistance (Anthony et al., 2003). Glucose clamp studies indicated that reduced placental size is responsible for fetal hypoglycemia, rather than altered placental glucose transport capacity (Wallace et al., 2003).

Administration of glucocorticoids

An important class of corticosteroid steroid hormones are glucocorticoids, which are synthesized and secreted by the adrenal gland. Nearly every tissue type in the body has glucocorticoid receptors, so they affect a variety of organ systems. These hormones are anti-inflammatory and control metabolism in muscle, fat, liver, and bone. Cortisol is amongst one of multiple glucocorticoids and while it's well known for its role in stress response, cortisol regulates multiple physiological processes throughout the body. This includes metabolism regulation of fats, proteins, and carbohydrates, regulates blood sugar levels by counteracting the effects of insulin, has anti-inflammatory actions, influences blood pressure changes, aids in the development of the fetus, and can play a role in triggering parturition.

During late gestation, increased concentrations of cortisol in fetal circulation have been associated in the maturation of several organ systems (Anthony et al., 2003). Women at risk of preterm delivery receive administration of synthetic glucocorticoids to stimulate fetal lung maturity due to complications of prematurity (Anthony et al., 2003). In spite of glucocorticoid administration used to treat underdeveloped fetal lungs, prenatal exposure to glucocorticoids can negatively impact fetal growth and the risk-to-benefit of this treatment is questionable. Experimental findings have repeatedly revealed fetal glucocorticoid exposure is associated with reduced fetal birth weight in humans, non-human primates, and sheep (Reinisch et al. 1978; Newnham et al., 2001; Novy & Walsh, 1983; Fowden et al., 1996) as well as reduced fetal head circumference, decreased thymus, adrenal, spleen, placental weight, and increased fetal liver weight (Novy & Walsh, 1983; Johnson et al. 1979; Newnham et al., 2001; Jensen et al., 2002). Fowden (1996) monitored the growth rate of sheep during late gestation after manipulating fetal plasma cortisol via exogenous infusion and fetal adrenalectomy. Control fetuses displayed normal prepartum increase in fetal plasma cortisol levels five days before birth. The adrenalectomized fetuses did not display the cortisol surge days before delivery and were larger in birth weight and crown-rump length compared to the controls. Infusion of cortisol for five days preterm increased fetal plasma cortisol levels and decreased CRL increments, which is similar to what is observed in untreated controls during the last 5 days before delivery. Long-term ramifications of the offspring from prenatal exposure to glucocorticoids during late gestation in rats have been reported and include hypertension, hyperglycemia, and hyperinsulinemia (Seckl, 2001). Furthermore, lambs exposed to single or repeated maternal or fetal glucocorticoid treatments display insulin resistance

(Newnham et al., 2001b) and weekly maternal doses resulted in delayed myelination of the central nervous system and altered blood pressure (Newnham, 2001b).

Maternal administration of glucocorticoids during late gestation in sheep are used to investigate how this circumstance results in fetal consequences, which has resulted in reduced fetal growth (Jobe et al., 1998; Moss et al., 2001) and some evidence of reduced placental weight (Jensen et al., 2002). While these instances mimic human FGR pregnancies, fetal blood gases and glucose concentrations are not altered that would more closely recapitulate what has been observed in human FGR pregnancies (Sloboda et al., 2000; Jensen et al., 2002). Furthermore, uterine and umbilical blood flow or placental nutrient transport has not been evaluated in this model (Anthony et al., 2003). Since we don't have blood flow or nutrient transport data to make a confident assumption on the impact of fetal growth from altered placental function and nutrient transfer, we can infer that prenatal exposure to glucocorticoids in late gestation negatively affects fetal growth directly (Anthony et al., 2003). Administration of glucocorticoids in early gestation, 27 dGA, did not affect fetal or placental growth, yet long term ramifications on the offspring, like hypertension, were a consequence (Dodic et al., 2002). While maternal administration of glucocorticoids is useful in understanding baseline aspects of fetal growth, it's not a sufficient model to interpret the deeper context of human FGR pregnancies without placental insufficiency. A more useful application for these studies could be geared towards the increased stress response observed in pre-eclamptic pregnancies (Anthony et al., 2003).

Utero-placental embolism

The utero-placental embolism model creates stunted placental function by reducing uteroplacental blood flow, resulting in reduced placental weights that produce fetal growth restriction (Anthony et al., 2003). Uterine perfusions in humans increases from 50 ml/min during week 10 of gestation to 1,300 ml/min at the end of gestation (Lang et al., 2000). Impaired uteroplacental perfusion is considered to be the most likely cause of FGR due to reduced substrate and oxygen delivery to the fetus (Lang et al. 2000). The techniques involved in creating this model include infusion of microspheres into the catheterized fetal descending aorta that end up embed into placental capillaries, vessel occlusion, and ligation of the umbilical artery (Anthony et al., 2003; Beede et al., 2019). Infusion of microspheres resulted in nearly a 40% increase in placental vascular resistance and reduces blood flow by 33%, resulting in reduced placental and fetal growth (Beede et al., 2019). A common downfall of this experimental design is necrosis of placental tissue and embolization-induced uterine blood flow restriction. The resulting effects of reduced placental substrate exchange can't be distinguished from the original effects of embolization of the uteroplacental vasculature with microspheres (Lang et al., 2000). Vascular occlusion is performed in the last third gestational period and uses an externally adjustable vascular occludar that controls uterine perfusion. This allows for regulation of uteroplacental perfusion through the end of gestation and the ability to study the effects of reduced uterine blood flow on fetal growth (Lang et al., 2000). Resulting fetal weights were reduced by 15% (Lang et al., 2000). Single umbilical artery ligation is performed early in the last third gestational period and has shown to reduce fetal growth by ~22% (Supramaniam et al., 2006). Reduced blood flow, fetal hypoxia and hypoglycemia, reduced oxygen and glucose fetal uptake, and fetal hypertension resulting

from the utero-placental embolism model are components that recapitulate human FGR pregnancies (Anthony et al., 2003). These techniques provide a step towards inducing placental insufficiency by reducing utero-placental blood flow compared to the previously mentioned models. Although, the reduction of blood flow is artificial in this model whereas placental insufficiency truly is derived from the progression of the placenta itself (Anthony et al., 2003). Furthermore, the degree of blood flow reduction is variable, which may be a result from variation in gestational age, degree of embolism achieved in, and site of embolism or occlusion (Anthony et al., 2003).

Carunclectomy

Another method to induce FGR in sheep pregnancies is to perform a carunclectomy, which is the surgical removal of uterine caruncles (placental attachment sites) 10 weeks before mating (Alexander, 1964; Anthony et al., 2003; Beede et al., 2019). To gain an appreciation for the drastic number of caruncles removed, these procedures typically leave behind only 4 functional caruncles resulting in reduced fetal growth of ~26% near term (Robinson et al., 1979; Zhang et al., 2016; Beede et al., 2019). This results in significant placental and fetal weight reductions, but not every instance results in FGR, which means the placenta is able to compensate (Owens et al., 1987, 1989). The FGR pregnancies produce fetuses that are hypoxic and hypoglycemic, have reduced umbilical uptake of oxygen and glucose, and the pregnancies overall have reduced uterine and umbilical blood flow (Owens et al., 1987; 1989). This model also recapitulates components that mimic human FGR pregnancies, but more information is needed to infer if the results are solely based from placental attachment site reductions or nutrient and

substrate transport deficits (Anthony et al., 2003). Furthermore, the manipulation of placental attachment sites does not provide an understanding of structural or functional changes in the placentas developed in these pregnancies. Further information would be beneficial in this model of FGR to investigate any altered placental vasculature and if there are differences between the placentas of carunclectomized FGR pregnancies versus carunclectomized pregnancies that result in normal fetal growth (Anthony et al., 2003). No changes in fetal arterial pressure may indicate that umbilical artery blood flow is not altered in these FGR pregnancies as they are in human FGR pregnancies (Edwards et al., 1999; Anthony et al., 2003). However, this established model of FGR has been beneficial in providing understanding effects of fetal programming (Anthony et al., 2003).

Exposure to heat stress

Hyperthermic induction of FGR in pregnant ewes has produced a well-characterized sheep model of FGR as it repeatedly creates placental insufficiency. Therefore, recreating a model closely mimicking human FGR pregnancies (Beede et al., 2019). This model was originally developed in 1971 and exposes the ewe to high ambient temperatures from early to late gestation, which results in severe cases of FGR (Alexander and Williams, 1971). Regular experimental use of this model began in the late 1980's and the parameters have since been refined to 35-40 °C and ~35% relative humidity for 50 to 60 consecutive days of gestation beginning by 40 dGA (Beede et al. 2019). The degree of FGR can be manipulated by exposing the pregnant ewes to heat stress for fewer days during their gestational period (Beede et al., 2019). Exposing pregnant ewes to heat stress from mid- to late gestation (64 to 136-141 dGA) resulted in

20%-30% fetal weight reductions and ~60% placental weight reductions (Bell et al., 1989; Early et al., 1991). Heat stress exposure during early to late gestation (33-39 to 112-135 dGA) resulted in even greater reductions of fetal weight from 50%-60% and similar reductions in placental weights as seen in heat exposure from mid- to late gestation (Ross et al., 1996; Anderson et al., 1997; Thureen et al., 1992). Similar results of fetal and placental growth reductions were observed when the ewes were exposed to heat for just 55 days from 37 dGA to 93 dGA (Galan et al., 1999). This model consistently reduces placental mass by up to 64% and fetal growth by 30% to 60% by the end of gestation (Anthony et al., 2003; Limesand et al., 2018). The hyperthermic exposure timeframe is used to correspond with peak placental growth to regulate FGR with placental insufficiency rather than fetal hyperthermia (Beede et al. 2019). Evidence of this is seen when FGR and the associated pathologies are not observed at the end of the hyperthermic period (90 to 105 dGA), but present afterwards and progressively worsen as the fetus is attempting to develop in the stunted placenta (Limesand et al., 2018). Fetal growth in these pregnancies is asymmetric, rather than symmetric, which is indicated by a greater biparietal diameter: abdominal circumference ratios and reduced ponderal indices (Galan et al., 1999; Regnault et al., 1999).

Furthermore, the fetuses from these studies are hypoxic and hypoglycemic, which resulted from reduced umbilical oxygen and glucose uptake (Anthony et al., 2003). Uterine and umbilical blood flows are also reduced and umbilical vein blood flow when normalized to fetal size is reduced (Anthony et al., 2003). Hypo- and hyper-glycemic clamp studies revealed reduced capacity for placental glucose transport in this model of FGR (Thureen et al., 1992). Reduced transport of nutrients and substrates is not directly

related to reduced placental mass as placental transport was still reduced when normalized to placental mass (Anthony et al., 2003). In addition, placental angiogenesis alterations are associated with early versus late onset FGR and expression of a variety of angiogenic growth factors and their respective receptors are altered in the placenta from this model (Anthony et al., 2003). The resulted changes in placental vascular architecture, umbilical Doppler velocity waveforms, and increased fetal arterial blood pressure are evidence of the altered angiogenic growth factors. The data resulting from this model of FGR in sheep is able to closely recapitulate major hallmarks of human FGR pregnancies with dysfunctions in both placental structure and function.

Lentivirus mediated RNA interference

More recently, the use of *in vivo* RNA interference (RNAi) to specifically target the placenta and reduce specific gene products has provided a method to investigate placental insufficiency (Purcell et al., 2009; Baker et al., 2016). RNAi is a mechanism by which double-stranded RNA (dsRNA) activates sequence-specific gene silencing (Purcell et al., 2009). The basis behind shRNA-mediated RNAi is based on our understanding of how micro-RNA (miRNA) are generated, processed, and function (Tanner et al. 2022). miRNAs are transcribed endogenously by RNA polymerase II as long primary miRNA transcripts (pre-miRNAs) that contain a stem-loop structure, which contains the mature miRNA (Sheu-Gruttadauria & MacRae, 2017). Pre-miRNA transcripts are processed in the nucleus by the RNase III activity of Drosha, the primary nuclease that initiates miRNA processing in the nucleus, by removing the pre-miRNA hairpin from the stem loop (Sheu-Gruttadauria & MacRae, 2017; Tanner et al. 2022). This yields a pre-miRNA with a hairpin-

loop structure that is exported out of the nucleus. The pre-miRNA is then processed in the cytoplasm by the cytoplasmic RNase III enzyme Dicer, producing an RNA duplex of 22 nucleotides long, mature miRNA (Sheu-Gruttadauria & MacRae, 2017; Tanner et al. 2022). The mature miRNA duplex is a guide sequence that incorporates into the RNA-induced silencing complex (RISC), a ribonucleoprotein that functions in gene silencing and is ultimately responsible for the gene silencing phenomenon of RNAi, and is loaded into Argonaute, cleaving the mRNA and retaining a single strand of the duplex (Sheu-Gruttadauria & MacRae, 2017; Tanner et al. 2022). This strand functions as the guide strand for targeted mRNA degradation (Sheu-Gruttadauria & MacRae, 2017; Tanner et al. 2022).

When shRNA is introduced into cells the shRNA is processed in the cell like endogenous miRNA, providing targeted-degradation or translational repression of that mRNA (Tanner et al. 2022). Within the targeting vector, the shRNA-encoding cassette is inserted downstream of an RNA polymerase III promoter (Tanner et al. 2022). The construct used by Purcell et al., (2009), LentiLox 3.7 (LL3.7), contains an enhanced green fluorescent protein (EGFP) expression cassette, which allows for *in vitro* visualization of successful infection by the lentivirus construct. The construct also contains an insertion site for the shRNA cassette downstream of the mouse U6 promoter, which is an RNA polymerase III promoter (Tanner et al. 2022). Previously reported in Baker et al., (2016), the LL3.7 vector was modified by replacing the mouse U6 promotor with the human U6 promotor and was found to be more efficient (Jeckel et al., 2018; Ali et al., 2020a; Tanner et al., 2021a,b). The use of lentiviral vectors to create transgenic animals has been previously reported in other species (Park, 2007) and more recently has been applied to

a variety of genes expressed in the developing sheep conceptus throughout gestation (Purcell et al., 2009; Baker et al., 2016; Jeckel et al., 2018; Ali et al., 2020a; Tanner et al., 2021a,b). *In vivo* RNAi within sheep placenta has been developed to generate models of altered placental function and allows for the direct assessment of the effect of specific gene product deficiencies throughout gestation (Tanner et al., 2021a,b).

Lentivirus mediated RNAi is used by infecting the trophectoderm of a hatched blastocyst, which in sheep would be 8 to 9 dGA, with a lentiviral construct expressing a gene specific shRNA (Purcell et al., 2009; Baker et al., 2016). Lentivirus is replication-deficient, so it is incapable of replicating outside of the trophectoderm (Tanner et al., 2022). Therefore, only the cells of the trophectoderm that surround the blastocyst are infected with the lentivirus during culture, avoiding any alterations to the inner cell mass (Tanner et al., 2022). Another benefit of this method is that lentivirus is stably integrated into the host genome, allowing expression of the shRNA to continue throughout gestation within the placenta (Tanner et al., 2022). Therefore, the impact of lentivirus gene silencing is long-term compared to other methods like the use of morpholino oligonucleotide mediated gene silencing in which the lasting effects of this method are lost as the conceptus and placenta proliferates (Dunlap et a., 2006). Purcell et al. (2009) demonstrated the vigor of lentivirus mediated RNAi in sheep placenta by targeting proline-rich 15 (PRR15) mRNA for degradation to investigate the role of PRR15 in sheep conceptus development during early gestation (Purcell et al., 2009). Embryos treated with lentivirus expressing shRNA against PRR15 mRNA did not elongate and most did not survive compared to the control embryos infected with a lentivirus expressing no shRNA (Purcell et al., 2009). Although, the efficacy of lentivirus mediated RNAi has been shown

to be more robust *in vitro* than it is *in vivo*. *In vitro*, the targeted mRNA is reduced ~90% and an 80%-90% reduction in the specific protein compared to 50%-60% mRNA reductions and 30%-40% placental protein reductions *in vivo* (Tanner et al., 2022). While the degree of product reduction within the placenta is slightly variable, it has led to significant phenotypic changes inducing FGR in sheep with placental insufficiency (Baker et al., 2016; Tanner et al., 2021b). This methodology allows for a new approach to learn from steady-state *in vivo* assessment of maternal-placental-fetal interactions and provides new insight into ruminant placental function (Tanner et al., 2022).

CHORIONIC SOMATOMAMMOTROPIN

Another critical function of the placenta is its capabilities to produce placental derived hormones. One of the most abundant placental-derived hormones is chorionic somatomammotropin (CSH), also known as placental lactogen (PL). CSH is a protein hormone with similar homologies in its chemical and biological properties to growth hormone (GH) and prolactin (PRL) (Handwerger, 1991). In humans it has been established that CSH is produced by the cytotrophoblasts during the first 6 weeks of gestation, then synthesized and secreted by the syncytiotrophoblast cells (Hoshina et al., 1982; Maruo et al., 1992). CSH is secreted into fetal and maternal circulation, has been identified in the amnion, and can be detected in maternal serum as early as 3 weeks of gestation (Kappes et al., 1992; Anthony et al., 1995; Anthony et al., 1998). Maternal concentrations substantially increase during the first trimester and then display more gradual increases to term (Anthony et al., 1998). CSH has both lactogenic and somatotropic effects, being in the growth hormone and prolactin family (Kappes et al.,

1992). In 1936, Ehrhardt was able to demonstrate prolactin activity in extracts of human placenta, which provided the first evidence for CSH (Ehrhardt, 1936). It wasn't until 1962 when CSH was successfully isolated from human term placentas and from maternal blood, which at the time was termed human placental lactogen (hPL) (Josimovich and MacLaren, 1962). Although it was established that CSH is produced by the placenta, its exact role during pregnancy was not well established. In 1976, women with pregnancies complicated by FGR had serum CSH concentrations measured in comparison to normal pregnancies and the FGR pregnancies displayed significant reductions in maternal CSH concentrations along with significantly reduced fetal and placental weights. This was the first indication of the possible importance that CSH is involved with regulating normal fetal and placental development (Spellacy et al., 1976). A later study in 1979 supported these findings when reduced birth weight and placental weight from FGR pregnancies displayed reduced maternal venous CSH. An unexpected finding was that CSH was lower in some pregnancies that had normal birth weight neonates. The presumed normal neonates had low ponderal indices (PI) or short crown-heel length (CHL), suggesting that normal birth weight found in concurrence with reduced CSH, low PI, and short CHL. This suggested that despite the pregnancies presenting as normal, there could be underlying complications representing unsuspected FGR related to reduced CSH and reduced CSH isn't always the causal factor of low birth weight (Daikoku et al., 1979).

Ruminant CSH

Studies involving ruminant animal models to investigate CSH established CSH is also produced in ovine placentas (Martal and Dijane, 1975). Within the sheep placenta,

CSH is produced by the chorionic binucleate cells, which are comparable to the syncytiotrophoblast cells CSH is produced by in humans, and can be detected by 16 dGA in the trophectoderm of sheep embryos (Anthony et al., 1995). Chorionic binucleate cells migrate from the chorionic epithelium and fuse with the uterine epithelium to form the syncytium. This provides a method of delivery for CSH to get to maternal circulation from the placenta (Anthony et al., 1998). The secretion of CSH into maternal and fetal circulations seems to be regulated separately (Kappes et al., 1992). Furthermore, there is not an established understanding of how CSH is delivered into fetal circulation but may be due to a population of binucleate cells that don't migrate into the maternal-fetal interface of the placentome (Wooding, 1992; Kappes et al., 1992). CSH is detectable in sheep maternal serum by 50 dGA and concentrations increase throughout gestation to term (Anthony et al., 1998). This increase in CSH concentrations is a function of the relative increase in chorionic binucleate cells throughout gestation rather than an increase in CSH production by individual binucleate cells throughout gestation (Anthony et al., 1998). Within fetal blood, serum concentrations of CSH peak by mid-gestation and plateau or decline to term. The plateau or decline is misleading because the entry rate of CSH into umbilical circulation continues to increase throughout gestation, but is masked by total fetal size and fetal blood volume (Schoknecht et al. 1992) and not necessarily that the secretion of CSH into fetal circulation plateaus (Anthony et al., 1998). Supporting this theory is evidence of increased fetal cotyledonary CSH mRNA up until 120 dGA followed by a decline in cotyledonary CSH mRNA at 135 dGA (Kappes et al., 1992).

In correlation to the findings in human FGR pregnancies, CSH measured in sheep maternal and fetal plasma samples revealed maternal CSH was correlated to the fetal

birth weight (Taylor et al., 1980). Similarly, Kappes reported maternal serum CSH and fetal cotyledonary CSH mRNA levels were correlated with total fetal weight. Moreso, by collecting fetal and maternal blood samples across gestation, Kappes calculated standard partial regression coefficients to evaluate the effects of maternal serum CSH and fetal cotyledonary CSH mRNA on total fetal weight. This resulted in a statistically robust correlation of maternal CSH and cotyledon CSH mRNA with total fetal weight and together accounted for 80.6% of the variation found in fetal weight (Kappes et al., 1992).

Biological actions of CSH

The biological actions of CSH have been thought to stimulate gonadal steroidogenesis, modulate maternal metabolic pathways, and augment the development of mammary glands to produce milk. (Anthony et al., 1998). Luteotropic actions have been established in the mouse and bovine, but in humans and sheep support of the corpus luteum (CL) by CSH is sparse. Assessment of luteotropic activity of mouse CSH (mCSH) at mid-gestation revealed a dose-dependent increase in progesterone of mouse ovarian cells treated with mCSH. *In vivo* studies using mice that were hysterectomized on day 9 of pregnancy received injections of pregnancy serum obtained from pregnant mice on day 10 of pregnancy in an attempt to maintain circulating progesterone concentrations similar to the concentrations observed prior to the hysterectomy. Serum progesterone concentrations were maintained and depletion of mCSH from 10 dGA pregnancy serum by affinity chromatography on an anti-mCSH column removed all luteotropic activity compared to the nonspecific control column that did maintain progesterone production. Therefore, this provided evidence that CSH in mice supports progesterone production at

mid-gestation (Galosy and Talamantes, 1995). During day 0-21 of the estrous cycle, Cattle treated with bovine CSH (bCSH) produced larger CL and higher plasma progesterone compared to controls after day 9 of the estrous cycle (Lucy et al., 1994). In contrast, women treated with human CSH (hCSH) did not have success extending the luteal phase of the menstrual cycle, after ovulation and before menstruation, while human chorionic gonadotropin resulted in an extended luteal phase, postponing the onset of menstruation (Stock et al., 1971). Infusion of ovine CSH (oCSH) into the arterial supply of the sheep ovary for 2 hours on day 12 of the estrous cycle, when progesterone production starts decreasing and PGF2α rises to regresses the CL, did not result in increased progesterone nor did it prevent luteal regression during PGF2α administration (Schramm et al., 1984). Sheep do not require progesterone production from the CL throughout the entirety of gestation, so luteal support by CSH throughout gestation would not exist (Anthony et al., 1998).

CSH regulation of metabolism

CSH is considered to regulate nutrient supply for fetal growth. The hypothesis that CSH affects fetal growth by acting as the “growth hormone of pregnancy” was derived from the understanding that CSH has both growth hormone-like and prolactin-like activity. (Handwerger, 1991). Handwerger further established CSH acts on the fetal and maternal systems in distinguishable ways, which divulges the complex nature of this placental hormone.

CSH regulation of maternal metabolism

It has previously been suggested that CSH plays a role in increasing maternal insulin-like growth factor 1 (IGF1) concentrations that are observed in the third trimester of pregnancy (Handwerger, 1991). This theory arose from evidence that ovine CSH (oCSH) stimulates IGF1 production in hypophysectomized rats, increased IGF1 concentrations during pregnancy in rats parallels the increase in human CSH (hCSH) concentrations, and lastly, pregnant rats and sheep maintain normal plasma IGF1 concentrations after hypophysectomy until after the placenta is delivered (Hurley et al., 1977; Breuer, 1969; Daughaday and Kapadia, 1978; Handwerger 1991). After delivery of the placenta, IGF1 concentrations in the hypophysectomized animals rapidly decreased to concentrations observed in growth hormone deficient animals (Handwerger, 1991).

Mimicking comparable actions of GH, CSH has effects on carbohydrate and protein metabolism (Handwerger, 1991). Evidence of CSH's involvement in nutrient manipulation and metabolism has developed the idea that hCSH promotes insulin production, impairs glucose tolerance, and stimulates lipolysis (Handwerger, 1991). Administration of CSH to pregnant women enhanced insulin secretion and impaired glucose tolerance (Handwerger, 1991). hCSH has been reported to stimulate insulin synthesis and secretion in islet cells of the pancreas, where insulin and glucagon are produced, from rats (Martin and Friesen, 1969) and hCSH administration to isolated adipose cells from rats stimulated lipolysis (Felber et al., 1972). Due to the drastic increase in CSH during gestation, Grumbach et al. (1968) hypothesized that CSH has a major role during pregnancy to act as a maternal "growth hormone" during the second half of pregnancy and acts as an antagonist of insulin by inducing glucose tolerance, lipolysis, and proteolysis (Handwerger, 1991).

CSH regulation of fetal metabolism

The effects of CSH on fetal metabolism originally were thought to act indirectly by changes in maternal metabolism and not by the direct effects of CSH on the fetus (Handwerger, 1991). Previous studies have indicated that CSH affects fetal growth by acting directly on fetal tissues (Handwerger, 1991). In support of the hypothesis that CSH plays an important role in regulating fetal growth, oCSH was shown to stimulate rat ornithine decarboxylase (ODC) activity in fetal liver after intraperitoneal administration (Butler et al., 1978). ODC is the rate limiting step in the polyamine biosynthesis of cells and polyamines are essential for cell proliferation, protein, RNA, and DNA synthesis (Handwerger, 1991). CSH has shown to stimulate amino acid transport in fetal muscle (Handwerger, 1991) as well as the diaphragm of fetal rats (Freemark and Handwerger, 1983). On day 20 of gestation, oCSH stimulated a dose-dependent increase in amino acid uptake, supporting the hypothesis that CSH has a direct role in regulating fetal amino acid and protein metabolism (Freemark and Handwerger, 1983). oCSH is also involved in stimulating glycogen synthesis in isolated hepatocytes of fetal rats and lambs (Freemark and Handwerger, 1984; 1986). Using hepatocytes from 20-day old fetal rats cultured *in vitro*, oCSH stimulated dose-dependent increases in glycogen (Freemark and Handwerger, 1984). Cultured ovine fetal hepatocytes also demonstrated dose-dependent increases in glycogen and total cellular glycogen content by stimulation of oCSH (Freemark and Handwerger, 1986). CSH promotes glycogen storage in the fetal liver, which the accumulation of hepatic glycogen in the fetus is a critical source of glucose of the neonate brain and red blood cells (Handwerger, 1991). Handwerger (1991) proposed

that many of the somatogenic activities of CSH are mediated through IGF's. *In vitro* studies using cultured rat embryo fibroblasts incubated with oCSH stimulated DNA synthesis and IGF production, specifically IGF2 (Adams et al., 1983a). They observed a 2-fold increase in IGF2 levels after incubation with oCSH for 48 hours, appearing to be a result from stimulating IGF2 synthesis rather than the inhibition of IGF2 degradation (Adams et al., 1983a). IGF2 degradation occurs via the IGF type 2 receptor (IGF2R), which solely interacts with IGF2 and not IGF1, and functions to internalize and degrade IGF2. IGF2 is heavily associated with proper fetal growth and development in rats (DeChiara et al., 1990). Furthermore, nonlactating dairy cows injected with bCSH for 5 days produced increased concentrations of IGF1 and IGF2 (Byatt et al., 1992). When oCSH was infused into fetal sheep vasculature at the end of gestation (122-135 dGA), fetal serum IGF1 concentrations increased (Schoknecht et al., 1992). Finally, human fetal fibroblasts released various components of the IGF axis when stimulated by hCSH including IGF1, IGF2, and IGF binding proteins (IGFBP's) (Hill et al., 1989). This data investigating IGF production via the stimulation of CSH provided further evidence of an important mechanism CSH uses to regulate fetal growth.

While a helpful amount of data exists to investigate the role of CSH during pregnancy in a variety of *in vitro* and *in vivo* studies, it has only scratched the surface of what CSH is capable of. Surprisingly, as we know the placenta produces CSH in considerable amounts, the priority to investigate the possible involvement of CSH on placental function is lacking. Furthermore, with the understanding that CSH clearly has growth-promoting effects and affects maternal and fetal metabolism independently, it would be safe to assume there's a considerable chance CSH potentiates the placenta's

ability to transfer nutrients and produce growth promoting factors. Developing an animal model to allow for the use of an *in vivo* assessment of CSH function throughout pregnancy would allow for a more accurate and in-depth investigation of the effects of CSH on placental function.

Development of CSH RNAi

While there have been previous attempts to investigate the function of CSH *in vivo* using sheep models, some of these attempts used a methodology that only measured the impacts of CSH at a certain timepoint during gestation by infusing CSH into fetal circulation (Oliver et al., 1995; Schoknecht et al., 1996). These studies resulted in minimal effects. Since CSH is produced from nearly the very beginning of gestation to the very end, it's necessary to investigate its effect throughout the entirety of gestation and divulge if it's needed throughout gestation and why. Other methodologies have been developed to alter the bioavailability of CSH through immunization against CSH, which also resulted in minimal effects (Walters et al., 1985; Leibovich et al., 2000). Manipulation of a hormone's gene product would more effectively alter the effects *in vivo* and provide a more compelling approach to investigate the function of CSH.

The more recent development of lentiviral-mediated RNAi has been beneficial to target a specific gene and allow for the direct assessment of the effect of that specific gene product. As previously described in the sheep models of FGR section, the use of lentiviral-mediated RNAi to target gene function in the sheep placenta was developed by Purcell et al. (2009) using lentiviral pLL3.7 vectors. Since then, this methodology has been extensively utilized to study alterations caused by reduced CSH within the sheep

placenta and its effects on fetal growth, placental nutrient and substrate transfer, and utero-placental blood flow alterations (Baker et al., 2016; Jeckel et al., 2018; Tanner et al., 2021b). The generation of CSH RNAi applied in the sheep placenta was first reported in Baker et al. (2016) with the objective to investigate CSH gene product deficiency throughout gestation and predicted CSH deficiency could reduce fetal growth and development. Creation of lentivirus target and control groups involved the construct of lentiviral vectors, specifically pLentiLox3.7 (hLL3.7) with a CSH-targeting sequence (hLL3.7 CSH shRNA) and a control non-targeting sequence (hLL3.7 NTS shRNA). A differentiating component of these lentiviral constructs compared to the pLL3.7 vectors used in Purcell et al. (2009) was the removal of the original mouse RNA polymerase III U6 promoter and exchanging it with the human RNA polymerase III U6 promoter. To generate CSH RNAi pregnancies, donor ewes underwent estrus synchronization, were bred by intact rams, and expanded hatched blastocysts were collected at 9 dGA (Baker et al., 2016). Blastocysts were infected with the hLL3.7 constructs, either the hLL3.7 CSH shRNA or the hLL3.7 NTS shRNA control, cultured for ~5 hours with the lentivirus, then washed and surgically transferred into synchronized recipient ewes (Baker et al., 2016). At 135 dGA, the pregnancies were terminated and ewes were euthanized for tissue harvest (Baker et al., 2016).

Baker et al. (2016) demonstrated that *in vivo* RNAi successfully assessed the function of CSH throughout gestation to term and further established CSH is critical for fetal growth and placental function. Pregnancies that exhibited significant placental and fetal growth restriction (hLL3.7 CSH shRNA) resulted in a 52% reduction in placental weight and a 32% reduction in fetal weight compared to the control pregnancies (hLL3.7

NTS shRNA) (Baker et al., 2016). The drastic reduction on placental mass due to CSH RNAi was not an expected outcome for this study. This started the interest in how CSH impacts placental growth and development, leading to FGR in these pregnancies. Furthermore, CSH RNAi pregnancies resulted in another phenotype other than FGR. The pregnancies that exhibited placental and fetal weights that were 2 standard deviations (SD) below the mean of the NTS RNAi controls were distinguished as placental insufficiency-FGR (PI-FGR). The other phenotype resulted in pregnancies with similar placental and fetal weights as the controls. These CSH RNAi pregnancies were distinguished as non-FGR pregnancies (Baker et al., 2016). This disparity in CSH-deficient phenotypes has been reported in human studies and while this disparity has been established, it raises the question as to how some CSH deficient pregnancies result in severe FGR and others result in normal fetal growth (Alexander et al., 1982; Barbeiri et al., 1986; Borody and Carlton, 1981; Daikoku et al., 1979; Lindberg and Nilsson, 1973; Rygaard et al., 1998; Sideri et al., 1983; Simon et al., 1986). The PI-FGR pregnancies also produced reduced fetal liver weights, fetuses associated with hypoinsulinemia, reduced fetal crown-rump lengths, and reduced cotyledonary *CSH* mRNA concentrations (Baker et al., 2016). Furthermore, the PI-FGR pregnancies had a significantly elevated uterine artery to vein glucose gradient, a significant reduction in umbilical artery IGF1 concentrations of 62%, and significant reductions in I*GF1*, *IGF2*, *IGFBP2*, and *IGFBP3* mRNA concentrations in fetal liver tissue (Baker et al., 2016). Jeckel et al. (2018) further examined the impact of CSH RNAi, but this study observed the effects at 50 dGA instead of term. This study also reported significant reductions in fetal weight and fetal liver weight as well as a ~40% reduction in uterine vein CSH concentrations. Although, there was less

of an effect on placental mass at 50 dGA (Jeckel et al., 2018). Placental *IGF1*, *IGF2*, *SLC2A1* (glucose transporter 1), and *SLC2A3* (glucose transporter 3) mRNA concentrations were also reduced, yet maternal IGF1 and insulin concentrations were not impacted by CSH RNAi at 50 dGA (Jeckel et al., 2018).

With recent reports of the steady-state assessment of blood flow, uterine, and umbilical nutrient uptakes, and uteroplacental utilization under steady-state, non-anesthetized and non-stressed conditions, Tanner and colleagues (2021a) were able to assess placental function with further *in vivo* assessment of CSH RNAi non-FGR pregnancies. In the CSH-deficient non-FGR pregnancies reported, uterine blood flow was significantly reduced by ~23% in these assumed “normal” pregnancies (Tanner et al., 2021a). This gave rise to the notion that while the non-FGR pregnancies don’t display reduced fetal or placental weight, CSH-deficiency is impacting placental function to some degree. Tanner et al. (2021a) also reported significantly increased uterine artery to uterine vein glucose gradients, similar to Baker et al. (2016). Uteroplacental glucose utilization and the fraction of uterine glucose uptake utilized by the placenta was significantly increased, while the fraction of uterine glucose uptake transferred to the fetus was decreased (Tanner et al., 2021a). This study of the non-FGR pregnancies from CSH RNAi established how normal fetal birth weight can still be associated with altered physiological changes and can predispose the fetus to an abnormal in utero environment.

Furthermore, Tanner et al. (2021b) reported the *in vivo* assessment of the CSH RNAi pregnancies with FGR. These FGR pregnancies resulted in fetal weights reduced by 30%, comparable to Baker et al. (2016), yet placental weight was only reduced by 21% (Tanner et al., 2021b). Uterine weight was reduced by 43%, uterine blood flow was

reduced by 42%, and umbilical blood flow relative to fetal weight was reduced (Tanner et al., 2021b). Uterine weight was not reported in the original Baker et al. (2016) study. The Tanner (2021b) study confirmed a repeated effect of CSH RNAi on uterine blood flow, leading to the idea that CSH may have direct actions in regulating uterine blood flow. Uterine glucose uptake, umbilical glucose uptake, and uteroplacental glucose utilization were all reduced 44%-47% (Tanner et al., 2021b). Additionally, oxygen uptakes, oxygen utilization, and essential amino acid uterine uptakes were significantly reduced (Tanner et al., 2021b). Ongoing studies have taken a further look into the group of CSH RNAi pregnancies with placental and fetal weights 2 SD below the mean of the controls (PI-FGR), which exhibited significant reductions in uterine and umbilical blood flows and global nutrient transfer deficiencies similar to what is reported in Tanner et al. (2021b).

The ability to conduct steady-state *in vivo* assessment of placental function with the use of catheterized pregnant sheep has provided a greater understanding of the role of CSH during pregnancy and its involvement in placental function and fetal development. Due to the novel use of *in vivo* RNAi and steady-state assessment of ruminant placental function, it has allowed for an opportunity, previously unfeasible to obtain, to reveal information undiscovered about the role of CSH and placental function. To no surprise, this has opened up more questions to investigate further into the depths of what CSH is capable of during pregnancy. Specifically, attempting to understand how CSH could be manipulating maternal and/or fetal growth factors throughout gestation.

INSULIN-LIKE GROWTH FACTOR AXIS

Insulin-like growth factor's (IGF's) were originally discovered from bone growth studies and are involved in many roles in physiology, growth, development, fetal growth, and placental function (Daughaday and Salmon, 1999; Forbes and Westwood, 2008). The IGF system is a complex network of two soluble ligand peptide hormones (IGF1 and IGF2), two cell surface transmembrane receptors (IGF1R and IGF2R), and six circulating 30 kDA proteins called binding proteins (IGFBP1-6). In the early 1970's, a compound termed sulfation factor was renamed somatomedin due to its somatotropin mediator effects and was grouped in the emerging classification of growth factors (Jones and Clemmons, 1995). At this time when somatomedin was being studied to further understand its biological actions, other studies were attempting to define other factors that stimulated insulin-like effects (Jones and Clemmons, 1995). These studies originally termed IGF's as nonsuppressible insulin-like activity (NSILA) and by 1970 after unsuccessful extraction of both NSILA and somatomedin, it was determined no organ existed that produced concentrated forms of these growth factors, unlike the ability to extract classical endocrine hormones from their tissue source (Jones and Clemmons, 1995). In 1976 the amino acid sequence of NSILA revealed to be 48% homologous with human proinsulin and therefore, was renamed insulin-like growth factor 1 (IGF1) (Rinderknecht and Humbel, 1978a). Sequencing of a second bioactive insulin-like factor revealed a similar sequence to IGF1, giving rise to the identification of IGF2 (Rinderknecht and Humbel, 1978b). Furthermore, sequencing of somatomedin-C revealed an identical sequence to IGF1 and established that the two growth factors were actually the same. From this point the nomenclature was consolidated under insulin-like growth factors (Jones and Clemmons, 1995).

In vitro cell culture studies using human placental cells discovered IGF1 interacted with a specific receptor that was distinct from the insulin receptor and differences in the binding affinity of both IGF1 and IGF2 suggested two different receptors existed (Marshall et al., 1974; Megyesi et al., 1975; Rechler et al., 1980). The ligand-receptor binding initiates the cellular action of IGF1 and IGF2. Most of the cellular effects of the IGF's are mediated by IGF1R and IGF2R is specific to binding IGF2 (Jones and Clemmons, 1995). Binding of IGF2 to IGF2R causes internalization and degradation of IGF2, inhibiting the actions of IGF2 that would be mediated through IGF1R (Jones and Clemmons, 1995). Furthermore, IGF1 and IGF2 are bound in circulation by IGFBP's as they act as transport proteins to control the outflow of IGF's from vasculature. Plasma binding proteins can prolong the half-life of the IGF's and regulate the availability of the IGF's to their specific target tissue and cells (Jones and Clemmons, 1995; Yoshimura, 2003). The IGFBP's also function to either potentiate or inhibit the actions of IGF1 and IGF2 depending on the binding proteins preference for IGF1 and IGF2 and by modulating the interaction with their receptors (Jones and Clemmons, 1995; Firth and Baxter, 2002; Yoshimura, 2003).

IGF1 and IGF2 molecular characteristics

IGF1 and IGF2 are low molecular weight 7.5 kDa single chain polypeptides and as polypeptides, the IGF's are synthesized by many cells and tissues, unlike classical hormones that are secreted by specific endocrine organs (Han and Fowden, 1994). Generally, IGF's rely on *de novo* synthesis for their release from tissues and do not require intracellular storage (Han and Fowden, 1994). While growth factors can be released into circulation, it is believed they do not utilize classical endocrine signaling, but rather

autocrine and paracrine signaling as many different cell types synthesize IGF's (Han and Fowden, 1994). The IGF's affect metabolism, mitogenesis, survival, and differentiation of a wide variety of cell types and therefore, they stimulate growth during fetal development and postnatal growth (Sferruzzi-Perri et al., 2017).

IGF1 and IGF2 biological actions in vitro

The IGF's act as progression factors in the cell cycle and increase DNA synthesis and cellular differentiation in cultured embryos and several fetal cell lines *in vitro* (Han and Fowden, 1994; Gardner et al., 1999). Using 3T3 fibroblasts, a cell line developed from mouse embryos, Stiles et al. (1979) investigated the mechanism of action by which IGF1 acts as a progression factor. IGF1 was required for the transition of quiescent cells from G_0 growth arrest through G_1 and continuation through the rest of the cell cycle, resulting in synthesis and cell proliferation (Stiles et al., 1979; Han and Fowden, 1994). cycle, resulting in synthesis and cell proliferation (Stiles et al., 1979; Han and Fowden, 1994). In addition, IGF1 has been shown to stimulate a mitogenic response in a wide variety of cell types, some of which include chondrocytes, osteoblasts, smooth muscle cells, skeletal muscle cells, neuronal cells, mammary epithelial cells, oocytes, granulosa cells, and several cancer cell lines (Jones and Clemmons, 1995). IGF1 is also a potent inducer of myoblast terminal differentiation, the embryonic precursor of muscle cells, and specifically, IGF1 turns on the expression of the myogenin gene, a muscle-specific transcription factor that coordinates skeletal muscle development (Florini et al., 1991a). Myoblasts also secrete IGF2, which can induce differentiation by autocrine actions of IGF2 (Florini et al., 1991b). IGF2 is also capable of promoting differentiation of

osteoclasts, chondrocytes, neural cells, and other cell types (Jones and Clemmons, 1995).

IGF1 and IGF2 hormone regulation in vitro

IGF1 and IGF2 function to regulate hormone secretion from a variety of cell types, like ovarian granulosa cells (Jones and Clemmons, 1995). In porcine granulosa cells, IGF1 stimulates pregnenolone and progesterone production while IGF2 only stimulates progesterone production (Giudice, 1992). IGF1 enhances follicle stimulating hormone (FSH)-stimulated progesterone release and increases luteinizing hormone (LH) receptor production synergistic with FSH in rat granulosa cells (Giudice, 1992). In human granulosa-luteal cells, IGF1 stimulates cell proliferation, but has little effect on progesterone synthesis (Giudice, 1992). In cultured pituitary somatotrophs, cells in the anterior pituitary that produce growth hormone (GH), IGF1 inhibits GH secretion (Yamasaki et al., 1991).

IGF1 and IGF2 biological actions during gestation

IGF1 and IGF2 play a critical role in regulating fetal growth by promoting proper placental development and function throughout gestation (Forbes and Westwood, 2008). Their metabolic, mitogenic, and differentiative actions are quite prominent in placental and fetal development (Jones and Clemmons, 1995). Both IGF1 and IGF2 have been found to be expressed in most tissues during fetal development, but predominantly mesenchymal originated cells (Han et al., 1987; Han et al., 1988). A study using mid-gestation human fetal tissues, IGF2 mRNA is expressed in the highest concentrations in

the liver, adrenal glands, and skeletal muscle while the placenta and stomach had the highest level of IGF1 mRNA (Han et al., 1988). Tissue IGF2 mRNA expression is substantially greater than IGF1 during fetal development in rodents at mid- to late gestation, sheep at 50 dGA to term, and humans at 14 to 16 weeks gestation (Hill, 1990; Delhanty and Han, 1993; Fowden, 2003). A study investigating IGF2 mRNA expression in sheep from 50 dGA to term and postnatal lambs from 2 days to 9 weeks postnatal age reported IGF2 was highest in ovine fetal tissue from early gestational ages and decreased with maturation, suggesting the involvement of IGF2 in fetal development during late gestation and postnatal life is not as prominent as IGF1 might be (Delhanty and Han, 1993).

IGF1 and IGF2 in fetal and maternal circulation

Furthermore, IGF1 and IGF2 are both detectable in fetal circulation during early gestation, yet IGF2 concentrations can be found at a concentration that is 3-10-fold higher than that of IGF1 in late gestation in humans, primates, ovine, bovine, porcine, and murine (Gluckman et al., 1983; Tarantal and Gargosky, 1995; Owens et al., 1994; Holland et al., 1997; Lee et al., 1993, Daughaday et al., 1982). Gluckman et al. (1983) suggested IGF1 and IGF2 are independently secreted into fetal circulation using samples of human umbilical cord serum and there was not a correlation between IGF1 and IGF2 concentrations. IGF1 concentrations increased with birth weight and gestational age while there was no association in IGF2 concentrations (Gluckman et al., 1983). Furthermore, IGF2 concentrations were similar to those found in adults at birth whereas IGF1 concentrations were significantly lower (Gluckman et al., 1983). Tarantal and Gargosky

(1995) reported increased fetal IGF1 and IGF2 in primates until the end of gestation whereas IGF1 slightly decreased at term and IGF2 continued to increase. In sheep, fetal plasma IGF1 concentrations were lower than that of IGF2 at 127 dGA, IGF1concentrations were 27% of that of IGF2 at 120 dGA and the ratio of IGF1 to IGF2 in plasma decreased with gestational age (Owens et al., 1994). IGF1 concentrations stayed relatively similar from 120 to 127 dGA while IGF1 in FGR fetuses decreased with advancing gestation (Owns et al., 1994). Circulating fetal IGF1 increases as gestation progresses from 60 dGA to term while circulating IGF2 increases from 60 dGA to 105 dGA then declines (Carr et al., 1995). Prior investigation of IGFI and IGF2 concentrations in the sheep fetus and neonate show that while IGFI concentrations at birth are similar those in the late gestation fetus, IGF2 concentrations fall threefold the week prior parturition and at birth, IGF2 values are similar to that in the adult (Gluckman Butler, 1983b). This study suggests IGF1 and IGF2 are not secreted in correlation in the sheep fetus (Gluckman Butler, 1983b).

IGF1 and IGF2 correlation to fetal birth weight

Concentrations of the IGF's in the fetus are positively correlated to birth weight per evidence in humans, primates, sheep, pigs, rabbits, and rodents (Daughaday et al., 1982; Gluckman et al., 1983; Lee et al., 1993; Carr et al., 1995; Tarantal and Gargosky, 1995; Kind et al., 1995; Thakur et al., 2000; Ong et al., 2000). Fetal plasma IGF1 levels from cord blood of neonates obtained immediately after delivery at 24 to 42 weeks of gestation correlated with birth weight, body length, and head circumference independent of gestational age and IGF1 levels rose with gestational age (Gluckman and Brinsmead,

1976). Furthermore, the comparison of IGF1 in maternal plasma collected from 8 weeks of gestation to term from normal pregnancies and FGR pregnancies reported significant reductions of maternal IGF1 concentrations in FGR pregnancies and placental weights were correlated to birth weights (Mirlesse et al., 1993). Serum obtained between 20 and 37 weeks of gestation in humans with normal pregnancies and FGR pregnancies reported both IGF1 and IGF2 increased to values two to three times higher at term in normal pregnancies compared to 33 weeks of gestation while IGF1 in FGR fetuses were significantly lower than normal fetuses of the same age (Lassarre et al., 1991). IGF1 levels were significantly higher in fetuses with weights above the mean for gestational age than in fetuses with weights below the mean, whereas IGF2 levels were similar in the two groups (Lassarre et al., 1991). This data suggests that during late gestation, IGF1 could be more involved in fetal growth in humans and not IGF2 (Lassarre et al., 1991). While both IGF1 and IGF2 exhibit structural similarities and are both significantly involved in fetal growth and development, each ligand can cause different outcomes due to their individual biological actions.

IGF1 in rodent fetal and placental development

A progression of publications of targeted mutagenesis of the genes encoding IGF1 and IGF2 have provided a model of the involvement the IGF's have in the development of fetal mice. Gene ablation of IGF1 in mice produced neonates that exhibited a birthweight 60% of normal at the end of gestation (Liu et al., 1993). Interestingly, the IGF1 heterozygous mice did not display phenotypic differences compared to the wild-type littermates (Liu et al., 1993). The neonates with IGF1 gene ablation were delivered to term

alive, but the majority of neonates were not viable past 6 hours after birth (Liu et al., 1993). No phenotypic abnormalities were observed in the IGF1 null mutants such as tissue disorganization and the apparent differences were viewed as developmental delays, emphasizing the role of IGF1 in promoting normal fetal development (Liu et al., 1993). This data provides evidence that IGF1 has a major involvement in embryonic growth and most likely cellular differentiation (Liu et al., 1993). The comparison of neonatal lethality in IGF1 null mutants and survivability in others was described in an accompanying study by Baker et al. (1993) investigating postnatal growth of the surviving IGF1 null mutants. Growth curves of the mutant embryos were analyzed throughout gestation and growth deficiency is noticeable by embryonic day (E) 13.5 (Baker et al., 1993). From this point of gestation onward the mutants continue to grow at a slower rate than the wild-type controls. While IGF1 mRNA is detectable in mouse embryos by E7.5, the level of IGF1 mRNA increases 9-fold between E11 and E13 in rat embryos, which could explain why growth deficiency isn't observed until E13.5 (Baker et al., 1993). Placental growth curves were documented and the IGF1 mutants did not show any impact on placental weight. Which in rodents, IGF2 is associated as the dominant growth promoting IGF in rodent placentas as it has been suggested that rodent placenta does not express IGF1 whereas many other species placentas express both IGF1 and IGF2 (Lee et al., 1990; Zhou and Bondy, 1992; Baker et al., 1993; Han and Carter, 2000; Fowden, 2003). Postnatal growth of the IGF1 mutants is lower after birth compared to the wild-type littermates and by 8 weeks of age their relative size was 30% of normal weight and remained at that growth deficiency from there on (Baker et al., 1993). Long bone ossification in adult IGF1 mutant mice was greatly reduced, reproductive organs were dramatically reduced, and the adults

are infertile (Baker et al., 1993). Supporting literature from human studies reported homozygous partial deletion of IGF1 is also associated with failure of growth in utero and postnatally (Fowden, 2003). Furthermore, infusion of human IGF1 into hypophysectomized rats stimulated carcass weight gain and organ growth levels (Thissen et al., 1991). Growth-arrested diabetic rats with reduced serum IGF1 levels received human IGF1 infusions, which restored their growth rate without normalization of blood glucose levels (Scheiwiller et al., 1986). Conversely, overexpression of IGF1 in transgenic mice resulted in dramatic postnatal growth and overgrowth could be observed by 4 weeks postnatal life (D'Ercole, 1993). The most dramatic organ growth observed was in the brain of IGF1 transgenic mice weighing 50% more than their normal littermates (D'Ercole, 1993). Furthermore, mice from lines selected for increased IGF1 concentrations had body weights 14% greater compared to mice from lines selected for low IGF1, produced significantly larger litters, fetuses with increased total weight, increased mammary gland weight, and had significantly increased total placental weights compared to the controls (Kroonsberg et al., 1989).

IGF2 in rodent fetal and placental development

The growth-promoting function of IGF2 is restricted to the period of embryogenesis in mice and furthermore, DeChiara et al. (1990, 1991) reported that the paternal IGF2 allele is expressed while the maternal allele is silent in most tissues. Studies investigating phenotypic changes in mice with an a paternally derived mutated IGF2 allele from male mice chimeras produced heterozygous progeny that were 60% of normal body weight compared to the wild-type littermates (DeChiara et al., 1990). The IGF2 heterozygous

mice were viable at birth and when they reached sexual maturity, males and females were fertile (DeChiara et al., 1990). Heterozygous progeny with the disrupted paternal IGF2 allele compared to the homozygous mutants are phenotypically indistinguishable with a body weight 60% that of the wild-type littermates (DeChiara et al., 1991). When the disrupted IGF2 allele is transmitted maternally, the offspring are phenotypically normal (DeChiara et al., 1991). Placental growth was impaired in mice with a paternally derived disrupted IGF2 allele and was detectible by E13.5, emphasizing IGF2's predominant role in placental growth and development (Baker et al., 1993). Placental development observations in complete gene ablation of IGF2 mice and mice with gene ablation of the placental specific IGF2 transcript alone (*IGF2*P0$^{+/-}$) revealed IGF2 null growth restriction occurs concurrently in gestation with placental growth restriction whereas the *IGF2*P0$^{+/-}$ mice had placental growth restriction that preceded fetal growth restriction (Coan et al., 2008). This study also compared structural properties of the null and *IGF2*P0$^{+/-}$ placentas. Disproportionate effects of IGF2 ablation occurred in placental compartments, which is not observed in the *IGF2*P0$^{+/-}$ placentas (Coan et al., 2008). This suggests complete IGF2 ablation results is disproportionate growth of the placenta and deletion of the placental specific IGF2 transcript creates proportional placental growth restriction (Coan et al., 2008). Similarly, Constância et al. (2002) reported on IGF2 ablation in the mouse placenta, specifically the IGF2 transcript expressed in the labyrinthine trophoblast of the placenta, resulted in reduced placental growth and fetal growth restriction. Transfer of nutrients through the placenta to the fetus was also decreased, suggesting that IGF2 is involved in controlling the placental supply of nutrients to the fetus (Constância et al., 2002). Conversely, fetal growth can be enhanced by over-expression of IGF2 by either

deleting IGF2R, the IGF2 clearance receptor, or by biallelic IGF2 expression in mice (Fowden, 2003). Over-expression of IGF2 in mice caused organomegaly, extra toes, cardiac abnormalities, and could be lethal at birth (Fowden, 2003). Similarly, placenta overgrowth occurs when IGF2 is overexpressed in mice (Fowden, 2003). Furthermore, generation of mice with gene ablation of both IGF1 and the paternal IGF2 allele were used to investigate how deficits of both genes are impacted in fetal growth. Growth deficient progeny with 30% normal body weight were born at term, but were not viable long after birth due to respiratory failure (Liu et al., 1993). It was concluded that the ablation of both IGF1 and IGF2 results in a compounded phenotype of growth deficiency and neonatal lethality and both IGF's are not essential for cellular proliferation as much as factors that are necessary for normal fetal growth (Baker et al., 1993).

IGF1 and IGF2 in sheep fetal and placental development

A rapid phase of placental growth precedes fetal growth, and in sheep, maximal placental growth occurs between 40 and 80 dGA whereas the majority of fetal growth occurs after 100 dGA (de Vrijer et al., 2006). In ruminants, the placenta is both the source of fetal plasma IGF2 and a site for IGF1 clearance from fetal circulation (Bassett et al., 1990; Holland et al., 1997). IGF1 and IGF2 mRNA are both expressed in fetal placentomes and maternal caruncles (Reynolds et al., 1997; Wathes et al., 1998). IGF1 mRNA expression was below the detectable limit in maternal caruncles following embryonic implantation and invasion of the fetal villi, suggesting locally produced IGF1 is not as important in the early stages of placental development and moderate levels of IGF2 mRNA were observed in maternal caruncles in early gestation (Reynolds et al., 1997;

Wathes, 1998). Previous studies also investigated IGF1 mRNA concentrations in cycling ewes uteri during the luteal phase versus pregnant uteri and no difference was found in IGF1 from the uterine horns, suggesting the IGF1 effects are from the fetus rather than maternal IGF1 production (Wathes, 1998). IGF2 mRNA in invading placentome villi has been shown to be expressed abundantly during early gestation (Reynolds et al., 1997). A hyperthermia-induced sheep model of placental insufficiency-FGR reported on IGF's in placental tissues at 55 and 90 dGA (de Vrijer et al., 2006). Maternal caruncle IGF1 mRNA was significantly increased at 90 dGA while cotyledon IGF1 concentrations stayed constant from 55 to 90 dGA (de Vrijer et al., 2006). In control cotyledons IGF2 mRNA concentrations increased significantly between 55 and 90 dGA and IGF2 mRNA in FGR cotyledons and were significantly increased at 55 dGA compared to control cotyledons (de Vrijer et al., 2006). This study suggested that aberrant changes in placental IGF expression in early and mid-gestation could predispose the pregnancy to placental insufficiency and therefore, FGR (de Vrijer et al., 2006).

To investigate if increased IGF1 promotes fetal growth in sheep during late gestation, fetal sheep were infused intravascularly with human IGF1 from 120 dGA to 130 dGA (Lok et al., 1996). IGF1 infusion increased plasma IGF1 concentrations by 140% and fetal liver, lungs, heart, kidneys, spleen, pituitary, and adrenal gland weights were all significantly increased by 16% to 50% (Lok et al., 1996). IGF1 administration also promoted skeletal maturation in the sheep fetus (Lok et al., 1996) While IGF1 promoted growth of major organs during late gestation, fetal and placental weight were not significantly changed (Lok et al., 1996). Furthermore, another study inducing fetuses with FGR via esophageal ligation at 90 dGA were administered IGF1 into the gut from 90 dGA

to 137 dGA and IGF1 treatment increased fetal weight in FGR fetuses (Kimble et al., 1999). Increased IGF2 expression in sheep produced *in vitro* or by cloning resulted in developmental abnormalities, muscle hypertrophy, and fetal overgrowth (Fowden, 2003). In fetal sheep, a switch occurs during late gestation from widespread local production of IGF's before birth to a selective pattern of expression after birth (Fowden, 2003). When the fetus transitions from nutrient delivery via the umbilicus to enteral nutrition at birth it seems there's a perinatal switch from local production of predominantly IGF2 to IGF1 production that's GH dependent. This could contribute to the change in growth regulatory mechanisms that ensure continued postnatal growth (Fowden, 2003).

The IGF axis relationship with CSH

CSH has been associated with stimulating IGF secretion in the fetus. This hypothesis was originally supported by *in vitro* studies. Using rat embryo fibroblasts cultures where IGF2 secretion was enhanced by CSH (Gluckman et al., 1986). CSH also enhanced IGF1 secretion in fibroblast cultures from adult rat tissues (Gluckman et al., 1986). *In vivo* oCSH increases the circulating concentrations of the IGF's in hypophysectomized rats and correlated increases in both IGF1 and CSH occur after 12 weeks of gestation to term in humans, further supporting that the somatotropic properties of oCSH may be in correlation with IGF1 and IGF2 (Adams et al., 1983b, 1983c; Hurley et al., 1977; Furlanetto et al., 1978). IGF1 activity can be maintained in pregnant hypophysectomized rats until delivery of the offspring, providing evidence that CSH can regulate maternal IGF secretion (Gluckman et al., 1986). CSH measured in serum of normal and FGR pregnancies from 20 to 37 weeks gestation in humans reported CSH

levels in fetal blood increased with gestational age in parallel with IGF1 and IGF2 levels after 33 weeks of gestation (Lassarre et al., 1991). This data provided further evidence that CSH has some regulatory role in IGF biosynthesis during gestation (Lassarre et al., 1991). In sheep during late gestation, CSH concentrations correlate with IGF2 concentrations, suggesting CSH as a regulator of IGF2 in the ewe (Gluckman et al., 1986). Furthermore, deficiency of CSH can perturb fetal and placental development, leading to FGR. Sheep induced with FGR by lentiviral mediated CSH RNAi resulted in pregnancies with significant reduction in fetal and placental weights (Tanner et al., 2021b). Maternal CSH concentrations were significantly reduced, but IGF1 concentrations were not affected (Tanner et al., 2021b). By contrast, fetal concentrations of IGF1 were significantly reduced (Baker et al., 2016; Tanner et al., 2021b). This data correlates reductions of maternal CSH impacting fetal growth and IGF1 secretion in the fetus, but not stimulating maternal IGF's. It has been postulated that CSH acts on the placenta in a paracrine fashion to impact fetal IGF's (Tanner et al., 2021b). Since CSH deficiency can result in both FGR and normal weight phenotypes, it was also reported that CSH deficiency reduces fetal IGF1 concentrations in the normal weight phenotypes as well (Ali et al., 2020b). CSH has a clear relationship with regulating IGF1 during pregnancy and reductions in CSH during gestation can alter the concentrations of IGF1.

The IGF axis regulation of placental substrates

In many cell types, the IGF's stimulate amino acid and glucose uptake and protein synthesis *in vitro* (Jones and Clemmons, 1995). IGF1 has insulin-like stimulatory effects on glucose uptake, glycolysis, and glycogen synthesis in rat skeletal muscle *in vitro*, but

does not stimulate glucose oxidation like insulin does (Dimitriadis et al., 1992). Fetal sheep in late gestation will acquire about 50% of its total energy and carbon requirements for growth from glucose and the placenta itself is a highly metabolic organ, consuming 40% to 60% of glucose and oxygen supplied to the uterus in late gestation (Bauer et al., 1998). Administration of IGF1 to either the fetus or ewe has been reported to alter the transfer and partitioning of glucose and amino acids between ovine fetal and uteroplacental tissues (Harding et al., 1994; Liu et al., 1994). Harding et al. (1994) studied the effects of IGF1 on fetal metabolism *in vivo* by infusing IGF1 in catheterized fetal sheep and reported an increase in fetal-placental amino acid uptake, decreased fetal and maternal blood glucose concentrations, and increased fetal-placental glucose uptakes. Liu et al. (1994) studied the effects of maternal IGF1 infusion in catheterized pregnant ewes and reported an increase in fetal blood glucose concentrations, decreased maternal plasma insulin, decreased maternal and fetal amino acid concentrations. Both studies did not observe any effects on placental blood flow by IGF1 infusions (Harding et al., 1994; Liu et al., 1994).

IGF's correlation to fetal nutrient availability

Since plasma IGF1 concentrations are positively correlated with birth weight, it is also correlated with fetal nutrient availability as reported in sheep (Iwamoto et al., 1992). Reducing the availability of both substrates and oxygen or of either substrate or oxygen alone lowers fetal IGF concentrations and nutrient restriction has shown to have a more noticeable effect on circulating IGF1 concentrations than it does on IGF2 (Iwamoto et al., 1992). Hypoxic induced catheterized fetal sheep from 115 to 124 dGA resulted in

significant reductions of plasma IGF1 concentrations, therefore decreasing IGF1 production and availability, but fetal weight was not reported (Iwamoto et al., 1992). In a state of maternal starvation, fetal plasma IGF1, IGF2, insulin, and blood glucose decreased and fetal glucose supplementation increased fetal plasma IGF1, IGF2, insulin, and blood glucose to near control values (Oliver et al., 1996). After 48 hours of maternal refeeding, fetal IGF1, IGF2, maternal IGF2, and fetal and maternal insulin and glucose returned to near control values (Oliver et al., 1996). This study suggests that fetal IGF1 and IGF2 are independently regulated and glucose plays an important role in the regulation of both (Oliver et al., 1996). Fetal IGF2 concentrations are mainly reduced in severe FGR or extreme nutrient depravation (Owens et al., 1994; Holmes et al., 1997) Therefore, IGF1 appears to be more responsive to changes in gestational nutrient conditions rather than IGF2 in the fetus during late gestation (Fowden, 2003).

IGF1 and placental facilitative glucose transporters

IG1 has also been shown to enhance the expression of the facilitative glucose transporter SLC2A1 in human trophoblast cells and the mouse placenta (Baumann et al., 2014; Jones et al., 2013). FGR induced by placental embolization in sheep results in fetal hypoxia, fetal hypoglycemia, has reduced SLC2A1 mRNA, and reduced placental glucose uptake (Wali et al., 2012). Intra-amniotic infusion of IGF-1 once-weekly into these fetuses promoted increased placental expression of SLC2A1 mRNA and increased growth of the fetuses (Wali et al., 2012). Impaired fetal nutrient supply is the secondary problem in FGR behind placental insufficiency and IGF1 treatment could provide a mechanism for increased substrate supply to the fetus (Wali et al., 2012).

The glucose transporter SLC2A3 is located on the maternal-facing, apical trophoblast membrane of the sheep placenta and likely plays a major role in the uptake of glucose into the trophoblast (Lynch et al., 2022). Lynch et al. (2022) investigated the effects of lentiviral mediated SLC2A3 RNAi, which resulted in significant reductions of placental SLC2A3 protein concentration at mid-gestation (75 dGA). This developed significant reductions in umbilical glucose concentrations, leading to fetuses that were hypoglycemic and also observed placental weight reductions of 21% (Lynch et al., 2022). Furthermore, this reduction in placental SLC2A3 resulted in significant reductions in uterine artery concentrations of IGF1 (Lynch et al., 2022). It is speculative as to what was driving reduced maternal IGF1 in response to reduced SLC2A3, but it may have resulted from reduction in trophoblast uptake of glucose, altering placental products that impact maternal IGF1 (Lynch et al., 2022). While uterine artery IGF1 was reduced, placental tissue mRNA concentrations of IGF1 were not affected at mid-gestation, but placental IGF2, IGF1R, and IGF2R mRNA were significantly increased (Lynch et al., 2022). Up-regulation of these IGF's were possibly a compensatory mechanism to stimulate placental growth to combat fetal hypoglycemia (Lynch et al., 2022). Equally important, after 48 hours of maternal starvation, infusion of glucose into fetal sheep with significantly reduced blood glucose results in elevated concentrations of IGF1 close to control values, suggesting a reciprocal relationship between IGF1 and glucose (Oliver et al., 1993). This data demonstrated that fetal glucose is an important regulator of circulating fetal IGF1 (Oliver et al., 1993). Furthermore, increased circulating fetal IGF1 increases amino acid transport across the placenta to the fetus and this suggests that elevated fetal glucose may indirectly lead to increased IGF-mediated amino acid transfer (Oliver et al., 1993).

IGF1R and IGF2R molecular characteristics

The actions of the IGF ligands are mediated by binding to IGF1R and IGF2R and the main signaling receptor is IGF1R, which binding of IGF1 or IGF2 activates the phosphoinositide-3 kinase (PI3K)-protein kinase B (AKT) and mitogen-activated protein kinase (MAPK) signaling pathways (Forbes, 2008; Sferruzzi et al., 2017). Activation of IGF1R results in the IGF's biological actions in cell proliferation, metabolism, mitogenesis, survival, migration, and differentiation (Denley et al., 2005). *In vitro* studies using human and rodent tumor cells with reduced IGF1R reduced IGF signaling and correlated with cellular apoptosis or programmed cell death (Bowman et al., 2010). Binding studies using 3T3 fibroblasts showed that IGF1R binds IGF1 with 15 to 20-fold higher affinity than IGF2 and IGF2R is specific to binding IGF2 with a 500-fold lower affinity for IGF1, which IGF2R may not interact with IGF1 at all (Germain-Lee et al., 1992; Jones and Clemmons, 1995). IGF1R binds IGF1 with a dissociation constant (K_D) range of of 0.2-1 nM and IGF2R binds IG2 with a K_D range of 0.017-0.7 nM (Jones and Clemmons, 1995). The K_D represents the inverse of the affinity of the receptor for its ligand, so the more readily available the receptor binds to the ligand, the lower the K_D is. IGF2R serves as the cation-independent mannose 6-phosphate receptor involved in lysosomal enzyme targeting by binding to mannose 6-phosphate residues on lysosomal enzymes and translocate newly synthesized lysosomal enzymes into endosomes (Liu et al., 1993; Jones and Clemmons, 1995). When IGF2R is cleaved, a soluble form of the receptor is released and when it's bound to IGF2, it results in degradation of IGF2 and inhibition of its mitogenic actions (Forbes, 2008). IGF2R doesn't seem to participate in a signaling pathway as blocking

IGF2R by antibodies in various cell cultures did not inhibit the mitogenic effect of IGF2 while blocking of IGF1R did impair IGF2 function (Liu et al., 1993). Since IGF2R functions to internalize and degrade IGF2, the absence of IGF2R would more-so increase the mitogenic effects of IGF2 rather than inhibit without a mechanism available to reduce the amount of IGF2 binding to IGF1R to control its mitogenic effects. Furthermore, IGF2R is encoded by the imprinted *Igf2R* gene, but in a reciprocal fashion to IGF2 as the expressed allele for IGF2R is maternal (Liu et al., 1993).

IGF1R and IGF2R presence in the placenta

The IGF's and their receptors are involved in pre- and postnatal growth and development and alteration in their activity is suggested to contribute to FGR. In the placenta, IGF1R is localized to all cell types including the trophoblast and IGF2R is expressed in the microvillous and plasma membranes of the trophoblast and in humans, IGF1R is potently expressed in the placenta by the middle of the first trimester (Forbes, 2008; Bowman et al., 2010). Immunohistochemistry studies reported IGF1R in human term placentas from both appropriately grown and FGR fetuses had normal localization and concentration of placental IGF1R (Holmes et al., 1999). However, IGF1R mRNA levels measured by quantitative PCR were significantly increased in human FGR placentas compared to normal term placentas (Abu-Amero et al., 1998). Conversely, FGR induced in rats by bilateral ligation of the uterine artery had reduced IGF1R mRNA and total protein levels (Reid, et al., 2002). Investigation of the IGF1R signal transduction system in human placentas with FGR reported reduced protein content of IGF1R and defective signaling intermediates, suggesting molecular defects in IGF1R signaling may

be impacting IGF1 responsiveness and proper development of the fetal-placental unit (Laviola et al., 2005). While IGF1R is expressed abundantly in the placenta, limited information is available on the IGF receptor signaling in placentas and how their regulation of expression occurs in FGR placentas. However, studies with gene manipulation of the receptors have provided further insight on the growth promoting role the receptors play in fetal development.

IGF1R and IGF2R in the ruminant placenta

Investigation of human IGF1 and ovine IGF2 binding to 95 dGA ovine placental membranes reported successful binding of the IGF's to IGF1R and IGF2R. Furthermore, it was observed that IGF1 could possibly be extracted from umbilical circulation by the placenta itself (Bassett et al., 1990). IGF1R had a 10-fold higher affinity for IGF1 than that observed by IGF2R and this provided early support for the role of IGF1 interacting with IGF1R and IGF2R in placental function or growth in the sheep placenta (Bassett et al., 1990). Radioreceptor assays tested the IGF2 binding sites on sheep placental membranes and with low cross-reactivity for IGF1, it showed a pattern of IGF2 specificity for IGF2R (Baxter and De Mellow, 1986). Characterization of IGF1R and IGF2R in the sheep placenta from 50 dGA to 140 dGA using membrane binding assays and immunohistochemistry was performed to further investigate their role in ovine placental development by (Lacriox et al., 1995). The binding affinity for IGF1R to IGF1 was similar to reports on placental membranes of pigs and humans (Lacriox et al., 1995) The concentrations of IGF1R and IGF2R varies depending on the developmental stage of the placenta. By 90 dGA, cotyledons expressed both receptors in high concentrations and

the number of IGF2R was greater than that of IGF1R. From 90 dGA onward, the concentration of IGF1R decreases more rapidly than the concentration of IGF2R (Lacriox et al., 1995). IGF1R was localized to the trophoblastic cells, determined by immunohistochemistry (Lacriox et al., 1995). Previous reports of IGF's down-regulating their receptors could help explain the decrease in IGF1R concentrations in the placenta observed after 100 dGA due to IGF1 concentrations being high (Lacriox et al., 1995). In sheep after 30 dGA, IGF1R mRNA reduces greatly where caruncles interdigitate with fetal cotyledons, but continues to express in caruncles near the tips of uterine horns where placentation fails to occur (Nayak and Giudice, 2003). High concentrations of IGF2 have been reported in the tips of invading fetal villi and there's speculation that IGF2 may be a factor for the reduction in IGF1R at the fetal-maternal interface (Nayak and Giudice, 2003). As previously mentioned in other animal models, this study identifies the ability for IGF1 and IGF2 to bind to IGF1R in the ovine placental membrane (Lacriox et al., 1995).

IGF1R regulation of fetal and placental development

Consistent expression of IGF1R occurs throughout rodent embryogenesis and mRNA levels of IGF1R in rat embryonic tissues decline significantly postnatally, implying that IGF1R is heavily involved in prenatal development (Werner et al., 1989; Liu et al., 1993). IGF1R heterozygous mice did not exhibit differences in growth or phenotype, suggesting a possible compensation mechanism to make up for a reduced gene dosage by half or half of the gene dosage of IGF1R is sufficient enough throughout mice gestation (Liu et al. 1993). IGF1R null mice reported in Liu et al. (1993) were severely growth restricted, more than the IGF1 or IGF2 knockout animals, and birth weights were 45% of

that of their wild-type littermates. Furthermore, the IGF1R null neonates were born alive, but not viable (Liu et al., 1993). These neonates presented with respiratory failure and examination of muscle groups revealed mutations in myocytes from the neck, limbs, and respiratory muscles. Failure of the null mice to breath could have been due to muscle hypoplasia (Liu et al., 1993). As previously discussed, IGF1 is a potent inducer of myocyte terminal differentiation and turns on expression of the myogenin gene (Florini et al., 1991a). Without IGF1R to bind to in these null mice, IGF1 loses its capability to perform its cellular differentiation actions. Culture of forebrain cells from E18.5 IGF1R null mice embryos had significantly fewer oligodendrocyte progenitors, cells that occur throughout the central nervous system, compared to controls (Liu et al., 1993). Oligodendrocytes occur as myelinating cells between nerve fibers and are involved in myelin formation and nutrition of neurons (Sommer and Schachner, 1981). IGF1R null mice had significantly increased cell densities in transverse sections of the spinal cord, suggesting crowding of neural cells resulting from a reduction in the surrounding neuroglial cell cytoplasm (Liu et al., 1993). This data suggests IGF1R plays a role in the development of neural cells in the central nervous system. IGF1R null mice also had translucent skin, developmental delays of cranial and facial bones and bones of the trunk and extremities (Liu et al., 1993). Comparing IGF1R null mutants to IGF1 and IGF1R double mutants did not appear to have distinguishable phenotypes, providing further evidence that IGF1 solely interacts with IGF1R (Liu et al., 1993). IGF2 and IGF1R double mutants were born at 30% of normal body weight and were not viable after birth (Liu et al., 1993). Similar underdevelopment of the epidermis and high cellular density of the central nervous system were similar to the IGF1R null mice, but the IGF2/IGF1R double mutants had

more pronounced bone developmental delays (Liu et al., 1993). With the disparity between neonatal lethality of the IGF1R mutant and the viability of the IGF2 mutants, the functions impaired by the IGF1R mutation may attribute to a disruption of the IGF1 interaction with IGF1R (Liu et al., 1993). Since some IGF1 null mice were able to survive to adulthood, it's possible that IGF2 may be able to partially compensate in the absence of IGF1 as long as IGF1R is present (Liu et al., 1993). This data concludes that IGF1R is essential for normal embryonic development and survival after birth and mediates the signaling of both IGF1 and IGF2. Examination of the growth kinetics to provide more evidence for the interaction between IGF1 and IGF2 with IGF1R revealed a significant difference in weight by E11 in IGF2/IGF1R mutants and the double mutants had a larger decrease in growth rate than the IGF1R mutants (Baker et al., 1993). There was no significant effect on placental weight in IGF1R null mice (Baker et al., 1993). Furthermore, placental growth is normal in double mutants of IGF1R and insulin receptors, which provided more evidence that IGF2's growth stimulatory effects in the placenta don't appear to be mediated by IGF1R and that IGF2 acts through IGF2R in a placenta specific manner (Fowden, 2003). Furthermore, mice containing null mutations in both IGF2 and IGF1R were more severely growth restricted than IGF1R null mice, providing evidence that IGF2 must mediate its growth stimulatory effects on fetal growth with IGF1R (Baker et al., 1993).

IGF2R regulation of fetal and placental development

Since the primary function of IGF2R is to clear IGF2 from circulation, mice with an IGF2R gene ablation have significantly increased birth weights than the wild-type

littermates (Lau et al., 1994). Reduced IGF2R stimulates growth by means of impaired clearance of IGF2, which provides evidence that IGF2R mediates IG2's fetal growth stimulatory effects by preventing excessive IGF2 growth effects (Lau et al., 1994). There are also reports that the molar ratio of IGF2 to soluble IGF2R is significantly related to placental weights and birth weights (Ong et al., 2000). Baker et al. (1993) also reported a possible IGF2R mouse mutant that was created by deletion of the T^{hp} gene, which eliminates IGF2R. Specific targeting of IGF2R was not available at the time. Previous studies have shown that embryos must inherit a functional T-associated maternal effect (*Tme*) locus through the maternal germ line for normal development to occur and a candidate gene for *Tme* is IGF2R, which has been mapped to the T^{hp} and t^{lub2} deletions (Johnson, 1974; 1975; Lau et al., 1994). Embryos that inherit a defective maternal *Tme* locus (chromosomal deletions T^{hp} or t^{lub2}) are oedemic and die at mid-gestation (Johnson 1974; Lau et al., 1994). Maternal transmission of the T^{hp} chromosome caused a lethal maternal effect and T^{hp} heterozygous embryos died in utero or later and are born with severe edema and polydactyly (Baker et al., 1993). The T^{hp} heterozygous mutants were 111% of normal birthweight and a double mutant of IGF1R/ T^{hp} were 93% of normal birthweight (Baker et al., 1993). Heterozygotes that inherited the deletion paternally were viable and can be identified phenotypically by a hairpin tail (Baker et al., 1993). Lau et al. (1994) further investigated whether paternal expression of IGF2R was necessary for early development in the mouse and if the lack of IGF2R expression would contribute to similarities seen in the *Tme* mutant phenotype. This study produced heterozygous offspring with a mutated IGF2R paternal allele are viable, reach adulthood, and are fertile, similar to the results reported in Baker et al. (1993) (Lau et al., 1994). They were able to

confirm that mice with a mutated maternal allele die at or near birth, are 25% to 30% larger than normal weight, have increased circulating IGF2 and IGFBP's, and exhibit the identifying tail kink (Lau et al., 1994). Embryonic death of these mice seemed to be from cardiac abnormalities, specifically overgrowth in the muscle layer of the ventricles and septal and valve defects (Lau et al., 1994). This data provides support that IGF2R plays a primary role in regulating IGF2, fetal growth, and heart development. Epigenetic changes in IGF2R in sheep have also been associated with fetal overgrowth (Young et al., 2001). Reduced fetal methylation and expression of ovine IGF2R was successful by 30% to 60% in large offspring (LO) fetuses compared to the controls and a 67% reduction in circulation IGF2R (Young et al., 2001). Fetuses were recovered at 125 dGA and LO fetuses weighted 5.5 kg or more while the largest control fetus weighted 4.5 kg (Young et al., 2001).

IGFBP's molecular characteristics

The IGFBP's share structural homology and have a higher affinity for IGF's than the IGF receptors, therefore the IGFBP's have a critical influence on the biological regulation of IGF1 and IGF2. Normal growth and development is achieved by the control of IGF availability to bind to IGF receptors through the binding proteins (Denley et al. 2005). IGFBP's in tissue potentiate or inhibit the effects of the IGF's by releasing the IGF's to bind to their respective receptor or sequester the IGF's from being able to bind to their receptors (Denley et al., 2005; Forbes and Westwood, 2008). The inhibitory actions of the binding proteins is achieved by competing with IGF1R for IGF binding and any IGFBP present in a molar excess eliminates IGF bioactivity (Wathes et al., 1998). Infusion of pure

IGFBP1 or -2 in rats demonstrated their half-lives are approximately 90 minutes (Jones and Clemmons, 1995). The stimulatory effects of IGFBP's are possibly achieved by the IGFBP's providing local increases in IGF concentration and delivering the IGF's to target cells (Wathes et al., 1998). IGFBP1 and -2 contain an amino acid integrin recognition sequence (Arg-Gly-Asp) to bind to cell surfaces. IGFBP3 has a different mechanism of cellular association by interacting with cell surface glycosaminoglycans and IGFBP5 binds to the extracellular matrix (Wathes et al., 1998). About 99% of IGF's in circulation are normally bound to binding proteins and bound IGFBP's have a lower affinity for IGF1 and IGF2 than circulating IGFBP's. This has been suggested as a possible mechanism to dissociate the IGF's from the binding proteins in favor of the receptors (Wathes et al., 1998; Denley et al., 2005). The IGF's are released from the binding protein complex by either proteolysis of IGFBP's or binding of IGFBP's to the extracellular matrix (Denley et al., 2005).

Binding proteins are capable of performing by IGF-dependent actions, but they can also have IGF-independent actions (Denley et al., 2005). IGF-independent actions of the binding proteins can be produced without IGF binding (Clemmons, 2018). IGF-independent actions also include altering IGF signaling through the receptors and this mechanism is mediated through protein and IGFBP interactions (Clemmons, 2018). Since these interactions require the use of the IGF receptors, it's not considered completely IGF independent (Clemmons, 2018). IGF-independent actions occur for IGFBP1, -2, -3 and possibly -5. These three binding proteins all bind to cell surfaces and it's possible their direct effects are mediated by cell surface receptors (Jones and Clemmons, 1995). However, there are some major differences in binding sites for these binding proteins.

IGFBP1 binds to an integrin receptor, IGFBP3 binds to an unknown cell surface molecule, and signal transduction pathways from their cell surface binding properties are not determined (Jones and Clemmons, 1995).

IGFBP1-3

Attempts to investigate the inhibitory and stimulatory effects of IGFBP's on IGF actions have been performed *in vitro* and *in vivo*. IGFBP1 has been shown to inhibit IGF stimulation in a variety of cell lines. IGF1 stimulation of DNA synthesis was inhibited by the addition of IGFBP1, -2, -3, and -4 to cell cultures (Jones and Clemmons, 1995). Other evidence supports IGFBP1's ability to inhibit IGF actions when addition of IGFBP1 to human granulosa/luteal cells hindered IGF DNA synthesis and inhibition of IGF receptor binding, although inhibition required excess concentrations of IGFBP1 (Jones and Clemmons, 1995). Human endometrial membrane IGF1 interaction with receptors was blocked by IGFBP1 and the recapitulation IGFBP1 inhibition of receptor association continues to suggest IGFBP1 is an inhibitor rather than a potentiator of IGF1 (Jones and Clemmons, 1995).

IGFBP1 is produced almost entirely by the liver and IGFBP1 is suggested to have a strong relationship with lipid metabolism and insulin. IGFBP1 crosses vasculature at higher concentrations if insulin is co-perfused, which suggests insulin may stimulate the translocation of IGFBP1 out of vasculature (Jones and Clemmons, 1995). Since IGF concentrations are positively correlated with insulin concentrations, reduced insulin in the presence of increased IGFBP1 suggests an inhibitory action of IGFBP1 on the IGF's as IGFBP1 inhibits insulin-like activity of IGF1 *in vitro* and this coincides with *in vivo* IGFBP1

inhibiting IGF-induced hypoglycemia (Han et al., 1994 ; Jones and Clemmons, 1995). This may be due to IGF complex formation with IGFBP1, preventing binding to receptors (Jones and Clemmons, 1995). Caloric restriction results in a significant increase of IGFBP1 and infusion of IGFBP1 in fasted rats resulted in an 11% increase in glucose 15 minutes after a bolus injection of IGFBP1, supporting the neutralization of IGFBP1 on insulin-like activity of IGF1. However, the levels of IGFBP1 observed were greater than levels in normal serum and it's speculative if such a change would occur in non-fasted, normal caloric animals (Jones and Clemmons, 1995). IGFBP1 also inhibited glucose incorporation into 3T3 fibroblasts (Jones and Clemmons, 1995). Furthermore, FGR induced by maternal fasting resulted in fetal hypoinsulinemia in which significant increases in IGFBP1 were observed (Han et al., 1994). This has been recapitulated in human studies as IGFBP1 was decreased in response to insulin and suppression of IGFBP1 occurred in response to carbohydrate ingestion (Clemmons, 2018). Similarly, hyperinsulinemic obese subjects are reported to have reduced IGFBP1 concentrations (Clemmons, 2018).

Conversely, stimulatory effects of IGFBP1 have also been observed in a conflicting fashion, but potentiation of IGF's may require the dephosphorylated form of IGFBP1 (Jones and Clemmons, 1995). IGFBP1 purified from human amniotic fluid enhanced IGF1's effect on DNA synthesis by a 5.5-fold increase in porcine smooth muscle cells, chick embryo fibroblasts, and mouse embryo fibroblasts (Jones and Clemmons, 1995). Furthermore, an IGF1 mutant mouse with impaired IGFBP1 binding had reduced DNA synthesis compared to normal (Jones and Clemmons, 1995). In conclusion, IGFBP1 functions to regulate free IGF1 concentrations and inhibits the ability of IGF1 to stimulate

differentiation but may stimulate IGF actions when it's dephosphorylated (Clemmons, 2018).

IGFBP2 been associated to inhibit IGF actions, particularly IGF2 as it has a 2-fold higher affinity for IGF2 (Jones and Clemmons, 1995; Carrick et al., 2001). Henceforth, purified IGFBP2 inhibited IGF2 stimulated DNA synthesis in human lung carcinoma cells and inhibited IGF1 and IGF2 binding to cell surfaces, limiting them from their receptors (Jones and Clemmons, 1995). IGFBP2 is an inhibitor of DNA synthesis in cultured fibroblasts and can inhibit IGF1 and IGF2 stimulated mitogenesis (Jones and Clemmons, 1995). Addition of IGFBP2 to human visceral pre-adipocyte cell cultures reported decreased fat cell differentiation and when IGFBP2 was silenced in adipocytes, adipogenesis was increased (Clemmons, 2018). Overexpression of IGFBP2 in human embryonic kidney fibroblasts inhibited cell proliferation and this effect was reversed with the addition of IGF's (Höflich et al. 1998). Another study supports this concept when IEC-6 intestinal epithelial cells transfected with an antisense IGFBP2 construct stimulated growth (Corkins et al. 1995). Furthermore, a mouse study using growth hormone transgenic mice with a 2- to 3-fold increased expression of IGF1 levels in serum were crossed with IGFBP2 transgenic mice and produced significantly reduced fetal growth. This data supports IGFBP2's inhibitory role to IGF1 (Hoeflich et al. 2001). IGFBP2 has been established to inhibit adipogenesis as mice overexpressing IGFBP2 were resistant to fat accumulation when increased caloric consumption attempted to induce adipogenesis and these mice were insulin sensitive (Clemmons, 2018). Furthermore, studies investigating transgenic mice with complete gene ablation of IGFBP2 were obese with a 30% larger body weight compared to normal (Clemmons, 2018). Reports of obese,

prepubertal children had significantly lower IGFBP2 concentrations compared to normal weight children (Clemmons, 2018). IGFBP2 shows a strong association in inhibiting IGF actions and has been suggested as a weak potentiator of IGF actions. IGFBP2 slightly enhanced IGF1 effects on glucose transport and bovine IGFBP2 added to porcine smooth muscle cells enhanced a response to IGF1 by 80% (Jones and Clemmons, 1995). In conclusion, IGFBP2 is a potent inhibitor of IGF stimulated mitogenesis, differentiation, cell proliferation, adipogenesis, and fetal growth.

In serum, about 75% of the IGF's circulate as a 150 kDa complex of IGF1 or IGF2 with IGFBP3, which is a 46-53 kDa protein (Jones and Clemmons, 1995). When IGF1 and IGF2 are complexed to IGFBP3 they are unable to cross capillary vasculature, which prolongs their half-life to 12-15 hours when free IGF1 has a half-life less than 10 minutes (Jones and Clemmons, 1995). However, the other binding proteins can leave circulation and target the IGF's to specific tissues (Wathes et al., 1998). IGFBP3 inhibits IGF actions by inhibiting IGF binding to their receptors and IGFBP3 has a significantly higher affinity for IGF1 and -2 than does IGF1R (Jones and Clemmons, 1995).

IGFBP3 plays a role in inhibiting IGF1 stimulated DNA synthesis in human skin fibroblasts and can inhibit the proliferation of human breast cancer cells independently of IGF stimulation (Jones and Clemmons, 1995). IGFBP3 reduced glucose incorporation into fat cells, inhibited IGF1 stimulated glycogenolysis and glucose oxidation in porcine fat cells (Jones and Clemmons, 1995). IGFBP3 can also potentiate IGF effects as evident in IGF1 enhanced cell replication of rat osteoblast cultures that produce IGFBP3 (Jones and Clemmons, 1995). Baby hamster kidney cells culture with IGF1 and IGFBP3 had a 90% increase in DNA synthesis compared to cells cultured with IGF1 alone (Jones and

Clemmons, 1995). Ultimately, it seems that cell surface association may be required for IGFBP3 to potentiate IGF action since localization to the cell surface results in significant reduction of the affinity of IGFBP3 for the IGF's (Jones and Clemmons, 1995). *In vivo* studies investigating the accuracy of IGFBP3's effects on IGF1 actions reported application of IGFBP3 and IGF1 accelerated wound healing and increased new tissue formation compared to IGF1 alone (Jones and Clemmons, 1995). Furthermore, administration of IGF1 to hypophysectomized animals can increase IGFBP3, but administration in humans only results in a minor increase (Jones and Clemmons, 1995). It has also been suggested that the ratio of IGF1 to IGFBP3 can correlate with alterations in lipid metabolism as IGF1 and IGFBP2 levels are positively correlated to LDL and total cholesterol (Clemmons, 2018).

IGFBP4-6

IGFBP4 is proteolytically cleaved in tissues and binding of IGF1 or -2 to IGFBP4 results in increased rate of proteolytic cleavage (Jones and Clemmons, 1995). IGFBP4 appears to inhibit IGF action in most conditions. IGFBP4 prevented IGF binding to cell surfaces in a study using an osteosarcoma cell line and increasing concentrations of IGF1 improved IGF availability to bind to cells surfaces as long as concentrations of IGFBP4 remain low (Jones and Clemmons, 1995). Furthermore, IGFBP4 inhibited IGF1 stimulated DNA and glycogen synthesis in human osteosarcoma cells, which required a 5-fold excess of IGFBP4 to inhibit IGF1, but not IGF2 (Jones and Clemmons, 1995). IGBP4 can also inhibit IGF1-stimulated angiogenesis as addition of IGFBP4 to adipose tissue inhibited sprouting angiogenesis, which is the growth of new capillary vessels out

of preexisting ones (Clemmons, 2018). As IGFBP4 mainly inhibits IGF action, it may be to protect cells from overstimulation by IGF's or to allow other transmembrane signaling pathways to activate that are inhibited by IGF's themselves (Jones and Clemmons, 1995).

In rats and humans, IGFBP5 and -6 are detected at extremely low concentrations and is unlikely to have physiological significance (Jones and Clemmons, 1995). IGFBP5 could inhibit IGF1 stimulated DNA and glycogen synthesis in human osteosarcoma cells, but IGFBP5 could also potentiate IGF1 cell growth effects as well as IGF2 action on mouse osteoblasts (Jones and Clemmons, 1995). Information on IGFBP6 actions are quite limited, but IGFBP6 may have an antigonadotropin effect in the ovary that is similar to IGFBP2, -3, -4, and -5 (Jones and Clemmons, 1995).

IGFBP's presence during gestation

In rodents, ungulates, humans, and primates, the most abundant IGFBP's in fetal plasma and tissue are IGFBP1, -2, -3, and -4 and fetal expression of these IGFBP's are tissue specific and developmentally regulated (Fowden, 2003). During pregnancy, the post-translational modification of IGFBP's is altered so that IGFBP affinity for the IGF's is reduced and the bioavailability of the IGF's is increased since the binding proteins have a higher affinity for the IGF's than the receptors (Forbes and Westwood, 2008). In human maternal serum from normal pregnancies, IGF1 bioavailability increased due to increased activity of IGFBP proteases (Giudice et al., 1990). After 6 weeks of gestation, IGFBP3, the dominant serum binding protein, significantly decreased, and this trend continued until term while returning to nonpregnant levels by day 5 postpartum (Giudice et al., 1990). IGFBP2 had a more gradual decrease throughout gestation and IGFBP1 levels increased

by the second trimester (Giudice et al., 1990). It was suggested that the reduction of IGFBP2 and IGFBP3 may have been due to an endogenous pregnancy-specific serum protease, yet it is not fully understood why IGFBP1 increased (Giudice et al., 1990). IGFBP1 is found in high concentrations in decidualized uterine endometrium stromal cells and the maternal decidua is a major site of IGFBP1 synthesis (Martina et al., 1997; Gupta, 2015). IGFBP2 has previously been shown to facilitate mitogenesis in porcine uterine endometrial epithelial cells *in vitro*. This study concluded that the IGFBP2 modulation of uterine cell growth may involve IGF-dependent and IGF-independent mechanisms and identify IGF2 and IGFBP2 as co-expressed in uterine epithelial cells (Badinga et al. 1999). Other studies that observed the IGFBP2 proliferative actions to be IGF-independent used overexpressed IGFBP2 mouse adrenocortical tumor cells. These cells displayed increased proliferation and increased cloning efficiency (Höflich et al. 2000). Höflich et al. proposed that the proliferative effect of IGFBP2 when overexpressed may have been IGF-independent due to observing the same mitogenic potency as IGF1 portrays when IGF1R was downregulated as well as decreased IGFBP2 interactions with an IGF1 analog. While the inhibitory IGFBP2 effects are possibly caused by sequestering IGF's, the proliferative effects of IGFBP2 seem to occur independently of IGF's (Oh et al., 1998).

IGFBP1 is a predominant IGFBP in fetal circulation and it is the most predominant IGFBP in amniotic fluid (Gupta, 2015; Bach, 2018). After examining the tissue distribution for IGFBP mRNA concentrations in fetal tissues, IGFBP1 is only expressed in human fetal livers out of all tissues examined, whereas all other IGFBP mRNA's were expressed in every tissue examined in the fetus (Han et al., 1996). In rats IGFBP2 is the major, and possibly the only, IGFBP in the fetus (Han and Fowden, 1994). Fetal sheep

concentrations of IGFBP1 and IGFBP2 are higher than those in the adult while IGFBP3 levels are lower (Wathes et al., 1998). Levels of IGFBP2 are highest in early gestation fetal sheep tissues and decreased with maturation, following the same pattern of IGF2 mRNA concentrations, suggesting both are synthesized together in fetal tissues where expression patterns paralleled (Delhanty and Han, 1993). In the sheep, IGFBP2 mRNA is expressed in every fetal tissue at least by 75 dGA and becomes limited to liver, kidney, and choroid plexus tissue in late gestation (Han and Fowden, 1994). A correlation between IGFBP2 and IGF2 mRNA levels were reported in fetal sheep tissues, which indicates a tissue-specific regulatory role for IGF2 and IGFBP2 during fetal development (Delhanty and Han, 1993). In sheep and humans, fetal body weight at term is positively correlated to plasma IGFBP3, but inversely related to plasma IGFBP1 (Fowden, 2003). Sheep uterine IGFBP3 mRNA is expressed in the caruncles, endometrial stroma and placentomes during gestation (Wathes et al.,1998).

Alterations of IGFBP's in FGR pregnancies

In FGR, fetal circulating levels of IGF1 are reduced, and IGFBP1 concentrations are increased, suggesting IGFBP1 inhibits IGF1 action and could play a role in restricted fetal growth (Gupta, 2015). Since it has been established that IGF1 is a critical regulator of fetal and placental development, altered IGFBP1 levels could change the bioavailability of IGF1 in pregnancies complicated by FGR, contributing to FGR. Decidua from pregnancies with abnormal embryonic development resulted in increased IGFBP1 mRNA levels compared to pregnancies with normal embryonic development (Fang et al., 2004). Abnormal embryonic development was defined as fetal development that did not match

gestational age or arrested embryonic development (Fang et al., 2004). Placentas from small for gestational age (SGA) neonates had reduced IGF1 mRNA and protein concentrations while IGFBP1, -2, and -3 levels were significantly higher compared to normal neonates (Nawathe et al., 2016). These same placentas reported to have increased methylation of the IGF1 promotor regions and reduced methylation of IGFBP1, -2, and -3 (Nawathe et al., 2016). Conversely, placentas from large for gestational age neonates had reduced IGFBP1, -2, and -3 compared to normal placentas (Nawathe et al., 2016). This data suggests that changes in methylation contribute to changes in in gene expression of the binding proteins that could contribute to aberrant fetal growth, specifically playing a role in the pathogenesis of SGA fetuses (Nawathe et al., 2016). Therefore, IGFBP1, -2, and -3 most likely play a critical role in fetal development as major regulators of the IGF's. Hyperthermia-induced (HT) placental insufficiency in sheep produced fetuses with significant weight reductions from 55 dGA to 90 dGA and investigation of placental mRNA concentrations of components of the IGF system at 55 dGA and 90 dGA were reported (de Vrijer et al., 2006). In control fetal cotyledons, IGFBP3 mRNA concentrations significantly decreased between 55 and 90 dGA, yet HT cotyledons only exhibited a minor reduction in IGFBP3 mRNA (de Vrijer et al., 2006). In maternal caruncles, IGFBP3 levels were consistent from 55 to 90 dGA and unaffected by treatment (de Vrijer et al., 2006). IGFBP1 was not detected in caruncle or cotyledon tissue in this study and IGFBP2 was detected in both placental tissues, but results were not suitable for analysis (de Vrijer et al., 2006). Hypoxia treatment resulting in FGR zebrafish resulted in significant increases of IGFBP1 mRNA and protein expression and when zebrafish embryonic cells were treated with IGFBP1, inhibition of IGF1 and IGF2 occurred, which

resulted in inhibition of cellular proliferation (Kajimura et al., 2005). Ovine fetal hypoxemia also resulted in increased IGFBP1 levels in plasma (Fowden, 2003). IGFBP expression during late gestation in the fetus can also be affected by nutritional conditions. Rat and sheep fetuses under nutrient restriction induced by maternal nutrient restriction, reduced uterine blood flow, or occlusion of the umbilical cord resulted in increased expression of IGFBP1 in both tissues and plasma (Fowden, 2003). Furthermore, FGR rat fetal livers reported increased expression of IGFBP1 compared to normal growth fetuses and FGR rat fetuses have markedly increased circulating IGFBP1 (Tapanainen et al., 1994). Overexpression of IGFBP1 and IGFBP3 in mice resulted in FGR, both in utero and postnatally (Fowden, 2003). Similarly, overexpression of IGFBP1 inhibits growth and developmental under normoxia in zebrafish (Kajimura et al., 2005).

Summary

While there is a clear necessity for the IGF axis in proper fetal and placental growth and development, the biological functions of every component of the IGF axis is quite complex. Adequate expression of IGF1 is clearly correlated to fetal growth due to its mitogenic and differentiation effects and appears to have a more prominent role than IGF2 in promoting cellular differentiation during embryonic development. IGF1's impact on fetal growth also plays into its role with regulating nutrient availability to the fetus. IGF2 seems to play a more predominant role in placental development as alterations in IGF2 impact placental growth more so than fetal growth, although IGF2 is still needed for proper fetal development. IGF1 and IGF2 would not be able to illicit their biological actions without IGF1R. IGF1R allows the IGF's to promote cell proliferation, metabolism, mitogenesis,

survival, migration, and differentiation in fetal and placental development while IGF2R serves a purpose to degrade IGF2 and regulate its mitogenic actions. Lastly, to add to the complexity of IGF1 and IGF2 just to function, involvement of the binding proteins regulating the bioavailability of the IGF's plays a critical role in whether normal fetal development can occur. Binding of these ligands can inhibit or potentiate their ability to regulate normal fetal growth and development and abnormal concentrations of binding proteins have a powerful relation to growth restricted fetuses. Furthermore, pregnancies with reduced CSH have a significant impact on IGF concentrations and in a state of reduced CSH in either normal or FGR pregnancies, IGF1 concentrations are reduced in the fetus in both phenotypes. Investigation into how the components of the IGF axis are expressed in CSH deficient pregnancies with both normal fetal development and fetal growth restriction would provide insightful context as to how the IGF axis relationship interacts with CSH. Therefore, this information could establish what determines a normal pregnancy phenotype versus a growth restricted phenotype.

CHAPTER II: IMPACT OF CHORIONIC SOMATOMAMMOTROPIN *IN VIVO* RNA INTERFERENCE PHENOTYPE ON UTEROPLACENTAL EXPRESSION OF THE IGF AXIS

INTRODUCTION

Fetal growth restriction (FGR), as a major pregnancy complication, is often characterized by abnormal placental development and function. Since the placenta is the interface between maternal and fetal circulation, it provides the means necessary for the exchange of nutrients and gases with the fetus. Consequently, abnormal placental development can hinder nutrient transfer to the fetus (Wilson *et al*., 2020). Insulin-like growth factors (IGF's) are known for their involvement and critical role in promoting growth of the fetus and placenta during gestation (Liu *et al*., 1993) and altered expression of the IGF's is associated with growth deficiency in both embryonic growth and placental growth (Baker *et al*., 1993, de Vrijer *et al*., 2006).

The IGF axis is comprised of insulin-like growth factor 1 (IGF1), IGF2, the type 1 IGF receptor (IGF1R), IGF2R, and 6 IGF-binding proteins (IGFBP1-6; Denley *et al*., 2005), many of which have been implicated in the regulation of fetal growth and development. The importance of both IGF1 and IGF2 for placental development was clearly demonstrated by placental-specific gene ablations, which resulted in fetal growth restriction and placental growth restriction (Sibley *et al*., 2004, Coan *et al*., 2008). In humans, reduced fetal plasma IGF-1 concentration is correlated with reduced birth weight (Ong *et al*., 2000), and IGF1 concentrations are reduced in FGR pregnancies (Larsen *et al*., 1996). Both IGF1 and IGF2 interact with IGF1R to illicit functional cellular

responses (Liu *et al*., 1993), whereas IGF2R acts as a clearance receptor for IGF2 (Baker *et al*., 1993, Ludwig *et al*., 1996). Mouse models carrying null mutations in IGF1R led to embryonic and placental growth deficiency (Baker *et al*., 1993) and an IGF2R knockout mouse model resulted in fetal overgrowth and perinatal lethality (Lau *et al*., 1994). The IGF binding proteins regulate IGF availability and IGF biological activity, although gene ablation studies have yet to demonstrate the necessity of the IGFBP's in placental and fetal growth. However, it is known that IGFBP1, 2, and 3 are expressed by the placenta and have the potential to either potentiate or inhibit activity of the IGF's (de Vrijer *et al*., 2006).

It has long been hypothesized that chorionic somatomammotropin/placental lactogen (CSH/PL) acts as a key regulator for fetal growth, in part through its potential stimulation of both maternal and fetal IGF production (Handwerger, 1991). Recently lentiviral mediated RNA interference (RNAi) induced CSH-deficient pregnancies have been reported (Baker *et al*., 2016, Jeckel *et al*., 2018, Tanner *et al*., 2021b), which result in fetal and placental growth restriction, and reductions in umbilical IGF1 concentrations (Baker *et al*., 2016, Tanner *et al*., 2021b), with no change in maternal IGF1. However, CSH RNAi results in two distinct pregnancy phenotypes (Tanner *et al*., 2022). The first being pregnancies with significant fetal and placental growth restriction (PI-FGR; Baker *et al*., 2016, Jeckel *et al*., 2018, Tanner *et al*., 2021b) and a second phenotype represented by pregnancies with normal fetal and placental weights (non-FGR), even though there are significant changes in placental and fetal characteristics (Ali *et al*., 2020, Tanner *et al*., 2021a). This disparity in CSH-deficient phenotypes, resulting from CSH RNAi, is supported by evidence in previous human studies (Daikoku *et al*., 1979,

Lindberg and Nilsson, 1973). We hypothesized that the impact of CSH RNAi could be mediated within the placenta through altering expression of various components of the IGF axis and that the expression of the IGF axis within the CSH RNAi placenta may help explain the varying CSH RNAi phenotypes observed.

MATERIALS AND METHODS

All animal procedures were approved by the Colorado State University Institutional Animal Care and Use committee (Protocol #1576), the Institutional Biosafety Committee (18-092B) and the University of Colorado Anschutz Medical Campus Institutional Animal Care and Use Committee (Protocol #00714).

Experimental Groups:

Two distinct cohorts of sheep were used as the basis of this study. The first cohort was previously reported by Tanner *et al*. (2021a) and consisted of 6 CSH RNAi pregnancies (2 males; 4 females) that did not exhibit fetal growth restriction, designated as CSH RNAi non-FGR, and only a tendency for restricted placental size, and a contemporary group of 6 NTS RNAi (non-targeting sequence controls) pregnancies (4 males; 2 females). The second cohort consisted of 5 CSH RNAi pregnancies (3 males; 2 females) that exhibited significant reductions in both fetal and placental weights, comparable to the report of Baker *et al*. (2016), designated as CSH RNAi PI-FGR, and 6 contemporary NTS RNAi pregnancies (3 males; 3 females).

Lentiviral Generation:

Generation of hLL3.7-CSH (CSH RNAi) and hLL3.7 NTS (non-targeting sequence; control RNAi) constructs and lentivirus was previously described in detail by Baker *et al*.

(2016) and further discussed in Tanner *et al*. (2022). Briefly, both the CSH and NTS sequences were cloned into pLL3.7, and in so doing, the mouse U6 promoter was replaced with the human U6 promoter. Virus generation and titration were completed in accordance with Baker *et al*. (2016). The shRNA sequences for CSH and the NTS control are provided in Table 1.

Table 1. Control and CSH-targeting short-hairpin RNA (shRNA) sequences.

Oligonucleotide	Sequence (5'-3')
Control (NTS) shRNA sense	GAGTTAAAGGTTCGGCACGAATTCAAGAGATTCGTGCCGAACCTTTAACTC
CSH-targeting shRNA sense	AAGGCCAAAGTACTTGTAGACTTCAAGAGAGTCTACAAGTACTTTGGCCTT

Generation of CSH RNAi Pregnancies:

All ewes (Dorper breed composition) were group housed in pens at the Colorado State University Animal Reproduction and Biotechnology Laboratory and provided access to hay, trace mineral, and water in order to meet or slightly exceed their National Research Council requirements (National Research Council 2007). Animal management, estrus synchronization, and embryo transfers were all performed as previously described (Baker *et al*., 2016; Tanner *et al*., 2021a, 2022). To summarize, after synchronization and breeding, expanded and hatched blastocysts were collected at 9 days gestational age (dGA) by flushing the uteri. Each blastocyst was then infected with either the NTS-control (hLL3.7 NTS shRNA) or CSH-targeting (hLL3.7 CSH shRNA) lentivirus. After a 5-hour incubation period, each blastocyst was washed in media and single blastocysts were surgically transferred into a synchronized recipient ewe. Each recipient ewe was

monitored daily for return to standing estrus and pregnancy was confirmed by ultrasound at 50 dGA.

Surgical instrumentation of Fetus and Ewe:

At approximately 115 dGA, pregnant recipient ewes were transported to the University of Colorado Anshutz Medical Campus, Perinatal Research Center (Aurora, CO, USA). All animals underwent surgical placement of fetal and maternal catheters to determine effects of CSH RNAi on blood flow and nutrient transport as previously described (Tanner *et al*., 2021a,b; 2022). Following catheterization, the ewes were given a minimum of 5 days to recover before the steady-state $^{3}H_2O$ transplacental diffusion technique was used to determine uterine and umbilical blood flows, and the repetitive sampling of maternal and fetal blood for the determination of uterine and umbilical uptakes of oxygen and glucose, as previously described (Tanner *et al*., 2021a,b; 2022). Uterine vein and umbilical vein concentrations of CSH were determined by radioimmunoassay, as previously described (Kappes *et al*., 1992, Baker *et al*., 2016; Jeckel *et al*., 2018, Tanner *et al*., 2021a,b). Maternal (uterine) and fetal (umbilical) concentrations of insulin, IGF1, and cortisol were assessed by enzyme-linked immunosorbent assay (ALPCO Immunoassays 80-INSOV-E01, 22-IGFHU-E01, and 11-CORHU-E01-SLV, respectively), as described previously (Andrews *et al*., 2015; Benjamin *et al*., 2017; Cilvik *et al*., 2021).

Tissue Collection:

Following the *in vivo* examination of these pregnancies, the pregnancies were terminated for tissue harvest. At necropsy the gravid uterus (uteroplacental unit) was removed and weighed, the fetus was removed for measurement and organ harvest, all

placentomes were trimmed from the endometrium, and the total number of placentomes and total placentome weight were recorded. Ten placentomes were randomly selected from each placenta and further separated into fetal cotyledons and maternal caruncles. The tissues were then snap frozen in liquid nitrogen (N_2) and stored at -80°C.

RNA Isolation:

Total cellular RNA was isolated from the maternal caruncles and fetal cotyledons using the RNeasy Mini Kit (Qiagen, Hilden, Germany) following the manufacturer's protocol. RNA concentration was quantified using the BioTek Synergy 2 Microplate Reader and the Gen5 version 3.09 Microplate Reader and Imager Software (BioTek, Winooski, VT). Quality was measured by the 260 to 280-nm absorbance ratio with a standard range of 2.0-2.3 requirement for all samples. Samples were then stored at -80°C.

Complimentary DNA Synthesis and Quantitative Real Time PCR (qPCR):

Complimentary DNA (cDNA) was synthesized from 2 μg of total cellular RNA using iScript Reverse Transcriptase Supermix fqPCR (BioRad Laboratories Inc., Hercules, CA.) according to the manufacturers protocol. An equal amount of cDNA (384 well plate: 2 ul/well) was used for each sample in the quantitative real-time PCR (qPCR) reaction. qPCR was performed using the CFX384 Real-Time System C1000 Thermal Cycler (BioRad Laboratories Inc.) and the protocol was followed as previously described (Baker *et al*., 2016). All primer sets for qRT-PCR were designed using Oligo software (Molecular Biology Insight, Cascade, CO) to amplify an intron-spanning product. Primer sequences and product sizes as well as PCR conditions are summarized in Table 2. A PCR product was generated for each gene using cDNA generated from 135 dGA fetal placenta

(cotyledons) and cloned into the StrataClone vector (Agilent Technologies, Santa Clara, CA). Each PCR product generated was sequenced (Colorado State University Proteomics and Metabolomics Facility) to verify amplification of the correct cDNA. Standard curves were then generated for each mRNA, using the PCR products amplified from the sequenced plasmids, from $1x10^{2}$ to $1x10^{-5}$ pg and were used to measure amplification efficiency. Starting quantities (pg) were normalized by dividing the starting quantity of mRNA of interest by the starting mRNA quantity (pg) of ribosomal S15 (*RPS15*), which has been previously determined not to be impacted by CSH RNAi (Jeckel et al., 2018).

Table 2. PCR primers, annealing temperatures, and product sizes for qPCR.

cDNA	Forward Primer (5'-3')	Reverse Primer (5'-3')	Anneal, °C	Product, bp
RPS15	atcattctgcccgagatggtg	tgctttacgggcttgtaggtg	58	124
IGF1	tcgcatctctcttctatctggccctgt	acagtacatctccagcctcctcaga	62	238
IGF2	gaccgcggcttctacttcag	aagaacttgcccacggggtat	62	202
IGFBP1	tgatgaccgagtccagtgag	gtccagcgaagtctcacac	62	247
IGFBP2	caatggcgaggagcactctg	tggggatgtgtagggaatag	55	330
IGFBP3	ctcagagcacagacaccca	ggcatatttgagctccac	54	335
IGF1R	aactgtcatctccaacctc	caagcctcccactatcaac	60	493
IGF2R	gacttgtgtccagaccagattc	gccgtcgtcctcactctcatc	58	674

RPS15, ribosomal protein S15; *IGF*, insulin-like growth factor; *IGFBP*, IGF-binding protein; *IGF1R* and *IGF2R*, Type 1 and Type 2 IGF receptor, respectively.

Statistical Analysis:

As the two separate CSH RNAi cohorts were not experimental contemporaries to each other because the pregnancies were generated and studied in different years, statistical comparisons were not made between the two cohorts. Furthermore, the pregnancy success rate limited the final number in each cohort, such that the study was not sufficiently powered to examine the effect of fetal sex. Accordingly, data derived from the respective cohort NTS RNAi and CSH RNAi pregnancies were statistically compared

by unpaired Students T-test using GraphPad Prism (version 9) to analyze the main effects of treatment. Statistical significance was set at $P \leq 0.05$ and a statistical tendency at $P \leq 0.10$. Data are reported as the mean ± standard error of the mean (SEM). The data figures are presented as bar graphs representing the mean, and the capped vertical lines representing the SEM.

RESULTS

Fetal and Placental Measurements:

As reported in Tanner *et al*. (2021a; Table 3) there were no significant differences ($P \geq 0.10$) due to CSH RNAi in the non-FGR pregnancies for fetal weight, fetal liver weight, crown-rump length, total placentome number, and umbilical blood flow. Placental weight tended to be reduced between treatment groups ($P \leq 0.10$; Table 3) while uterine blood flow was significantly reduced ($P \leq 0.05$; Table 3). While there was no effect of CSH RNAi in these non-FGR pregnancies on uterine and umbilical uptakes of glucose and oxygen (Table 3), uteroplacental utilization of glucose was increased, such that Tanner *et. al*. (2021a) reported that the fraction of glucose taken up by the uterus and utilized by the uteroplacental tissues was significantly ($P \leq 0.05$) increased, and the fraction utilized by the fetus was significantly ($P \leq 0.05$) decreased.

With the second cohort, the 5 CSH RNAi pregnancies exhibited fetal and placental weights that were >2 standard deviations below the mean of the NTS RNAi controls, similar to those reported by Baker *et al*. (2016) and were designated to CSH RANi PI-FGR pregnancies. As evidenced in Table 4, CSH RNAi PI-FGR pregnancies exhibited significant ($P < 0.05$) reductions in fetal weight, fetal liver weight, crown-rump length,

Table 3. Measurements collected at 135 dGA for CSH RNAi non-FGR pregnancies. *

	NTS RNAi	CSH RNAi non-FGR	P-Value	% Change
Fetal Weight, g	3738.5 ± 136.4	3817.7 ± 189.9	0.74	2.12
Fetal Liver Weight, g	110.1 ± 11.5	100.8 ± 7.1	0.51	8.51
Crown-Rump Length, cm	50.8 ± 0.6	52.2 ± 0.7	0.14	2.82
Placental Weight, g	431.6 ± 20.5	384.4 ± 12.5	0.08	10.9
Total Placentome Number	69.17 ± 4.59	65.67 ± 7.14	0.69	5.06
Uterine Blood Flow, ml/min	1870.1 ± 121.4	1451.8 ± 104.4	0.03	22.4
Umbilical Blood Flow, ml/min	799.1 ± 75.3	791.3 ± 54.23	0.94	0.97
Uterine Glucose Uptake, μmol/min	431.8 ± 44.3	479.1 ± 34.2	0.42	11.0
Umbilical Glucose Uptake, μmol/min	155.7 ± 12.4	139.3 ± 10.5	0.34	10.5
Uteroplacental Glucose Utilization, μmol/min	276.1 ± 40.4	339.8 ± 28.6	0.06	23.1
Uterine Oxygen Uptake, mmol/min	2.63 ± 0.18	2.48 ± 0.14	0.51	5.65
Umbilical Oxygen Uptake, mmol/min	1.47 ± 0.06	1.40 ± 0.08	0.53	4.43
Uteroplacental Oxygen Utilization, mmol/min	1.16 ± 0.17	1.08 ± 0.11	0.68	7.18

Data are shown as mean values ± SEM for all pregnancies in each treatment group.
*These data are derived from Tanner et al., 2021a

Table 4. Measurements collected at necropsy 135 dGA for CSH RNAi PI-FGR pregnancies.

	NTS RNAi	CSH RNAi PI-FGR	P-Value	% Change
Fetal Weight, g	4625.6 ± 37.1	3226.4 ± 325.8	≤0.01	30.2
Fetal Liver Weight, g	125.2 ± 3.32	74.2 ± 9.04	≤0.01	40.7
Crown-Rump Length, cm	51.4 ± 0.71	46.9 ± 1.37	0.02	8.76
Placental Weight, g	517.7 ± 28.6	247.9 ± 20.5	≤0.01	52.1
Total Placentome Number	70.5 ± 5.86	58.0 ± 1.79	0.09	17.7
Uterine Blood Flow, ml/min	2477.6 ± 501.9	1453.7 ±403.9	0.03	41.3
Umbilical Blood Flow, ml/min	854.6 ± 41.3	496.9 ± 170.1	≤0.01	41.9
Uterine Glucose Uptake, μmol/min	423.0 ± 84.6	266.9 ± 75.2	0.04	36.9
Umbilical Glucose Uptake, μmol/min	137.5 ± 9.85	78.4 ± 28.5	0.02	43.0
Uteroplacental Glucose Utilization, μmol/min	292.7 ± 62.0	188.1 ± 75.2	0.17	35.7
Uterine Oxygen Uptake, mmol/min	3.01 ± 0.56	1.79 ± 0.49	≤0.01	40.3
Umbilical Oxygen Uptake, mmol/min	1.53 ± 0.09	0.93 ± 0.33	0.02	39.5
Uteroplacental Oxygen Utilization, mmol/min	1.53 ± 0.30	0.94 ± 0.35	0.06	38.4

Data are shown as mean values ± SEM for all pregnancies in each treatment group.

placental weight, as well as reduced uterine and umbilical blood flow rates. Additionally, there were significant (P≤0.05) reductions in uterine and umbilical uptake of both glucose and oxygen (Table 4), and diminished uteroplacental utilization of both. While the umbilical uptake of both oxygen and glucose was significantly reduced (Table 4), umbilical vein (5.65±0.14 vs. 4.97±1.23 mmol/L; NTS RNAi vs. CSH RNAi PI-FGR; P=0.22) and artery (3.85±0.10 vs. 3.02±0.83 mmol/L; P=0.11) oxygen content was not statistically impacted, whereas umbilical vein (1.20±0.03 vs. 0.77±0.20 mmol/L; NTS RNAi vs. CSH RNAi PI-FGR; P=0.002) and artery (1.02±0.04 vs. 0.66±0.12 mmol/L; P=0.008) glucose concentrations were both significantly reduced.

135 dGA Plasma Hormone Concentrations:

Within the CSH RNAi non-FGR pregnancies reported by Tanner *et al*. (2021a), there were no effects of CSH RNAi on uterine or umbilical concentrations of insulin, IGF1 or cortisol (data not presented). For the CSH RNAi PI-FGR pregnancies, maternal CSH was significantly (P≤0.05) depressed (Table 5), whereas maternal concentrations of insulin, IGF1 and cortisol were not impacted by CSH RNAi. By contrast, umbilical concentrations of both insulin and IGF1 were significantly (P≤0.05) depressed (Table 5) in CSH RNAi PI-FGR pregnancies, umbilical cortisol concentrations were significantly (P≤0.05) elevated, and umbilical CSH concentration was not impacted.

135 dGA placental IGF's, IGFBP's, and IGFR's mRNA concentrations:

Fetal cotyledon and maternal caruncle mRNA harvested at 135 dGA were subjected to qPCR to determine *IGF1*, *IGF2*, *IGFBP1-3*, *IGF1R* and *IGF2R* mRNA concentrations. While the CSH RNAi non-FGR pregnancies did not differ in fetal cotyledon *IGF1* or *IGF2* mRNA concentrations (Figure 1A, 1C), the CSH RNAi PI-FGR

Table 5. 135 dGA Plasma Hormone Concentrations in CSH RNAi PI-FGR Pregnancies.

	NTS RNAi	CSH RNAi PI-FGR	P-Value	% Change
Uterine Vein CSH, ng/ml	371.2 ± 64.6	165.3 ± 49.5	0.05	55.5
Umbilical Vein CSH, ng/ml	32.0 ± 3.28	31.1±6.88	0.89	2.86
Uterine Artery Insulin, ng/ml	0.54 ± 0.14	0.67±0.19	0.60	22.5
Umbilical Artery Insulin, ng/ml	0.73 ± 0.10	0.35 ± 0.08	0.02	52.9
Uterine Artery IGF1, ng/ml	170.7 ± 13.8	151.9 ± 14.2	0.39	11.0
Umbilical Artery IGF1	167.5 ± 23.8	72.0 ± 20.13	0.02	57.0
Uterine Artery Cortisol, ng/ml	159.5 ± 62.2	121.3 ± 59.7	0.67	23.9
Umbilical Artery Cortisol, ng/ml	51.4 ± 4.66	129.2 ± 28.0	0.01	151.5

Data are shown as mean values ± SEM for each treatment group.

pregnancies exhibited reduced *IGF1* mRNA ($P \leq 0.05$; Figure 1B). While *IGF2* mRNA was not significantly impacted by treatment in the CSH RNAi PI-FGR pregnancies, it was reduced by 33.3% (Figure 1D). The mRNA concentration for *IGF1R* and *IGF2R* did not differ due to treatment in the non-FGR pregnancies (Figure 2A, 2C). In the CSH RNAi PI-FGR pregnancies, neither *IGF1R* (Figure 2B) or *IGF2R* (Figure 2D) mRNA were statistically impacted by treatment, although *IGF1R* mRNA concentration was 91.5% greater ($P=0.17$).

Within the maternal portion of the placenta (caruncles) there were no significant changes in *IGF1* or *IGF2* mRNA concentrations in either the CSH RNAi non-FGR or PI-FGR pregnancies (Figure 3). Maternal caruncle *IGF1R* mRNA was not impacted by CSH RNAi in either non-FGR or PI-FGR pregnancies (Figure 4 A and 4B, respectively), nor was *IGF2R* mRNA impacted in CSH RNAi non-FGR pregnancies (Figure 4C). However, *IGF2R* mRNA concentration was significantly increased in the maternal caruncles of CSH RNAi PI-FGR pregnancies (Figure 4D).

There were no significant changes in the mRNA concentrations of either *IGFBP1* or *IGFBP3* in either the fetal cotyledons or maternal caruncles because of CSH RNAi (data not presented). However, as evidenced in Figure 5A, *IGFBP2* mRNA concentration was significantly increased in the fetal cotyledons of CSH RNAi non-FGR placenta, whereas there was no effect of CSH RNAi on PI-FGR concentrations of *IGFBP2* mRNA (Figure 5B). In the maternal portion (caruncles) of the placenta, there was a statistical tendency ($P \leq 0.10$) for *IGFBP2* mRNA concentration to be increased in CSH RNAi non-FGR placenta (Figure 6A), and a similar tendency in CSH RNAi PI-FGR maternal caruncles (Figure 6B).

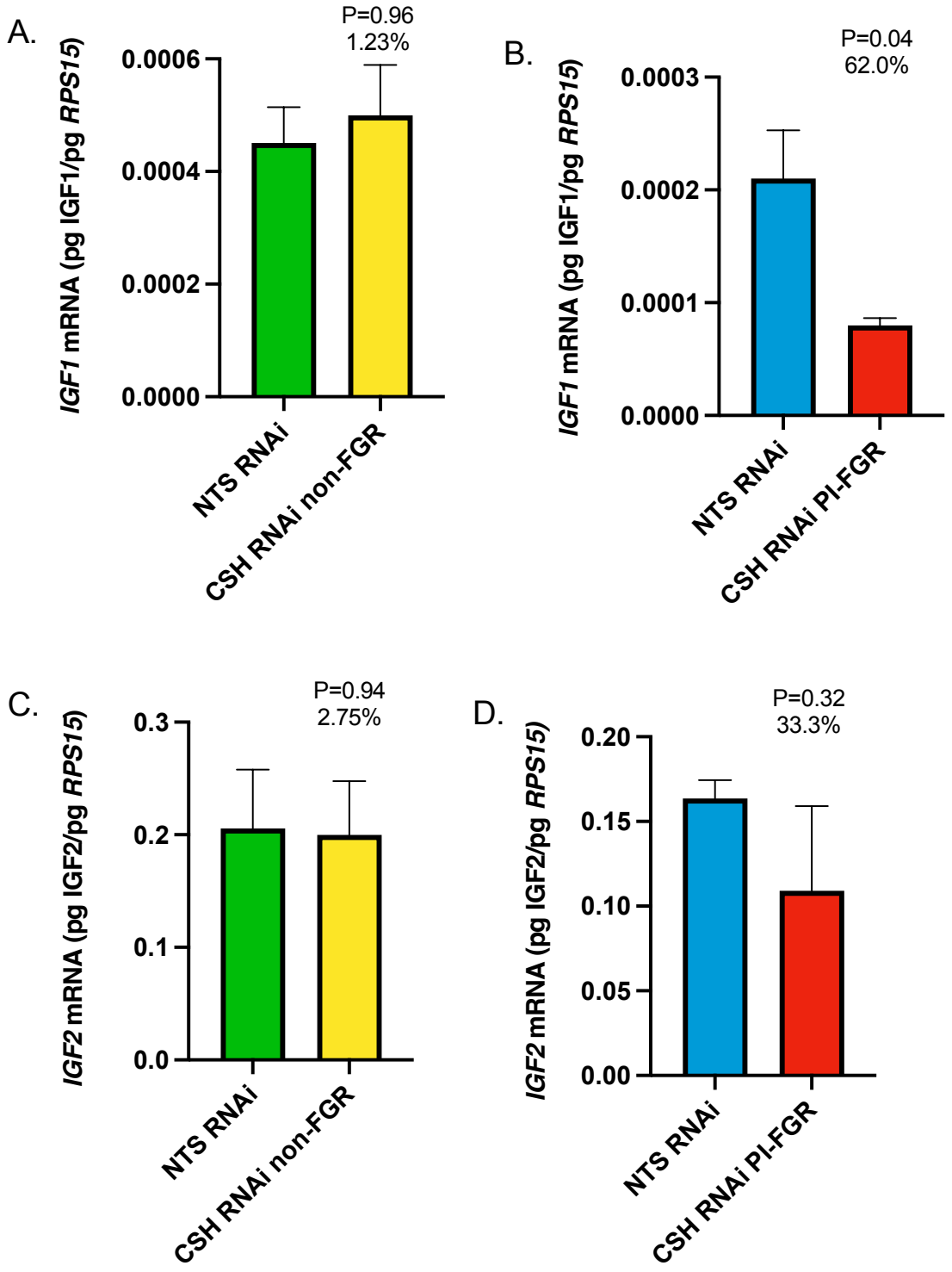

Figure 1. 135 dGA fetal cotyledon tissue (A) non-FGR *IGF1* mRNA concentrations, (B) PI-FGR *IGF1* mRNA concentrations, (C) non-FGR *IGF2* mRNA concentrations, and (D) PI-FGR *IGF2* mRNA concentrations. Data are shown as means ± SEM for the starting quantity of the mRNA of interest (pg) divided by the starting quantity (pg) of the housekeeping mRNA of *RPS15*.

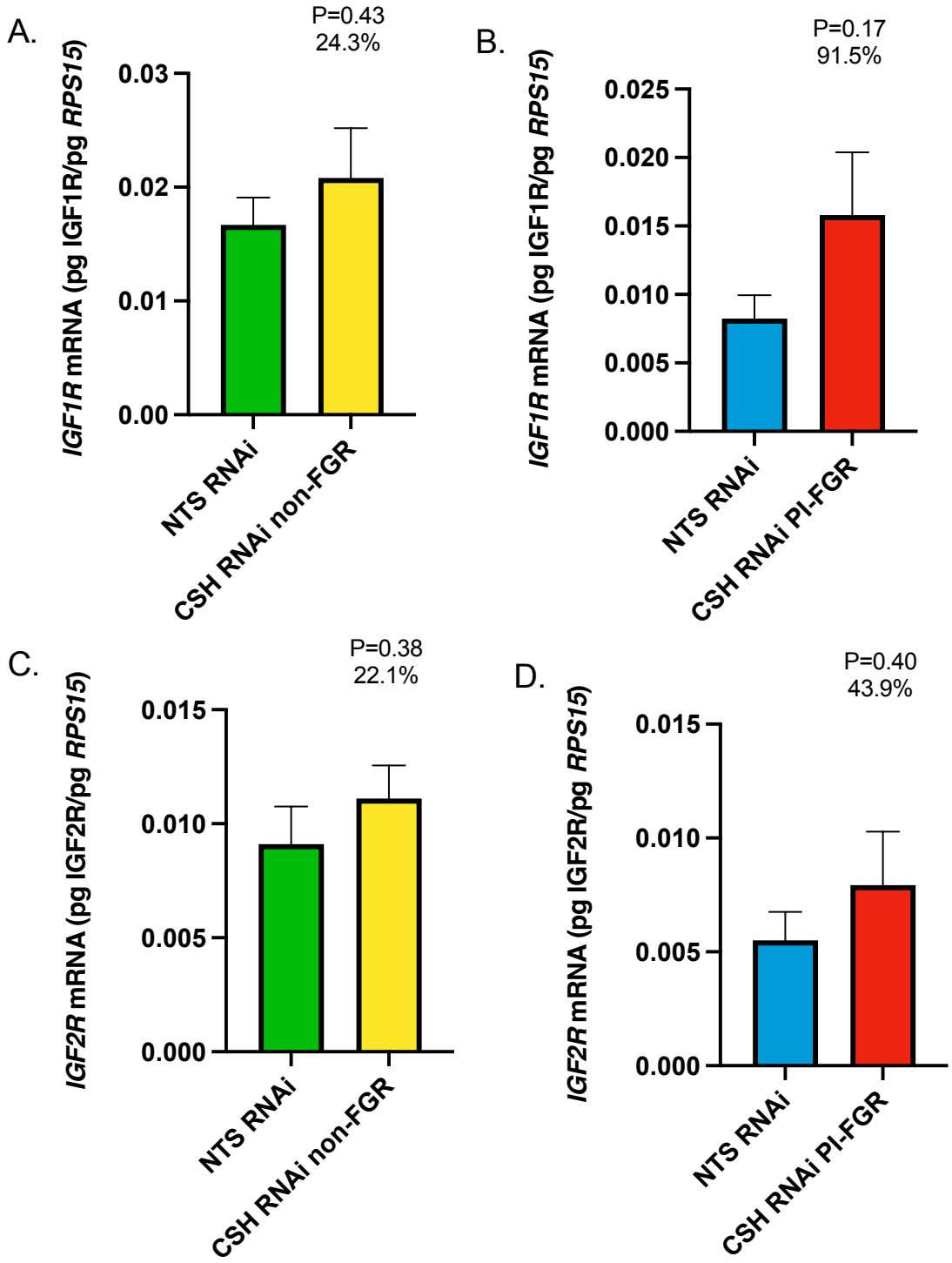

Figure 2. 135 dGA fetal cotyledon tissue (A) non-FGR *IGF1R* mRNA concentrations, (B) PI-FGR *IGF1R* mRNA concentrations, (C) non-FGR *IGF2R* mRNA concentrations, and (D) PI-FGR *IGF2R* mRNA concentrations. Data are shown as means ± SEM for the starting quantity of the mRNA of interest (pg) divided by the starting quantity (pg) of the housekeeping mRNA of *RPS15*.

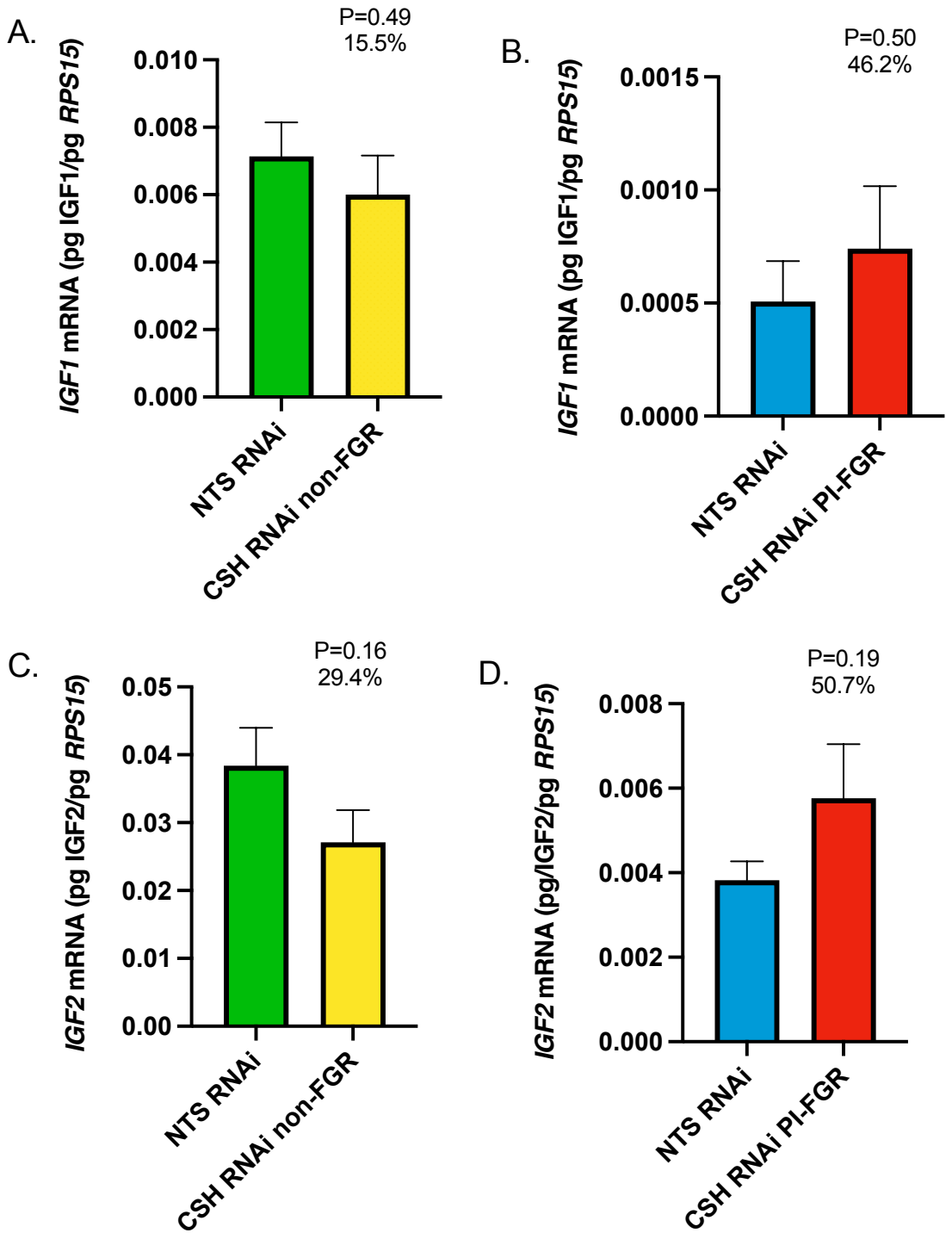

Figure 3. 135 dGA maternal caruncle tissue (A) non-FGR *IGF1* mRNA concentrations, (B) PI-FGR *IGF1* mRNA concentrations, (C) non-FGR *IGF2* mRNA concentrations, and (D) PI-FGR *IGF2* mRNA concentrations. Data are shown as means ± SEM for the starting quantity of the mRNA of interest (pg) divided by the starting quantity (pg) of the housekeeping mRNA of *RPS15*.

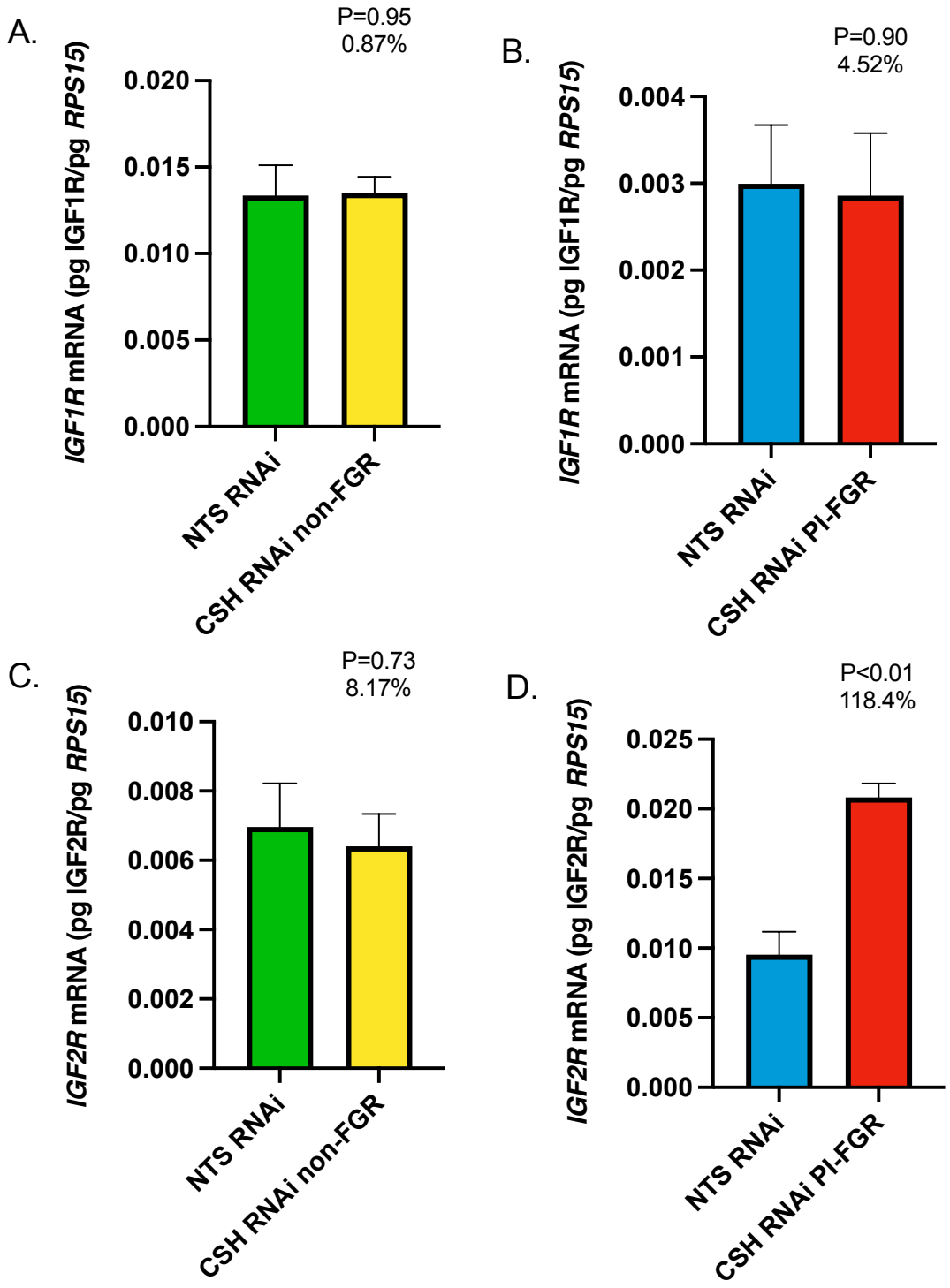

Figure 4. 135 dGA maternal caruncle tissue (A) non-FGR *IGF1R* mRNA concentrations, (B) PI-FGR *IGF1R* mRNA concentrations, (C) non-FGR *IGF2R* mRNA concentrations, and (D) PI-FGR *IGF2R* mRNA concentrations. Data are shown as means ± SEM for the starting quantity of the mRNA of interest (pg) divided by the starting quantity (pg) of the housekeeping mRNA of *RPS15*.

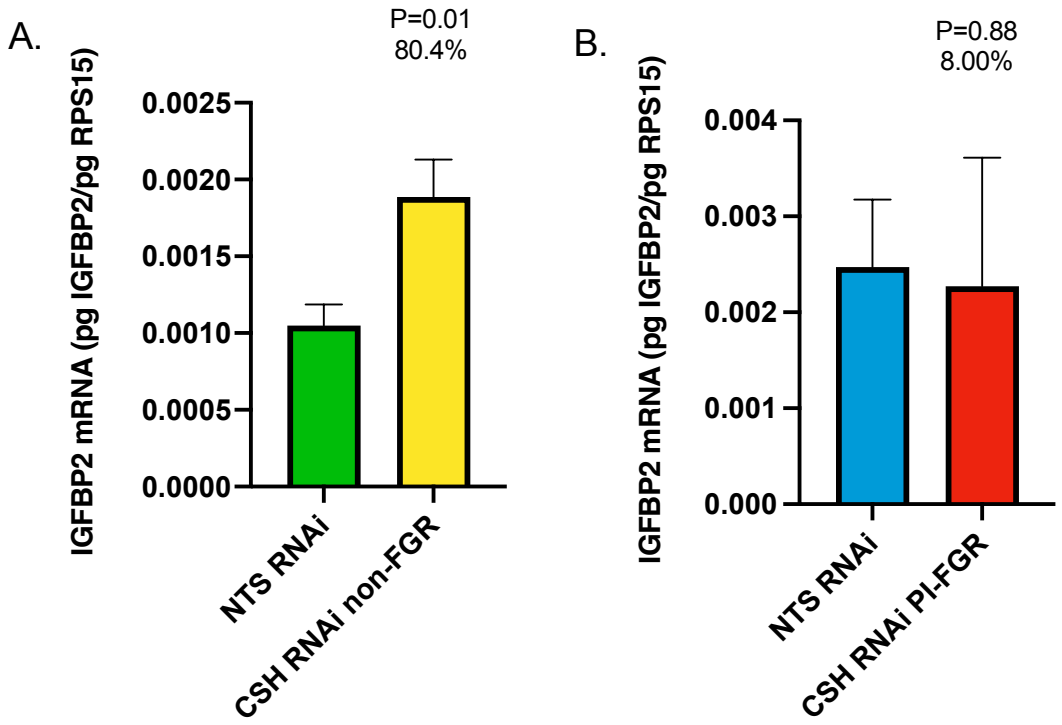

Figure 5. 135 dGA fetal cotyledon tissue (A) non-FGR *IGFBP2* mRNA concentrations, (B) PI-FGR *IGFBP2* mRNA concentrations. Data are shown as means ± SEM for the starting quantity of the mRNA of interest (pg) divided by the starting quantity (pg) of the housekeeping mRNA of *RPS15*.

DISCUSSION

Placental development and function are prerequisites for fetal growth, and it has been reported (Ghidini, 1996) that 60% of FGR pregnancies, with a normally formed fetus, result from altered placental function. The placenta is not only responsible for the uptake of maternal nutrients, and the partial transfer of these nutrients to the fetus, but is also a source of specific hormones that can impact maternal and fetal physiology. One such placental hormone is chorionic somatotropin/placental lactogen (CSH/PL) that has long been hypothesized to directly affect maternal and fetal metabolism and endocrine function (Handwerger, 1991). However, direct evidence supporting this hypothesis was not

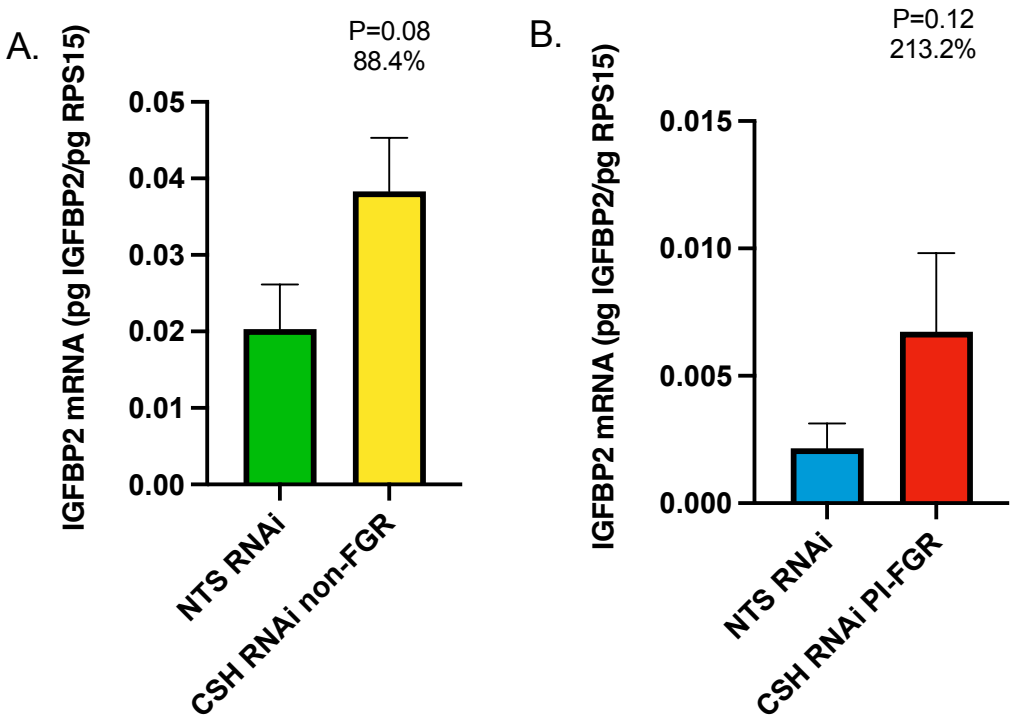

Figure 6. 135 dGA maternal caruncle tissue (A) non-FGR *IGFBP2* mRNA concentrations, (B) PI-FGR *IGFBP2* mRNA concentrations. Data are shown as means ± SEM for the starting quantity of the mRNA of interest (pg) divided by the starting quantity (pg) of the housekeeping mRNA of *RPS15*.

reported until Baker *et al*. (2016), using *in vivo* lentiviral-mediated RNAi demonstrated that CSH RNAi resulted in significant fetal and placental growth restriction (PI-FGR) near term. With the report of Baker *et al*. (2016), there were two unexpected results. The first was the large impact on placental mass, as a role for CSH augmenting placental growth had not been hypothesized previously. The second was that some of the pregnancies harvested near-term appeared to be normal, and the remainder exhibited placental and fetal weights ≥2 SD below the mean of the control pregnancies. However, further analysis of these (Baker *et al*., 2016) non-FGR pregnancies by Ali *et al*., (2020), revealed that there were significant differences with the non-FGR fetuses, even when birthweight was not impacted. The dichotomy in late-term phenotype observed with CSH RNAi is

supported by case reports of human pregnancies in which deletions or mutations in the *CSH* loci resulted in FGR or apparently normal outcomes (Alexander *et al*., 1982; Barbeiri *et al*., 1986; Borody and Carlton, 1981; Rygaard *et al*., 1998; Sideri *et al*., 1983; Simon *et al*., 1986). This is supported by the results of Daikoku *et al*. (1979) and Lindberg and Nilsson (1973), which observed both normal birth weights and FGR in pregnancies with suppressed maternal concentrations of CSH. Based on the observations of Ali *et al*. (2020), it raises the question as to how "normal" the outcome of the human pregnancies deficient in CSH without FGR are.

Herein, we report the impact of CSH RNAi on expression of the IGF axis in both the maternal and fetal components of the placenta, from a cohort of CSH RNAi non-FGR pregnancies (Tanner *et al*., 2021a) as well as a cohort of CSH RNAi PI-FGR pregnancies in which the impact on placental and fetal growth was very similar to that originally reported by Baker *et al.* (2016). With both CSH RNAi cohorts, the pregnancies underwent maternal and fetal cannulation, allowing the direct assessment of uterine and umbilical blood flows, uterine uptake of nutrients, uteroplacental nutrient utilization and umbilical nutrient uptakes, under non-stressed, non-anesthetized conditions (Tanner *et al*., 2021a,2022). As previously reported in Tanner *et al*. (2021a) and presented in Table 3, the primary difference in CSH RNAi non-FGR pregnancies and the NTS RNAi controls was the significant reduction in uterine blood flow, coupled with increased uteroplacental utilization of glucose, resulting in the fraction of glucose taken up by the uterus and utilized by the fetus being significantly reduced. By contrast, the CSH RNAi PI-FGR pregnancies (Table 4) exhibited an even greater difference in uterine blood flow, as well as a significant reduction in umbilical blood flow. Furthermore, the CSH RNAi PI-FGR pregnancies had

significant reductions in uterine uptake, uteroplacental utilization and umbilical uptake of both oxygen and glucose, resulting in significant reductions in fetal and placental weights (Table 4). Comparing these two CSH RNAi phenotypes suggests that a common consequence resulting from CSH RNAi is significantly reduced uterine blood flow, and the magnitude of this reduction in uterine blood flow may determine the degree by which placental and fetal weights were impacted, as a result of limiting nutrient uptake and transfer.

With the CSH RNAi PI-FGR pregnancies, uterine artery insulin, IGF1 and cortisol concentrations were not impacted, whereas umbilical concentrations of insulin and IGF1 were significantly reduced (Table 5), and umbilical artery cortisol was significantly increased. By contrast, these same hormones were not impacted in CSH RNAi non-FGR pregnancies, in either the uterine or umbilical circulation (data not presented). The impact of CSH RNAi in PI-FGR pregnancies on umbilical insulin, IGF1 and cortisol is similar to that reported by Tanner *et al*. (2021b). The significant reductions in umbilical insulin and IGF1 are often observed, in a variety of sheep models of FGR (Wallace *et al*., 2005; Morrison, 2008). Elevated umbilical cortisol concentrations during late gestation has also been observed during late-gestation in FGR sheep pregnancies, and can be viewed as a fetal stress response, even in response to short-term fasting (Fowden and Forhead, 2022), and glucocorticoid administration can induce fetal metabolic changes observed with FGR (Fowden and Forhead, 2004; Morrison, 2008).

The reduction in fetal cotyledon tissue CSH concentration resulting from CSH RNAi has been fairly consistent across studies (Baker *et al*., 2016; Tanner *et al*., 2021a,b), but the impact on uterine vein and umbilical vein CSH concentrations has been more

variable. With the original report of Baker *et al*. (2016), only a 24% reduction in uterine vein CSH concentration was observed, which did not reflect the reduction in fetal cotyledon tissue concentrations of *CSH* mRNA or CSH. By contrast, in the current study, we report an ≈56% reduction in uterine vein CSH concentration within the CSH RNAi PI-FGR pregnancies, which exhibited similar phenotypic changes to those reported by Baker *et al*. (2016). A major difference between these two studies is that the Baker *et al*. (2016) samples were collected during a terminal surgery, rather than following cannulation and numerous days to recover. Taylor *et al*. (1980) reported elevated concentrations of CSH for several days following surgery, and therefore collecting samples during a terminal surgery could "artificially" elevate CSH concentrations, potentially masking the impact of CSH RNAi, such that the current data collected under non-anesthetized non-stressed conditions is more reflective of the impact of CSH RNAi on maternal concentrations.

In contrast to the impact on uterine vein concentrations of CSH, in none of the CSH RNAi studies (Baker *et al*., 2016; Tanner *et al*., 2021a,b), including the current CSH RNAi PI-FGR pregnancies (Table 4), has a difference in umbilical vein CSH concentrations been observed. One possible explanation for this is that any reduction in the CSH entry rate into the umbilical circulation (Schoknecht et al. 1992), may have been masked by reductions in fetal size and blood volume. Another likely explanation for this resides with the fetal hypoglycemia reported (Tanner *et al*., 2021b), and observed in the current study. Freemark *et al*. (1992) reported that fasting induced fetal hypoglycemia resulted in significant elevations in umbilical CSH concentrations, which were "normalized" upon maternal glucose infusion. Recently, Lynch *et al*. (2022) reported that the fetal hypoglycemia observed at mid-gestation as a result of fetal placental SLC2A3 RNAi was

associated with a 70% increase in umbilical CSH concentrations, with no impact on uterine vein concentrations. As such, the fetal hypoglycemia resulting from CSH RNAi may have subsequently induced a relative elevation in umbilical CSH concentrations, potentially overcoming the deficit in CSH being secreted into the umbilical circulation as a result of CSH RNAi. Regardless of the exact explanation for these data, they highlight the complexity of the interactions amongst the three functional compartments (maternal, placental and fetal) of pregnancy (Hay *et al*., 1984), and the need to investigate all three simultaneously.

Following the initial report of Baker *et al*. (2016), Jeckel *et al*. (2018) examined the mRNA concentrations of the IGF axis in the fetal cotyledon portion of the placenta, from those pregnancies reported in Baker *et al*. (2016). At 135 dGA, Jeckel *et al.* (2018) reported significant reductions in fetal placenta *IGF1* and *IGF2* mRNA concentrations, with no significant changes in *IGFBP1, IGFBP2* and *IGFBP3* mRNA concentrations. Jeckel *et al*. (2018) did not assess *IGF1R* and *IGF2R* mRNA concentrations, nor did they assess IGF axis expression in the maternal placenta (caruncles) or either placental tissue derived from the non-FGR pregnancies reported by Baker *et al.* (2016). This led us to examine the IGF axis in both the maternal and fetal placenta, and from CSH RNAi non-FGR and PI-FGR pregnancies. Similar to what was reported by Jeckel *et al*. (2018), we observed a significant reduction in fetal placenta (cotyledon) *IGF1* mRNA concentrations in the CSH RNAi PI-FGR pregnancies, but no difference in *IGF1* mRNA concentrations was identified in the maternal placenta (caruncle) of the CSH RNAi PI-FGR pregnancies. Furthermore, *IGF1* mRNA concentration was not impacted by CSH RNAi in either the maternal or fetal placenta of non-FGR pregnancies. As reported herein (Table 5), and

previously (Baker *et al*., 2016; Tanner *et al*., 2022b), umbilical artery concentrations of IGF1 are significantly reduced in CSH RNAi PI-FGR pregnancies, which is associated with significantly smaller fetal livers and reduced concentrations of fetal liver *IGF1* mRNA concentrations. Infusion of CSH into fetal sheep for 14 days results in significantly increased fetal arterial concentrations of IGF1 (Schoknecht *et al*., 1996). Therefore, the reduction in fetal placenta *IGF1* mRNA in CSH RNAi PI-FGR pregnancies may well result from a lack of intra-placental CSH stimulation of *IGF1* transcription.

IGF1 has been shown to enhance the expression of the facilitative glucose transporter SLC2A1 in human trophoblast cells (Baumann *et al*., 2014) and the mouse placenta (Jones *et al.,* 2013). In sheep, IUGR induced by placental embolization (Wali *et al*., 2012) results in fetal hypoxia and hypoglycemia, and is associated with reductions in *SLC2A1* mRNA and placental glucose uptake, but intra-amniotic infusion of IGF1 into these fetuses increased placental expression of *SLC2A1* mRNA (Wali *et al*., 2012). The reduction in fetal placental *IGF1* mRNA, and presumably placental IGF1, may have played a role in the reduction in uterine and umbilical glucose uptake with the CSH RNAi PI-FGR pregnancies. However, Tanner *et al*. (2021a) reported a 25% reduction ($P \leq 0.10$) in fetal placenta SLC2A1 protein concentration, in the CSH RNAi non-FGR pregnancies which did not exhibit a reduction in fetal placenta *IGF1* mRNA (Figure 1A). There appears to be a reciprocal relationship between IGF1 and glucose, since infusion of fetal sheep with glucose results in elevated fetal concentrations of IGF1 (Oliver *et al*., 1993), such that the reduction in fetal placental *IGF1* mRNA may have been impacted by reduced uterine uptake and uteroplacental utilization of glucose in the CSH RNAi PI-FGR pregnancies. Beyond the interplay of glucose and IGF1, CSH is significantly elevated in

fetal circulation as a result of fetal hypoglycemia (Freemark *et al*., 1992; Lynch *et al.*, 2022). However, the increased umbilical CSH as a result of fetal hypoglycemia, was associated with the maintenance of fetal concentrations of IGF1. Consequently, the interplay of CSH, IGF1 and glucose between the three compartments of pregnancy may ultimately impact the degree of placental and fetal growth restriction that occurs in FGR pregnancies.

In contrast to what was reported by Jeckel *et al.* (2018), there were not significant changes in *IGF2* mRNA concentrations within the fetal placenta in either the CSH RNAi non-FGR or PI-FGR pregnancies. In the PI-FGR pregnancies there was a 33% reduction in *IGF2* mRNA, but this was not statistically different, whereas Jeckel *et al*. (2018) reported a statistically significant 53% reduction in *IGF2* mRNA. The characteristics of the current CSH RNAi PI-FGR pregnancies and those from which the Jeckel *et al.* (2018) samples were derived are very similar, except for sample collection methods. The fetal cotyledons examined by Jeckel *et al*. (2018) were collected following an 18 h fast and anesthesia (Baker *et al*., 2016), which may have impacted *IGF2* mRNA concentrations. In another sheep model of PI-FGR (de Vrijer *et al*., 2006) *IGF2* mRNA was significantly increased in the fetal placenta at 55 dGA, but not at 90 dGA, and near-term data was not reported. Interestingly, *IGF1* mRNA in the fetal placenta was not impacted at either gestational age, possibly indicating that IGF2 plays a predominant role in early placental development, with IGF1 being more important during late gestation. In mice, placenta-specific *Igf2* gene ablation (Coen *et al*., 2008) clearly demonstrated the importance of Igf2 in rodent placental growth and function. Since the rat placenta is thought to not express *Igf1* (Zhou and Bondy, 1992), and *Igf1* gene ablation in mice did not impact placenta size

(Baker *et al*., 1993), it appears that in rodents Igf2 drives placental growth and function rather than Igf1. However, it is possible that IGF1 plays the predominant role in non-rodent placental growth and function, especially during later gestation. Within the maternal placenta (caruncles), *IGF2* mRNA concentrations were not statistically different in either the CSH RNAi non-FGR or PI-FGR pregnancies, although *IGF2* mRNA was 29% lower in non-FGR pregnancies and 51% greater in PI-FGR pregnancies (Figure 3). The lack of a significant effect of CSH RNAi on *IGF2* mRNA within the maternal caruncles agrees with a lack of effect at either 55 or 90 dGA in the pregnancies reported by de Vrijer *et al*. (2006).

While there are two IGF receptors, IGF1R and IGF2R, both IGF1 and IGF2 activate IGF1R (Denley *et al*., 2005) resulting in enhanced cell proliferation, differentiation, and/or migration (Khandwala *et al*., 2000; Pollak *et al*., 2004). In contrast, IGF2R primarily modulates the availability of IGF2 (Denley *et al*., 2005) and acts as a clearance receptor for IGF2 (Scott and Firth, 2004). Within the CSH RNAi non-FGR pregnancies, there were no significant changes in *IGF1R* or *IGF2R* mRNA concentrations in either the fetal (Figure 2) or maternal (Figure 4) components of the placenta. However, in the fetal placenta of CSH RNAi PI-FGR pregnancies, *IGF1R* mRNA increased 92%, although this did not reach statistical significance (Figure 2), which may have been an attempt to upregulate IGF1R in the face of significantly reduced *IGF1* mRNA in the fetal placenta (Figure 1). In the maternal placenta of CSH RNAi PI-FGR pregnancies, neither the concentration of *IGF1* or *IGF1R* mRNA were impacted, collectively suggesting that IGF1 did not play a role in the maternal placenta response (e.g., reduced uterine blood flow, etc.) to CSH RNAi in either the non-FGR and PI-FGR pregnancies. Interestingly, the concentration of

IGF2R mRNA was significantly elevated (118%; Figure 4) within the maternal placenta of CSH RNAi PI-FGR pregnancies, potentially to offset the effects of any increased production of IGF2 by the maternal placenta (Figure 3). One possible explanation for the increased *IGF2R* mRNA in CSH RNAi PI-FGR pregnancies is a response to the significant reduction in uterine uptake of glucose. When fetal placenta glucose uptake is diminished in response to SLC2A3 RNAi (Lynch *et al.* 2022), similar increases in *IGF2* and *IGF2R* mRNA concentrations were reported as to what was observed in the current CSH RNAi PI-FGR maternal placenta. The physiological importance of this finding is not at all clear, but may suggest the need to maintain a fine balance between IGF1 and IGF2, and the actions they propagate through IGFR1, thereby inhibiting the autocrine and paracrine actions of IGF2 mediated through IGF1R (Kiess *et al*., 1987; Nolan *et al*., 1990; Kornfeld, 1992).

The IGFBPs can potentiate or inhibit the actions of IGFs, dependent on the binding proteins preference for IGF1 and IGF2 (Clemmons *et al*. 2001; Firth *et al*., 2002). Similar to what was reported by Jeckel *et al*. (2018), we did not observe any significant differences in the concentration of *IGFBP1* or *IGFBP3* mRNA in either the fetal or maternal placenta, in either the CSH RNAi non-FGR or PI-FGR pregnancies. However, in the CSH RNAi non-FGR pregnancies, the concentration of *IGFBP2* mRNA was significantly increased in both the fetal and maternal portions of the placenta (Figures 5 and 6), and a non-statistically significant increase within the maternal caruncles of CSH RNAi PI-FGR pregnancies. IGFBP2 is reported to inhibit IGF actions, particularly IGF2, as it has a 2-fold higher affinity for IGF2 (Jones *et al*., 1995; Carrick *et al*., 2001). When IGFBP2 is overexpressed in human embryonic kidney fibroblasts, cell proliferation was

inhibited and was reversed with the addition of IGF's (Höflich *et al*., 1998). However, other studies reported that IGFBP2 promoted tumor cell proliferation (Hoeflich *et al*. 2000), likely in an IGF-independent manner (Oh, 1998). In the pig, the uterine glandular epithelium co-express IGF2 and IGFPB2, and Badinga *et al*. (1999) demonstrated both IGF2-dependent and independent effects of IGFBP2 on glandular epithelial cell proliferation. The enhanced concentration of *IGFBP2* mRNA within the CSH RNAi non-FGR pregnancies may be one mechanism by which uterine and placental growth was maintained in these pregnancies. Since *IGFBP2* mRNA concentrations were also elevated in the maternal caruncles of CSH RNAi PI-FGR pregnancies, this might implicate the significantly reduced uterine blood flow that occurs in both the non-FGR and PI-FGR pregnancies as a potential driver of enhanced *IGFBP2* expression.

CONCLUSIONS

Investigating the interplay of the three compartments of pregnancy is complex but is necessary to understand the progression of normal pregnancies, as well as determine the etiology of pregnancy complications such as FGR. By combining *in vivo* lentiviral-mediated RNAi with *in vivo* steady-state assessment of blood flows, as well as nutrient uptakes and utilization by both maternal and fetal tissues, we were able to compare CSH RNAi pregnancies that do not result in FGR and those that result in PI-FGR. Clearly nutrient uptakes and utilization, as well as hormone secretion, are greatly impacted in PI-FGR pregnancies, but the non-FGR pregnancies are not truly "normal." This also allowed us to examine the mRNA concentrations of the IGF axis in both the maternal (caruncles) and fetal (cotyledons) placenta. As might be expected, the majority of differences

observed were within the PI-FGR pregnancies, especially the impact on fetal placental *IGF1* mRNA, which fits with CSH RNAi impacts on fetal liver and umbilical concentrations of IGF1. However, in non-FGR pregnancies, enhanced *IGFBP2* mRNA within both maternal and fetal placenta may suggest a compensatory mechanism that allowed the maintenance of uterine and placental growth. This potentially IGF-independent action of IGFBP2 to promote placental growth, needs further investigation, along with the potential role of reduced uterine blood flow inducing *IGFBP2* mRNA.

LITERATURE CITED

Abu-Amero, S. N., Ali, Z., Bennett, P., Vaughan, J. I., & Moore, G. E. (1998). Expression of the insulin-like growth factors and their receptors in term placentas: a comparison between normal and IUGR births. *Molecular reproduction and development*, *49*(3), 229–235.

Adams, S. O., Nissley, S. P., Handwerger, S., & Rechler, M. M. (1983a). Developmental patterns of insulin-like growth factor-I and -II synthesis and regulation in rat fibroblasts. *Nature*, *302*(5904), 150–153.

Adams, S. O., Nissley, S. P., Greenstein, L. A., Yang, Y. W., & Rechler, M. M. (1983b). Synthesis of multiplication-stimulating activity (rat insulin-like growth factor II) by rat embryo fibroblasts. *Endocrinology*, *112*(3), 979–987.

Adams, S. O., Nissley, S. P., Kasuga, M., Foley, T. P., Jr, & Rechler, M. M. (1983c). Receptors for insulin-like growth factors and growth effects of multiplication-stimulating activity (rat insulin-like growth factor II) in rat embryo fibroblasts. *Endocrinology*, *112*(3), 971–978.

Alexander, G. (1964). Studies on the placenta of the sheep (Ovis Aries L.) Effect of surgical reduction in the number of caruncles. *Journal of Reproduction and Fertility*, *7*, 307–322.

Alexander, I., Anthony, F., & Letchworth, A. T. (1982). PLacental protein profile and glucose studies in a normal pregnancy with extremely low levels of human placental lactogen. Case report. *British journal of obstetrics and gynaecology*, *89*(3), 241–243.

Alexander, G., & Williams, D. (1971). Heat stress and development of the conceptus in domestic sheep. *The Journal of Agricultural Science*, *76*(1), 53-72.

Ali, A., Stenglein, M. D., Spencer, T. E., Bouma, G. J., Anthony, R. V., & Winger, Q. A. (2020a). Trophectoderm-Specific Knockdown of LIN28 Decreases Expression of Genes Necessary for Cell Proliferation and Reduces Elongation of Sheep Conceptus. *International journal of molecular sciences*, *21*(7), 2549.

Ali, A., Swanepoel, C. M., Winger, Q. A., Rozance, P. J., & Anthony, R. V. (2020b). Chorionic somatomammotropin RNA interference alters fetal liver glucose utilization. *The Journal of endocrinology*, *247*(3), 251–262.

Alexander, I., Anthony, F., & Letchworth, A. T. (1982). PLacental protein profile and glucose studies in a normal pregnancy with extremely low levels of human placental lactogen. Case report. *British journal of obstetrics and gynaecology*, *89*(3), 241–243.

Anderson, A. H., Fennessey, P. V., Meschia, G., Wilkening, R. B., & Battaglia, F. C. (1997). Placental transport of threonine and its utilization in the normal and growth-restricted fetus. *The American journal of physiology*, *272*(5 Pt 1), E892–E900.

Andrews, S. E., Brown, L. D., Thorn, S. R., Limesand, S. W., Davis, M., Hay, W. W., Jr, & Rozance, P. J. (2015). Increased adrenergic signaling is responsible for decreased glucose-stimulated insulin secretion in the chronically hyperinsulinemic ovine fetus. *Endocrinology*, *156*(1), 367–376.

Anthony, R. V., Pratt, S. L., Liang, R., & Holland, M. D. (1995). Placental-fetal hormonal interactions: impact on fetal growth. *Journal of animal science*, *73*(6), 1861–1871.

Anthony, R.V., Limesand, S.W., Fanning, M.D., Liang, R. (1998). Placental lactogen and growth hormone. In: Bazer FW, ed. Endocrinology of pregnancy. Totowa, NJ: Humana Press, 461–490.

Anthony, R. V., Scheaffer, A. N., Wright, C. D., & Regnault, T. R. (2003). Ruminant models of prenatal growth restriction. *Reproduction (Cambridge, England). Supplement*, *61*, 183–194.

Bach L. A. (2018). IGF-binding proteins. *Journal of molecular endocrinology*, *61*(1), T11–T28.

Badinga, L., Song, S., Simmen, R. C., Clarke, J. B., Clemmons, D. R., & Simmen, F. A. (1999). Complex mediation of uterine endometrial epithelial cell growth by insulin-like growth factor-II (IGF-II) and IGF-binding protein-2. *Journal of molecular endocrinology*, *23*(3), 277–285.

Baker, C. M., Goetzmann, L. N., Cantlon, J. D., Jeckel, K. M., Winger, Q. A., & Anthony, R. V. (2016). Development of ovine chorionic somatomammotropin hormone-deficient pregnancies. *American journal of physiology. Regulatory, integrative and comparative physiology*, *310*(9), R837–R846.

Baker, J., Liu, J. P., Robertson, E. J., & Efstratiadis, A. (1993). Role of insulin-like growth factors in embryonic and postnatal growth. *Cell*, *75*(1), 73–82.

Barbieri, F., Botticelli, A., Consarino, R., Genazzani, A. R., & Volpe, A. (1986). Failure of placenta to produce hPL in an otherwise uneventful pregnancy: a case report. *Biological research in pregnancy and perinatology*, *7*(3), 131–133.

Barker, D. J., & Clark, P. M. (1997). Fetal undernutrition and disease in later life. *Reviews of reproduction*, *2*(2), 105–112.

Barry, J. S., & Anthony, R. V. (2008). The pregnant sheep as a model for human

pregnancy. *Theriogenology*, *69*(1), 55–67.
Bauer, M. K., Harding, J. E., Bassett, N. S., Breier, B. H., Oliver, M. H., Gallaher, B. H., Evans, P. C., Woodall, S. M., & Gluckman, P. D. (1998). Fetal growth and placental function. *Molecular and cellular endocrinology*, *140*(1-2), 115–120.

Baumann, M. U., Schneider, H., Malek, A., Palta, V., Surbek, D. V., Sager, R., Zamudio, S., & Illsley, N. P. (2014). Regulation of human trophoblast GLUT1 glucose transporter by insulin-like growth factor I (IGF-I). *PloS one*, *9*(8), e106037.

Baxter, R. C., & de Mellow, J. S. (1986). Measurement of insulin-like growth factor-II by radioreceptor assay using ovine placental membranes. *Clinical endocrinology*, *24*(3), 267–278.

Beede, K. A., Limesand, S. W., Petersen, J. L., & Yates, D. T. (2019). Real supermodels wear wool: summarizing the impact of the pregnant sheep as an animal model for adaptive fetal programming. *Animal frontiers : the review magazine of animal agriculture*, *9*(3), 34–43.

Bell, A. W., McBride, B. W., Slepetis, R., Early, R. J., & Currie, W. B. (1989). Chronic heat stress and prenatal development in sheep: I. Conceptus growth and maternal plasma hormones and metabolites. *Journal of animal science*, *67*(12), 3289–3299.

Benjamin, J. S., Culpepper, C. B., Brown, L. D., Wesolowski, S. R., Jonker, S. S., Davis, M. A., Limesand, S. W., Wilkening, R. B., Hay, W. W., Jr, & Rozance, P. J. (2017). Chronic anemic hypoxemia attenuates glucose-stimulated insulin secretion in fetal sheep. *American journal of physiology. Regulatory, integrative and comparative physiology*, *312*(4), R492–R500.

Borody, I. B., & Carlton, M. A. (1981). Isolated defect in human placental lactogen synthesis in a normal pregnancy. Case report. *British journal of obstetrics and gynaecology*, *88*(4), 447–449.

Bowman, C. J., Streck, R. D., & Chapin, R. E. (2010). Maternal-placental insulin-like growth factor (IGF) signaling and its importance to normal embryo-fetal development. *Birth defects research. Part B, Developmental and reproductive toxicology*, *89*(4), 339–349.

Breuer C. B. (1969). Stimulation of DNA synthesis in cartilage of hypophysectomized rats by native and modified placental lactogen and anabolic hormones. *Endocrinology*, *85*(6), 989–999.

Bruin, C., Damhuis, S., Gordijn, S., & Ganzevoort, W. (2021). Evaluation and Management of Suspected Fetal Growth Restriction. *Obstetrics and gynecology clinics of North America*, *48*(2), 371–385.

Butler, S. R., Hurley, T. W., Schanberg, S. M., & Handwerger, S. (1978). Ovine placental lactogen stimulation of ornithine decarboxylase activity in brain and liver of neonatal rats. *Life sciences*, *22*(23), 2073–2078.

Byatt, J. C., Eppard, P. J., Veenhuizen, J. J., Sorbet, R. H., Buonomo, F. C., Curran, D. F., & Collier, R. J. (1992). Serum half-life and in-vivo actions of recombinant bovine placental lactogen in the dairy cow. *The Journal of endocrinology*, *132*(2), 185–193.

Carr, J. M., Owens, J. A., Grant, P. A., Walton, P. E., Owens, P. C., & Wallace, J. C. (1995). Circulating insulin-like growth factors (IGFs), IGF-binding proteins (IGFBPs) and tissue mRNA levels of IGFBP-2 and IGFBP-4 in the ovine fetus. *The Journal of endocrinology*, *145*(3), 545–557.

Carrick, F. E., Forbes, B. E., & Wallace, J. C. (2001). BIAcore analysis of bovine insulin-like growth factor (IGF)-binding protein-2 identifies major IGF binding site determinants in both the amino- and carboxyl-terminal domains. *The Journal of biological chemistry*, *276*(29), 27120–27128.

Chandler, K. D., Leury, B. J., Bird, A. R., & Bell, A. W. (1985). Effects of undernutrition and exercise during late pregnancy on uterine, fetal and uteroplacental metabolism in the ewe. *The British journal of nutrition*, *53*(3), 625–635.

Cilvik, S. N., Wesolowski, S. R., Anthony, R. V., Brown, L. D., & Rozance, P. J. (2021). Late gestation fetal hyperglucagonaemia impairs placental function and results in diminished fetal protein accretion and decreased fetal growth. *The Journal of physiology*, *599*(13), 3403–3427.

Clemmons D. R. (2001). Use of mutagenesis to probe IGF-binding protein structure/function relationships. *Endocrine reviews*, *22*(6), 800–817.

Clemmons D. R. (2018). Role of IGF-binding proteins in regulating IGF responses to changes in metabolism. *Journal of molecular endocrinology*, *61*(1), T139–T169.

Coan, P. M., Fowden, A. L., Constancia, M., Ferguson-Smith, A. C., Burton, G. J., & Sibley, C. P. (2008). Disproportional effects of Igf2 knockout on placental morphology and diffusional exchange characteristics in the mouse. *The Journal of physiology*, *586*(20), 5023–5032.

Constância, M., Hemberger, M., Hughes, J., Dean, W., Ferguson-Smith, A., Fundele, R., Stewart, F., Kelsey, G., Fowden, A., Sibley, C., & Reik, W. (2002). Placental-specific IGF-II is a major modulator of placental and fetal growth. *Nature*, *417*(6892), 945–948.

Corkins, M. R., Vanderhoof, J. A., Slentz, D. H., MacDonald, R. G., & Park, J. H. (1995).

Growth stimulation by transfection of intestinal epithelial cells with an antisense insulin-like growth factor binding protein-2 construct. *Biochemical and biophysical research communications*, *211*(3), 707–713.

Crocker, I. P. (2011). Placental Origins of Intrauterine Growth Restriction. In H. H. Kay, D. M. Nelson, Y. Wang (Eds.), *The Placenta: From Development to Disease* (pp. 237-245). Wiley-Blackwell.

Daikoku, N. H., Tyson, J. E., Graf, C., Scott, R., Smith, B., Johnson, J. W., & King, T. M. (1979). The relative significance of human placental lactogen in the diagnosis of retarded fetal growth. *American journal of obstetrics and gynecology*, *135*(4), 516–521.

Daughaday, W. H., & Kapadia, M. (1978). Maintenance of serum somatomedin activity in hypophysectomized pregnant rats. *Endocrinology*, *102*(4), 1317–1320.

Daughaday, W. H., Parker, K. A., Borowsky, S., Trivedi, B., & Kapadia, M. (1982). Measurement of somatomedin-related peptides in fetal, neonatal, and maternal rat serum by insulin-like growth factor (IGF) I radioimmunoassay, IGF-II radioreceptor assay (RRA), and multiplication-stimulating activity RRA after acid-ethanol extraction. *Endocrinology*, *110*(2), 575–581.

DeChiara, T. M., Efstratiadis, A., & Robertson, E. J. (1990). A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. *Nature*, *345*(6270), 78–80.

DeChiara, T. M., Robertson, E. J., & Efstratiadis, A. (1991). Parental imprinting of the mouse insulin-like growth factor II gene. *Cell*, *64*(4), 849–859.

Delhanty, P. J., & Han, V. K. (1993). The expression of insulin-like growth factor (IGF)-binding protein-2 and IGF-II genes in the tissues of the developing ovine fetus. *Endocrinology*, *132*(1), 41–52.

Denley, A., Cosgrove, L. J., Booker, G. W., Wallace, J. C., & Forbes, B. E. (2005). Molecular interactions of the IGF system. *Cytokine & growth factor reviews*, *16*(4-5), 421–439.

D'Ercole A. J. (1993). Expression of insulin-like growth factor-I in transgenic mice. *Annals of the New York Academy of Sciences*, *692*, 149–160.

de Vrijer, B., Davidsen, M. L., Wilkening, R. B., Anthony, R. V., & Regnault, T. R. (2006). Altered placental and fetal expression of IGFs and IGF-binding proteins associated with intrauterine growth restriction in fetal sheep during early and mid-pregnancy. *Pediatric research*, *60*(5), 507–512.

Dimitriadis, G., Parry-Billings, M., Bevan, S., Dunger, D., Piva, T., Krause, U., Wegener,

G., & Newsholme, E. A. (1992). Effects of insulin-like growth factor I on the rates of glucose transport and utilization in rat skeletal muscle in vitro. *The Biochemical journal*, *285 (Pt 1)*(Pt 1), 269–274.

Dunlap, K. A., Palmarini, M., Varela, M., Burghardt, R. C., Hayashi, K., Farmer, J. L., & Spencer, T. E. (2006). Endogenous retroviruses regulate periimplantation placental growth and differentiation. *Proceedings of the National Academy of Sciences of the United States of America*, *103*(39), 14390–14395.

Early, R. J., McBride, B. W., Vatnick, I., & Bell, A. W. (1991). Chronic heat stress and prenatal development in sheep: II. Placental cellularity and metabolism. *Journal of animal science*, *69*(9), 3610–3616.

Edwards, L. J., Simonetta, G., Owens, J. A., Robinson, J. S., & McMillen, I. C. (1999). Restriction of placental and fetal growth in sheep alters fetal blood pressure responses to angiotensin II and captopril. *The Journal of physiology*, *515 (Pt 3)*(Pt 3), 897–904.

Ehrhardt, K. (1936). Ueber das Laktationshormon des Hypophysenvorderlappens. *Münch. med. Wschr*, 29, 1163-1164.

Fang, Q., Wang, Y. X., & Zhou, Y. (2004). Insulin-like growth factor binding protein 1 and human embryonic development during 6 - 10 gestational weeks.

Felber, J. P., Zaragoza, N., Benuzzi-Badoni, M., & Genazzani, A. R. (1972). The double effect of human chorionic somatomammotropin (HCS) and pregnancy on lipogenesis and on lipolysis in the isolated rat epididymal fat pad and fat pad cells. *Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme*, *4*(4), 293–296.

Firth, S. M., & Baxter, R. C. (2002). Cellular actions of the insulin-like growth factor binding proteins. *Endocrine reviews*, *23*(6), 824–854.

Florini, J. R., & Ewton, D. Z. (1990). Highly specific inhibition of IGF-I-stimulated differentiation by an antisense oligodeoxyribonucleotide to myogenin mRNA. No effects on other actions of IGF-T. *The Journal of biological chemistry*, *265*(23), 13435–13437.

Florini, J. R., Ewton, D. Z., & Roof, S. L. (1991a). Insulin-like growth factor-I stimulates terminal myogenic differentiation by induction of myogenin gene expression. *Molecular endocrinology (Baltimore, Md.)*, *5*(5), 718–724.

Florini, J. R., Magri, K. A., Ewton, D. Z., James, P. L., Grindstaff, K., & Rotwein, P. S. (1991b). "Spontaneous" differentiation of skeletal myoblasts is dependent upon autocrine secretion of insulin-like growth factor-II. *The Journal of biological chemistry*, *266*(24), 15917–15923.

Forbes, K., & Westwood, M. (2008). The IGF axis and placental function. a mini review. *Hormone research*, *69*(3), 129–137.

Ford, S. P., Hess, B. W., Schwope, M. M., Nijland, M. J., Gilbert, J. S., Vonnahme, K. A., Means, W. J., Han, H., & Nathanielsz, P. W. (2007). Maternal undernutrition during early to mid-gestation in the ewe results in altered growth, adiposity, and glucose tolerance in male offspring. *Journal of animal science*, *85*(5), 1285–1294.

Fowden A. L. (2003). The insulin-like growth factors and feto-placental growth. *Placenta*, *24*(8-9), 803–812.

Fowden, A. L., & Forhead, A. J. (2004). Endocrine mechanisms of intrauterine programming. *Reproduction (Cambridge, England)*, *127*(5), 515–526.

Fowden, A. L., & Forhead, A. J. (2022). Endocrine regulation of fetal metabolism towards term. *Domestic animal endocrinology*, *78*, 106657.

Fowden, A. L., Szemere, J., Hughes, P., Gilmour, R. S., & Forhead, A. J. (1996). The effects of cortisol on the growth rate of the sheep fetus during late gestation. *The Journal of Endocrinology*, *151*(1), 97–105.

Freemark, M., & Handwerger, S. (1983). Ovine placental lactogen, but not growth hormone, stimulates amino acid transport in fetal rat diaphragm. *Endocrinology*, *112*(1), 402–404.

Freemark, M., & Handwerger, S. (1984). Ovine placental lactogen stimulates glycogen synthesis in fetal rat hepatocytes. *The American journal of physiology*, *246*(1 Pt 1), E21–E24.

Freemark, M., & Handwerger, S. (1986). The glycogenic effects of placental lactogen and growth hormone in ovine fetal liver are mediated through binding to specific fetal ovine placental lactogen receptors. *Endocrinology*, *118*(2), 613–618.

Freemark, M., Keen, A., Fowlkes, J., Mularoni, T., Comer, M., Grandis, A., & Kodack, L. (1992). The placental lactogen receptor in maternal and fetal sheep liver: regulation by glucose and role in the pathogenesis of fasting during pregnancy. *Endocrinology*, *130*(2), 1063–1070.

Furlanetto, R. W., Underwood, L. E., Van Wyk, J. J., & Handwerger, S. (1978). Serum immunoreactive somatomedin-C is elevated late in pregnancy. *The Journal of clinical endocrinology and metabolism*, *47*(3), 695–698.

Galosy, S. S., & Talamantes, F. (1995). Luteotropic actions of placental lactogens at midpregnancy in the mouse. *Endocrinology*, *136*(9), 3993–4003.

Gagnon R. (2003). Placental insufficiency and its consequences. *European Journal of Obstetrics, Gynecology, and Reproductive Biololgy*, *110 Suppl 1*, S99-S107.

Galan, H. L., Hussey, M. J., Barbera, A., Ferrazzi, E., Chung, M., Hobbins, J. C., & Battaglia, F. C. (1999). Relationship of fetal growth to duration of heat stress in an ovine model of placental insufficiency. *American journal of obstetrics and gynecology*, *180*(5), 1278–1282.

Gardner, R. L., Squire, S., Zaina, S., Hills, S., & Graham, C. F. (1999). Insulin-like growth factor-2 regulation of conceptus composition: effects of the trophectoderm and inner cell mass genotypes in the mouse. *Biology of reproduction*, *60*(1), 190–195.

Germain-Lee, E. L., Janicot, M., Lammers, R., Ullrich, A., & Casella, S. J. (1992). Expression of a type I insulin-like growth factor receptor with low affinity for insulin-like growth factor II. *The Biochemical journal*, *281 (Pt 2)*(Pt 2), 413–417.

Ghidini A. (1996). Idiopathic fetal growth restriction: a pathophysiologic approach. *Obstetrical & gynecological survey*, *51*(6), 376–382.

Giudice, L. C., Farrell, E. M., Pham, H., Lamson, G., & Rosenfeld, R. G. (1990). Insulin-l ike growth factor binding proteins in maternal serum throughout gestation and in the puerperium: effects of a pregnancy-associated serum protease activity. *The Journal of clinical endocrinology and metabolism*, *71*(4), 806–816.

Giudice L. C. (1992). Insulin-like growth factors and ovarian follicular development. *Endocrine reviews*, *13*(4), 641–669.

Gluckman, P. D., & Brinsmead, M. W. (1976). Somatomedin in cord blood: relationship to gestational age and birth size. *The Journal of clinical endocrinology and metabolism*, *43*(6), 1378–1381.

Gluckman, P. D., Johnson-Barrett, J. J., Butler, J. H., Edgar, B. W., & Gunn, T. R. (1983). Studies of insulin-like growth factor -I and -II by specific radioligand assays in umbilical cord blood. *Clinical endocrinology*, *19*(3), 405–413.

Gluckman, P.D., Bassett, N. S., & Butler, J. H. (1986). The somatotrophic axis in the fetus and newborn. In *Perinatal Endocrinology* (pp. 1-21). Perinatol. Press, Ithaca, NY.

Grumbach, M. M., Kaplan, S. L., Sciarra, J. J., & Burr, I. M. (1968). Chorionic growth hormone-prolactin (CGP): secretion, disposition, biologic activity in man, and postulated function as the "growth hormone" of the 2d half of pregnancy. *Annals of the New York Academy of Sciences*, *148*(2), 501–531.

Gude, N. M., Roberts, C. T., Kalionis, B., & King, R. G. (2004). Growth and function of the normal human placenta. *Thrombosis research*, *114*(5-6), 397–407.

Gupta M. B. (2015). The role and regulation of IGFBP-1 phosphorylation in fetal growth restriction. *Journal of cell communication and signaling*, *9*(2), 111–123.

Han, V. K., D'Ercole, A. J., & Lund, P. K. (1987). Cellular localization of somatomedin (insulin-like growth factor) messenger RNA in the human fetus. *Science (New York, N.Y.)*, *236*(4798), 193–197.

Han, V. K., Lund, P. K., Lee, D. C., & D'Ercole, A. J. (1988). Expression of somatomedin/insulin-like growth factor messenger ribonucleic acids in the human fetus: identification, characterization, and tissue distribution. *The Journal of clinical endocrinology and metabolism*, *66*(2), 422–429.

Han, V. K. M., and Fowden, A. L. (1994). Paracrine regulation of fetal growth. In 'Early Fetal Growth and Development'. (Eds R. H. T. Ward, S. K. Smith and D. Donnai.) pp. 275-91. (Royal College of Obstetrics and Gynaecology Press: London.)

Han, V. K., Matsell, D. G., Delhanty, P. J., Hill, D. J., Shimasaki, S., & Nygard, K. (1996). IGF-binding protein mRNAs in the human fetus: tissue and cellular distribution of developmental expression. *Hormone research*, *45*(3-5), 160–166

Han, V. K., & Carter, A. M. (2000). Spatial and temporal patterns of expression of messenger RNA for insulin-like growth factors and their binding proteins in the placenta of man and laboratory animals. *Placenta*, *21*(4), 289–305.

Handwerger S. (1991). Clinical counterpoint: the physiology of placental lactogen in human pregnancy. *Endocrine reviews*, *12*(4), 329–336.

Hay, W. W., Jr, Sparks, J. W., Battaglia, F. C., & Meschia, G. (1984). Maternal-fetal glucose exchange: necessity of a three-pool model. *The American journal of physiology*, *246*(6 Pt 1), E528–E534.

Hill, D. J., Camacho-Hubner, C., Rashid, P., Strain, A. J., & Clemmons, D. R. (1989). Insulin-like growth factor (IGF)-binding protein release by human fetal fibroblasts: dependency on cell density and IGF peptides. *The Journal of endocrinology*, *122*(1), 87–98.

Hill D. J. (1990). Relative abundance and molecular size of immunoreactive insulin-like growth factors I and II in human fetal tissues. *Early human development*, *21*(1), 49–58.

Holland, M. D., Hossner, K. L., Williams, S. E., Wallace, C. R., Niswender, G. D., &

Odde, K. G. (1997). Serum concentrations of insulin-like growth factors and placental lactogen during gestation in cattle. I. Fetal profiles. *Domestic animal endocrinology*, *14*(4), 231–239.

Holmes, R., Montemagno, R., Jones, J., Preece, M., Rodeck, C., & Soothill, P. (1997). Fetal and maternal plasma insulin-like growth factors and binding proteins in pregnancies with appropriate or retarded fetal growth. *Early human development*, *49*(1), 7–17.

Holmes, R., Porter, H., Newcomb, P., Holly, J. M., & Soothill, P. (1999). An immunohistochemical study of type I insulin-like growth factor receptors in the placentae of pregnancies with appropriately grown or growth restricted fetuses. *Placenta*, *20*(4), 325–330.

Hoeflich, A., Fettscher, O., Lahm, H., Blum, W. F., Kolb, H. J., Engelhardt, D., Wolf, E., & Weber, M. M. (2000). Overexpression of insulin-like growth factor-binding protein-2 results in increased tumorigenic potential in Y-1 adrenocortical tumor cells. *Cancer research*, *60*(4), 834–838.

Hoeflich, A., Nedbal, S., Blum, W. F., Erhard, M., Lahm, H., Brem, G., Kolb, H. J., Wanke, R., & Wolf, E. (2001). Growth inhibition in giant growth hormone transgenic mice by overexpression of insulin-like growth factor-binding protein-2. *Endocrinology*, *142*(5), 1889–1898.

Höflich, A., Lahm, H., Blum, W., Kolb, H., & Wolf, E. (1998). Insulin-like growth factor-binding protein-2 inhibits proliferation of human embryonic kidney fibroblasts and of IGF-responsive colon carcinoma cell lines. *FEBS letters*, *434*(3), 329–334.

Hoshina, M., Boothby, M., & Boime, I. (1982). Cytological localization of chorionic gonadotropin alpha and placental lactogen mRNAs during development of the human placenta. *The Journal of cell biology*, *93*(1), 190–198.

Hurley, T. W., D'Ercole, A. J., Handwerger, S., Underwood, L. E., Furlanetto, R. W., & Fellows, R. E. (1977). Ovine placental lactogen induces somatomedin: a possible role in fetal growth. *Endocrinology*, *101*(5), 1635–1638.

Jacob, R., Barrett, E., Plewe, G., Fagin, K. D., & Sherwin, R. S. (1989). Acute effects of insulin-like growth factor I on glucose and amino acid metabolism in the awake fasted rat. Comparison with insulin. *The Journal of clinical investigation*, *83*(5), 1717–1723.

Jeckel, K. M., Boyarko, A. C., Bouma, G. J., Winger, Q. A., & Anthony, R. V. (2018). Chorionic somatomammotropin impacts early fetal growth and placental gene expression. *The Journal of endocrinology*, *237*(3), 301–310.

Jensen, E. C., Gallaher, B. W., Breier, B. H., & Harding, J. E. (2002). The effect of a

chronic maternal cortisol infusion on the late-gestation fetal sheep. *The Journal of endocrinology*, *174*(1), 27–36.

Jobe, A. H., Wada, N., Berry, L. M., Ikegami, M., & Ervin, M. G. (1998). Single and repetitive maternal glucocorticoid exposures reduce fetal growth in sheep. *American journal of obstetrics and gynecology*, *178*(5), 880–885.

Johnson, J. W., Mitzner, W., London, W. T., Palmer, A. E., & Scott, R. (1979). Betamethasone and the rhesus fetus: multisystemic effects. *American journal of obstetrics and gynecology*, *133*(6), 677–684.

Jones, J. I., & Clemmons, D. R. (1995). Insulin-like growth factors and their binding proteins: biological actions. *Endocrine reviews*, *16*(1), 3–34.

Jones, H. N., Crombleholme, T., & Habli, M. (2013). Adenoviral-mediated placental gene transfer of IGF-1 corrects placental insufficiency via enhanced placental glucose transport mechanisms. *PloS one*, *8*(9), e74632.

Josimovich, J. B., & Maclaren, J. A. (1962). Presence in the human placenta and term serum of a highly lactogenic substance immunologically related to pituitary growth hormone. *Endocrinology*, *71*, 209–220.

Kajimura, S., Aida, K., & Duan, C. (2005). Insulin-like growth factor-binding protein-1 (IGFBP-1) mediates hypoxia-induced embryonic growth and developmental retardation. *Proceedings of the National Academy of Sciences of the United States of America*, *102*(4), 1240–1245.

Kappes, S. M., Warren, W. C., Pratt, S. L., Liang, R., & Anthony, R. V. (1992). Quantification and cellular localization of ovine placental lactogen messenger ribonucleic acid expression during mid- and late gestation. *Endocrinology*, *131*(6), 2829–2838.

Khandwala, H. M., McCutcheon, I. E., Flyvbjerg, A., & Friend, K. E. (2000). The effects of insulin-like growth factors on tumorigenesis and neoplastic growth. *Endocrine reviews*, *21*(3), 215–244.

Kiess, W., Haskell, J. F., Lee, L., Greenstein, L. A., Miller, B. E., Aarons, A. L., Rechler, M. M., & Nissley, S. P. (1987). An antibody that blocks insulin-like growth factor (IGF) binding to the type II IGF receptor is neither an agonist nor an inhibitor of IGF-stimulated biologic responses in L6 myoblasts. *The Journal of biological chemistry*, *262*(26), 12745–12751.

Kimble, R. M., Breier, B. H., Gluckman, P. D., & Harding, J. E. (1999). Enteral IGF-I enhances fetal growth and gastrointestinal development in oesophageal ligated fetal sheep. *The Journal of endocrinology*, *162*(2), 227–235.

Kind, K. L., Owens, J. A., Robinson, J. S., Quinn, K. J., Grant, P. A., Walton, P. E., Gilmour, R. S., & Owens, P. C. (1995). Effect of restriction of placental growth on expression of IGFs in fetal sheep: relationship to fetal growth, circulating IGFs and binding proteins. *The Journal of endocrinology*, *146*(1), 23–34.

Kornfeld S. (1992). Structure and function of the mannose 6-phosphate/insulinlike growth factor II receptors. *Annual review of biochemistry*, *61*, 307–330.

Kroonsberg, C., McCutcheon, S. N., Siddiqui, R. A., Mackenzie, D. D., Blair, H. T., Ormsby, J. E., Breier, B. H., & Gluckman, P. D. (1989). Reproductive performance and fetal growth in female mice from lines divergently selected on the basis of plasma IGF-1 concentrations. *Journal of reproduction and fertility*, *87*(1), 349–353.

Lang, U., Baker, R. S., Khoury, J., & Clark, K. E. (2000). Effects of chronic reduction in uterine blood flow on fetal and placental growth in the sheep. *American journal of physiology. Regulatory, integrative and comparative physiology*, *279*(1), R53–R59.

Larsen, T., Main, K., Andersson, A. M., Juul, A., Greisen, G., & Skakkebaek, N. E. (1996). Growth hormone, insulin-like growth factor I and its binding proteins 1 and 3 in last trimester intrauterine growth retardation with increased pulsatility index in the umbilical artery. *Clinical endocrinology*, *45*(3), 315–319.

Lassarre, C., Hardouin, S., Daffos, F., Forestier, F., Frankenne, F., & Binoux, M. (1991). Serum insulin-like growth factors and insulin-like growth factor binding proteins in the human fetus. Relationships with growth in normal subjects and in subjects with intrauterine growth retardation. *Pediatric research*, *29*(3), 219–225.

Lau, M. M., Stewart, C. E., Liu, Z., Bhatt, H., Rotwein, P., & Stewart, C. L. (1994). Loss of the imprinted IGF2/cation-independent mannose 6-phosphate receptor results in fetal overgrowth and perinatal lethality. *Genes & development*, *8*(24), 2953–2963.

Laviola, L., Perrini, S., Belsanti, G., Natalicchio, A., Montrone, C., Leonardini, A., Vimercati, A., Scioscia, M., Selvaggi, L., Giorgino, R., Greco, P., & Giorgino, F. (2005). Intrauterine growth restriction in humans is associated with abnormalities in placental insulin-like growth factor signaling. *Endocrinology*, *146*(3), 1498–1505.

Lee, C. Y., Chung, C. S., & Simmen, F. A. (1993). Ontogeny of the porcine insulin-like growth factor system. *Molecular and cellular endocrinology*, *93*(1), 71–80.

Lee, J. E., Pintar, J., & Efstratiadis, A. (1990). Pattern of the insulin-like growth factor II gene expression during early mouse embryogenesis. *Development (Cambridge, England)*, *110*(1), 151–159.

Leibovich, H., Gertler, A., Bazer, F. W., & Gootwine, E. (2000). Active immunization of ewes against ovine placental lactogen increases birth weight of lambs and milk production with no adverse effect on conception rate. *Animal reproduction science*, *64*(1-2), 33–47.

Leiser, R., Krebs, C., Ebert, B., & Dantzer, V. (1997). Placental vascular corrosion cast studies: A comparison between ruminants and humans. *Microscopy Research and Technique*, *38*(1–2), 76–87.

Limesand, S. W., Camacho, L. E., Kelly, A. C., & Antolic, A. T. (2018). Impact of thermal stress on placental function and fetal physiology. *Animal reproduction*, *15*(Suppl 1), 886–898.

Lindberg, B. S., & Nilsson, B. A. (1973). Human placental lactogen (HPL) levels in abnormal pregnancies. *The Journal of obstetrics and gynaecology of the British Commonwealth*, *80*(12), 1046–1053.

Liu, J. P., Baker, J., Perkins, A. S., Robertson, E. J., & Efstratiadis, A. (1993). Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). *Cell*, *75*(1), 59–72.

Lok, F., Owens, J. A., Mundy, L., Robinson, J. S., & Owens, P. C. (1996). Insulin-like growth factor I promotes growth selectively in fetal sheep in late gestation. *The American journal of physiology*, *270*(5 Pt 2), R1148–R1155.

Ludwig, T., Eggenschwiler, J., Fisher, P., D'Ercole, A. J., Davenport, M. L., & Efstratiadis, A. (1996). Mouse mutants lacking the type 2 IGF receptor (IGF2R) are rescued from perinatal lethality in Igf2 and Igf1r null backgrounds. *Developmental biology*, *177*(2), 517–535.

Lucy, M. C., Byatt, J. C., Curran, T. L., Curran, D. F., & Collier, R. J. (1994). Placental lactogen and somatotropin: hormone binding to the corpus luteum and effects on the growth and functions of the ovary in heifers. *Biology of reproduction*, *50*(5), 1136–1144.

Lynch, C. S., Kennedy, V. C., Tanner, A. R., Ali, A., Winger, Q. A., Rozance, P. J., & Anthony, R. V. (2022). Impact of Placental SLC2A3 Deficiency during the First-Half of Gestation. *International journal of molecular sciences*, *23*(20), 12530.

Marshall, R. N., Underwood, L. E., Voina, S. J., Foushee, D. B., & Van Wyk, J. J. (1974). Characterization of the insulin and somatomedin-C receptors in human placental cell membranes. *The Journal of clinical endocrinology and metabolism*, *39*(2), 283–292.

Martal, J., & Djiane, J. (1975). Purification of a lactogenic hormone in sheep placenta. *Biochemical and biophysical research communications*, *65*(2), 770–778.

Martin, J. M., & Friesen, H. (1969). Effect of human placental lactogen on the isolated islets of Langerhans in vitro. *Endocrinology*, *84*(3), 619–621.

Martina, N. A., Kim, E., Chitkara, U., Wathen, N. C., Chard, T., & Giudice, L. C. (1997). Gestational age-dependent expression of insulin-like growth factor-binding protein-1 (IGFBP-1) phosphoisoforms in human extraembryonic cavities, maternal serum, and decidua suggests decidua as the primary source of IGFBP-1 in these fluids during early pregnancy. *The Journal of clinical endocrinology and metabolism*, *82*(6), 1894–1898.

Maruo, T., Ladines-Llave, C. A., Matsuo, H., Manalo, A. S., & Mochizuki, M. (1992). A novel change in cytologic localization of human chorionic gonadotropin and human placental lactogen in first-trimester placenta in the course of gestation. *American journal of obstetrics and gynecology*, *167*(1), 217–222.

Megyesi, K., Kahn, C. R., Roth, J., Neville, D. M., Jr, Nissley, S. P., Humbel, R. E., & Froesch, E. R. (1975). The NSILA-s receptor in liver plasma membranes. Characterization and comparison with the insulin receptor. *The Journal of biological chemistry*, *250*(23), 8990–8996.

Mellor D. J. (1983). Nutritional and placental determinants of foetal growth rate in sheep and consequences for the newborn lamb. *The British veterinary journal*, *139*(4), 307–324.

Mellor, D. J., & Murray, L. (1982). Effects of long term undernutrition of the ewe on the growth rates of individual fetuses during late pregnancy. *Research in veterinary science*, *32*(2), 177–180.

Mirlesse, V., Frankenne, F., Alsat, E., Poncelet, M., Hennen, G., & Evain-Brion, D. (1993). Placental growth hormone levels in normal pregnancy and in pregnancies with intrauterine growth retardation. *Pediatric research*, *34*(4), 439–442.

Morrison J. L. (2008). Sheep models of intrauterine growth restriction: fetal adaptations and consequences. *Clinical and experimental pharmacology & physiology*, *35*(7), 730–743.

Moss, T. J., Sloboda, D. M., Gurrin, L. C., Harding, R., Challis, J. R., & Newnham, J. P. (2001). Programming effects in sheep of prenatal growth restriction and glucocorticoid exposure. *American journal of physiology. Regulatory, integrative and comparative physiology*, *281*(3), R960–R970.

National Research Council (US). Committee on Nutrient Requirements of Small Ruminants, National Research Council, Committee on the Nutrient Requirements of Small Ruminants, Board on Agriculture, Division on Earth, & Life Studies.

(2007). *Nutrient requirements of small ruminants: sheep, goats, cervids, and new world camelids*.

Nawathe, A. R., Christian, M., Kim, S. H., Johnson, M., Savvidou, M. D., & Terzidou, V. (2016). Insulin-like growth factor axis in pregnancies affected by fetal growth disorders. *Clinical epigenetics*, *8*, 11.

Nayak, N. R., & Giudice, L. C. (2003). Comparative biology of the IGF system in endometrium, decidua, and placenta, and clinical implications for foetal growth and implantation disorders. *Placenta*, *24*(4), 281–296.

Newnham J. P. (2001a). Is prenatal glucocorticoid administration another origin of adult disease?. *Clinical and experimental pharmacology & physiology*, *28*(11), 957–961.

Newnham, J. P., & Moss, T. J. (2001b). Antenatal glucocorticoids and growth: single versus multiple doses in animal and human studies. *Seminars in neonatology: SN*, *6*(4), 285–292.

Nolan, C. M., Kyle, J. W., Watanabe, H., & Sly, W. S. (1990). Binding of insulin-like growth factor II (IGF-II) by human cation-independent mannose 6-phosphate receptor/IGF-II receptor expressed in receptor-deficient mouse L cells. *Cell regulation*, *1*(2), 197–213.

Novy, M. J., & Walsh, S. W. (1983). Dexamethasone and estradiol treatment in pregnant rhesus macaques: effects on gestational length, maternal plasma hormones, and fetal growth. *American journal of obstetrics and gynecology*, *145*(8), 920–931.

Oh Y. (1998). IGF-independent regulation of breast cancer growth by IGF binding proteins. *Breast cancer research and treatment*, *47*(3), 283–293.

Oliver, M. H., Harding, J. E., Breier, B. H., Evans, P. C., Gallaher, B. W., & Gluckman, P. D. (1995). The effects of ovine placental lactogen infusion on metabolites, insulin-like growth factors and binding proteins in the fetal sheep. *The Journal of endocrinology*, *144*(2), 333–338.

Oliver, M. H., Harding, J. E., Breier, B. H., Evans, P. C., & Gluckman, P. D. (1993). Glucose but not a mixed amino acid infusion regulates plasma insulin-like growth factor-I concentrations in fetal sheep. *Pediatric research*, *34*(1), 62–65.

Oliver, M. H., Harding, J. E., Breier, B. H., & Gluckman, P. D. (1996). Fetal insulin-like growth factor (IGF)-I and IGF-II are regulated differently by glucose or insulin in the sheep fetus. *Reproduction, fertility, and development*, *8*(1), 167–172.

Ong, K., Kratzsch, J., Kiess, W., Costello, M., Scott, C., & Dunger, D. (2000). Size at

birth and cord blood levels of insulin, insulin-like growth factor I (IGF-I), IGF-II, IGF-binding protein-1 (IGFBP-1), IGFBP-3, and the soluble IGF-II/mannose-6-phosphate receptor in term human infants. The ALSPAC Study Team. Avon Longitudinal Study of Pregnancy and Childhood. *The Journal of clinical endocrinology and metabolism*, *85*(11), 4266–4269.

Owens, J. A., Falconer, J., & Robinson, J. S. (1987). Effect of restriction of placental growth on oxygen delivery to and consumption by the pregnant uterus and fetus. *Journal of developmental physiology*, *9*(2), 137–150.

Owens, J. A., Kind, K. L., Carbone, F., Robinson, J. S., & Owens, P. C. (1994). Circulating insulin-like growth factors-I and -II and substrates in fetal sheep following restriction of placental growth. *The Journal of endocrinology*, *140*(1), 5–13.

Owens, J. A., Falconer, J., & Robinson, J. S. (1989). Glucose metabolism in pregnant sheep when placental growth is restricted. *The American journal of physiology*, *257*(2 Pt 2), R350–R357.

Park F. (2007). Lentiviral vectors: are they the future of animal transgenesis?. *Physiological genomics*, *31*(2), 159–173.

Pollak, M. N., Schernhammer, E. S., & Hankinson, S. E. (2004). Insulin-like growth factors and neoplasia. *Nature reviews. Cancer*, *4*(7), 505–518.

Purcell, S. H., Cantlon, J. D., Wright, C. D., Henkes, L. E., Seidel, G. E., Jr, & Anthony, R. V. (2009). The involvement of proline-rich 15 in early conceptus development in sheep. *Biology of reproduction*, *81*(6), 1112–1121.

Rechler, M. M., Zapf, J., Nissley, S. P., Froesch, E. R., Moses, A. C., Podskalny, J. M., Schilling, E. E., & Humbel, R. E. (1980). Interactions of insulin-like growth factors I and II and multiplication-stimulating activity with receptors and serum carrier proteins. *Endocrinology*, *107*(5), 1451–1459.

Reed, S. A., Ashley, R., Silver, G., Splaine, C., Jones, A. K., Pillai, S. M., Peterson, M. L., Zinn, S. A., & Govoni, K. E. (2022). Maternal nutrient restriction and over-feeding during gestation alter expression of key factors involved in placental development and vascularization. *Journal of animal science*, *100*(6), skac155.

Regnault, T. R., Orbus, R. J., Battaglia, F. C., Wilkening, R. B., & Anthony, R. V. (1999). Altered arterial concentrations of placental hormones during maximal placental growth in a model of placental insufficiency. *The Journal of endocrinology*, *162*(3), 433–442.

Regnault, T. R., Galan, H. L., Parker, T. A., & Anthony, R. V. (2002). Placental

development in normal and compromised pregnancies-- a review. *Placenta*, *23 Suppl A*, S119–S129.

Reid, G. J., Flozak, A. S., & Simmons, R. A. (2002). Placental expression of insulin-like growth factor receptor-1 and insulin receptor in the growth-restricted fetal rat. *Journal of the Society for Gynecologic Investigation*, *9*(4), 210–214.

Reinisch, J. M., Simon, N. G., Karow, W. G., & Gandelman, R. (1978). Prenatal exposure to prednisone in humans and animals retards intrauterine growth. *Science (New York, N.Y.)*, *202*(4366), 436–438.

Reynolds, T. S., Stevenson, K. R., & Wathes, D. C. (1997). Pregnancy-specific alterations in the expression of the insulin-like growth factor system during early placental development in the ewe. *Endocrinology*, *138*(3), 886–897.

Rinderknecht, E., & Humbel, R. E. (1978a). The amino acid sequence of human insulin-like growth factor I and its structural homology with proinsulin. *The Journal of biological chemistry*, *253*(8), 2769–2776.

Rinderknecht, E., & Humbel, R. E. (1978b). Primary structure of human insulin-like growth factor II. *FEBS letters*, *89*(2), 283–286.

Robinson, J. S., Kingston, E. J., Jones, C. T., & Thorburn, G. D. (1979). Studies on experimental growth retardation in sheep. The effect of removal of a endometrial caruncles on fetal size and metabolism. *Journal of developmental physiology*, *1*(5), 379–398.

Roseboom, T. J., van der Meulen, J. H., Ravelli, A. C., Osmond, C., Barker, D. J., & Bleker, O. P. (2001). Effects of prenatal exposure to the Dutch famine on adult disease in later life: an overview. *Molecular and cellular endocrinology*, *185*(1-2), 93–98.

Ross, J. C., Fennessey, P. V., Wilkening, R. B., Battaglia, F. C., & Meschia, G. (1996). Placental transport and fetal utilization of leucine in a model of fetal growth retardation. *The American journal of physiology*, *270*(3 Pt 1), E491–E503.

Rossetti, L., Frontoni, S., Dimarchi, R., DeFronzo, R. A., & Giaccari, A. (1991). Metabolic effects of IGF-I in diabetic rats. *Diabetes*, *40*(4), 444–448.

Rygaard, K., Revol, A., Esquivel-Escobedo, D., Beck, B. L., & Barrera-Saldaña, H. A. (1998). Absence of human placental lactogen and placental growth hormone (HGH-V) during pregnancy: PCR analysis of the deletion. *Human genetics*, *102*(1), 87–92.

Scheiwiller, E., Guler, H. P., Merryweather, J., Scandella, C., Maerki, W., Zapf, J., &

Froesch, E. R. (1986). Growth restoration of insulin-deficient diabetic rats by recombinant human insulin-like growth factor I. *Nature*, *323*(6084), 169–171.

Schocknecht, P. A., McGuire, M. A., Cohick, W. S., Currie, W. B. & Bell, A. W. (1992) The effect of placental lactogen infusion on serum IGF-1 and IGFBP-2 concentrations in the late gestation ovine fetus. J. Anim. Sci. 70:212 (abs).

Schoknecht, P. A., McGuire, M. A., Cohick, W. S., Currie, W. B., & Bell, A. W. (1996). Effect of chronic infusion of placental lactogen on ovine fetal growth in late gestation. *Domestic animal endocrinology*, *13*(6), 519–528.

Schramm, W., Friesen, H. G., Robertson, H. A., & McCracken, J. A. (1984). Effect of exogenous ovine placental lactogen on luteolysis induced by prostaglandin F-2 alpha in sheep. *Journal of reproduction and fertility*, *70*(2), 557–565.

Scott, C. D., & Firth, S. M. (2004). The role of the M6P/IGF-II receptor in cancer: tumor suppression or garbage disposal?. *Hormone and metabolic research = Hormon- und Stoffwechselforschung = Hormones et metabolisme*, *36*(5), 261–271.

Seckl J. R. (2001). Glucocorticoid programming of the fetus; adult phenotypes and molecular mechanisms. *Molecular and cellular endocrinology*, *185*(1-2), 61–71.

Sferruzzi-Perri, A. N., Sandovici, I., Constancia, M., & Fowden, A. L. (2017). Placental phenotype and the insulin-like growth factors: resource allocation to fetal growth. *The Journal of physiology*, *595*(15), 5057–5093.

Shani, J., Zanbelman, L., Khazen, K., & Sulman, F. G. (1970). Mammotrophic and prolactin-like effects of rat and human placentae and amniotic fluid. *The Journal of endocrinology*, *46*(1), 15–20.

Sheu-Gruttadauria, J., & MacRae, I. J. (2017). Structural Foundations of RNA Silencing by Argonaute. *Journal of molecular biology*, *429*(17), 2619–2639.

Sibley, C. P., Coan, P. M., Ferguson-Smith, A. C., Dean, W., Hughes, J., Smith, P., Reik, W., Burton, G. J., Fowden, A. L., & Constância, M. (2004). Placental-specific insulin-like growth factor 2 (Igf2) regulates the diffusional exchange characteristics of the mouse placenta. *Proceedings of the National Academy of Sciences of the United States of America*, *101*(21), 8204–8208.

Sideri, M., De Virgiliis, G., Guidobono, F., Borgese, N., Sereni, L. P., Nicolini, U., & Remotti, G. (1983). Immunologically undetectable human placental lactogen in a normal pregnancy. Case report. *British journal of obstetrics and gynaecology*, *90*(8), 771–773.

Simon, P., Decoster, C., Brocas, H., Schwers, J., & Vassart, G. (1986). Absence of human

chorionic somatomammotropin during pregnancy associated with two types of gene deletion. *Human genetics*, *74*(3), 235–238.

Sloboda, D. M., Newnham, J. P., & Challis, J. R. (2000). Effects of repeated maternal betamethasone administration on growth and hypothalamic-pituitary-adrenal function of the ovine fetus at term. *The Journal of endocrinology*, *165*(1), 79–91.

Sommer, I., & Schachner, M. (1981). Monoclonal antibodies (O1 to O4) to oligodendrocyte cell surfaces: an immunocytological study in the central nervous system. *Developmental biology*, *83*(2), 311–327.

Spellacy, W. N., Buhi, W. C., & Birk, S. A. (1976). Human placental lactogen and intrauterine growth retardation. *Obstetrics and gynecology*, *47*(4), 446–448.

Steyn, C., Hawkins, P., Saito, T., Noakes, D. E., Kingdom, J. C., & Hanson, M. A. (2001). Undernutrition during the first half of gestation increases the predominance of fetal tissue in late-gestation ovine placentomes. *European journal of obstetrics, gynecology, and reproductive biology*, *98*(2), 165–170.

Stiles, C. D., Capone, G. T., Scher, C. D., Antoniades, H. N., Van Wyk, J. J., & Pledger, W. J. (1979). Dual control of cell growth by somatomedins and platelet-derived growth factor. *Proceedings of the National Academy of Sciences of the United States of America*, *76*(3), 1279–1283.

Stock, R. J., Josimovich, J. B., Kosor, B., Klopper, A., & Wilson, G. R. (1971). The effect of chorionic gonadotrophin and chorionic somatomammotrophin on steroidogenesis in the corpus luteum. *The Journal of obstetrics and gynaecology of the British Commonwealth*, *78*(6), 549–553.

Supramaniam, V. G., Jenkin, G., Loose, J., Wallace, E. M., & Miller, S. L. (2006). Chronic fetal hypoxia increases activin A concentrations in the late-pregnant sheep. *BJOG : an international journal of obstetrics and gynaecology*, *113*(1), 102–109.

Tanner, A. R., Kennedy, V. C., Lynch, C. S., Hord, T. K., Winger, Q. A., Rozance, P. J., & Anthony, R. V. (2022). In vivo investigation of ruminant placenta function and physiology-a review. *Journal of animal science*, *100*(6), skac045.

Tanner, A. R., Lynch, C. S., Ali, A., Winger, Q. A., Rozance, P. J., & Anthony, R. V. (2021a). Impact of chorionic somatomammotropin RNA interference on uterine blood flow and placental glucose uptake in the absence of intrauterine growth restriction. *American journal of physiology. Regulatory, integrative and comparative physiology*, *320*(2), R138–R148.

Tanner, A. R., Lynch, C. S., Kennedy, V. C., Ali, A., Winger, Q. A., Rozance, P. J., &

Anthony, R. V. (2021b). CSH RNA Interference Reduces Global Nutrient Uptake and Umbilical Blood Flow Resulting in Intrauterine Growth Restriction. *International journal of molecular sciences*, *22*(15), 8150.

Tapanainen, P. J., Bang, P., Wilson, K., Unterman, T. G., Vreman, H. J., & Rosenfeld, R. G. (1994). Maternal hypoxia as a model for intrauterine growth retardation: effects on insulin-like growth factors and their binding proteins. *Pediatric research*, *36*(2), 152–158.

Tarantal, A. F., & Gargosky, S. E. (1995). Characterization of the insulin-like growth factor (IGF) axis in the serum of maternal and fetal macaques (Macaca mulatta and Macaca fascicularis). *Growth regulation*, *5*(4), 190–198.

Taylor, M. J., Jenkin, G., Robinson, J. S., Thorburn, G. D., Friesen, H., & Chan, J. S. (1980). Concentrations of placental lactogen in chronically catheterized ewes and fetuses in late pregnancy. *The Journal of endocrinology*, *85*(1), 27–34.

Thakur, A., Sase, M., Lee, J. J., Thakur, V., & Buchmiller, T. L. (2000). Ontogeny of insulin-like growth factor 1 in a rabbit model of growth retardation. *The Journal of surgical research*, *91*(2), 135–140.

Thissen, J. P., Underwood, L. E., Maiter, D., Maes, M., Clemmons, D. R., & Ketelslegers, J. M. (1991). Failure of insulin-like growth factor-I (IGF-I) infusion to promote growth in protein-restricted rats despite normalization of serum IGF-I concentrations. *Endocrinology*, *128*(2), 885–890.

Thureen, P. J., Trembler, K. A., Meschia, G., Makowski, E. L., & Wilkening, R. B. (1992). Placental glucose transport in heat-induced fetal growth retardation. *The American journal of physiology*, *263*(3 Pt 2), R578–R585.

Turco, M. Y., & Moffett, A. (2019). Development of the human placenta. *Development (Cambridge, England)*, *146*(22), dev163428.

Wali, J. A., de Boo, H. A., Derraik, J. G., Phua, H. H., Oliver, M. H., Bloomfield, F. H., & Harding, J. E. (2012). Weekly intra-amniotic IGF-1 treatment increases growth of growth-restricted ovine fetuses and up-regulates placental amino acid transporters. *PloS one*, *7*(5), e37899.

Wallace, J. M., Aitken, R. P., & Cheyne, M. A. (1996). Nutrient partitioning and fetal growth in rapidly growing adolescent ewes. *Journal of Reproduction and Fertility*, *107*(2), 183–190.

Wallace, J. M., Bourke, D. A., & Aitken, R. P. (1999a). Nutrition and fetal growth: Paradoxical effects in the overnourished adolescent sheep. *Journal of Reproduction and Fertility. Supplement*, *54*, 385–399.

Wallace, J. M., Bourke, D. A., Aitken, R. P., & Cruickshank, M. A. (1999b). Switching

maternal dietary intake at the end of the first trimester has profound effects on placental development and fetal growth in adolescent ewes carrying singleton fetuses. *Biology of Reproduction*, *61*(1), 101–110.

Wallace, J. M., Bourke, D. A., Aitken, R. P., Leitch, N., & Hay, W. W., Jr (2002). Blood flows and nutrient uptakes in growth-restricted pregnancies induced by overnourishing adolescent sheep. *American journal of physiology. Regulatory, integrative and comparative physiology*, *282*(4), R1027–R1036.

Wallace, J. M., Bourke, D. A., Aitken, R. P., Milne, J. S., & Hay, W. W., Jr (2003). Placental glucose transport in growth-restricted pregnancies induced by overnourishing adolescent sheep. *The Journal of physiology*, *547*(Pt 1), 85–94.

Wallace, J. M., Da Silva, P., Aitken, R. P., & Cruickshank, M. A. (1997). Maternal endocrine status in relation to pregnancy outcome in rapidly growing adolescent sheep. *The Journal of Endocrinology*, *155*(2), 359–368.

Wallace, J. M., Regnault, T. R., Limesand, S. W., Hay, W. W., Jr, & Anthony, R. V. (2005). Investigating the causes of low birth weight in contrasting ovine paradigms. *The Journal of physiology*, *565*(Pt 1), 19–26.

Wallace, L. (1948). The growth of lambs before and after birth in relation to the level of nutrition. *The Journal of Agricultural Science, 38*(3), 243-302.

Wathes, D. C., Reynolds, T. S., Robinson, R. S., & Stevenson, K. R. (1998). Role of the insulin-like growth factor system in uterine function and placental development in ruminants. *Journal of dairy science*, *81*(6), 1778–1789.

Waters, M. J., Oddy, V. H., McCloghry, C. E., Gluckman, P. D., Duplock, R., Owens, P. C., & Brinsmead, M. W. (1985). An examination of the proposed roles of placental lactogen in the ewe by means of antibody neutralization. *The Journal of endocrinology*, *106*(3), 377–386.

Werner, H., Woloschak, M., Adamo, M., Shen-Orr, Z., Roberts, C. T., Jr, & LeRoith, D. (1989). Developmental regulation of the rat insulin-like growth factor I receptor gene. *Proceedings of the National Academy of Sciences of the United States of America*, *86*(19), 7451–7455.

Wilson, R. L., Owens, K., Sumser, E. K., Fry, M. V., Stephens, K. K., Chuecos, M., Carrillo, M., Schlabritz-Loutsevitch, N., & Jones, H. N. (2020). Nanoparticle mediated increased insulin-like growth factor 1 expression enhances human placenta syncytium function. *Placenta*, *93*, 1–7.

Wooding F. B. (1992). Current topic: the synepitheliochorial placenta of ruminants: binucleate cell fusions and hormone production. *Placenta*, *13*(2), 101–113.

Yamasaki, H., Prager, D., Gebremedhin, S., & Melmed, S. (1991). Insulin-like growth factor-I (IGF-I) attenuation of growth hormone is enhanced by overexpression of pituitary IGF-I receptors. *Molecular endocrinology (Baltimore, Md.)*, *5*(7), 890–896.

Yoshimura Y. (2003). Insulin-like growth factors and their binding proteins: Potential relevance to reproductive physiology. *Reproductive medicine and biology*, *2*(1), 1–24.

Young, L. E., Fernandes, K., McEvoy, T. G., Butterwith, S. C., Gutierrez, C. G., Carolan, C., Broadbent, P. J., Robinson, J. J., Wilmut, I., & Sinclair, K. D. (2001). Epigenetic change in IGF2R is associated with fetal overgrowth after sheep embryo culture. *Nature genetics*, *27*(2), 153–154.

Zapf, J., Hauri, C., Waldvogel, M., & Froesch, E. R. (1986). Acute metabolic effects and half-lives of intravenously administered insulinlike growth factors I and II in normal and hypophysectomized rats. *The Journal of clinical investigation*, *77*(6), 1768–1775.

Zhang, S., Barker, P., Botting, K. J., Roberts, C. T., McMillan, C. M., McMillen, I. C., & Morrison, J. L. (2016). Early restriction of placental growth results in placental structural and gene expression changes in late gestation independent of fetal hypoxemia. *Physiological reports*, *4*(23), e13049.

Zhou J, Bondy C. (1992). Insulin-like growth factor-II and its binding proteins in placental development. *Endocrinology*, 131(3), 1230-1240.