THESIS

THE IMPACT OF PLACENTAL SLC2A3 (GLUT-3) RNA INTERFERENCE ON FETAL GROWTH AND PHYSIOLOGY AT MID-GESTATION IN SHEEP

Submitted By

Cameron S. Lynch

Department of Biomedical Sciences

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Master's Committee:

Advisor: Russell V. Anthony

Dawit Tesfaye
Terry Engle

ABSTRACT

THE IMPACT OF PLACENTAL SLC2A3 (GLUT-3) RNA INTERFERENCE ON FETAL GROWTH AND PHYSIOLOGY AT MID-GESTATION IN SHEEP

Glucose is the primary energy substrate for fetal oxidative processes and growth. In order for glucose to be transported from maternal to fetal circulation in the ruminant placenta, it must be sequentially transported by SLC2A1 (GLUT-1) on the maternal-fetal syncytial layer, then by SLC2A3 (GLUT-3) on the apical trophoblast membrane, and again by SLC2A1 on the basolateral trophoblast membrane. SLC2A1 is the most abundant placental facilitative glucose transporter, and as such, is believed to be the primary glucose transporter in human and sheep placenta. However, SLC2A3 exhibits a five-fold greater affinity and transport capacity for glucose. As such, in addition to its location on the apical trophoblast membrane, any deficiency in SLC2A3 could impact trophoblast glucose uptake and placental transfer of glucose to the fetus, thus potentially altering placental development and setting the stage for fetal hypoglycemia and intrauterine growth restriction (IUGR). It was our objective to use placenta-specific RNA interference (RNAi) to diminish SLC2A3, and determine the impact at mid-gestation (75 dGA) in sheep.

The resulting pregnancies underwent a terminal surgery at 75 dGA. SLC2A3 RNAi resulted in a 37% reduction ($p \leq 0.05$) in placental SLC2A3 concentration. SLC2A3-deficiency resulted in decreased fetal growth as evident by reduced fetal weight ($p \leq 0.10$), head circumference ($p \leq 0.05$), femur length ($p \leq 0.05$), and tibia length ($p \leq 0.05$). While there were no significant reductions in maternal glucose or

insulin concentrations, the SLC2A3 RNAi pregnancies had decreased umbilical vein ($p \leq 0.05$) and umbilical artery ($p \leq 0.05$) glucose concentrations, as well as reduced umbilical artery insulin ($p \leq 0.10$). Additionally, apparent attempts at compensation for SLC2A3-deficiency, by increasing SLC2A1, CSH, and *IGF-2*, were unable to prevent fetal hypoglycemia and the impacts on fetal development. Placental SLC2A1 concentration were increased ($p \leq 0.10$), however this increase in expression was unable to prevent fetal hypoglycemia. The significant increase in umbilical vein CSH concentrations ($p \leq 0.05$) appeared to preserve fetal liver weight and circulating umbilical concentrations of IGF-1, both of which are commonly decreased in IUGR pregnancies. SLC2A3-deficiency also resulted in a significant increase in *IGF-2* ($p \leq 0.05$), *IGF1R* ($p \leq 0.05$), and *IGF2R* ($p \leq 0.05$) expression. This suggests an apparent attempt to increase placental growth via IGF-2 acting through IGF1R, while IGF2R, which primarily acts to sequester and degrade IGF-2, doesn't allow placental growth to be overstimulated.

While it has been suggested that SLC2A3 is predominantly important in late gestation, our data indicate that SLC2A3 is important for normal fetal development and appears to be a rate limiting glucose transporter during the first-half of gestation. A deficiency in SLC2A3 impacts trophoblast glucose uptake and subsequently glucose transfer to the fetus, and appears to set the stage during early gestation for the development of IUGR.

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CHAPTER I: LITERATURE REVIEW

Intrauterine growth restriction (IUGR), also referred to as fetal growth restriction (FGR), is often an idiopathic disease that has a multitude of potential etiologies that are linked to IUGR, such as genetic abnormalities, environmental cues (i.e., infection, drugs), antihypertensive medications, alcohol, and cigarette smoking (Crocker, 2011). IUGR and small-for-gestational age (SGA) can often be seen in the literature as exchangeable terms, but they are not exchangeable. Small-for-gestational age more reflects newborn birth weights below the 10^{th} percentile for an uncomplicated obstetric population (Crocker, 2011). Meanwhile, IUGR is more reflective of the fetus failing to achieve its genetically pre-determined growth potential (Crocker, 2011). Clinicians are able to assess if a mother has an IUGR pregnancy via serial ultrasounds that monitor fetal weight, disproportionate asymmetric growth (i.e., increased head to abdominal ratio), reductions in amniotic fluid volume, and abnormal Doppler waveforms in the umbilical artery, ductus venosus, and middle cerebral artery (Crocker, 2011). Doppler velocimetry is a valuable tool used to assess pregnancies for fetal growth restriction. Some common findings that are indicative of IUGR are: increased uterine artery flow resistance, increased pulsatility index that progresses to absent or reverse end diastolic flow, aberrant doppler flow velocity waveforms of the umbilical arteries, reversed flow in the ductus venosus, or changes in cerebral circulation (Crocker, 2011).

IUGR is the second-leading cause of perinatal mortality and impacts up to 6% of all human pregnancies (Gagnon, 2003). Through the epidemiological work of Barker and his colleagues, along with other epidemiological studies, it has been well

established that neonates born from an IUGR pregnancy are more likely to develop diseases such as hypertension, cardiovascular disease, and type 2 diabetes, in adulthood (Crocker, 2011; Fowden et al., 2010; Gluckman et al., 2008). The increased risk to develop these adult-onset diseases appears to stem from the poor in utero environment the fetus is subjected to (i.e., undernutrition, placental insufficiency, etc.), and when these insults happen during sensitive developmental periods of early life, they seem to cause permanent adaptations on the structure, physiology, and metabolism of the fetus (Godfrey & Barker, 2001).

However, due to various etiologies of IUGR, and the varying degrees of severity, it can be difficult to investigate and understand the placenta's role in the pathophysiology of intrauterine growth restriction. Additionally, due to obvious ethical reasons, many of the questions remaining about the progression of human pregnancy and the progression of IUGR pregnancies cannot be addressed experimentally in humans. Thus, animal models can be a valuable tool in addressing these questions experimentally. Throughout the literature, some common characteristics of human IUGR pregnancies are: reduced placental and fetal mass, reduced fetal oxygenation and glycemia, reduced umbilical vein oxygen, amino acid, and glucose uptake, reduced umbilical vein blood flow, increased fetal blood pressure, and increased umbilical artery pulsatility index (Anthony et al., 2003). However, it should be noted that not all IUGR pregnancies are the same, and some of these listed characteristics may not be present.

Sheep and Human Placenta Comparison

In studying normal and IUGR pregnancies, laboratory rodents, non-human primates, and domestic ruminants are some of the common animal models used. While each of

these animal models have provided insightful knowledge on normal and compromised pregnancies, the domestic ruminants, more specifically the sheep, have been an invaluable model towards our current understanding of placental and fetal physiology due to our ability to surgically place and maintain catheters in both the maternal and fetal vasculature, thus allowing repeated sampling under non-stressed, steady state conditions *in vivo* (Barry & Anthony, 2008). While no animal model truly recapitulates human pregnancy, the pregnant sheep has provided considerable insight for the past 50 years on maternal-fetal interactions and on placental nutrient transfer and utilization. The pregnant sheep is an ideal model for human pregnancy due to the commonalities between each species placental development, metabolic function, and nutrient transport.

In some aspects of placental development, the sheep and human placenta are quite different. Humans have a discoid, hemochorial placenta and the human conceptus truly invades into the uterine lining. Conversely, sheep have a cotyledonary, synepitheliochorial placenta with a multitude of specialized non-glandular, well vascularized endometrium attachment sites, comprised of fetal cotyledon and maternal caruncle, throughout their placenta, and the sheep conceptus does not undergo invasive implantation (Wooding, 1992). Additionally, human and sheep fetal villous trees also differ in form and size; however, the architecture of stem, intermediate, and terminal villi, and the fetal vessels/capillary complex, are structurally analogous to each other (Barry & Anthony, 2008; Leiser et al., 1997). Since the human placenta can only be studied morphologically after birth, the ruminant placenta offers a valid model to study microvascular research during all stages of normal and compromised

pregnancies. One placental structural abnormality observed in human and sheep IUGR pregnancies is alterations to the villous tree, such as reductions in placental villous number, diameter, and surface area (Salafia et al., 1997; Krebs et al., 1996; Macara et al., 1996; Mayhew et al., 2003), and reductions in villous arterial number, diameter, and degree of branching (Barry & Anthony, 2008; Lee & Yeh, 1986; Giles et al., 1985). These alterations observed in IUGR pregnancies cannot be thoroughly studied with the term human placenta, thus the sheep has been a useful model in elucidating how changes in vasculogenesis and angiogenesis during early placental development may set the stage for abnormal placental vascular structure and impaired placental function described in human and sheep IUGR placenta (Barry & Anthony, 2008). Thus, while there are contrasting features between the human and sheep placenta, the similarities between fetal vascular structure makes the sheep a useful model to study the progression of placental vascular development and placental nutrient exchange in normal and compromised pregnancies.

In terms of metabolic function and nutrient transport, the human and sheep placenta have many similarities. One of the main duties of the placenta is to transport nutrients from maternal circulation to fetal circulation. Fetal growth is dependent on the placental supply of oxygen and nutrients provided by either simple diffusion (i.e., oxygen) or transporter-mediated transfer (i.e., glucose and amino acids) (Barry & Anthony, 2008). The major nutrients needed for fetal growth are glucose and amino acids (Hay & Sparks, 1985; Regnault et al., 2002). As gestation advances, placental and fetal growth increases dramatically, and it has been demonstrated that placental size is directly correlated with fetal growth in both humans and sheep (Barry & Anthony, 2008; Fowden

et al., 2006). Additionally, while there are obvious ethical concerns about studying placental metabolic function and nutrient transport of pregnancy in humans, the chronically catheterized fetal sheep and ewe, along with the application of the Fick principle and transplacental diffusion technique, has provided a useful model for studying placental physiology, placental metabolism, and placental transfer function and capacity.

One of the key substrates for the placenta and fetus is oxygen, which is able to readily diffuse from maternal circulation, across the placenta, and into fetal circulation (Illsley et al., 2010). As the placenta is a highly metabolic organ, it consumes a large fraction of the total oxygen being diffused across the maternal circulation. For example, in sheep at mid-gestation, 80% of the total oxygen taken up by the uterus was being consumed by the placenta (Bell et al., 1986). The remaining oxygen is transported into fetal circulation where the vast majority will be utilized for oxidative metabolism of various substrates (Philipps, 2011). Thus, oxygen is a critical substrate for the placenta and fetus. In the human and sheep, the predominant factors responsible for oxygen transport across the placenta include: uterine and umbilical blood flow, placental surface area and permeability, the quantity of oxygen consumed by the placenta, fetal and maternal blood oxygen carrying capacity, and hemoglobin oxygen binding affinity (Battaglia & Meschia, 1986; Barry & Anthony, 2008). Despite all the factors that play into placental oxygen transport, blood flow is the major determinant of fetal oxygen delivery (Barry & Anthony, 2008). During early gestation, the conceptus develops in a hypoxic environment until the maturation of the utero-placental vasculature, and villous structures allows for measurable gas transfer to the fetus (Barry & Anthony, 2008). After

which, uterine blood flow increases similarly throughout the latter half of gestation in both humans and sheep (Meschia, 1984; Konje et al., 2003). Additionally, the placental oxygen and fetal oxygen consumption rates are similar between humans and sheep. When assessing term human and sheep placental and fetal oxygen consumption rates, placental oxygen consumption has been reported as 37 ml/kg/min for humans and 34 ml/kg/min for sheep (Meschia et al., 1980; Bonds et al., 1986), while fetal oxygen consumption in both humans and sheep has been reported to be between 0.25 and 0.35 mmol/min/kg (Pardi & Cetin, 2006). However, in some human and sheep IUGR pregnancies, depending on the severity of IUGR, fetal hypoxemia can be found as a result of restricted placental oxygen transfer and restricted uterine-umbilical blood flows (Barry & Anthony, 2008). In human IUGR pregnancies, Pardi et al. (1992) demonstrated blood in the uterine vein of IUGR pregnancies had a higher PO_2 than the control, as well as a lower uterine oxygen extraction rate. Similar findings have been demonstrated in sheep IUGR pregnancies with reductions in umbilical venous PO_2 and reductions in relative umbilical blood flows resulting in fetal hypoxemia (Regnault et al., 2003; Regnault et al., 2007).

Glucose is the primary substrate for the human and sheep placenta and fetus (Barry & Anthony, 2008; Marconi & Paolini, 2008). As the fetus has essentially no ability for glucose production until near term, the fetal glucose supply is dependent upon circulating maternal glucose concentrations (Barry & Anthony, 2008). Placental glucose uptake and transfer to the fetus is mediated by facilitative glucose transporter proteins on the trophoblast's plasma membranes (Barry & Anthony, 2008). Currently, in human and sheep placental tissues, only SLC2A1 (GLUT1) and SLC2A3 (GLUT3) have been

localized to locations on the trophoblast plasma membranes that would allow for glucose transport (Wooding et al., 2005; Illsley, 2000). A shared commonality in both the human and sheep is that the maternal to fetal glucose gradient is the major driving force of fetal blood glucose concentration, and in both species, the transplacental glucose gradient is increased near term as a result of decreasing fetal glucose concentrations (Hay et al., 1990; Bozzetti et al., 1988; Hay, 1991). In both human and sheep IUGR pregnancies, a common observation is fetal hypoglycemia (Economides et al., 1989; Marconi et al., 1996; Anthony et al., 2003; Barry et al., 2006). It has been suggested that the severity of IUGR may impact the degree and mechanism of fetal hypoglycemia (Barry & Anthony, 2008).

As for amino acids, in both human and sheep pregnancies, the concentration of amino acids is higher in fetal circulation than maternal circulation, thus amino acids must cross the placenta by active, energy-dependent transport systems mediated by specific transporters on the syncytiotrophoblast plasma membranes (Cetin, 2001; Jansson, 2001). The current amino acid transport systems that have been identified can be broadly grouped into neutral, cationic, and anionic transport systems (Jansson, 2001; Hay et al., 2011). When assessing amino acid transport and metabolism, it can be quite complex as there are a multitude of factors that can be involved, such as: placental consumption and metabolism, interconversion of amino acids within the placenta, relative concentrations of the amino acids in maternal and fetal plasma, and the placental-fetal hepatic amino acid metabolic cycles (Barry & Anthony, 2008; Hay et al., 2011). It has been demonstrated, in pregnancies with intrauterine growth restriction, that there are reductions in total amino acid concentrations (Hay et al., 2011; Barry &

Anthony, 2008); however, there are pregnancies with intrauterine growth restriction in which fetal amino acid concentrations are maintained despite decreased placental transport (Hay et al.,2011). It is important to note that reductions in amino acid concentrations are often interpreted as a reduced supply and utilization of amino acids, but those values actually represent a balance among the rates of amino acids supply, synthesis into protein, release by protein catabolism, and oxidation (Hay et al., 2011). In human IUGR pregnancies, fetal amino acid concentrations are found to be decreased in comparison with control pregnancies (Cetin et al., 1988; Cetin et al., 1996). Additionally, in humans with more severe IUGR, defined by an increased umbilical arterial pulsatility index, amino acid transport activity decreases (Glazier et al., 1997). Meanwhile, in sheep, moderate IUGR results in decreased amino acid concentrations, but more severe IUGR sheep pregnancies have been shown to increase the amino acid concentration in fetal circulation possibly due to protein being catabolized and utilized as metabolic fuel (Barry & Anthony, 2008). Overall, depending on the severity of growth restriction, human and sheep IUGR pregnancies exhibit reduced placental amino acid transport with various impacts on fetal amino acid concentrations. The commonalities between human and sheep pregnancies, from the perspective of placental development, metabolic function, and nutrient transport, make the pregnant sheep a useful model to study normal and compromised pregnancies as it relates to human pregnancy.

Sheep Models of IUGR

While a great deal has been learned from clinical studies of human IUGR, animal models are essential to addressing the mechanistic questions related to the

development of IUGR. While the degree of restriction is variable, there are various sheep models that are capable of inducing IUGR that also share similar characteristics to human IUGR. Fetal growth restriction in sheep can be induced in a variety of methods, including maternal nutrient restriction or excess, administration of glucocorticoids, uteroplacental embolization, restriction of placental attachment sites (carunclectomy), or maternal hyperthermia (Anthony et al., 2003).

As the maternal circulation is the source for the majority of nutrients utilized by the placenta and fetus, altering the concentration of nutrients via maternal feed intake (i.e., undernutrition or overnutrition) can influence placental and fetal growth (Wallace et al., 1999b). The cohort of women who lived through the Dutch famine presented a unique opportunity to study the impacts of maternal undernutrition during gestation on the developing fetus. Roseboom et al. (2001) reported that maternal undernutrition during gestation increases the risk of the offspring to have long-lasting deleterious health effects in adult life, however the effects seem to depend upon if any organs or systems were undergoing critical periods in development at the time of undernutrition. After Wallace (1948) demonstrated the ability to restrict the growth of the sheep fetus by maternal undernutrition, the pregnant sheep has been used as a model to understand the effects of maternal undernutrition during various time points across gestation. In a study done on nutrient restriction from early to mid-gestation, Ma et al. (2011) reported fetal weight and total placentome weight were decreased at mid-gestation. When nutrient restriction occurs from early to late gestation, there are significant reductions in both fetal and placental mass (Mellor & Murray, 1982; Wallace, 1948; Ma et al., 2011). However, when nutrient restriction occurs only during late gestation, fetal and placental

growth may or may not be impacted depending on the mother's body composition at the onset of nutrient restriction (Mellor, 1983; Chandler et al., 1985). In the maternal nutrient restriction studies done in sheep, it appears that nutrient restriction at any time during gestation can have long term ramifications on the health and well-being of the offspring. For example, maternal undernutrition can impact fetal cardiovascular development as demonstrated by Hoet and Hanson (1999), in which maternal undernutrition occurred during the first thirty days of gestation, and resulted in a transition from fetal hypotension during late gestation to hypertension during postnatal life.

Conversely, maternal overnutrition can also influence placental and fetal growth. By overnourishing singleton-bearing adolescent sheep throughout pregnancy, Wallace et al. (1996, 1997, 1999a) demonstrated a significant reduction in placental growth and, consequently, a significant decrease in offspring birthweight as compared to moderately fed, normally growing adolescent sheep of equivalent age. It became apparent in this experimental paradigm that the increased maternal concentrations of insulin and insulin-like growth factor 1 (IGF-1) during early gestation, in response to maternal overnutrition, ensured anabolic drive to promote maternal tissue synthesis at the expense of the nutrient requirements of the gravid uterus (Wallace et al., 1997, 1999a). This observation was further confirmed when Wallace et al. (1999b) reported the carcass of overnourished dams had an increase in body fat deposition with a less pronounced increase in body protein. It has been suggested that the impaired placental growth is the primary constraint to fetal growth in the maternal overnutrition model (Wallace et al., 1999a, 2002). Additionally, the overnourished sheep model recapitulates what has been observed in human IUGR pregnancies, in that the fetuses of overnourished ewes have

reduced uterine and umbilical blood flows, decreased uterine oxygen uptake, decreased placental glucose transport capacity, and the fetuses are hypoxic, hypoglycemic, and hypoinsulinemic (Wallace et al., 2002; Wallace et al., 2003; Anthony et al., 2003). Similar to the maternal undernutrition model, the ramifications of maternal overnutrition on placental and fetal growth seem to depend on the point in gestation the dams are overnourished. In the nutritional switch over studies done by Wallace et al. (1999b), when a dam was started on a moderate diet and subsequently switched to a high intake diet at the end of the first trimester (i.e., 50 dGA), the deleterious effects on placental development and fetal growth were still observed. However, if a high intake diet dam was reduced to a moderate diet at the end of the first trimester, placental growth was stimulated and was found to enhance pregnancy outcome. Thus, the maternal nutrient intake at key developmental timepoints during gestation, whether in regard to undernutrition or overnutrition, can influence the outcome of pregnancy and postnatal productivity.

Another model utilized to experimentally induce IUGR is through the administration of glucocorticoids. In normal pregnancies, fetal glucocorticoid concentrations are lower than maternal concentrations as the placental enzyme 11β-hydroxsteroid dehydrogenase type 2 (11β-HSD2) catalyzes cortisol and corticosterone to inert 11-keto steroids, cortisone, and 11-dehydrocorticosterone, thus preventing maternal glucocorticoids from reaching fetal circulation (Seckl, 2001). During late gestation, there are increasing concentrations of cortisol in fetal circulation which is believed to be involved in the maturation of several organ systems (Anthony et al., 2003). For women at risk of preterm delivery, it is a common clinical practice to administer prenatal

glucocorticoids to decrease the incidence of respiratory distress syndrome, or other complications of prematurity, but most notably, alter the maturation rates of various organs such as the fetal lung (Jobe et al., 1998; Seckl, 2001; Anthony et al., 2003). It has been repeatedly demonstrated that administration of exogenous glucocorticoids to pregnant mothers can negatively impact fetal growth as lowered offspring weight at birth has been reported in pregnant humans, non-human primates, rats, and sheep administered exogenous glucocorticoids (Reinisch et al., 1978, Novy & Walsh, 1983; Mosier et al., 1982; Berry et al., 1997; Fowden et al., 1996). When synthetic glucocorticoids are administered to rodents during the last week of pregnancy, it resulted in the offspring having decreased birth weight, elevated blood pressure, and glucose intolerance in the adult offspring (Levit et al., 1996; Nyirenda et al., 1998). It has also been shown that glucocorticoid exposure in late trimester rats leads to reduced offspring birth weight, as well as lifelong hypertension, hyperglycemia, and hyperinsulinemia (Seckl, 2001). Maternal administration of various doses of glucocorticoids in late gestation sheep have also reported reduced fetal growth, elevated fetal blood pressure, postnatal glucose intolerance, and in one case, reduced placental size (Jobe et al., 1998; Moss et al., 2001; Jensen et al., 2002; Anthony et al., 2003). Another approach to increase the fetal exposure to maternal glucocorticoids is by inhibiting placental 11β-HSD2 activity. Through this approach, it has been demonstrated that the offspring have reduced birth weights, as well as postnatal development of hyperglycemia and elevated blood pressure (Lindsay et al., 1996a; Lindsay et al., 1996b). In this model, it is apparent that glucocorticoids affect fetal growth directly rather than through alterations in placental function and nutrient transfer (Anthony et al., 2003).

This is supported by Dodic et al. (2002) in which maternal administration of glucocorticoids at day 27 of gestation resulted in adult hypertension in the offspring, but no effects on placental or fetal growth. Thus, fetal exposure to excess glucocorticoids can affect the growth, metabolism, and cardiovascular function of the fetus.

Another model used to experimentally induce IUGR is utero-placental embolism. During pregnancy in humans and sheep, maternal cardiac output increases by 30-40% due to an increase in heart rate and stroke volume, as well as a decrease in systemic and uteroplacental vascular resistance (Ueland et al., 1969; Clapp, 1985; Metcalfe & Parer, 1966). Subsequently, uterine perfusion in humans and sheep increases from approximately 50 ml/min in week ten of gestation, to as much as 1,500 ml/min at the end of gestation (Lang et al., 2000). This significant increase in uteroplacental blood flow is necessary for the continuous delivery of adequate nutrients and oxygen for placental and fetal growth (Lang et al., 2000). However, inadequate uteroplacental perfusion during pregnancy can result in a deficiency in nutrient and oxygen delivery to the placenta and fetus, thus potentially setting the stage for IUGR. In this model, utero-placental embolism can be created either by restriction of uterine or umbilical blood perfusion, either by infusion of microspheres, vessel occlusion, or ligation of a single uterine artery during late gestation (Anthony et al., 2003). Inherent to this model, uterine and umbilical artery blood flows are reduced; however, depending on the gestational age, degree of embolism achieved, and the site of embolism or occlusion results in a variability in the amount of reduction observed (Anthony et al., 2003). The utero-placental embolism model can closely recapitulate some events that occur in human IUGR, as sheep utero-placental embolism pregnancies have observed reductions in

placental and fetal mass (Lang et al., 2000; Oyama et al., 1992), the fetus is hypoxic and hypoglycemic (Louey et al., 2000; Clapp et al., 1981), and in some instances, fetal uptake of oxygen and glucose are reduced (Clapp et al., 1981). Additionally, the fetus can display signs of brain sparing and asymmetric fetal growth restriction (Lang et al., 2000). The utero-placental embolism model is useful for examining the impacts of reduced utero-placental blood flow on fetal growth; however, the primary shortcoming of this model is utero-placental blood flow is artificially modified rather than from the placenta developing functional insufficiency itself (Anthony et al., 2003).

Another method for generating IUGR pregnancies in sheep is a caruncle ctomy, or as originally described by Alexander (1964), the surgical removal of uterine caruncles before mating. Placental nutrient exchange can be impacted by several factors: exchange area, thickness and permeability of the placental barrier, and placental perfusion (Owens et al., 1986). By sufficiently reducing the number of endometrial caruncles in the uterus before breeding, it effectively diminishes placental exchange area and transport capacity. It should be noted that not all pregnancies generated with this method result in IUGR; however, when IUGR ensues, this model mimics many similar characteristics of human IUGR. For example, placental and fetal weights are reduced in this model (Owens et al., 1989), and nutrient supply is consequently restricted resulting in fetal hypoxemia, hypoglycemia, and hypoinsulinemia (Robinson et al., 1979; Harding et al., 1985). Uterine and umbilical blood flows are also significantly reduced in this model (Owens et al., 1987). When directly assessed, this model results in decreased supply of oxygen to the pregnant uterus and fetus, as well as reduced utero-placental oxygen consumption (Owens et al., 1987). Additionally, when assessing

glucose, Owen et al. (1986) reported reduced glucose delivery to the pregnant uterus and fetus, as well as reduced utero-placental consumption on a relative weight basis (per kilogram of placenta). While this model has provided considerable insight into IUGR, it is not clear what structural and functional changes occur within the placenta.

Lastly, a common model used to induce IUGR in sheep is through exposing pregnant ewes to high ambient temperatures during gestation. While exposing pregnant ewes to high ambient temperatures from mid-gestation to late gestation does result in reduced placental and fetal mass (Bell et al., 1989; Early et al., 1991), greater reductions in placental and fetal mass, and even some of the most severe cases of IUGR, are observed when pregnant ewes are exposed to high ambient temperatures from early to late gestation (Alexander & Williams, 1971; Anderson et al., 1997; Thureen et al., 1992). In this model of inducing IUGR in sheep, the placental insufficiency that ensues from pregnant ewes being exposed to heat stress recapitulates many similar characteristics of human IUGR pregnancies. This model results in significantly reduced placental mass, an asymmetrically grown fetus, as well as reduced uterine and umbilical blood flows (Galan et al., 1999; Bell et al., 1987). Additionally, due to the reduced umbilical oxygen and glucose uptake, the fetus is hypoxic and hypoglycemic (Bell et al., 1987; Thureen et al., 1992). Abnormal umbilical arterial and aortic Doppler velocimetry, and reduced transplacental amino acid flux are also observed in this model (Galan et al., 1999; Ross et al., 1996; Anderson et al., 1997). It has been suggested that the significant reduction in placental growth observed in this model is the primary effect of chronic maternal heat stress, and the associated reduction in fetal growth can be accredited to the decreased placental ability to supply oxygen and nutrients, thus

making this a valuable model to examine placental insufficiency that results in IUGR (Bell et al., 1987; Regnault et al., 2002).

IGF Axis in the Mammalian Placenta

The insulin-like growth factor (IGF) system is a complex network of two soluble ligands, six binding proteins, several cell surface transmembrane receptors, and numerous IGF-related binding proteins. The insulin-like growth factors, IGF-1 and IGF-2, are small, highly homologous single chain mitogenic polypeptides that are structurally similar to proinsulin (Forbes & Westwood, 2008; Denley et al., 2005). Both IGF-1 and IGF-2 play a role in placental and fetal development (Forbes & Westwood, 2008; Owens, 1991; Kaur et al., 2021). While neither maternal growth hormone, IGF-1, or IGF-2 cross the placenta (Laron et al., 1966; Fholenhag et al., 1994; Wang et al., 1991), maternal IGFs can influence maternal tissue growth and metabolism, thereby modulating nutrient availability for placental and fetal growth. Additionally, in human pregnancy, dysregulated expression of the IGFs and their associated signaling components are often reported in the placenta of pregnancies with abnormal fetal growth (Abu-Amero et al., 1998; Sheikh et al., 2001; Laviola et al., 2005; Borzsonyi et al., 2011; Nawathe et al., 2016). When measured in cord blood from healthy newborns, fetal IGF-1 levels are positively correlated with birth weight (Klauwer et al., 1997; Osorio et al., 1996; Ong et al., 2000). Conversely, when cord blood is collected and measured from IUGR pregnancies, IGF-1 concentrations are significantly reduced (Larsen et al., 1996; Giudice et al., 1995; Koutsaki et al., 2011).

The role of the IGFs in pregnancy and placental and fetal growth have been primarily studied in two ways. First, they have been given exogenously to either

placental cultures *in vitro* or to pregnant animals *in vivo.* In elucidating the role of the IGFs on the placenta, *in vitro* studies with trophoblast cell lines from humans, pigs, and mice have demonstrated both IGF-1 and IGF-2 have a role in preventing apoptosis, enhancing cellular proliferation, migration and invasion, and promoting overall placental growth (Kaur et al., 2021; Miller et al., 2005; Kim et al., 2008; Sferruzzi-Perri et al., 2017). Secondly, the *Igf* genes, their receptors, and key molecules in the downstream signaling pathways have been knocked-out and over-expressed in genetically modified mice (Sferruzzi-Perri et al., 2017). Both IGF-1 and IGF-2 are important during pregnancy as when either *Igf-1* or *Igf-2* are deleted in mice, the resulting offspring have significantly reduced birth weights as compared to their wild-type littermates (Sferruzzi-Perri et al., 2011). Additionally, if both genes are deleted simultaneously, the deleterious effects on fetal growth are additive (Efstratiadis, 1998). In homozygous IGF-1 gene deletion (*Igf-1*$^{-/-}$), greater than 95% of the pups die perinatally (Powell-Braxton et al., 1993), with each fetus often presenting with severe embryonic and postnatal growth restriction (Woods et al., 1996). When the *Igf-1* gene is mutated, the offspring are often growth restricted (Baker et al., 1993; DeChiara et al., 1990; Woods et al., 1996; Louiv et al., 1997), however placental weight is not impacted (Baker et al., 1993). In contrast, IGF-1 administration has been shown to increase fetal weight in normal pregnancy (Sferruzzi-Perri et al., 2006) or in IUGR pregnancies where IGF-1 is administered either into the amniotic fluid (Eremia et al., 2007) or as an adenoviral mediated transgene delivered directly into the placenta (Jones et al., 2013).

In elucidating IGF-2 role in placental and fetal growth, it is important to note that in the mouse, and not in the human, the *Igf-2* gene is subject to parental imprinting, with

only the paternal allele being expressed (Sferruzzi-Perri et al., 2017). Additionally, there are several promoters for the *Igf-2* gene that can be mutated and cause placental specific or placental and fetal IGF-2 effects. Total ablation of the *Igf-2* gene from the fetoplacental unit results in disproportional growth of placental compartments, whereas deleting the placental specific transcript of IGF-2 alone results in proportional placental growth restriction (Coan et al., 2008). The P0 promoter for the *Igf-2* gene is specific to the placenta (Moore et al., 1997), as it is only expressed in the labyrinthine trophoblast cells of the mouse placenta (Sferuzzi-Perri et al., 2017). Thus, IGF-2 is reduced specifically in the placenta, while fetal IGF-2 expression is not impacted. When the P0 promoter is deleted, placental growth is reduced as early as embryonic day 12, with no change in fetal weight (Constancia et al., 2002). Constancia et al. (2002) observed that fetal growth continued normally until embryonic day 16, which after, fetal growth was subsequently reduced in the P0 mutant mice at embryonic day 19 and at birth. Additionally, the passive permeability of nutrients to the mutant placenta were decreased, but secondary active placental amino acid transport was upregulated. Constancia et al. (2002) suggested that the upregulation of secondary active placenta amino acid transport was able to compensate for the reduced placental size and passive permeability, and was able to maintain fetal growth up to embryonic day 16. This study demonstrated that deleting the placental specific *Igf-2* promoter impairs placental-specific IGF-2 production, thus reducing placental growth and nutrient transfer capacity, ultimately resulting in reduced fetal growth.

In further assessing the P0 mutant placenta, Constancia et al. (2005) assessed glucose and amino acid transporter expression and observed increased placental

transport of glucose and amino acids via increased expression of *Slc2a3* and *Slc38a4* (SNAT4). However, in null mutants, in which both fetal and placental *Igf-2* is deleted, there is no upregulation of either transporter system (Constancia et al., 2005). Collectively, these findings suggest that the P0 mutant placenta is able to compensate for impaired development by upregulating its nutrient transport systems, and can minimize the degree of fetal growth restriction relative to the *Igf-2* null placenta. Additionally, in the P0 and null mutant placentas, the loss of *Igf-2* results in reduced labyrinthine zone volume, exchange surface area, and vascularization (Sferruzzi-Perri et al., 2017). Thus, placental *Igf-2* also appears to regulate the development of the diffusional exchange characteristic of the mouse placenta (Sibley et al., 2004). Alternatively, overexpression of the *Igf-2* gene results in fetal overgrowth (Constancia et al., 2002), and can be accomplished by activating the normally silent maternal gene copy in the H19 mouse model, increasing IGF-2 availability via *Igf2r* ablation, or deleting the PI3K signaling inhibitor (Leighton et al., 1995; Ludwig et al., 1996; Ripoche et al., 1997; Church et al., 2012). In the H19 mouse model, fetal and placental weights are increased, despite reduced expression of *Slc2a3* and *Slc38a4* and reduced placental transport of glucose and system A amino acids (Angiolini et al., 2011). When the P0 promoter is mutated in the H19 placenta, fetal overgrowth is maintained, the overgrowth of the placenta is decreased, and system A amino acid transport is upregulated (Angiolini et al., 2011). Thus, IGF-2 in the placenta appears to be important for fine-tuning the nutrient supply for fetal and placental growth.

There are six different insulin-like growth factor binding proteins (IGFBP-1 to IGFBP-6) that share significant structural homology and contain specific amino acids in

their amino and carboxyl terminus that mediate their high affinity binding for IGF-1 and IGF-2 (Sferruzzi-Perri et al., 2017; Clemmons, 2018). The actions of IGF-1 and IGF-2 are regulated by the IGFBP's, as they regulate the bioavailability of IGF-1 and IGF-2 to their receptors. While individual IGFBPs can have IGF-independent activity (Bach, 2018), a few of the main functions of the IGFBPs are to increase the half-life of the IGFs in circulation, delivery of the IGFs to tissues, and blocking or promoting access of the IGFs to their receptors (Guler et al., 1989; Forbes & Westwood, 2008; Kaur et al., 2021). The majority of IGF in circulation is bound by IGFBP-3 in complex with the acid labile subunit (ALS) (Forbes & Westwood, 2008). Additionally, there are IGFBP proteases, such as pregnancy-associated plasma protein A (PAPP-A), that cleave IGFBPs to release IGFs, thus IGFBP proteases also play a role in the regulation of IGF bioavailability (Kaur et al., 2021). It is important to note that the IGFBPs have a higher affinity for IGF-1 and IGF-2 than the IGF receptors, and therefore can sequester IGFs and function as IGF inhibitors (Forbes & Westwood, 2008; Denley et al., 2005). The IGFBPs can also undergo post-transcriptional modification, such as phosphorylation and proteolysis, that consequently alters the affinity of IGFBPs for IGF-1 and IGF-2 (Firth & Baxter, 2002; Forbes & Westwood, 2008).

During pregnancy, IGF-1 and IGFBP-1 play a major role in regulating fetal growth (Nawathe et al., 2016). Additionally, during normal pregnancy, the activity of the IGFBP proteases increases, leading to increased IGF-1 bioavailability (Giudice et al., 1990). However, in compromised pregnancies, IGFBPs, most notably IGFBP-1, become dysregulated and can serve as an IGF inhibitor (Denley et al., 2005), and subsequently suppressing fetal growth by inhibiting the binding of IGF-1 to its receptors on the

placenta (Iwashita et al., 1992; Lee et al., 1993). In human and animal models, low birth weight has been negatively correlated with high circulating levels of IGFBP-1 (Watson et al., 2006). When assessed in one human case of IUGR pregnancy, IGFBP-1 was 7-fold higher in fetal cord serum as compared to normal weight pregnancies (Klauwer et al., 1997; Giudice et al., 1995). Thus, it has been suggested that increased IGFBP-1 levels in IUGR pregnancies are sequestering IGF-1 and inhibiting the mitogenic effects of the IGFs (Watson et al., 2006). Additionally, the lone actions of IGFBP-1 can be sufficient to cause alterations in fetal growth, as in a transgenic mouse line where IGFBP-1 was overexpressed in the fetal liver, Watson et al. (2006) reported no changes in circulating levels of IGF-1, but significant increased concentrations of IGFBP-1, and fetal growth restriction.

The endocrine, paracrine, and autocrine actions of the IGFs are mediated through two receptors, IGF1R and IGF2R (Kaur et al., 2021). For the IGFs to elicit their effects, both IGF-1 and IGF-2 act primarily through the binding and activation of IGF1R (Baker et al., 1993; Forbes & Westwood, 2008). IGF1R has a high binding affinity for IGF-1, lower affinity for IGF-2, and little affinity for insulin (ED_{50} nM: 0.2, 0.6, and >100, respectively) (Denley et al., 2005). IGF2R has a high binding affinity for IGF-2, a very low affinity for IGF-1, and does not bind insulin (K_D nM: $0.4x10^{-3}$, 0.2, and no binding, respectively) (Denley et al., 2005). Additionally, IGF1R shares structural homology with the insulin receptor, and a functional hybrid receptor can form between IGF1R and either insulin receptor-α (IR-A) or insulin receptor-β (IR-B) (Denley et al., 2005; Hakuno & Takahashi, 2018). IGF1R and IGF2R are structurally and functionally unrelated. IGF1R is a heterotetrametric transmembrane glycoprotein, that upon IGF binding,

causes the activation of the receptor and the autophosphorylation of intracellular tyrosine residues and subsequent activation of downstream signaling (Forbes & Westwood, 2008; Walenkamp et al., 2006). IGF2R, also known as the cation-independent mannose 6-phosphate receptor, is a single transmembrane protein that consists of a large extracellular domain and a small cytoplasmic tail that does not have tyrosine kinase activity (Efstratiadis, 1998; Reynolds et al., 1997). While IGF2R can bind the phosphomannosyl recognition marker of lysosomal hydrolases, in mammals it also interacts with IGF-2 (Ludwig et al., 1996). Due to the absence of an autophosphorylation site or tyrosine kinase activity, IGF2R has no intrinsic signaling transduction capability (Denley et al., 2005). Instead, in the context of the IGF system, IGF2R has been demonstrated to primarily sequester IGF-2 from potential receptor activation interactions and internalize extracellular IGF-2, via receptor mediated endocytosis, and degrade it (Oka et al., 1985; Kiess et al., 1987; Nolan et al., 1990; Denley et al., 2005).

Both IGF1R and IGF2R are localized on distinct placental surfaces, thus enabling maternal and/or fetal IGF-1, IGF-2, and insulin to bind to them (Hiden et al., 2009). IGF1R is localized to all cell types of the placenta (Holmes et al., 1999; Fang et al., 1997). As demonstrated in humans and mice, abnormalities in IGF1R can impact fetal growth. There are several reports of human infants being born with heterozygous missense mutations in the *IGF1R* gene, with each infant presenting with severe IUGR, postnatal growth failure, and postnatal failure to thrive (Walenkamp et al., 2006; Abuzzahab et al., 2003; Kawashima et al., 2005). In *Igf1r* null mice, the resulting offspring are growth restricted as their birth weight is reduced between 45-60% as

compared to the normal weight littermates (Baker et al., 1993; Efstratiadis, 1998; Sferruzzi-Perri et al., 2011), and in some cases, the offspring dies soon after birth due to multiple physiological abnormalities and respiratory failure (Hakuno & Takahashi, 2018). In heterozygous *Igf1r* knockout mice, the resulting offspring also display low birth weight and postnatal growth deficits (Hakuno & Takahashi, 2018). Thus, this data suggests that IGF1R is critical for prenatal and postnatal development and growth.

Conversely, if *Igf2r* is deleted in mice, it results in fetal overgrowth with the resulting offspring being 25-30% larger than their normal weight littermates and have elevated circulating levels of IGF-2 (Efstratiadis, 1998; Ludwig et al., 1996; Lau et al., 1994). It is important to note that in mice, though largely not in humans, the *Igf2r* gene is paternally imprinted, but in a reciprocal fashion to *Igf-2* with expression from the maternal allele (Monk et al., 2006; Sferruzzi-Perri et al., 2017). Thus, if the offspring inherit a non-functional IGF2R gene from their fathers, the offspring are still viable and develop normally into adults; however, if the offspring inherits the mutated allele from their mother, the offspring die around birth as a consequence of major cardiac abnormalities (Lau et al., 1994). Additionally, when mice mutants inherit a maternally targeted disruption of the imprinted *Igf2r* gene, it results in increased serum and tissue levels of IGF-2, fetal overgrowth, and the offspring usually die perinatally, potentially due to excess IGF-2 overstimulating IGF1R (Ludwig et al., 1996). Interestingly, Igf2r mutants can be completely rescued when they carry a second mutation that eliminates either IGF-2 or IGF1R. *Igf2r*/*Igf-2* double mutants only survive a few weeks postnatally, while *Igf1r*/*Igf2r* double mutants only differ from their wild-type siblings in their pattern of postnatal growth (Ludwig et al., 1996). Thus, the role of IGF2R appears to be

preventing excessive IGF-2 affects in the placenta as in mice that have abolished *Igf2r* gene, the offspring has 7- to 11-fold higher levels of IGF-2, consequently causing somatic overgrowth, visceromegaly, and placentomegaly (Eggenschwiler et al., 1997).

Glucose and the Placenta

The placenta is an active metabolic organ tasked with a multitude of duties to perform, such as disposing of waste products, transporting nutrients from maternal to fetal circulation, hormone production, and regulating its own metabolism. Over gestation, the placenta evolves and grows via a variety of proliferating cell types and requires high levels of nutrient supply, which is supplied by the maternal circulation. While the placenta utilizes a significant part of the nutrients it takes up from maternal circulation for its own metabolic processes, the placenta must adapt and balance its own metabolic needs while also providing enough nutrients to sustain fetal growth (Illsley, 2011). Glucose is the primary substrate for placental energy generation and fetal oxidative processes (Illsley, 2011). Until near term, the only source of glucose for the placenta and the fetus is what is within maternal circulation. The high concentration of glucose in maternal circulation, and the low concentration of glucose in fetal circulation, sets up and drives glucose down a concentration gradient from maternal to fetal circulation. In blood samples collected simultaneously from humans, fetal plasma glucose concentrations are significantly lower than those observed in the mother across gestation (Aynsley-Green et al., 1985; Bozzetti et al., 1988; Ashmead et al., 1993). Additionally, the linear relationship that is observed between maternal and fetal glucose concentrations throughout gestation is observed during euglycemia, maternal

hyperglycemia, and fetal hypoglycemia (Bozzetti et al., 1988; Whaley et al., 1966; Kalhan & Parimi, 2011).

The placenta has a very high rate of glucose consumption. When assessed in the sheep placenta, it has been documented that at mid-gestation the placenta consumes roughly 80% of the glucose it takes up from maternal circulation (Hauguel et al., 1983; Bell et al., 1986), while the late-gestation sheep placenta consumes approximately 72% of the glucose it takes up from maternal circulation (Meschia et al., 1980; Bell et al., 1999). The glucose taken up by the placenta can be utilized by the placenta for oxidation, glycogen synthesis, or lactate production (Kalhan & Parimi, 2011). How the placenta distributes and utilizes this glucose, via various metabolic pathways, also changes across gestation. Earlier in pregnancy, about 75% of glucose is metabolized through the glycolytic pathway, 15% via non-triose phosphate pathways (i.e., glycogen synthesis), and 10% via the pentose phosphate pathway (Illsley, 2011). By term, 90% of glucose consumption is via the glycolytic pathway, while the non-triose phosphate pathways and pentose phosphate pathway account for 5%, each (Illsley, 2011).

Glucose does not passively transfer through the placenta by simple diffusion, but instead is transported by facilitated diffusion by glucose transporters (GLUTs) found in the placenta. While it was once thought glucose was transferred across the placenta by simple diffusion, the work done by W.F. Widdas (1952) demonstrates that simple diffusion could not provide an adequate explanation for the quantitative and qualitative circumstances of placental glucose transfer in the sheep, and instead proposed that the observed kinetic relationships were more consistent with a carrier system. Additionally,

it is clear now, through the work done in human and animal studies, that the only source of glucose for the fetus is from the maternal circulation until near term (Simmons et al., 1979; Hay et al., 1984; Hay, 1991). Human and animal studies have clearly demonstrated that the fetus in normal pregnancies is unable to perform gluconeogenesis in utero, even in scenarios of maternal fasting (Kalhan & Parimi, 2000). In a study done with normal and diabetic pregnant women at term, Kalhan et al. (1979) demonstrated, through the use of isotopic tracer infusions (i.e.,^{13}C-glucose) into maternal circulation and simultaneous maternal and umbilical blood sampling, after a brief maternal fast, the fetal and maternal glucose pools were in equilibrium with each other. They did not observe a lower ^{13}C-glucose enrichment in fetal blood and thus demonstrated that a brief maternal fast did not initiate systemic glucose production in the human fetus (Kalhan et al., 1979). Marconi et al. (1993), performed a similar isotopic tracer study except with pregnant women that had pregnancies complicated by IUGR. A common characteristic of the IUGR fetus is they are hypoglycemic (Marconi & Paolini, 2008), so it was thought that the hypoglycemic environment would trigger fetal glucogenesis. However, Marconi and colleagues (1993) clearly demonstrated, by comparing steady state maternal and fetal glucose enrichments, no differences in tracer enrichment in maternal and fetal compartments, thus again confirming that in humans, maternal glucose is the only source of glucose for the fetus. Studies done with sheep allow the ability to assess where glucose is going in much more detail. That is, there is the ability to measure umbilical glucose uptake, fetal glucose utilization, the umbilical arterial and venous gradient, umbilical blood flow, and simultaneous tracer isotopic infusion to the mother and fetus (Kalhan & Parimi, 2011). Several studies have

demonstrated that in normoglycemic late gestation sheep pregnancies, as well as insulin-induced maternal hypoglycemic late gestation sheep pregnancies, with the use of isotopic infusion techniques, have clearly demonstrated that maternal glucose is the only source of fetal glucose in sheep pregnancies as well as absent or negligible fetal glucose production (Anand et al., 1980; Hay et al., 1981). However, while the induction of hepatic gluconeogenesis normally occurs at birth in human and sheep neonates, early activation of fetal hepatic glucose production has been demonstrated in the IUGR fetus, depending on the severity of the IUGR (Wesolowski & Hay, 2016).

Blood flow is also an important thing to consider when talking about nutrient delivery from maternal circulation, through the placenta, and into fetal circulation. Uterine blood flow increases as gestation advances, which also results in an increased delivery of glucose to the fetus (Gilbert et al., 1984). This makes sense as the third/later part of gestation is accompanied by rapid fetal growth, which would require copious amounts of glucose for that growth. However, a disturbance in blood flow, as what is observed in IUGR pregnancies, can interfere with glucose transport to fetal circulation. For example, Wilkening et al. (1985) utilized pregnant sheep to study how altering uterine blood flow would impact the rate of glucose transfer across the placenta and into umbilical/fetal circulation. When this group reduced uterine blood flow from 600 to 300 ml/min per kg of fetus (via a cuff-type occluder) and maintaining constant maternal glucose concentration (via a glucose clamp), this reduction did not have any effect on fetal glucose uptake. However, at blood flow rates less than 300 ml/min/kg, fetal glucose uptake was significantly decreased and even became negative in one instance. So, in normal physiological range of uterine blood flow, fetal glucose uptake is not

impacted, but when it falls outside of normal physiological ranges, as it can in IUGR, it can alter glucose uptake. Additionally, any alterations in umbilical blood flow, which can be observed in IUGR pregnancies, can impact nutrient uptake as demonstrated in sheep, through the use of single umbilical artery ligation, in which reductions in umbilical blood flow resulted in a decrease in fetal glucose uptake (Oh et al., 1975).

Facilitative Glucose Transporters in the Mammalian Placenta

Not only in the placenta, but throughout the body, the transport of glucose across cell membranes is largely energy-independent and carried out by facilitative diffusion by the glucose transporters (GLUTs). In total, there are 14 structurally related GLUT proteins that are encoded by the solute carrier 2A (SLC2A) gene family, which are expressed in a tissue-specific manner (Mueckler & Thorens 2013; Simmons, 2011). The various GLUT proteins are categorized into three classes based on sequence similarity: Class 1 consists of GLUT1-4 and GLUT-14; Class 2 consists of GLUT-5, GLUT-7, GLUT-9, and GLUT-11; and Class 3 consists of GLUT-6, GLUT-8, GLUT-10, and GLUT-12, and the proton driven myoinositol transporter HMIT1, or GLUT-13 (Mueckler & Thorens 2013; Simmons, 2011; Joost et al., 2002; Augustin, 2010). It was originally presumed that all the GLUT proteins catalyzed hexose transport into and out of cells. While this is the case with the Class 1 GLUT proteins, some Class 2 and Class 3 GLUT proteins don't primarily function in catalyzing glucose transport, but instead have alternative substrates and functions within their physiological settings (Holman, 2020).

Each glucose transporter consists of 12 transmembrane segments, an intracytoplasmic hydrophilic loop, an exofacial loop bearing a single site of N-linked glycosylation, and both the amino-terminal (N-terminal) and carboxyl-terminal (C-

terminal) are exposed in the cytoplasm (Mueckler & Thorens 2013; Simmons, 2011). To transport glucose, the four trimer substructures that make up a glucose transporter move relative to each other and alternate opening and closing a cleft to either the internal or external side of the membrane (Holman, 2020). While GLUT proteins have varied physiological functions related to their principal substrates and the cell types they are expressed in, the amino-terminal and carboxyl-terminal sequences are unique for each different isoform, and additionally may contribute to isoform-specific properties, such as kinetics, hormone sensitivity, and regulatory properties (Holman 2020; Mueckler & Thorens 2013; Simmons, 2011). Additionally, while each GLUT protein has regions that are highly conserved between each isoform, subtle differences in amino acid side chains can radically alter substrate preference and interaction (Holman, 2020).

While the X-ray crystal structures of GLUT proteins are not available, there are certain sites of the amino acid sequence that have been proposed to explain the varying affinity for substrate of the various glucose transporters. For example, GLUT-3, a low K_m/high affinity glucose transporter, has small amino acids (i.e., glycine) and residues capable of hydrogen bonding to glucose (i.e., serine and asparagine) at the constriction of the pore in TM9 and TM11 that are suggested to contribute to its transport kinetics. Meanwhile, in comparison to GLUT-3, lower affinity glucose transporters, such as GLUT-1 and GLUT-2, have bulkier amino acids at the constriction of the pore in TM9 and TM11 (i.e., alanine, isoleucine, and phenylalanine) that may alter the kinetic properties and cause the increase in K_m and may contribute to the differences in transport kinetics (Simpson et al., 2008).

The two classical methods utilized to assess the kinetic parameters (K_m) of Class 1 glucose transporters expressed in *Xenopus* oocytes were 2-deoxyglucose (2-DG) uptake or by measuring the equilibrium exchange of 3-*O*-methylglucose. With either method, GLUT-3 exhibited a lower K_m as compared to the other Class 1 glucose transporters. The K_m values that have been reported utilizing 2-DG uptake are as follows: GLUT-1: 6.9 mM, GLUT-2: 11.2 mM, GLUT-4: 4.6 mM, and GLUT-3: 1.4mM (Arbuckle et al., 1996; Gould & Holman, 1993). The K_m values that have been reported utilizing 3-*O*-methylglucose are as follows: GLUT-1: 20.9 mM, GLUT-2: 42.3 mM, GLUT-4: 4.3 mM, and GLUT-3: 10.6 mM (Gould & Holman, 1993; Nishimura et al., 1993). Additionally, while GLUT-3 has a higher affinity for glucose than GLUT-1, -2, and -4, it also has a five-fold greater transport capacity than GLUT-1 and GLUT-4 (Simpson et al., 2008).

Human Placenta

In the human placenta, GLUT-1, -3, -4, -8, -9, -10, and 12 have been reported to be expressed (Illsley & Baumann, 2020). GLUT-1 is ubiquitously expressed in the human placenta and has been reported to be expressed in the syncytiotrophoblast, cytotrophoblast, endothelial cells, and the villous stromal elements (Jansson et al., 1993; Jansson et al., 1995; Ericsson et al., 2005; Illsley & Baumann, 2020). Throughout gestation, GLUT-3 has been localized to the microvillous membrane of the syncytiotrophoblast in human placenta (Ericsson et al., 2005; Brown et al., 2011) and has been reported to be localized in cytotrophoblasts in term human placentas (Janzen et al., 2013). GLUT-3 has also been reported to be localized to the fetal endothelium, however, it is not believed to have a role in transplacental transport of glucose

(Hauguel-de Mouzon et al., 1997; Illsley & Baumann, 2020). While it is not clear if the placenta is an insulin-regulatable tissue, GLUT-4 protein and mRNA have been identified in trophoblast homogenates (Ericsson et al., 2005). In first trimester villous tissue, GLUT-4 has been localized to the cytosol of syncytiotrophoblast; however, when assessed in term human placental tissue, GLUT-4 appears to be localized to intravillous stromal cells (Ericsson et al., 2005; Xing et al., 1998). Despite this discrepancy, when GLUT-4 expression is assessed between first trimester and term placental tissues, GLUT-4 expression is markedly lower at term (Ericsson et al., 2005). Thus, suggesting GLUT-4 role in the human placenta is more in the first trimester than at term.

Sheep Placenta

Based on the refined localization study by Wooding et al. (2005), the facilitative glucose transporters are localized similarly in cattle and sheep. GLUT-3 is localized on the apical microvillous membrane of the trophoblast cells, whereas GLUT-1 is localized at the maternal-fetal syncytial layer around the maternal blood capillaries as well as on the basolateral membrane of the trophoblast cells. This arrangement of the facilitative glucose transporters in the ruminant placenta infers, that in order for glucose to be transported from maternal to fetal circulation, GLUT-1 and GLUT-3 must be utilized sequentially. Due to the localization and K_m of GLUT-3, it can be viewed as the rate limiting glucose transporter in the ruminant placenta and adequate levels of GLUT3 would be required for trophoblast glucose uptake. Additionally, while not consistent, Wooding et al. (2005) also reported a sporadic labeling of GLUT-8 in the cytoplasm of trophoblast cells.

GLUT-1

GLUT-1 was the first glucose transporter isoform to be identified, purified, and cloned (Kaasahara & Hinkle, 1977; Zoccoli et al., 1978). GLUT-1 is a ubiquitous isoform present in the majority of human tissues and is responsible for transfer of its principal substrate, glucose, however GLUT-1 is also capable of transporting mannose, galactose, glucosamine, and reduced ascorbate (Mueckler & Thorens, 2013). Since GLUT-1 is present in so many tissues, homozygous GLUT-1 gene deletion (*SLC2A1*$^{-/-}$) is embryonic lethal and, in the murine model, results in the conceptus not surviving past embryonic day 14 (Wang et al., 2006; Heilig et al., 2003). Murine offspring with heterozygous GLUT-1 gene deletion (*Slc2a1*$^{+/-}$) are viable; however, their phenotype mimics what is seen with human GLUT-1 deficiency syndrome, in that the offspring display development delays, infantile seizures, acquired microcephaly, hypoglycorrhachia, and decreased brain glucose uptake (Wang et al., 2006; Augustin, 2010). Additionally, GLUT-1 plays an important role in the pre-implantation embryo survival. Decreased GLUT-1 expression, via antisense oligoprobes, results in reduced glucose transport and high rates of apoptosis at the blastocyst stage (Chi et al., 2000).

In the human placenta, GLUT-1 has been localized to the microvillous membrane and the basal membrane of syncytiotrophoblast cells (Jansson et al., 1993; Barros et al., 1995). In the trophoblast cells, GLUT-1 expression increases as gestation advances, with GLUT-1 expression reaching its highest levels in the third trimester of gestation (Jansson et al., 1993; Sakata et al., 1995; Currie et al., 1997; Carruthers et al., 2009; Brown et al., 2011). This continual increase in GLUT-1 expression corresponds with the increasing demand for energy substrates for fetal growth in the

second half of pregnancy. When assessed in placental samples across gestation, the microvillous and basal membrane GLUT-1 expression increases at varying rates (Brown et al., 2011; Jansson et al., 1993). In the late second trimester, basal membrane GLUT-1 expression increases 2-fold and remains unaltered thereafter to term (Jansson et al., 1993). When assessed in term human placentas, GLUT-1 expression at the microvillous membrane has been reported to be 3-fold higher than the basal membrane GLUT-1 expression (Jansson et al., 1993; Barros et al., 1995). As the placental surface area grows, due to the folding of the villi, the 3-fold difference in GLUT-1 expression at the microvillous membrane correlates to approximately a 20-fold higher glucose transport capacity (Jansson et al., 1993; Stanirowski et al., 2021; Teasdale & Jean-Jacques, 1985). Due to the localization and asymmetric distribution of GLUT-1 in the human syncytiotrophoblast cells, GLUT-1 in the basal membrane has been suggested to be the rate limiting step in transplacental glucose transfer (Illsley, 2000). Additionally, it has been reported that the basal membrane GLUT-1 expression is correlated with fetal birth weight in non-diabetic, normal pregnant women (Borges et al., 2019). Taken together, the localization and expansive abundance of GLUT-1 in the mammalian placenta has led to GLUT-1 being considered the major glucose transporter isoform in the mammalian placenta (Illsley, 2000).

The results of studies on placental expression of GLUT-1 during pregnancies affected by IUGR have been conflicting due to varying etiologies of the IUGR pregnancy. In preterm and term IUGR placentas, with IUGR being defined as birthweight two standard deviations below the population mean, GLUT-1 expression was unaltered when measured in both the microvillous membrane and basal membrane

of human placentas (Jansson et al., 1993; Jansson et al., 2002). Additionally, in human placentas affected by idiopathic full-term IUGR, no differences were observed in GLUT-1 protein in the basal plate (maternal side) or the chorionic surface (fetal side) (Janzen et al., 2013). In monochorionic twin pregnancies with fetal growth restriction, defined as the presence of inter-twin birth weight discordance of greater than 25% and the smaller twin with a birth weight less than the 10th percentile in the third trimester, *GLUT-1* gene expression was not significantly altered between appropriate for gestational age and growth restricted fetuses (Chang et al., 2021). No change in *GLUT-1* expression was also seen if the pregnancies were further separated based on normal umbilical artery doppler versus abnormal umbilical artery Doppler with persistently absent or reverse end-diastolic flow (Chang et al., 2021).

The conflicting results are also seen in various experimental animal models of IUGR. In a rat model of maternal hypothyroxinemia, while placental weights were not altered, fetal growth was significantly reduced as well as GLUT-1 expression (Pickard et al., 1999). In the glucocorticoid model, administration of glucocorticoids has been demonstrated to induce IUGR as well as alter placental glucose transporter expression. In human term placental trophoblast cells that were exposed to varying concentrations of glucocorticoid (i.e., triamcinolone), or in the placentas of pregnant rats that received a single intraperitoneal injection of triamcinolone, Hahn et al. (1999) reported GLUT-1 to be significantly down-regulated in response to glucocorticoid administration. Conversely, when pregnant rats were administered subcutaneous dexamethasone from day 15 of gestation to day 21, while placental and fetal weights were reduced, there was an increase in GLUT-1 protein expression in the dexamethasone treated groups

(Langdown & Sugden, 2001). In rats that underwent uterine artery ligation, Das et al. (1998) reported a 50% decline in placental GLUT-1 protein levels, along with a decline in fetal glucose and insulin concentrations and placental and fetal body weights. In the undernutrition model, a 50% reduction in the maternal diet spanning from mid- to late-gestation in mice resulted in decreased placental and fetal weights, but no change in placental GLUT-1 expression (Ganguly et al., 2012). Additionally, in pregnant rats that were feed 50% of their diet during the last week of gestation resulted in reduced fetal weights, no change in placental weight, and no effect on placental expression of GLUT-1 (Lesage et al., 2002). In pregnant ewes fed 50% of their NRC dietary requirements from early to mid-gestation (d28 to d78) resulted in significantly decreased placental and fetal weights as well as increased GLUT-1 mRNA and protein levels at mid-gestation (Ma et al., 2011). However, when the nutrient restricted ewes were fed to 100% of their NRC dietary requirements from mid- to late-gestation (d78 to d135), the differences in placental and fetal weight as well as GLUT-1 mRNA and protein expression were no longer observed (Ma et al., 2011). Then, in a primate model, Kavitha et al. (2014) fed baboons 70% of the maternal control diets and demonstrated decreased GLUT-1 expression in the maternal villous membrane at term.

A common characteristic of IUGR pregnancies is they can be deficient in the transport of oxygen and glucose, and how GLUT-1 expression is impacted in these scenarios has also been investigated, but again with conflicting results. GLUT-1 expression in response to hypoglycemia and hyperglycemia has mainly been tested *in vitro*. In syncytial cells, which did not express GLUT-3, GLUT-1 expression was enhanced following incubation (24 h) in the absence of glucose (0 mM), not altered

when incubated in 1 or 12 mM glucose, and GLUT-1 expression was reduced in high concentrations of glucose (20 mM) (Illsley et al., 1998). Additionally, Illsley et al. (1998) also reported villous fragments incubated in high concentrations of glucose (20 mM), resulted in a limited suppression of GLUT-1 expression. Conversely, in JAR or JEG-3 choriocarcinoma cells, low or high extracellular glucose concentrations did not impact GLUT-1 expression (Illsley et al., 1998). In trophoblast cells isolated from term human placentas, hyperglycemia reduced GLUT-1 mRNA and protein expression (Hahn et al., 1998). Additionally, Das et al. (1998) reported GLUT-1 expression in late gestation pregnant ewes exposed to chronic maternal glucose and insulin infusions. In response to maternal and fetal hypoglycemia, there was a 30-50% decline in placental GLUT-1 protein levels. In response to maternal and fetal hyperglycemia, there was a 3-fold increase in GLUT-1 protein at 48 h, followed by a persistent decline in GLUT-1 protein between 10 to 21 days. These data suggest that in response to hypoglycemia, GLUT-1 expression is increased; however, in response to hyperglycemia, GLUT-1 expression is decreased. GLUT-1 expression has also been assessed in response to hypoxic conditions. As measured in BeWo choriocarcinoma cells and human placental villous tissue explants, hypoxic conditions resulted in increased GLUT-1 mRNA and protein expression (Hayashi et al., 2004; Baumann et al., 2007). In contrast, when GLUT-1 expression is measured in placentas from high-altitude pregnant women exposed to low oxygen concentrations (>3,000 m), basal membrane GLUT-1 protein expression was significantly reduced (Zamudio et al., 2006; Zamudio et al., 2010). However, the reduced GLUT-1 expression in the human samples may be attributable to other

modulating factors and not merely the reduced oxygen concentration (Zamudio et al., 2010).

GLUT-3

GLUT-3, encoded by the *SLC2A3* gene, was first isolated and cloned from a human fetal skeletal muscle cell line, and found to share a 64.4% sequence identity with *SLC2A1* (Kayano et al., 1988; Mueckler & Thorens, 2013). GLUT-3 has been described to be highly expressed in the brain, while also being present in the placenta, kidney, colon, small intestine, stomach, and subcutaneous fat (Simpson et al., 2008). Other than glucose, GLUT-3 has also been shown to be able to transport substrates such as galactose, mannose, and xylose, but not fructose (Simpson et al., 2008; Mueckler & Thorens, 2013).

In the differentiating embryo, GLUT-3 has been shown to be expressed as early as the late four-cell stage, through the blastocyst stage, and onwards. GLUT-3 has been localized to the apical membrane of the trophectoderm in the compact morula (Pantaleon et al., 1997). In determining the role of GLUT-3 in the developing embryo, Pantaleon et al. (1997) utilized antisense oligodeoxynucleotides to inhibit GLUT-3 protein expression and assess its role as a functional transporter on the apical membrane of the trophectoderm. Pantaleon et al. (1997) demonstrated that GLUT-3 expression is necessary for blastocyst formation as there was a significant decrease in the number of embryos forming to be blastocysts by 48 hours when they were cultured with GLUT-3 antisense oligonucleotides. Additionally, the resulting antisense-cultured blastocysts had significantly reduced glucose transport activity as compared with the blastocysts cultured with sense oligonucleotides (Pantaleon et al., 1997).

Homozygous deletion of the *Slc2a3* gene (*Slc2a3*$^{-/-}$) is embryonic lethal and results in complete embryonic/fetal loss by day 12.5 dGA in mice, thus demonstrating GLUT-3 is essential for embryonic development (Ganguly et al., 2007; Schmidt et al., 2009). In contrast, when a single allele is mutated (i.e., *Slc2a3*$^{+/-}$), Ganguly et al. (2007) reported post-implantation survival of the pups. Despite the normal placental weights, GLUT-3-mediated transplacental glucose transport was decreased in the heterozygous mice and resulted in significant growth restriction of the pups. While GLUT-1 expression was unaltered, there was an apparent compensatory increase in system A amino acid placental transport, which has also been reported in another cohort of *Slc2a3*$^{+/-}$ mice (Ganguly et al., 2012).

In assessing GLUT-3 protein expression in human placental samples across gestation, Brown et al. (2011) collected first (8-13 weeks), second (14-17 weeks), and term (39 weeks) placental samples, and analyzed GLUT-3 protein expression in the microvillous membrane of each trimester placental samples. Brown et al. (2011) reported GLUT-3 protein expression is highest in the first trimester, and decreases as gestation advances. Having been demonstrated that GLUT-3 expression is highest in early gestation and decreases as gestation advances, it has been proposed that GLUT-3 is more important in early gestation than late gestation (Simpson et al., 2008; Brown et al., 2011). Brown et al. (2011) and others have suggested that due to the high affinity and high transport capacity for glucose, GLUT-3 would make an ideal glucose transporter in the first trimester of pregnancy. Early in the first trimester, the trophoblast cells must invade the maternal spiral arteries in the decidua before establishing intervillous blood flow (Brown et al., 2011). Thus, in an environment where there is

limited glucose, it is argued that this environment clearly favors the kinetic characteristics of GLUT-3 to mediate glucose uptake to the trophoblast cell and developing fetus.

Similar to GLUT-1, GLUT-3 expression can be modified in states of maternal hyperglycemia and hypoglycemia. Das et al. (2000) utilized late gestation, chronically catheterized ewes to assesses the physiological regulation of GLUT-3 during maternal hyperglycemia and hypoglycemia. To establish maternal hyperglycemia, the ewes were chronically administered a dextrose solution by intravenous infusion into the maternal circulation. To establish maternal hypoglycemia, insulin was chronically infused into the maternal circulation. Das et al. (2000) observed GLUT-3 protein expression to decline at 17-20 days in response to chronic maternal hyperglycemia, while also reporting increased uterine and uteroplacental uptake rates of glucose, but normal fetal glucose uptake rates. So, in response to maternal hyperglycemia, it appears that GLUT-1 protein expression decreases earlier than GLUT-3 protein expression (Das et al., 1998; Das et al., 2000). Das et al. (2000) suggested that the decrease in GLUT-1 and GLUT-3 protein concentrations would lead to a diminution of the total placental glucose transporter pool, which may be beneficial in protecting the fetus from glucose toxicity, fetal hypoxia, and tissue acidosis. In response to chronic maternal hypoglycemia, Das et al. (2000) observed no significant changes in GLUT-3 protein expression, but did note that the uterine, uteroplacental, and fetal net glucose uptake rates were decreased. So, in response to chronic maternal hypoglycemia, GLUT-1 protein expression decreases while GLUT-3 protein expression is not altered, thus ultimately increasing GLUT-3 relative contribution to the total glucose transporter pool (Das et al., 1998; Das

et al., 2000). Das et al. (2000) suggested that, the resulting increase of GLUT-3 in the glucose transporter pool, along with GLUT-3 higher affinity for glucose and its localization on the apical membrane of trophoblast cells in the sheep placenta, would perhaps ensure an adequate glucose supply to the placenta.

In a cohort of human placentas collected from women affected by idiopathic full term IUGR, Janzen et al. (2013) observed, on the maternal aspect of the placenta, increased GLUT3 protein expression in the trophoblast of the IUGR placentas as compared to the gestational age matched healthy controls. Additionally, they reported no differential methylation of the GLUT-3 promoter in the IUGR placentas as compared to the controls, and found increased activation of placental HIF-1α. Janzen et al. (2013) hypothesized that hypoxia, through the actions of HIF-1α, may play a role in the up-regulation of GLUT-3. In assessing GLUT-3 localization in placental samples via immunohistochemistry, Janzen et al. (2013) noted increased GLUT-3 in the trophoblast villi that were adjacent to fibrinoid deposition. When the trophoblast is damaged, it can lead to an increased local activation of a coagulation cascade in the intervillous space that will result in the deposition of perivillous fibrin which, when fibrinoid embeds the placental villi, it can impair transplacental gas and nutrient exchange (Janzen et al., 2013). Thus, Janzen et al. (2013) suggested that increased fibrinoid deposition leads to hypoxia, causing the activation of HIF-1α, and resulting in increased trophoblast expression of GLUT-3. Additionally, in a study of monochorionic twin pregnancies with selective fetal growth restriction and abnormal umbilical Doppler, Chang et al., (2021) reported HIF-1α and GLUT-3 gene expression were up-regulated in these fetuses, and suggested the growth restricted fetus would be experiencing significant hypo-perfusion,

thus leading to increased upregulation of HIF-1α that subsequently causes increased expression of GLUT-3. This potential interaction, between HIF-1α and GLUT-3, has also been demonstrated in BeWo cells, in which following 24 h exposure to 3% oxygen, resulted in markedly upregulated GLUT-3 expression (Baumann et al., 2007). However, this upregulation was completely inhibited by HIF-1α antisense oligonucleotides, thus demonstrating the potential for the HIF-1 pathway to play a role in oxygen-mediated regulation of GLUT-3 (Baumann et al., 2007).

While Janzen et al. (2013) proposed hypoxia regulates GLUT-3 expression, a mechanism has since been proposed for how GLUT-3 expression is regulated by hypoxia through the HIF-1α transcription factor. Lauer et al. (2020) identified a novel long noncoding RNA referred to as noncoding intergenic co-induced transcript (NICI), and found it to be co-expressed with *SLC2A3*. They observed a lack of direct interaction between the HIF binding site and the *SLC2A3* promoter, but a strong association between HIF-binding and expression of *SLC2A3* and NICI, in which there was delayed induction of *SLC2A3* compared to NICI. To further elucidate what was occurring, Lauer et al. (2020) utilized antisense oligonucleotides against NICI, and demonstrated a knockdown of NICI with a comparable reduction of *SLC2A3* mRNA expression. Conversely, when the induced transcription of NICI, in a HIF-independent manner occurs, increased expression of NICI as well as increased levels of *SLC2A3* mRNA was observed. Additionally, upon knocking out NICI with CRISPR/Cas-9-mediated deletion of the NICI transcript, Lauer et al. (2020) observed significantly reduced recruitment of RNA polymerase 2 to the *SLC2A3* promoter. Thus, Lauer et al. (2020) proposed a

mechanism in which NICI is transcriptionally activated by the HIF-1α transcription factor, and in turn regulates hypoxic GLUT-3 expression.

GLUT-8

GLUT-8, formerly referred to as GLUTX1, is a class 3 glucose transporter that is expressed predominantly in the testis, but has also been reported to be expressed in the brain, liver, heart, skeletal muscle, adipose tissue, spleen, and placenta (Gawlik et al., 2008). The amino acid sequence of GLUT-8 shares 29.4% sequence identity to GLUT-1 (Schmidt et al., 2009), and exhibits a unique dileucine motif at the amino-terminal cytoplasmic domain, similar to the dileucine motif present at the carboxyl-terminus of GLUT-4, that results in the internalization of GLUT-8 (Ibberson et al., 2000; Lisinski et al., 2001; Sandoval et al., 2000; Schmidt et al., 2006). When GLUT-8 was still a novel glucose transporter, Ibberson et al. (2000) set out to further elucidate GLUT-8 role as a functional glucose transporter. In doing so, Ibberson et al. (2000) injected synthetic mRNAs, transcribed from full-length rat cDNA, into Xenopus oocytes. After two days, the 2-deoxy-D-glucose (2-DOG) uptake was measured and found not to be different from the controls. Ibberson et al. (2000) suggested that the lack of transport activity was due to the amino-terminal dileucine motif, thus potentially preventing the plasma membrane expression of GLUT-8. Subsequently, Ibberson et al. (2000) mutated two leucine amino acids into alanines in the dileucine motif, and injected oocytes with the corresponding mutant mRNA, repeated the experiment, and observed the transport activity to be strongly stimulated as the mutated GLUT-8 had been translocated from intracellular compartments to the plasma membrane. In a similar study, where two leucine amino acids were mutated into alanines in the dileucine motif, Lisinski et al.

(2001) reported similar findings in that the mutation of GLUT-8's dileucine motif led to its expression on the plasma membrane. To understand if GLUT-8 underwent constitutive recycling between intracellular compartments and the plasma membrane, Lisinski et al. (2001) inhibited endocytosis by co-expressing a dominant-negative dynamin mutant (dynamin-K44A), which inhibits endocytosis of a variety of membrane proteins, and observed GLUT-8 accumulating on the plasma membrane. Thus, Lisinki et al. (2001) proposed GLUT-8 may recycle in a dynamin-dependent manner between internal membranes and the plasma membrane in rat adipose cells. However, GLUT-8 appears to remain an intracellular glucose transporter unless the dileucine motif is mutated.

While GLUT-8 appears to be primarily an intracellular glucose transporter under basal conditions, it has been observed to be expressed on the plasma membrane. Carayannopoulous et al. (2000) reported murine preimplantation blastocysts exhibit insulin-stimulated glucose uptake; however, preimplantation blastocysts do not express the insulin-regulated glucose transporter, GLUT-4. Instead, Carayannopoulous et al. (2000) demonstrated that GLUT-8 was responsible for the insulin-stimulated glucose uptake they observed as it translocated from intracellular compartments to at or near the plasma membrane of the trophectoderm cells upon insulin exposure. While GLUT-1 and GLUT-3 may contribute to the blastocyst's glucose uptake, as they are both found on the plasma membrane of the trophectoderm in murine blastocysts (Winterhager & Gellhaus, 2017), Carayannopoulous et al. (2000) effectively decreased GLUT-1 and GLUT-3 protein expression via antisense oligoprobes and observed no effect on insulin-stimulated glucose uptake as compared to the controls. Carayannopoulous et al. (2000) further confirmed GLUT-8 role in the preimplantation blastocyst by using GLUT-8

antisense oligoprobes and demonstrating that insulin-stimulated glucose uptake was inhibited in blastocysts exposed to GLUT-8 antisense oligonucleotides. Additionally, Pinto et al. (2002) further demonstrated the importance of GLUT-8 translocating to the plasma membrane for blastocyst survival by culturing murine blastocysts for 72 hours with GLUT-8 antisense oligonucleotides and demonstrating that decreased GLUT-8 expression during preimplantation development induced increased apoptosis of murine blastocysts, as well as observing higher rates of resorption and consequently lower pregnancy rates compared to blastocysts cultured with GLUT-8 sense oligonucleotides. However, it should be noted, that GLUT-8 ability to translocate from intracellular compartments to the plasma membrane, whether stimulated by insulin or not, has not been documented in any other cell type unless there was a purposeful disruption in intracellular trafficking mechanisms (Lisinski et al., 2001; Ibberson et al., 2000; Gawlik et al., 2008). Thus, at least in the preimplantation blastocyst, GLUT-8 appears to be vital for blastocyst survival as it takes over the role of GLUT-4 for insulin-stimulated glucose uptake.

In a study done by Piroli et al. (2002), they found GLUT-8, through the use of immunoblot analysis of rat hippocampal membrane-containing fractions, to be expressed in high density microsomes (HDM) and low-density microsomes (LDM) under basal conditions. Upon finding GLUT-8 expressed in these microsomal fractions, Piroli et al. (2002) utilized immunogold electron microscopy to further assess and define more accurately the localization of GLUT-8 in the microsomal compartments of hippocampal neurons and demonstrated that GLUT-8 was associated with the rough endoplasmic reticulum and the cytoplasm. In a different study, where *Slc2a8* null mice were created,

the subsequent offspring appeared normal (i.e., normal growth, body weight development, and glycemic control); however, upon further investigation, the spermatozoa of the offspring demonstrated lower ATP levels, reduced mitochondrial membrane potential, and significant reductions in sperm motility (Gawlik et al., 2008). Despite demonstrating the necessity of GLUT-8 in intracellular compartments of spermatozoa, the normal glycemic control of the *Slc2a8* null mice suggests that GLUT-8 does not play a significant role in the regulation of whole-body energy balance and glucose homeostasis (Gawlik et al., 2008; Schmidt et al., 2009). Instead, GLUT-8 stands to regulate the influx or efflux of glucose from intracellular organelles. As GLUT-8 has been associated with the rough endoplasmic reticulum, GLUT-8 may transport glucose molecules that are liberated from oligosaccharides during protein glycosylation events and shuttle that glucose into the cytoplasm where it can be converted to glucose-6-phospahte or re-enter glycolytic pathways (Piroli et al., 2002). The glucose could also be converted to UDP-glucose in the cytoplasm and re-enter the rough endoplasmic reticulum, via GLUT-8, to be used in oligosaccharide synthesis (Piroli et al., 2002). As GLUT-8 has also been associated with lysosomes, GLUT-8 may mediate the transport of glucose molecules from glycoproteins that were subjected to lysosomal degradation (Schmidt et al., 2009). Thus, it can be suggested that GLUT-8 intracellular transport role may be to catalyze the transport of glucose, and other potential sugar molecules, into or out of intracellular organelles (Schmidt et al., 2009).

Janzen et al. (2018) reported, in human idiopathic late-term IUGR pregnancies, GLUT-8 mRNA and protein are increased on the maternal aspect of the placenta, but are not altered on the fetal aspect of the placenta. Additionally, GLUT-8

immunofluorescence staining of the IUGR-affected pregnancies also demonstrated a significant increase in GLUT-8 expression (Janzen et al., 2018). Similar to the human, when assessing cotyledon GLUT-8 mRNA in IUGR sheep pregnancies generated by CSH-deficiency, GLUT-8 mRNA expression was not different from the controls at 50 dGA, but there was a significant increase in GLUT-8 mRNA at 135 dGA in the CSH-deficient pregnancies (Jeckel et al., 2018). Conversely, Limesand et al. (2004) assessed GLUT-8 expression in PI-IUGR sheep pregnancies generated via the hyperthermic model, and reported GLUT-8 mRNA and protein concentrations were decreased. However, in sheep, when cotyledon GLUT-8 mRNA was assessed in normal day 90 and 135 pregnancies, GLUT-8 mRNA increased as gestation advances (Limesand et al., 2004). From these studies, it appears when glucose uptake by the placenta is decreased, GLUT-8 may upregulate to maintain placental metabolism.

In Vivo RNA Interference

While none of the models mentioned fully recapitulate human IUGR pregnancies, the sheep models of IUGR have helped develop our understanding of IUGR pregnancies tremendously. While ruminant models have provided a strong understanding of *in vivo* placental function, the ability to create transgenic pregnancies to assess the function of specific genes expressed by the placenta has been problematic and lagging behind other species, most notably the rodent (Anthony et al., 2010). In one attempt, Dunlap et al. (2006) injected morpholino antisense oligonucleotides into the uterine lumen on day eight of pregnancy to examine the impact of inhibiting the expression of endogenous Jaagsiekte sheep retrovirus within the sheep conceptus. While this method was successful, morpholino oligonucleotide mediated

gene knockdown relies on transient transfection, and the efficacy is lost as the sheep conceptus proliferates (Anthony et al., 2010), making this approach unsuitable for long-term studies. One method that holds the potential to assess gene function in the ruminant placenta is through lentiviral-mediated RNA interference, which can be achieved in the placenta through viral infection of the trophectoderm layer of hatched blastocysts. Purcell et al. (2009) demonstrated the viability of this approach as they were able to inhibit conceptus elongation in the sheep conceptus by knocking down proline-rich 15 expression *in vivo* using lentiviral-mediated short-hairpin RNA (shRNA) constructs.

When shRNAs are introduced into cells, they are processed in the same fashion as endogenous micro-RNA (miRNA) (Paddison et al., 2002). miRNAs are small RNAs that negatively regulate gene expression at the post-transcriptional level and can feed into the RNA interference (RNAi) pathway (He & Hannon, 2004). RNAi is an evolutionary conserved response to exogenous double-stranded RNA (dsRNA) that appears to be an endogenous defense mechanism against viral infection, as well as endogenous gene regulation (He & Hannon, 2004; Anthony et al., 2010). Our current understanding of the biogenesis and post-transcriptional suppression of miRNA is as follows. In the nucleus, primary miRNA transcripts are processed by the ribonuclease-III (RNase III) enzyme Drosha, yielding a ~70-nucleotide pre-miRNA with a characteristic hairpin loop structure. The pre-miRNA is exported into the cytoplasm where it is further processed by another RNase III enzyme, Dicer, which removes the loop sequence and yields a 21-25 base pair duplex, or mature miRNA. Only one strand of the duplex is preferentially assembled into the RNA-induced silencing complex (RISC), and depending on the level

of complementarity between the small RNA and its target, subsequently leads to either mRNA cleavage or translational repression. If there is perfect complementarity, the RNase H activity of Argonaute, a component of RISC, cleaves the mRNA and targets it for nuclease-mediated degradation. If there is imperfect complementarity, Argonaute may not cleave the target, but instead trigger translational repression rather than mRNA cleavage (He & Hannon, 2004; Anthony et al., 2010). Additionally, this pathway can be exploited experimentally. A shRNA can be introduced into the nuclei of target cells using a targeting vector, commonly either adenovirus or lentivirus vectors. While there are strengths and weaknesses to both types of targeting vectors, adenoviral vectors only provide for transient expression while lentiviral vectors provide stable integration into the host genome (Anthony et al., 2010). Thus, adenoviral vectors "effect" may dissipate over time in rapidly proliferating tissues, such as the ruminant placenta, while lentiviral vectors are ideal for long-term transgenesis (Anthony et al., 2010). The shRNA is then synthesized in the nucleus of the transfected cell, and as mentioned, is processed in the same fashion as endogenous miRNA, thus leading to either targeted mRNA degradation or mRNA translational repression. Ultimately, the shRNA construct can cause the "knock-down" of a specific gene, leading to reduced protein expression. The use of this method has been demonstrated several times in the ruminant placenta (Baker et al., 2016; Jeckel et al., 2018; Tanner et al., 2021a; Tanner et al., 2021b). With this approach, the ability to target the expression of specific placental nutrient transporters within the placenta would provide considerable insight into the role of individual placental transport mechanisms.

Summary

For the majority of gestation, until near term, the fetus has a very low capacity for glucose production. As such, the two major facilitative glucose transporter isoforms in the mammalian placenta, SLC2A1 and SLC2A3, mediate glucose transfer, down its concentration gradient, from maternal circulation, across the placenta, and into fetal circulation. As previously noted, SLC2A1 is the most abundant glucose transporter isoform in the mammalian placenta, while SLC2A3 has a five-fold greater transport capacity and affinity for glucose. Additionally, in sheep, SLC2A3 is the only reported glucose transporter isoform present on the apical microvillous trophoblast membrane, and as such, SLC2A3 mediates trophoblast glucose uptake. Due to the distinct localization of SLC2A1 and SLC2A3 in the sheep placenta, the sheep stands to be a unique model to examine the relative importance of SLC2A1 and SLC2A3 in placental glucose uptake and transfer, as well as assessing how perturbations in either facilitative glucose transporter may play a role in pathologic conditions, such as IUGR. Thus, by utilizing placenta-specific RNAi, to effectively diminish the translation of *SLC2A3*, the impact of SLC2A3 deficiency on placental uptake and transfer of glucose, placental development and function, and fetal development can be assessed, as well as any placental or fetal compensatory mechanisms that may arise to combat SLC2A3 deficiency.

Chapter II: THE *IN VIVO* PHYSIOLOGICAL RAMIFICATIONS OF PLACENTAL SLC2A3 DEFICIENCY IN SHEEP

INTRODUCTION

The placenta is tasked with the transport of oxygen, glucose, and amino acids from the maternal circulation to fetal circulation to support fetal growth and development. Of these nutrients, glucose is the predominant energy substrate for fetal oxidative processes and growth (Hay, 2006). Additionally, the placenta also consumes the majority of glucose it takes up, especially during the first half of gestation (Bell et al., 1986). Functional placental insufficiency is an underlying cause of intrauterine growth restriction (IUGR), and while the specific causes of placental insufficiency are not well characterized, the transport of glucose can be deficient in IUGR pregnancies as the fetuses are often hypoglycemic (Marconi & Paolini, 2008). However, when assessed in human placental samples collected at term, the abundance of the facilitative glucose transporters is not reduced in the IUGR placenta thus leaving the root cause behind the fetal hypoglycemia in IUGR pregnancies unresolved (Jansson et al., 2002; Janzen et al., 2013). Although, the abundance of the facilitative glucose transporters in the term placenta may not be reflective of the glucose transport capacity of the IUGR placenta throughout gestation. Any deficits in placental glucose transport during the first-half of pregnancy could impact placental development and function, resulting in functional placental insufficiency leading to fetal hypoglycemia and IUGR. Due to obvious ethical concerns, this cannot be addressed in the human and requires the use of relevant animal models.

The pregnant sheep has been a valuable model for human pregnancy and has provided considerable insight into *in vivo* placental nutrient uptake, utilization, and

transfer to the developing fetus. The two main facilitative glucose transporters in the mammalian placenta are SLC2A1 (GLUT-1) and SLC2A3 (GLUT-3), and their localization in the sheep placenta offers a unique opportunity to study their relative roles in glucose transport, and how a deficiency in either glucose transporter may impact placental development and function. In the sheep, in order for glucose to be transported from maternal to fetal circulation, glucose travels down a concentration gradient and must be sequentially transported by SLC2A1 on the maternal-fetal syncytial layer, then by SLC2A3 on the apical trophoblast membrane, and again by SLC2A1 on the basolateral membrane of the trophoblast (Wooding et al., 2005). SLC2A1 is the most abundant placental glucose transporter, and as such is believed to be the primary glucose transporter in the human and sheep placenta (Jansson et al., 1993; Hauguel-De Mouzon et al., 1994). However, SLC2A3 has a 5-fold greater affinity and transport capacity for glucose (Simpson et al., 2008). Due to SLC2A3 being located on the apical (maternal-facing) trophoblast membrane, and its greater affinity and transport capacity for glucose, any deficiency in SLC2A3 may significantly impact trophoblast glucose uptake and transfer to the fetus, thus potentially altering placental development and consequently setting the stage for IUGR.

In mice, it has been demonstrated that functional ablation studies of either SLC2A3 or SLC2A1 results in embryonic lethality (Ganguly et al., 2007; Schmidt et al., 2009; Wang et al., 2006). This suggests that both glucose transporters are vital for embryonic and fetal survival; however, it is not clear if perturbations in either SLC2A1 or SLC2A3 can results in placental insufficiency and IUGR. However, with the use of lentiviral-mediated RNA interference, this method can be utilized to specifically

knockdown specific gene products in the placenta and can be used to assess the *in vivo* physiological ramifications of placental glucose transporter deficiencies throughout gestation. Thus, it was our objective to use placenta-specific RNA interference to diminish SLC2A3, and determine the impact at mid-gestation (75 dGA) in sheep.

MATERIAL AND METHODS

Lentivirus Vector Construction and Lentivirus Generation

Lentiviral infection was used to stably integrate and express shRNA targeting *SLC2A3* mRNA in the host cell. Lentiviral vectors were constructed and generated using the protocol previously described extensively (Baker et al., 2016). In summary, lentiviral vector construction was done by cloning SLC2A3 targeting shRNA or scramble control (SC) shRNA sequence (Table 1) into the pLKO.1 vector (Addgene, Cambridge, MA), which contained the human U6 promoter upstream of cloning sites for shRNA cassettes. The human U6 promoter and downstream SLC2A3 or SC shRNA sequence within pLKO.1 were then PCR amplified, with the resulting PCR amplicon being gel purified and cloned into the StrataClone PCR cloning vector (Agilent Technologies, Santa Clara, CA). The human U6 promoter and SLC2A3 or SC DNA fragment were digested from the StrataClone PCR cloning vector, with the subsequent DNA fragment being ligated into the lentiviral vector pLentiLox3.7 (pLL3.7). All PCR products and constructs, as well as the insertion of the human U6 promoter and SLC2A3 or SC shRNA sequence into pLL3.7, were verified by DNA sequencing during and after construction.

Table 1. Scramble control and SLC2A3-targeting shRNA sequences.

Oligonucleotide	Sequence (5'-3')
SC shRNA sense	GAGTTAAAGGTTCGGCACGAATTCAAGAGATTCGTGCC GAACCTTTAACTC
SLC2A3 shRNA sense	GCGCAACTCAATGCTTATTGTTTCAAGAGAACAATAAGCA TTGAGTTGCGC

To generate the lentivirus, 293FT cells (Invitrogen, Carlsbad, CA) were grown to 60-70% confluency on a 150-mm tissue culture dish. On the day of transfection, for each 150-mm dish, 8.82 μg of SLC2A3 or SC targeting plasmid DNA, 6.66 μg of psPAX2 packaging plasmid DNA (Addgene), and 2.70 μg of pMD2.G envelope plasmid DNA (Addgene) were mixed with 180 μl of polyfect transfection reagent (Qiagen Inc., Germantown, MD) and brought to a final volume of 675 μl using serum-free, antibiotic-free, high-glucose DMEM media. This mixture was incubated for 10 min at room temperature to allow for complex formation. Immediately before treating cells with the transfection mixture, medium was gently aspirated from cells and replaced with 18 ml of fresh complete medium (10% heat inactivated fetal bovine serum (FBS) and 1x penicillin-streptomycin-amphotericin B (PSA)). Then, 885 μl of transfection mixture was added to each 150-mm dish of 293FT cells dropwise for even distribution. After a 4-6 h incubation at 37°C and 5% CO_2, the transfection media was replaced with fresh complete medium. After 72 h, the medium containing lentiviral particles was collected and ultra-centrifuged over a 20% sucrose cushion at 47,000 *g* for 2 h at 4°C. After ultra-centrifugation, lentiviral pellets were resuspended in either 1X PBS, for cell transfection, or CDM-2, for day 9 blastocysts, and stored in aliquots at -80°C. To titer the lentiviral aliquots, frozen aliquots were thawed, initially diluted 1:40 and then serially diluted 10-

fold with high-glucose, complete DMEM media. Serial dilutions of virus ranging from 10^{-2} to 10^{-7} and 8 μg of polybrene (Sigma-Aldrich, St. Louis, MO) per milliliter of media were added to a 70% confluent 6-well tissue culture plate of human embryonic kidney-293 (HEK-293) cells. Cells were incubated with viral dilutions overnight, then washed with PBS and fresh complete medium was added. At 48 h post-infection, the number of green fluorescent positive cells were counted in the last well containing fluorescent cells. The titer was then calculated by dividing the number of live cells by the serial dilution factor from which the live cells were counted and multiplying this number by the initial dilution factor used to dilute the original stock of virus.

Cell Lines

Immortalized ovine trophoblast (iOTR) cells (Ali et al., 2020) were utilized to test the degree of knockdown of the SLC2A3 shRNA construct. To infect cells, a frozen viral aliquot of SLC2A3 or SC lentivirus was resuspended in 500 μl of appropriate media with 8 μg/ml polybrene (Sigma-Aldrich). The iOTR cells were incubated with lentiviral particles at a multiplicity of infection (MOI) of 100 or 500 for 8 h at 37°C and 5% CO_2, after which the media was replaced with fresh complete media. After 72 h of culture, cells were assessed for the percentage of green fluorescent positive cells. The subsequent cells were passaged up to a 150-mm tissue culture plate and were pelleted if the cells within that plate were >95% green fluorescent positive. The cell pellet was then stored in -80°C until further analysis.

Generation of Pregnancies

All Dorper ewes were grouped housed in pens at Colorado State University Animal Reproduction and Biotechnology Laboratory, and were provided access to hay,

water, and trace mineral in order to meet or slightly exceed their National Research Council (National Research Council, 2007) requirements. Animal management, estrus synchronization, and embryo transfers were done as previously described (Baker et al., 2016). In summary, after synchronization, donor ewes displaying standing estrus were bred by intact rams over a 24 h period. Nine days after breeding, donor ewes were euthanized using pentobarbital sodium (88 mg/kg iv, Euthasol; VetOne, Boise, ID), and a complete hysterectomy was performed. The uteri were then flushed using DMEM-F-12 (1:1) medium supplemented with 0.25% BSA. Fully expanded and hatched blastocysts were collected and incubated in a 100 μl drop, overlaid with mineral oil, containing 100,000 transducing units of either control RNAi (SC RNAi) or SLC2A3 RNAi lentivirus, 50 ng of polybrene (5 ng/μl; Sigma-Aldrich), and chemically defined medium (CMD-2) for late-stage embryos. Drops containing single blastocysts were incubated in 5% CO_2-5% O_2-90% N_2 at 37°C for approximately 5 h. Each blastocyst was then thoroughly washed in HEPES-buffered chemically defined medium (HCDM-2), and a single blastocyst was surgically transferred into the uterine horn ipsilateral to the corpus luteum of a synchronized recipient Dorper ewe. The recipient ewe was then monitored daily for return to standing estrus and confirmed pregnant at 50 days of gestation age (dGA) by ultrasonography (Mindray Medical Equipment, Mahway, NJ). At 70 dGA, all pregnancies underwent Doppler velocimetry assessment as described previously (Tanner et al., 2021b).

Tissue Collection

At 75 dGA, 6 control (SC RNAi) and 6 SLC2A3 RNAi pregnancies underwent a terminal surgery as previously described (Baker et al., 2016). Pregnant recipient ewes

were food restricted for 18 h before surgery. Pregnant recipient ewes were sedated using ketamine (12.5 mg/kg iv, Ketacine; VetOne) and diazepam (0.125 mg/kg iv; Hospira), intubated with an endotracheal tube, and maintained on 2 L/min O_2 and 1-3% isoflurane (Flurios; VetOne). Once the fetus and umbilical cord were exposed, fetal blood was collected from the umbilical artery and vein, and maternal blood was collected from the uterine artery and vein ipsilateral to the fetus, with the resulting serum stored in -80°C until further analysis. The fetus was then euthanized using pentobarbital sodium by intravenous administration through the umbilical vein (88 mg/kg, Euthasol; VetOne) and was excised. Fetal sex, body weight, crown-rump length, head and abdominal circumference, and femur and tibia length were recorded for the recovered fetus. The fetal liver and pancreas were harvested, weighed, and stored in a 50-ml conical that was then snap-frozen in liquid nitrogen. A complete hysterectomy was performed and all placentomes were excised and recorded for total placentome number and weight. Thirty placentomes were randomly selected and placed into a 50-ml conical tube and snap-frozen in liquid nitrogen. The resulting tissue was pulverized using a mortar and pestle and the resulting pulverized tissue was kept at -80°C for later use.

Biochemical Analysis of Blood Samples

Plasma glucose and lactate were measured by Yellow Springs Instrument 2900 (YSI Incorporated, Yellow Springs, OH), and plasma amino acids were measured by HPLC as described previously (Tanner et al., 2021a). Maternal and fetal plasma concentrations of insulin and IGF-1 were assessed by enzyme-linked immunosorbent assay (ALPCO Immunoassays 80-IN-SOV-E01 and 22-IGFHU-E01, respectively) as described previously (Andrews et al., 2015; Benjamin et al., 2017; Tanner et al., 2021a).

The concentration of plasma CSH was assessed by radioimmunoassay (RIA) as previously described (Baker et al., 2016).

Western Blot Analysis

Protein isolation and analysis was done in accordance with methods described in (Tanner et al., 2021a; Tanner et al., 2021b). Pulverized placentome tissue (75 mg) was lysed in 500 μl of lysis buffer (0.48 M Tris, pH 7.4; 10 mM EGTA, pH 8.6; 10 mM EDTA, pH 8; 0.1 mM PMSF; 0.1 mM ABESF; 0.0015 mM pepstatin A; 0.0014 mMe-64; 0.004 mM bestatin; 0.002 mM leupeptin; and 0.00008 mM aprotinin) and sonicated on ice. For placental SLC2A3 analysis, 25 μg of protein from each sample were electrophoresed through NuPAGE 4-12% Bis-Tris gels (Life Technologies, Carlsbad, CA), and transferred to a 0.45-um pore nitrocellulose membrane. For iOTR cell SLC2A3 analysis, 10 μg of protein from each sample were electrophoresed through NuPAGE 4-12% Bis-Tris gels (Life Technologies), and transferred to a 0.45-um pore nitrocellulose membrane. The resulting blots were stained with Ponceau S (Sigma-Aldrich) to assess total protein/lane using the ChemiDoc XRS+ (BioRad, Hercules, California). The resulting blots were then blocked (5% non-fat dry milk/1X Tris-Bis solution + 1% Tween) for 24 h at 4°C. To visualize SLC2A3, the blots were incubated in a 1:1000 dilution of rabbit-α-SCL2A3 (Tanner et al., 2021a) for 24 h at 4°C. After washing, the blots were incubated in a 1:5000 dilution of goat α-rabbit IgG conjugated to horse radish peroxidase (ab97051; Abcam) on a shaker platform for 4 h at room temperature. The immunoblots were developed using an ECL Western Blotting Detection Reagent chemiluminescent kit (Amersham, Pittsburgh, PA) and imaged using the ChemiDoc XRS+ (BioRad). Densitometry calculations were performed using Image Lab software

(version 6.1; BioRad) and densitometry of SLC2A3 was normalized on total protein/lane. To account for technical error between immunoblots, a common sample was included in each immunoblot and densitometry measurements were adjusted based on the average densitometry measurements of the common sample.

For analysis of placental SLC2A1, 5 μg of protein from each sample were electrophoresed through a 4-15% Tris-Glycine stain-free gel (BioRad) and transferred to a 0.45-um pore nitrocellulose membrane. After transfer, the nitrocellulose membrane was imaged using the ChemiDoc XRS+ chemiluminescence system (BioRad) to assess total protein/lane to use for normalization. After blocking for 24 h at 4°C, SLC2A1 was visualized by incubating the blots with a 1:40000 dilution of rabbit α-SLC2A1 (07-1401; EMD Millipore, Burlington, MA) for 24 h at 4°C. After washing, the blots were incubated in a 1:80000 dilution of goat α-rabbit IgG conjugated to horse radish peroxidase (ab205718; Abcam) on a shaker platform for 4 h at room temperature. As described for SLC2A3, densitometry analysis of SLC2A1 was performed using Image Lab software (version 6.1; BioRad) and normalized on total protein/lane.

For analysis of placental concentrations of SLC2A8, 20 μg of each sample were electrophoresed through 4-15% Tris-Glycine stain-free gels (BioRad), and transferred and analyzed as described for SLC2A1. SLC2A8 was detected using a 1:2000 dilution of rabbit α-SLC2A8 (LS-C757596; LifeSpan BioSciences, Seattle, WA) and a 1:10000 dilution of goat α-rabbit IgG conjugated to horse radish peroxidase (ab97051; Abcam). For analysis of fetal liver concentrations of insulin receptor beta (IR-β), 25 μg of each sample were electrophoresed through 4-15% Tris-Glycine stain-free gels (BioRad), and transferred and analyzed as described for SLC2A1. IR-β was detected using a 1:1000

dilution of mouse α-IR-β (ab69058; Abcam) and a 1:5000 dilution of goat α-mouse IgG conjugated to horse radish peroxidase (ab6789; Abcam).

RNA Isolation

Total cellular RNA was isolated from 75 dGA pulverized placentome samples using the RNeasy Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's protocol. RNA concentration was quantified using the BioTek Synergy 2 Microplate Reader (BioTek, Winooski, VT), and RNA quality was measured by the 260/280 nm absorbance ratio. Samples were stored at -80°C until use.

cDNA Synthesis and Quantitative Real-Time PCR

cDNA was generated from 2 μg of total cellular RNA using iScript Reverse Transcription Supermix (BioRad) according to the manufacturer's protocol. To control for variance in efficiency of the reverse transcription reaction, cDNA was quantified using the Quant-iT OliGreen ssDNA Assay Kit (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol, and quality was measured by the 260/280 absorbance ratio. An equal mass of cDNA (96 well plate: 25 ng/μl; 384 well plate: 10 ng/μl) were used for each sample in the quantitative real-time PCR (qRT-PCR). qRT-PCR was performed using either the CFX96 or CFX384 Real-Time System (BioRad). Forward and reverse primers for qRT-PCR were designed using Oligo software (Molecular Biology Insights, Cascade, CO) to amplify an intron-spanning product. Primer sequences and amplicon size are summarized in Table 2. Standard curves were generated as described previously (Jeckel et al., 2018). Briefly, a PCR product for each gene was generated using cDNA from 135 dGA fetal placenta as a template and cloned into the StrataClone vector (Agilent Technologies), and each PCR product was

sequenced to verify amplification of the correct cDNA. Using the PCR products amplified from the sequenced plasmids, standard curves were generated for each mRNA from $1x10^2$ to $1x10^{-5}$, and were used to measure amplification efficiency. The starting quantity (pg) was normalized by dividing the starting quantity of mRNA of interest by the starting mRNA quantity (pg) of ribosomal protein S15 (RPS15).

Table 2. Primers and product sizes for cDNA used in qRT-PCR

cDNA	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product, bp
SLC2A8	ATG TGT TTC ATG CCC GAG ACC C	TGG ATG ACA CCC ACG ACG ACT	314
RPS15	ATC ATT CTG CCC GAG ATG GTG	TGC TTG ACG GGC TTG TAG GTG	134
IGF1	TCG CAT CTC TTC TAT CTG GCC CT	ACA GTA CAT CTC CAG CCT CCT CA	240
IGF2	GAC CGC GGC TTC TAC TTC AG	AAG AAC TTG CCC ACG GGG TAT	203
IGFBP1	TGA TGA CCG ACT CCA GTG AG	GTC CAG CGA AGT CTC ACA C	248
IGFBP2	CAA TGG CGA GGA GCA CTC TG	TGG GGA TGT GTA GGG AAT AG	330
IGFBP3	CTC AGA CGA CAG ACA CCC A	GGC ATA TTT GAG CTC CAC	336
IGF1R	AAC TGT CAT CTC CAA CCT C	CAA GCC TCC CAC TAT CAA C	493
IGF2R	GAC TTG TGT CCA GAC CAG ATT C	GCC GTC GTC CTC ACT CTC ATC	674

PRS15, ribosomal protein S15; IGF, insulin-like growth factor; IGFBP, IGF-binding protein

Statistical Analysis

Data were analyzed by 2-way analysis of variance using GraphPad Prism (version 9) to analyze the main effects of treatment and fetal sex, as well as the treatment x sex interaction. There were no treatment by fetal sex interactions, therefore

the data are presented as the main effect of treatment only. Statistical significance was set at P ≤ 0.05 and a statistical tendency at P ≤ 0.10. Data are reported as the mean ± standard error of the mean (SEM). The data figures are presented as bar and scatter plots, with the capped vertical lines representing the SEM.

RESULTS

Knockdown of SLC2A3 in iOTR cells

To assess the effectiveness of the SLC2A3 shRNA construct, iOTR cells were infected at a MOI of 500, approximately the MOI used for blastocyst infection, and SLC2A3 concentration were measured. In the SLC2A3 shRNA-infected iOTR cells, SLC2A3 RNAi resulted in a 91% reduction in SLC2A3 concentration ($p \leq 0.05$; Figure 1) as compared to SC RNAi-infected iOTR cells.

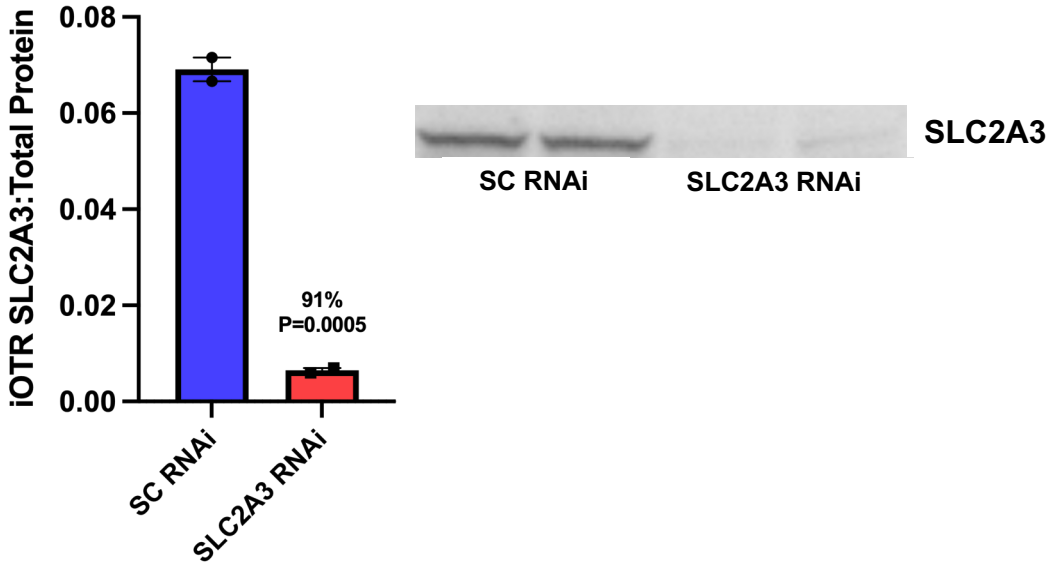

Figure 1. iOTR SLC2A3 protein concentrations as measured by Western blot. Data are shown as means ± SEM.

Fetal and placental measurements

As assessed by Doppler velocimetry, at 70 dGA, pulsatility indices, resistance indices, systolic/diastolic ratios, fetal heart rate, umbilical artery cross-sectional area, and cross-sectional diameter did not differ between treatment ($p \geq 0.10$; Table 3). As assessed by ultrasonography, crown-rump length and abdominal circumference did not differ between treatment ($p \geq 0.10$; Table 3). Binocular distance had a tendency to be reduced in the SLC2A3 RNAi pregnancies ($p \leq 0.10$; Figure 2), while femur and tibia length were significantly reduced in the SLC2A3 RNAi pregnancies ($p \leq 0.05$; Figure 2). This was again observed at necropsy at mid-gestation (75 dGA) when the six SC and six SLC2A3 RNAi pregnancies were harvested. Head circumference, femur length, and tibia length were significantly reduced in the SLC2A3 RNAi pregnancies ($p \leq 0.05$; Figure 3). Crown-rump length and head circumference did not differ between treatment ($p \geq 0.10$; Table 4). Fetal body weight tended to be reduced ($p \leq 0.10$; Figure 4), and placentome weight was reduced in the SLC2A3 RNAi pregnancies, however it did not reach statistical significance ($p \geq 0.10$; Figure 4). Additionally, while fetal liver weight was not different between treatments ($p \geq 0.10$; Figure 5), fetal pancreas weight was significantly reduced in the SLC2A3 RNAi pregnancies ($p \leq 0.05$; Figure 5).

SLC2A3 RNAi resulted in a 37% reduction of SLC2A3 concentration ($p \leq 0.05$; Figure 6), as well as a tendency for SLC2A1 concentration to be increased ($p \leq 0.10$; Figure 6) in the SLC2A3 RNAi pregnancies. Additionally, there was no difference in SLC2A8 concentration between treatment.

Table 3. 70 dGA fetal measurements and Doppler velocimetry measurements.

	SC RNAi	SLC2A3 RNAi	P-Value	% change
Crown-rump length, cm	14.88 ± 1.13	14.31 ± 0.40	0.58	3.83
Abdominal circumference, cm	13.09 ± 0.55	12.20 ± 0.46	0.24	6.82
Pulsatility Index	2.80 ± 0.27	2.97 ± 0.15	0.59	6.15
Resistance Index	0.86 ± 0.05	0.85 ± 0.04	0.92	0.79
Systolic: Diastolic	16.18 ± 6.60	11.04 ± 3.96	0.52	31.76
Fetal heart rate, bpm	204.72 ± 1.91	200.03 ± 8.69	0.61	2.29
Umbilical artery cross-sectional area, cm^2	0.09 ± 0.007	0.104 ± 0.01	0.44	11.52
Umbilical artery cross-sectional diameter, cm	0.34 ± 0.01	0.35 ± 0.02	0.74	2.37

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

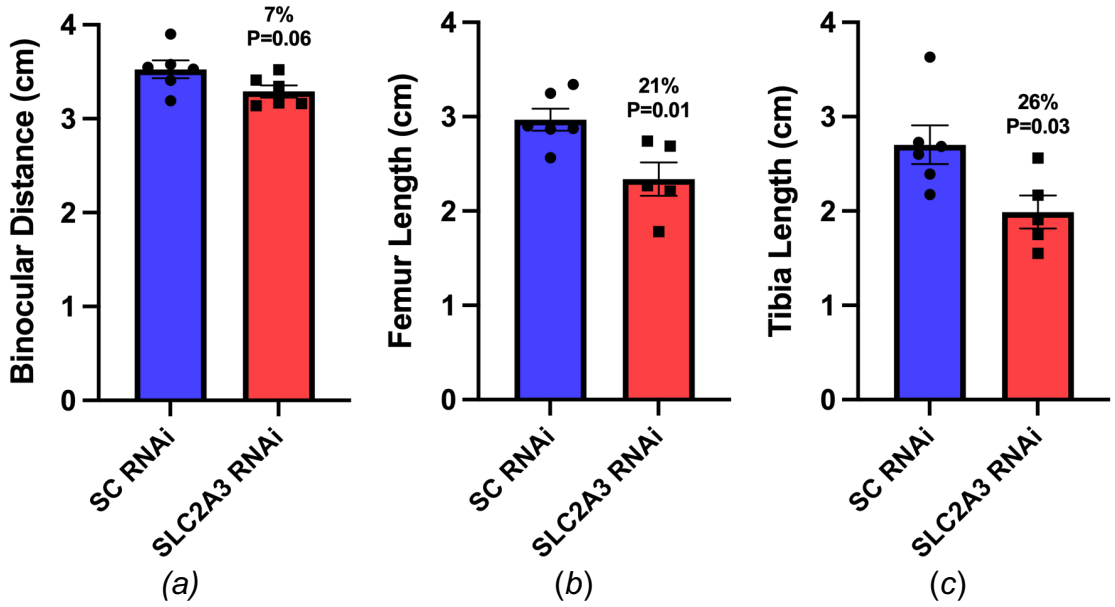

Figure 2. Fetal measurements taken via ultrasonography at 70 dGA: (**a**) binocular distance in cm, (**b**) femur length in cm, (**c**) tibia length in cm. Data are shown as means ± SEM for all pregnancies in each treatment group.

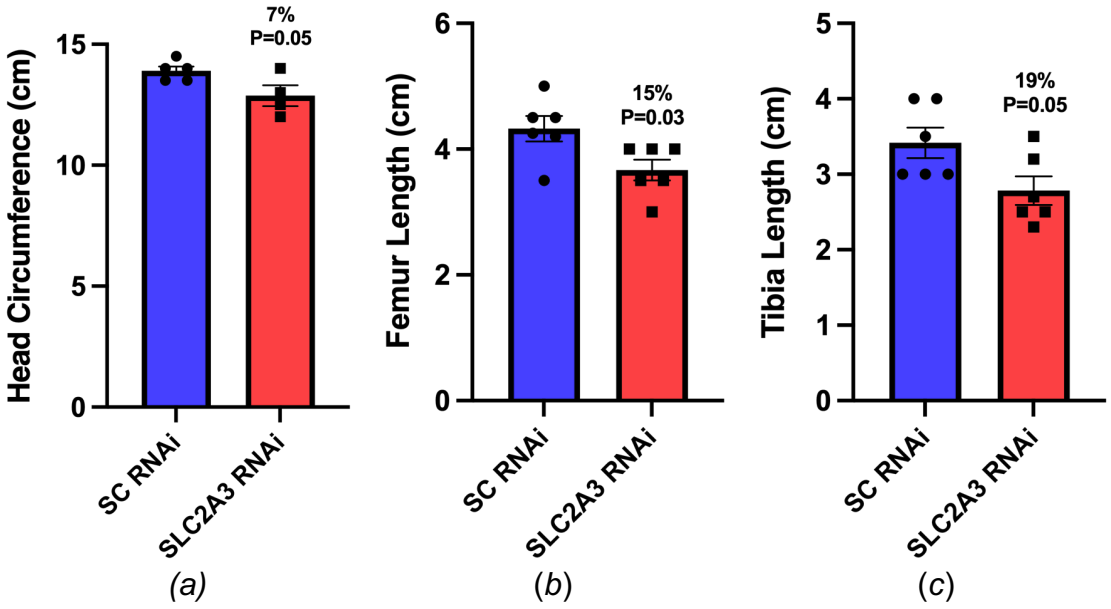

Figure 3. Fetal measurements taken at necropsy at 75 dGA: (**a**) head circumference in cm, (**b**) femur length in cm, (**c**) tibia length in cm. Data are shown as means ± SEM for all pregnancies in each treatment group.

Table 4. Fetal measurements collected at necropsy (75 dGA).

	SC RNAi	SLC2A3 RNAi	P-Value	% change
Placentome number	81.83 ± 5.51	73.33 ± 7.17	0.37	10.39
Crown-rump length, cm	19.33 ± 0.4	19.17 ± 0.36	0.76	0.86
Abdominal circumference, cm	13.62 ± 0.51	12.92 ± 0.35	0.28	5.14

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

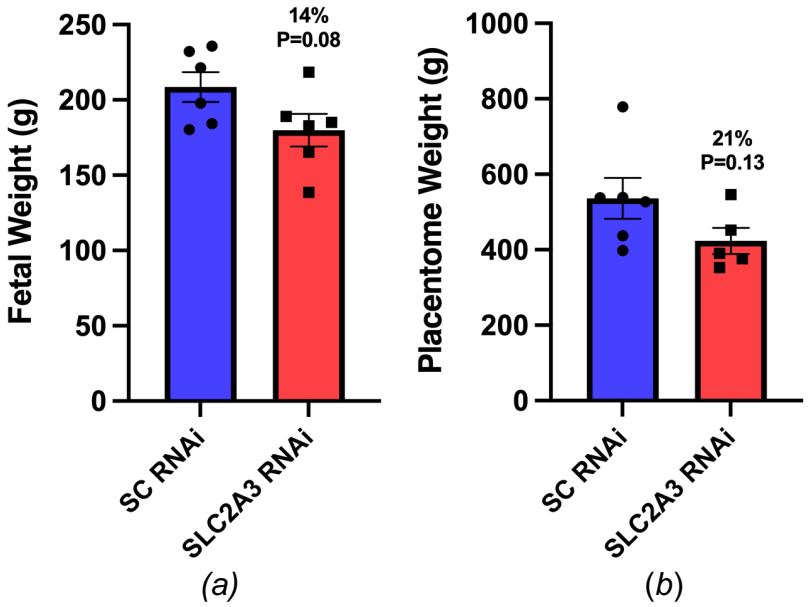

Figure 4. Measures of fetal and placentome mass at 75 dGA: (**a**) fetal weight in g, (**b**) placentome weight in g. Data are shown as means ± SEM for all pregnancies in each treatment group.

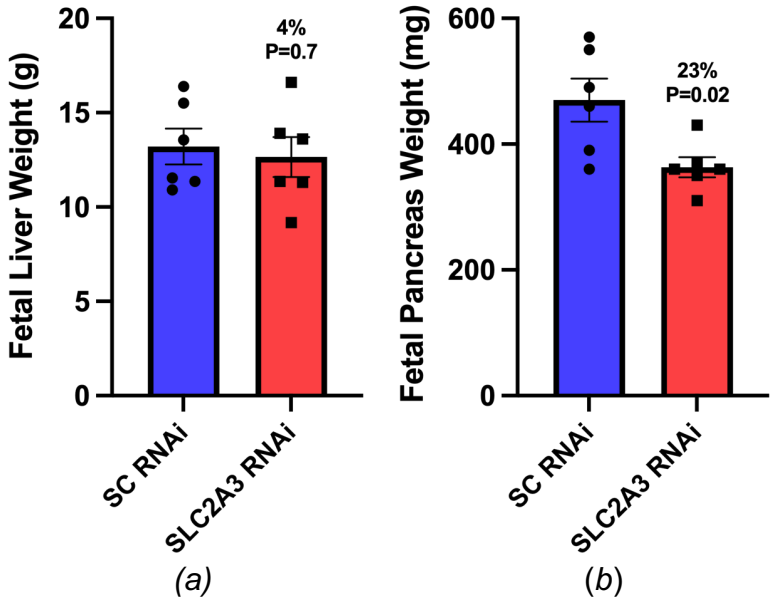

Figure 5. Measures of fetal liver and fetal pancreas mass at 75 dGA: (**a**) fetal liver weight in g, (**b**) fetal pancreas weight in mg. Data are shown as means ± SEM for all pregnancies in each treatment group.

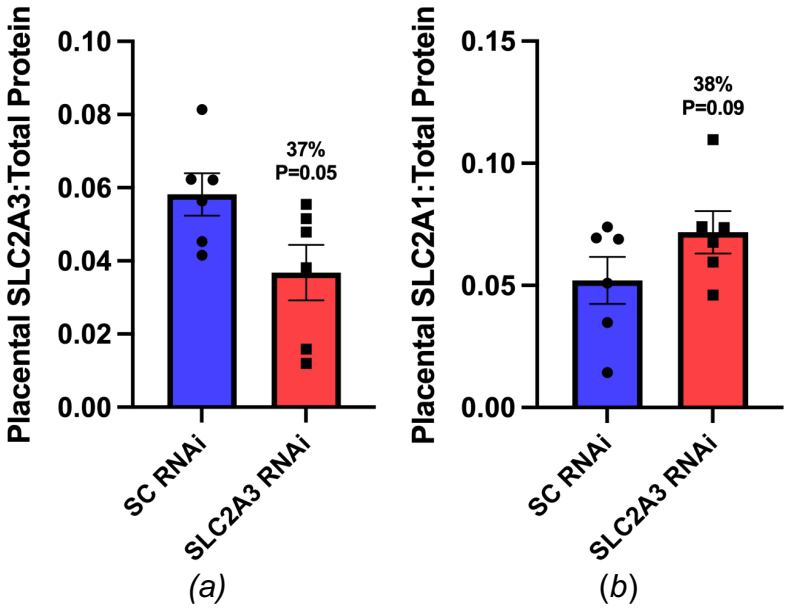

Figure 6. Placental SLC2A3 and SLC2A1 protein concentrations as measured by Western blot at 75 dGA: (**a**) placental SLC2A3 protein concentration, (**b**) placental SLC2A1 protein concentration. Data are shown as means ± SEM for all pregnancies in each treatment group.

Day 75 plasma measurements

Day 75 uterine artery and vein concentrations of glucose (Figure 7) and insulin (Figure 8) were not impacted by SLC2A3 RNAi treatment ($p \geq 0.10$). In contrast, umbilical vein and artery glucose concentrations were significantly reduced in the SLC2A3 RNAi pregnancies ($p \leq 0.05$; Figure 7) by 42% and 46%, respectively. No differences were observed in lactate concentrations in either uterine or umbilical circulations ($p \geq 0.10$; Figure 9). Additionally, other than a few exceptions, in general, there did not appear to be an overall impact on uterine (Table 5) or umbilical (Table 6) artery and vein concentrations of amino acids. Umbilical artery insulin concentrations tended to be reduced ($p \leq 0.11$; Figure 8) by 44% in SLC2A3 RNAi pregnancies; however, there was no difference in fetal liver IR-β concentration between treatments.

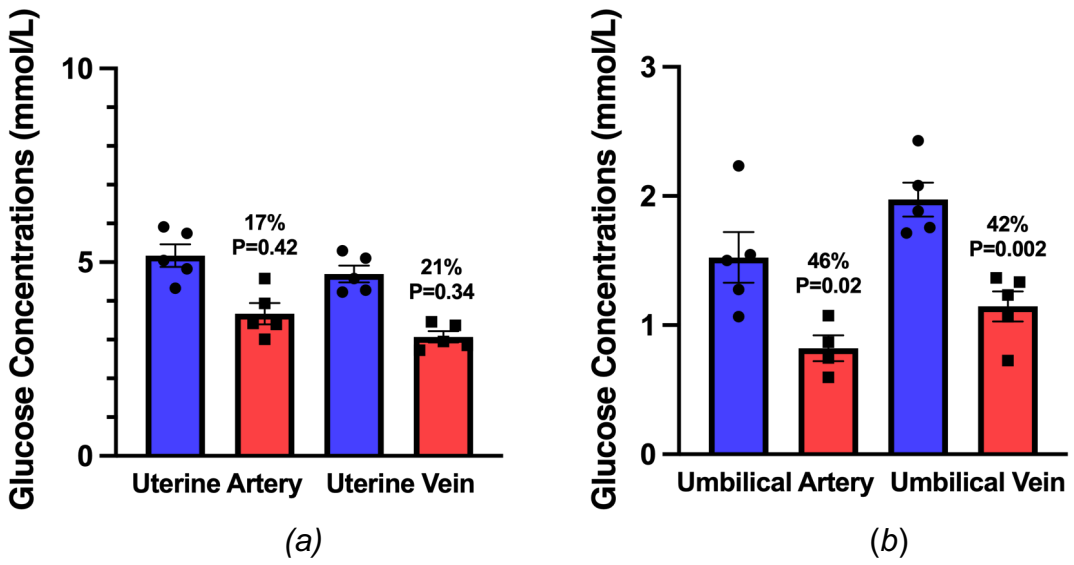

Figure 7. Uterine and umbilical plasma glucose concentrations at 75 dGA: (**a**) uterine artery and vein plasma glucose concentrations in mmol/L, (**b**) umbilical artery and vein plasma glucose concentrations in mmol/L. Data are shown as means ± SEM for all pregnancies in each treatment group.

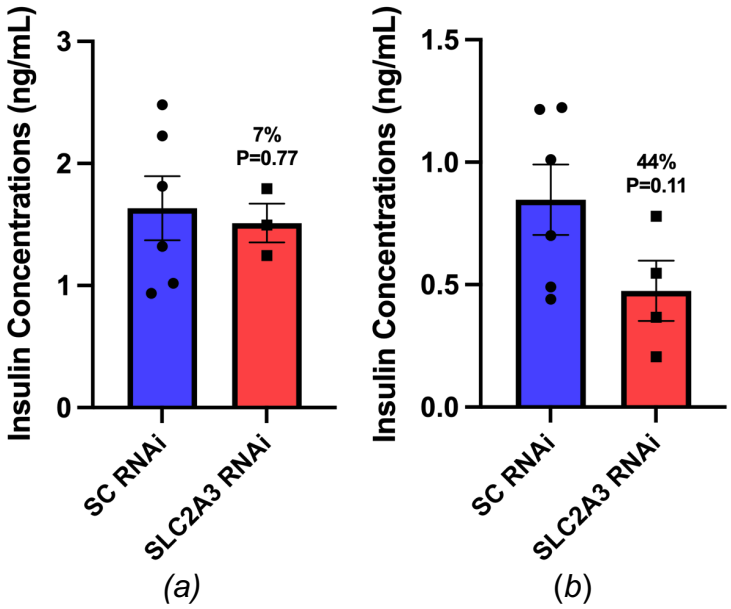

Figure 8. Uterine and umbilical artery plasma insulin concentrations at 75 dGA: (**a**) uterine artery plasma insulin concentrations in ng/ml, (**b**) umbilical artery plasma insulin concentrations in ng/ml. Data are shown as means ± SEM for all pregnancies in each treatment group.

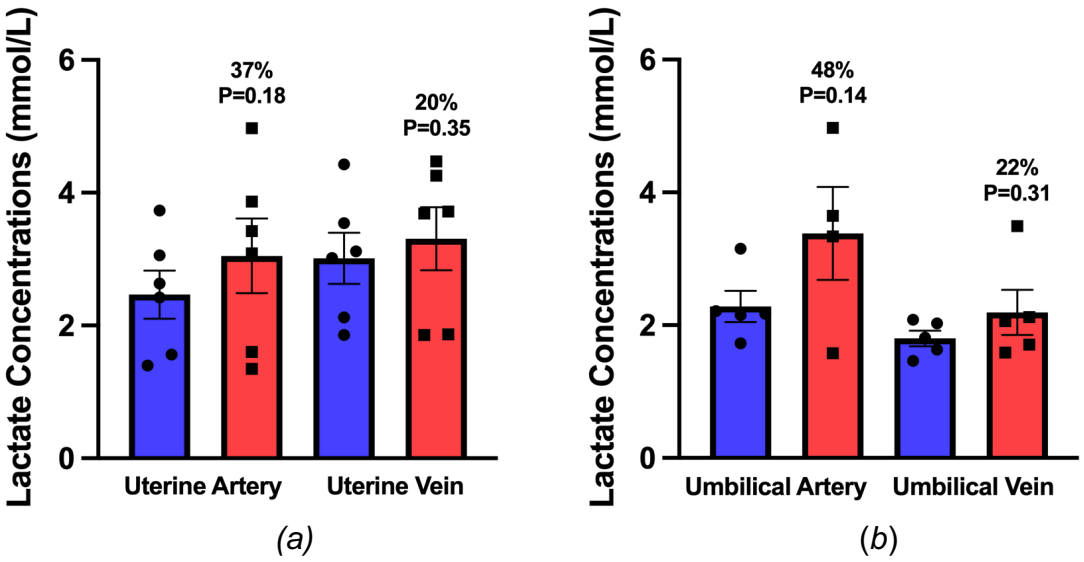

Figure 9. Uterine and umbilical plasma lactate concentrations at 75 dGA: (**a**) uterine artery and vein plasma lactate concentrations in mmol/L, (**b**) umbilical artery and vein plasma lactate concentrations in mmol/L. Data are shown as means ± SEM for all pregnancies in each treatment group.

Table 5. Maternal plasma amino acid concentrations (75 dGA).

	SC UtA	472 UtA	P-Value	% change	SC UtV	472 Utv	P-Value	% change
TAU	43.50 ± 8.70	49.55 ± 8.83	0.64	13.91	78.85 ± 17.57	74.16 ± 17.13	0.98	0.92
ASP	18.53 ± 2.66	17.65 ± 2.58	0.82	4.78	27.42 ± 3.50	34.10 ± 10.13	0.55	24.38
THR	108.92 ± 15.67	147.58 ± 17.81	0.13	35.50	153.15 ± 45.77	151.47 ± 19.38	0.97	1.10
SER	63.61 ± 6.28	75.04 ± 7.41	0.27	17.97	109.98 ± 50.80	71.67 ± 8.15	0.47	34.84
ASN	26.70 ± 3.15	42.09 ± 3.93	0.01	57.67	32.27 ± 5.42	42.23 ± 5.13	0.21	30.85
GLU	105.34 ± 11.72	106.04 ± 6.40	0.96	0.66	158.64 ± 12.88	172.02 ± 34.99	0.73	8.43
GLN	309.41 ± 17.70	317.86 ± 25.19	0.79	2.73	308.16 ± 30.39	328.82 ± 28.26	0.63	6.70
PRO	78.25 ± 9.15	81.99 ± 5.96	0.74	4.78	84.70 ± 6.03	89.16 ± 6.52	0.63	5.27
GLY	628.54 ± 53.55	578.48 ± 32.09	0.44	7.96	630.56 ± 63.84	687.23 ± 66.76	0.55	8.99
ALA	198.69 ± 14.19	219.90 ± 10.41	0.26	10.68	219.66 ± 19.76	245.43 ± 13.65	0.31	11.73
CIT	188.30 ± 23.08	256.7 ± 34.01	0.13	36.32	171.15 ± 19.28	253.27 ± 35.50	0.07	47.98
VAL	197.76 ± 13.18	231.82 ± 13.41	0.10	17.22	201.25 ± 24.21	219.92 ± 12.70	0.51	9.28
CYS	21.92 ± 2.88	17.99 ± 3.70	0.42	17.93	11.94 ± 4.15	21.28 ± 4.84	0.17	78.20
MET	22.15 ± 1.96	27.58 ± 2.98	0.16	24.51	33.38 ± 10.20	28.49 ± 3.39	0.66	14.65
ILEU	102.26 ± 3.79	115.23 ± 6.93	0.13	12.69	94.24 ± 4.77	104.06 ± 3.18	0.12	10.42
LEU	120.45 ± 4.05	134.11 ± 9.62	0.22	11.33	114.62 ± 9.81	125.2 ± 6.66	0.39	9.23
TYR	39.20 ± 3.28	49.47 ± 5.61	0.14	26.22	52.23 ± 12.11	54.75 ± 7.68	0.86	4.82
PHE	34.06 ± 1.89	38.38 ± 3.34	0.29	12.66	44.80 ± 10.34	42.56 ± 4.15	0.84	4.99
TRP	34.27 ± 1.78	36.78 ± 2.54	0.44	7.30	35.72 ± 4.65	39.95 ± 1.99	0.96	0.65
ORNI	94.49 ± 6.14	115.83 ± 8.20	0.06	22.58	97.07 ± 17.58	105.10 ± 8.02	0.69	8.27
LYS	103.41 ± 6.97	144.09 ± 17.23	0.05	39.33	117.46 ± 15.91	148.69 ± 19.41	0.24	26.59
HIS	59.89 ± 1.70	61.65 ± 3.16	0.63	2.94	57.60 ± 2.77	62.99 ± 3.74	0.27	9.38
ARG	210.83± 9.11	233.19 ± 20.86	0.35	10.61	219.04 ± 11.37	231.31 ± 23.04	0.64	5.60

Data are shown as mean values ± SEM for all ewes in each treatment group. Tau, taurine; Asp, aspartic acid; Thr, threonine; Ser, serine, Asn, asparagine; Glu, glutamic acid; Gln, glutamine; Pro, proline; Gly, glycine; Ala, alanine; Cit, citrulline; Val, valine; Cys, cystine; Met, methionine; Ileu, isoleucine; Leu, leucine; Tyr, tyrosine; Phe, phenylalanine; Trp, tryptophan; Orni, ornithine; Lys, lysine, His, histidine; Arg, arginine

Table 6. Fetal plasma amino acid concentrations (75 dGA).

	SC UmbA	472 UmbA	P-Value	% change	SC UmbV	472 UmbV	P-Value	% change
TAU	132.41 ± 16.08	141.54 ± 19.40	0.73	6.89	132.14 ± 13.98	144.70 ± 23.33	0.64	9.50
ASP	43.10 ± 4.27	41.54 ± 3.05	0.80	3.63	40.87 ± 3.80	32.67 ± 2.58	0.12	20.06
THR	453.57 ± 32.87	561.20 ± 66.67	0.15	23.73	497.44 ± 24.57	531.22 ± 57.58	0.58	6.79
SER	375.99 ± 25.84	420.26 ± 48.66	0.40	11.77	328.51 ± 15.49	338.54 ± 38.41	0.80	3.05
ASN	52.01 ± 2.62	66.88 ± 5.40	0.02	28.60	76.66 ± 2.62	86.04 ± 6.95	0.21	12.24
GLU	182.77 ± 23.49	181.57 ± 15.68	0.97	0.65	49.03 ± 3.88	40.59 ± 8.67	0.37	17.22
GLN	453.66 ± 36.48	495.05 ± 38.65	0.47	9.12	591.28 ± 28.78	589.07 ± 32.74	0.96	0.37
PRO	114.12 ± 14.32	136.71 ± 9.92	0.28	19.80	143.05 ± 12.36	163.25 ± 9.12	0.24	14.12
GLY	465.10 ± 46.10	458.02 ± 48.14	0.92	1.52	550.47 ± 30.18	477.85 ± 26.96	0.11	13.19
ALA	319.75 ± 28.75	349.43 ± 50.04	0.59	9.28	417.10 ± 18.71	384.69 ± 17.40	0.24	7.77
CIT	151.75 ± 16.05	184.56 ± 19.39	0.23	21.62	152.28 ± 16.16	168.38 ± 19.32	0.54	10.57
VAL	263.06 ± 19.26	313.77 ± 42.27	0.25	19.28	318.44 ± 26.91	332.76 ± 36.17	0.75	4.50
CYS	16.52 ± 1.99	19.53 ± 2.05	0.34	18.25	12.72 ± 2.86	12.39 ± 1.50	0.93	2.59
MET	79.89 ± 6.43	89.07 ± 11.02	0.46	11.48	98.43 ± 6.07	103.53 ± 4.21	0.53	5.18
ILEU	75.66 ± 4.25	91.57 ± 11.79	0.18	21.03	106.30 ± 5.92	107.65 ± 10.54	0.91	1.27
LEU	130.62 ± 7.42	150.52 ± 17.26	0.26	15.24	188.21 ± 9.92	181.61 ± 14.04	0.70	3.51
TYR	104.88 ± 3.21	116.85 ± 14.31	0.35	11.42	134.05 ± 9.35	136.81 ± 14.15	0.87	2.05
PHE	92.42 ± 2.39	108.34 ± 11.15	0.13	17.22	123.35 ± 5.79	127.38 ± 7.21	0.67	3.27
TRP	50.73 ± 1.83	45.51 ± 4.21	0.23	10.28	59.73 ± 2.75	55.44 ± 2.72	0.30	7.18
ORNI	160.01 ± 15.14	196.74 ± 43.89	0.38	22.95	165.92 ± 11.63	186.65 ± 39.12	0.59	12.49
LYS	202.84 ± 16.16	210.34 ± 19.88	0.78	3.70	260.43 ± 15.82	270.66 ± 30.56	0.76	3.93
HIS	50.08 ± 3.82	52.69 ± 4.12	0.66	5.20	68.09 ± 1.68	66.35 ± 7.37	0.81	2.55
ARG	251.54 ± 15.84	193.81 ± 9.20	0.03	22.95	313.01 ± 19.29	246.22 ± 15.08	0.03	21.34

Data are shown as mean values ± SEM for all ewes in each treatment group. Tau, taurine; Asp, aspartic acid; Thr, threonine; Ser, serine, Asn, asparagine; Glu, glutamic acid; Gln, glutamine; Pro, proline; Gly, glycine; Ala, alanine; Cit, citrulline; Val, valine; Cys, cystine; Met, methionine; Ileu, isoleucine; Leu, leucine; Tyr, tyrosine; Phe, phenylalanine; Trp, tryptophan; Orni, ornithine; Lys, lysine, His, histidine; Arg, arginine

While IGF-1 concentrations in umbilical circulation were not different between treatments ($p \geq 0.10$; Figure 10), uterine artery concentrations of IGF-1 were reduced ($p \leq 0.05$; Figure 10) by 26% in SLC2A3 RNAi pregnancies. Uterine vein concentrations of CSH were not impacted by treatment ($p \geq 0.10$; Figure 11); however, umbilical vein concentrations of CSH were significantly increased by 70% ($p \leq 0.05$; Figure 11) in SLC2A3 RNAi pregnancies.

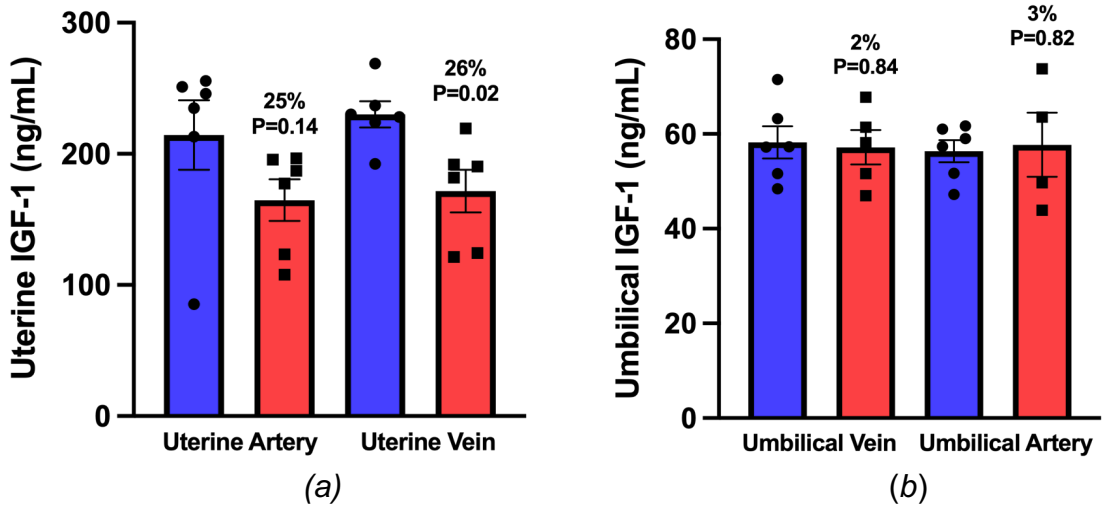

Figure 10. Uterine and umbilical plasma IGF-1 concentrations at 75 dGA: (**a**) uterine artery and vein plasma IGF-1 concentrations in ng/ml, (**b**) umbilical vein and artery plasma IGF-1 concentrations in ng/ml. Data are shown as means ± SEM for all pregnancies in each treatment group.

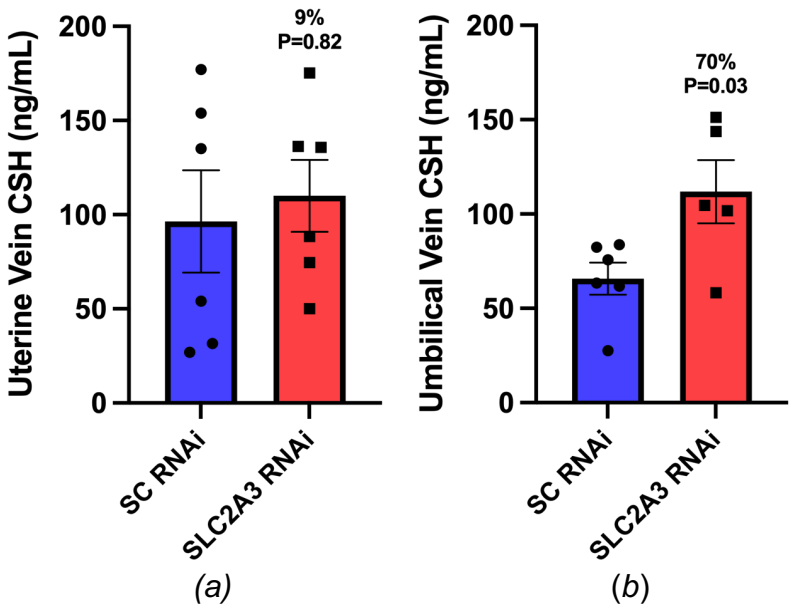

Figure 11. Uterine and umbilical plasma CSH concentrations at 75 dGA: (**a**) uterine vein plasma CSH concentrations in ng/ml, (**b**) umbilical vein plasma CSH concentrations in ng/ml. Data are shown as means ± SEM for all pregnancies in each treatment group.

IGF, IGFBP, and IGF receptor mRNA concentrations

Day 75 placental tissues were assessed for *IGF*, *IGFBP*, and *IGFR* (IGF receptor) mRNA concentration. While there were no differences in placental *IGF-1* mRNA concentration ($p \geq 0.10$; Table 7), placental *IGF-2* mRNA concentration was increased by 71% ($p \leq 0.05$; Figure 12) in SLC2A3 RNAi pregnancies. Additionally, in SLC2A3 RNAi pregnancies, placental *IGF-1R* and *IGF-2R* mRNA concentration were increased by 40% and 69% ($p \leq 0.05$; Figure 12), respectively. There were no differences in placental *IGFBP-1*, *IGFBP-2*, or *IGFBP-3* mRNA concentration between treatments ($p \geq 0.10$; Table 7).

Table 7. Placental insulin-like growth factor mRNA concentrations (75 dGA)

Gene	SC RNAi	SLC2A3 RNAi	P-Value	% change
IGF-1, pg/pg	0.0011 ± 0.0002	0.0011 ± 0.00013	0.88	3.18
IGFBP-1, pg/pg	0.00018 ± 0.000076	0.00013 ± 0.000066	0.64	26.81
IGFBP-2, pg/pg	0.00051 ± 0.000082	0.00057 ± 0.000057	0.57	11.58
IGFBP-3, pg/pg	0.025 ± 0.0056	0.028 ± 0.0049	0.69	12.19

Data are shown as mean values ± SEM for the starting quantity of the mRNA of interest (pg) divided by the starting quantity (pg) of the housekeeping mRNA.

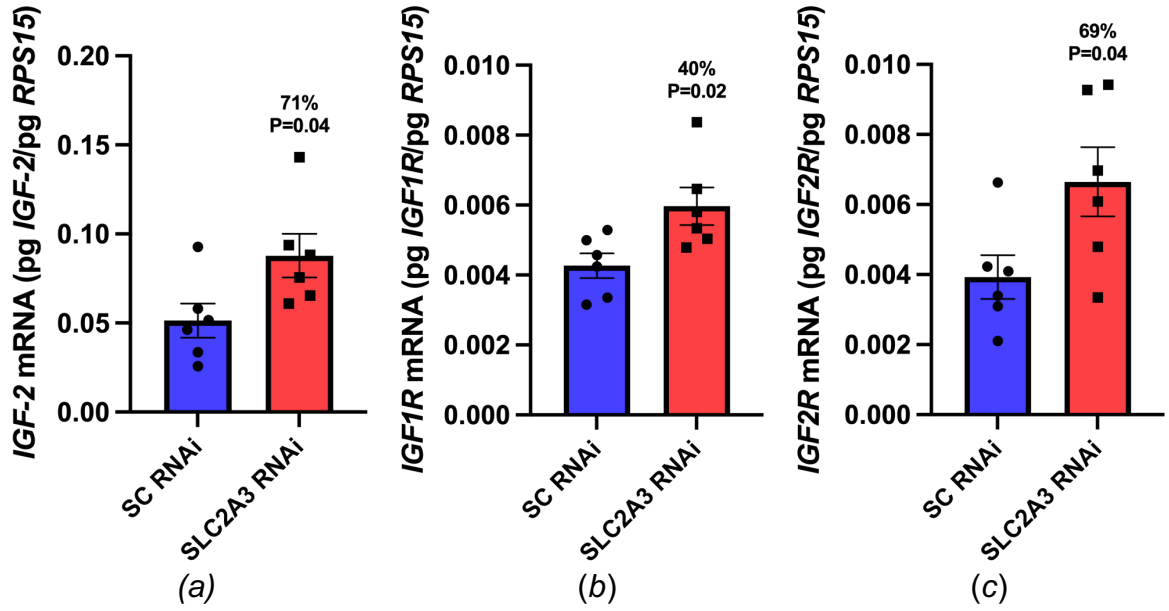

Figure 12. (**A**) Placental *IGF-2* mRNA concentrations at 75 dGA, (**B**) placental IGF-1R mRNA concentrations at 75 dGA, and (**C**) placental IGF-2R mRNA concentrations at 75 dGA. Data are shown as means ± SEM for the starting quantity of the mRNA of interest (pg) divided by the starting quantity (pg) of the housekeeping mRNA.

DISCUSSION

Glucose is the principal energy substrate for the placenta and fetus, and is essential for normal fetal metabolism and growth (Hay, 2006). At mid-gestation, the majority of glucose transfer from the placenta to the fetus is due to the maternal-fetal glucose concentration gradient (Hay, 2006), in which placental glucose uptake and transfer is mediated by two facilitative glucose transporters, SLC2A1 and SLC2A3. In most mammalian placentae, due to SLC2A1 location and overall abundance, SLC2A1 has been considered the primary placental glucose transporter (Jansson et al., 1993; Hauguel-De Mouzon et al., 1994; Hahn & Desoye, 1996). However, kinetically, SLC2A3 would be more efficient than SLC2A1 at maintaining glucose transport at low maternal blood glucose concentrations due to its five-fold greater affinity and transport capacity for glucose (Simpson et al., 2008; Hay, 2006). A common characteristic of IUGR pregnancies is relative fetal hypoglycemia (Marconi & Paolini, 2008). Additionally, the IUGR placenta is often decreased in size, which may then decrease the number of glucose transporters as a result of decreased placental membrane surface area. However, *in vivo* studies in humans and sheep would suggest that the placental glucose transporters operate well below saturation, thus inferring placental glucose transport capacity is much greater than actual transport rates, and any small change in placental glucose transporter expression may have little or no effect on the rate of placental glucose transport (Hay et al., 1990; Hay & Meznarich, 1989; Stacey et al., 1978). However, there is a lack of experimental evidence that assess the relative role of SLC2A1 and SLC2A3 in transplacental glucose transport. Subsequently, the sheep placenta, due to the distinct location of SLC2A1 and SLC2A3, provides an opportunity

to assess the relative importance of apical microvilli transport versus basolateral transport of glucose, as well as the relative role of each placental glucose transporter on transplacental glucose transfer, placental development and function, and fetal growth. The distribution of SLC2A3 has important implications for glucose flux across the sheep placenta, as SLC2A3 is found primarily on the maternal-facing apical trophoblast membrane (Wooding et al., 2005), where it might act to specifically take up glucose into the trophoblast cells. Thus, any deficiency in SLC2A3 could result in impaired placental development, and consequently, fetal growth.

SLC2A3 RNAi resulted in a significant 37% reduction in placental SLC2A3 protein concentration at 75 dGA. Subsequently, while there were no changes in uterine artery or uterine vein glucose concentrations, umbilical vein and umbilical artery glucose concentrations were significantly decreased (42% and 46%, respectively). As SLC2A3 is the only reported glucose transporter isoform on the apical trophoblast membrane in the sheep placenta, this data would suggest that placental uptake and transfer of glucose is impaired when SLC2A3 protein is diminished, thus giving rise to fetal hypoglycemia. Additionally, a reduction in the uptake of glucose could also impair placental development and function, as the placenta utilizes a considerable amount of glucose for its own metabolic processes (Hay, 1995). At mid-gestation, the placenta consumes approximately 83% of the glucose transferred to the utero-placental unit, while in late gestation, the placenta consumes approximately 72% of the glucose transferred to the utero-placental unit (Bell et al., 1986; Meschia et al., 1980). Thus, with the relative amount of glucose the placenta utilizes for its own metabolic needs, any deficit in placental glucose uptake could impact placental development and function. As

SLC2A3 is the sole facilitative glucose transporter on the apical membrane of the sheep placenta, any deficiencies in SLC2A3 would impact glucose uptake, thus potentially altering placental development and consequently setting the stage during early gestation for the development of IUGR.

Conversely, it has been argued that the glucose transport capacity of the placenta is greater on the fetal than the maternal trophoblast membrane (Hay et al., 1990). Thus suggesting, as SLC2A1 has been localized to the basal membrane of the trophoblast cell in the sheep placenta, SLC2A1 may be the more important of the two placental glucose transporters (Ehrhardt & Bell, 1997; Wooding et al., 2005). However, in the SLC2A3-deficient placenta, placental SLC2A1 had a statistical tendency to be increased (38%), but was unable to prevent fetal hypoglycemia. Collectively, these data suggest, at least in the sheep placenta, that SLC2A3 is the more important glucose transporter in the sheep placenta at mid-gestation. Additionally, it has been demonstrated, that in response to fetal hypoglycemia, there is an increase in fetal amino acid oxidation to help maintain fetal oxidative metabolism (Limesand et al., 2009). However, when amino acid concentrations in uterine and umbilical artery and vein samples were assessed, despite a few exceptions, there did not appear to be an overall impact on uterine or umbilical concentrations of amino acid in the SLC2A3-deficient pregnancies. While it is not apparent how these few differences arouse, and since amino acid uptake cannot be assessed due to not knowing relative blood flow, it appears that SLC2A3 RNAi, at mid-gestation, solely results in a deficiency in glucose transport with no perturbations to amino acid transport.

Insulin secretion has been repeatedly demonstrated to be decreased in IUGR pregnancies (Hay, 2006; Wallace et al., 2006; Limesand et al., 2005; Nieto-Diaz et al., 1996). As insulin is a regulator of fetal growth, it has been suggested that the fetal β-cell function coordinates anabolic hormone concentrations to fetal nutrient supply (Thorn et al., 2011; Rozance et al., 2006; Hay et al., 2016). However, in the IUGR fetus, where nutrient supply is often decreased, insulin secretion is subsequently decreased as an adaptive response to limit fetal growth in response to the reduced nutrient supply (Thorn et al., 2011). Although, while reducing insulin secretion allows glucose supply to continue to support vital organs, primarily the brain and the heart, it consequently sacrifices the growth of other fetal organs and protein synthesis in fetal skeletal muscle (Thorn et al., 2011). Fetal measurements taken at mid-gestation would suggest that the SLC2A3-deficient fetus, potentially in response to hypoglycemia and hypoinsulinemia, had reduced fetal growth as evident by the reductions in head circumference, femur length, and tibia length. Additionally, as characteristically seen in IUGR pregnancies, SLC2A3 RNAi resulted in a statistical tendency for fetal weight to be reduced, and while not statistically significant, placentome weight was also reduced.

When assessing the fetal liver in PI-IUGR fetuses, decreased fetal liver growth is often reported, while the insulin receptor is upregulated as a compensatory response to low levels of insulin (Thorn et al., 2009; Thorn et al., 2011; Gentilli et al., 2009). Additionally, when fetal liver growth is decreased in either sheep or human IUGR pregnancies, a decrease in IGF-1 concentrations is often observed (Iniguez et al., 2006; Leger et al., 1996; Lassarre et al., 1991; Harper et al., 1987; Economides et al., 1991; Macko et al., 2013; Hay et al., 2016). In normal pregnancies, as measured in fetal

sheep, two-thirds of the umbilical blood flow supplies the fetal liver, while the remaining one-third passes through the ductus venosus (Tchirikov et al., 2006). However, in growth-restricted human fetuses, umbilical blood supply to the fetal liver is reduced, while ductus venosus shunting is increased (Tchirikov et al., 2006). Thus, it has been suggested that the decrease in fetal liver growth in IUGR pregnancies may be due to a compensatory mechanism where the ductus venosus dilates, thus increasing the proportion of umbilical blood that bypasses the fetal liver and allows the preferential supply of oxygen and nutrient rich blood to key organs, such as the fetal heart and brain (Hay et al., 2016; Tchirikov et al., 2006). However, in response to SLC2A3 RNAi, fetal liver weight, insulin receptor concentration, and circulating IGF-1 concentrations were unaffected. These lack of changes may be due in part to the significant increase (70%) in circulating CSH concentrations observed in the SLC2A3-deficient pregnancies. A similar increase in CSH concentrations was reported in late gestation pregnant ewes that were fasted for 72 h, producing maternal and fetal hypoglycemia and hypoinsulinemia, in which Freemark et al. (1992) reported a 50% increase in the concentration of CSH in fetal plasma. When fasting was reversed, either by refeeding the ewes or by intravenous administration of dextrose at a rate sufficient to maintain maternal euglycemia during fasting, the increased fetal plasma glucose and insulin concentrations appeared to prevent the rise in fetal plasma CSH concentrations that occurred during fasting (Freemark et al., 1992). Additionally, the increase in circulating CSH concentrations may have preserved fetal liver weight and function. In response to CSH-deficient sheep pregnancies, fetal liver weight is decreased in early (Jeckel et al., 2018) and late gestation sheep pregnancies, as well as significant decreases in

circulating IGF-1 concentrations (Baker et al., 2016; Tanner et al., 2021b). Moreover, it has been suggested that CSH may stimulate the production of IGF-1. While short-term fetal infusion (24 hr) studies of CSH into late gestation lambs show no effect on IGF-1 concentration (Oliver et al., 1995), chronic fetal infusion of CSH (2 weeks) into late gestation lambs significantly increased circulating IGF-1 concentrations in fetal plasma, as well as increased hepatic glycogen deposition (Schoknecht et al., 1996). Altogether, these data suggest that the increased circulating CSH concentrations, in the hypoglycemic, hypoinsulinemic SLC2A3-deficient pregnancies, preserved fetal liver weight and function.

In the SLC2A3-deficient pregnancies, *IGF-2*, *IGF1R*, and *IGF2R* mRNA concentrations were significantly increased. Targeted mutagenesis studies done in mice, specifically for the placental gene encoding IGF-2, have demonstrated that overexpression of *Igf-2* results in placental overgrowth (Constancia et al., 2002; Ludwig et al., 1996; Louvi et al., 1997), whereas total ablation of the *Igf-2* gene results in placental growth restriction (Coan et al., 2008; Sferruzzi-Perri et al., 2017; DeChiara et al., 1991). This data suggests that placental IGF-2 stimulates placental growth, which would primarily be mediated through IGF1R, as IGF2R has been demonstrated to be a clearance receptor for IGF-2 (Reynolds et al., 1997; Baker et al., 1993; Eggenschwiler et al., 1997; Denley et al., 2005; Okamoto et al., 1990; Ludwig et al., 1996). When *Igf2r* is knocked-out in mice, fetal and placental overgrowth ensues due to elevated circulating levels of IGF-2 overstimulating placental IGF1R (Zhou & Bondy, 1992; Lau et al., 1994; Ludwig et al., 1996). In addition, in sheep IUGR pregnancies, induced either by hyperthermia or the removal or uterine caruncles, demonstrated a decrease in

placental mass and increased placental *IGF-2* mRNA concentration (Zhang et al., 2016; de Vrijer et al., 2006). Moreover, in a cohort of ewes in moderate condition and the smallest placentas, thus supporting more mass of fetus per gram of placenta, had the greatest expression of *IGF-2* as compared to the controls (Osgerby et al., 2003). Thus, it appears the increase in *IGF-2* and *IGF1R* mRNA concentrations may be a compensatory mechanism to stimulate placental growth to increase the total nutrient exchange surface area to combat the SLC2A3 deficiency, while *IGF2R* mRNA concentrations were increased to prevent the overstimulation of IGF1R and the potential overgrowth of the placenta by IGF-2.

In the SLC2A3-deficient pregnancies, there was an unexpected decrease in circulating maternal IGF-1 concentrations. More recently, IGFs in the maternal circulation have been demonstrated to regulate fetal growth via their actions on the mother and the placenta. Maternal IGF-1 appears to alter maternal tissue growth and metabolism to modulate nutrient availability for conceptus growth, while maternal IGF-2 appears to act on the placenta to influence substrate transport to the fetus (Sferruzzi-Perri et al., 2011). In pregnant rats, it has been reported that maternal IGF-1 decreases during the second half of pregnancy as compared to nonpregnant control rats (Donovan et al., 1991). Palmer et al. (1996) hypothesized that the decrease in maternal IGF-1 concentration was associated with the repartitioning of maternal nutrients, specifically protein, to the growing fetus. However, in sheep, plasma IGF-1 is unaltered over the course of pregnancy (Sferruzzi-Perri et al., 2011; Wallace et al., 1997; de Boo et al., 2008). Contradictory to the rat, when maternal IGF-1 is increased exogenously in early and late sheep pregnancies, increased maternal nutrient concentrations have been

reported, along with occasional increases in fetal weight (de Boo et al., 2008; Wallace et al., 2006; Stelwagen et al., 1994; Jenkinso et al., 1999; Wallace et al., 2004; Costine et al., 2005; Koch et al., 2010). However, it should be noted that the extent of IGF-1 actions on the mother is dependent on whether it is a singleton or twin pregnancy, gestational age, and maternal age and nutrition status (Costine et al., 2005; Wallace et al., 2006; Kenyon et al., 2007; Wright et al., 2008; Koch et al., 2010). Thus, there appears to be no prior evidence to support why maternal IGF-1 concentrations were reduced in SLC2A3-deficient pregnancies. However, this finding suggests, that in response to SLC2A3 RNAi, the placenta is secreting, or failing to secrete, some hormone or protein that, in turn, modulates maternal IGF-1 concentrations.

SUMMARY

Although a large majority of IUGR fetuses are hypoglycemic, assessment of near-term or term IUGR placentas would infer there is not a deficit in placental glucose transport as there is not a deficiency in the concentration of the primary placental facilitative glucose transporters. However, the abundance of the placental glucose transporters in placentas harvested at or near term may not reflect the glucose transport capacity of the IUGR placenta throughout gestation. Instead, any deficit in placental glucose transport during the first-half of pregnancy could impact placental development and function, thus potentially resulting in functional placental insufficiency and setting the stage for fetal hypoglycemia and IUGR. Thus, it was our objective to use lentiviral mediated RNAi to attenuate the expression of facilitative glucose transporter SLC2A3, only in the placenta, and assess its relative importance in placental glucose transport. For obvious ethical reasons, this cannot be assessed in humans, and thus requires the

use of a relevant animal model. As such, the sheep has been an invaluable model towards our current understanding of placental and fetal physiology and, due to the distinct location of SLC2A1 and SLC2A3 in the sheep placenta, provides a unique opportunity to assess the relative importance of apical (SLC2A3) transport versus basolateral (SLC2A1) membrane transport on placental development and function and fetal growth. As SLC2A3 is located on the apical trophoblast membrane of the sheep placenta, and its five-times greater affinity and transport capacity for glucose, we hypothesized that SLC2A3 deficiency would result in impaired placental development and significant IUGR by mid-gestation (75 dGA).

SLC2A3 RNAi resulted in a 37% reduction ($p \leq 0.05$) in placental SLC2A3 concentration. Subsequently, while there were no significant reductions in maternal glucose or insulin concentrations, the SLC2A3 RNAi pregnancies had decreased umbilical vein ($p \leq 0.05$) and umbilical artery ($p \leq 0.05$) glucose concentrations, as well as reduced umbilical artery insulin ($p \leq 0.10$). SLC2A3 RNAi also resulted in decreased fetal growth as evident by reduced fetal weight ($p \leq 0.10$), head circumference ($p \leq 0.05$), femur length ($p \leq 0.05$), and tibia length ($p \leq 0.05$). Additionally, in an apparent attempt at compensation for SLC2A3-deficiency, SLC2A1 concentrations were increased by 38% ($p \leq 0.10$), umbilical vein CSH concentrations were increased by 70% ($p \leq 0.05$), and placental *IGF-2* ($p \leq 0.05$), *IGF1R* ($p \leq 0.05$), and *IGF2R* ($p \leq 0.05$) mRNA were increased; however, they were unable to prevent fetal hypoglycemia and the impacts on fetal development. Ehrhardt & Bell (1997) reported that as pregnancy progresses in sheep, from mid- to late-gestation, SLC2A3 mRNA and protein levels increase, and thus suggested that SLC2A3 is more important as pregnancy advances.

However, the substantial differences we observed in fetal glucose concentrations, fetal insulin concentrations, and fetal measurements in this cohort of SLC2A3-deficient pregnancies suggests that SLC2A3 plays an important role for normal fetal development during the first-half of gestation. Additionally, due to its location on the apical trophoblast membrane, coupled with our results, may suggest that SLC2A3 is the rate-limiting glucose transporter during the first-half of gestation.

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