DISSERTATION

INVESTIGATING THE RAMIFICATIONS OF PLACENTAL SLC2A3 (GLUT3) GLUCOSE TRANSPORT DEFICIENCY IN SHEEP

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ABSTRACT

INVESTIGATING THE RAMIFICATIONS OF PLACENTAL SLC2A3 (GLUT3) GLUCOSE TRANSPORT DEFICIENCY IN SHEEP

Glucose, the primary energy substrate for fetal oxidative processes and growth, is transferred from maternal to fetal circulation down a concentration gradient by placental facilitative glucose transporters. In sheep, SLC2A1 and SLC2A3 are the primary transporters available in the placental epithelium, with SLC2A3 located on the maternal-facing apical trophoblast membrane and SLC2A1 located on the fetal-facing basolateral trophoblast membrane. We have previously reported that impaired placental SLC2A3 glucose transport resulted in smaller, hypoglycemic fetuses with reduced umbilical artery insulin and glucagon concentrations, in addition to diminished pancreas weights.

These findings led us to subject RNA derived from *SLC2A3*-RNAi (RNA interference) and NTS-RNAi (non-targeting sequence) fetal pancreases to qPCR followed by transcriptomic analysis. We identified a total of 771 differentially expressed genes (DEGs). Upregulated pathways were associated with fat digestion and absorption, particularly fatty acid transport, lipid metabolism, and cholesterol biosynthesis, suggesting a potential switch in energetic substrates due to hypoglycemia. Pathways related to molecular transport and cell signaling in addition to pathways influencing growth and metabolism of the developing pancreas were also impacted. A few genes directly related to gluconeogenesis were also differentially expressed. Our results suggest that fetal hypoglycemia during the first half of gestation impacts fetal pancreas development and function that is not limited to β cell activity.

Our results at mid-gestation led us to hypothesize that the placenta could compensate for a deficiency in SLC2A3 during the second half of gestation to maintain or recover fetal growth and development. To investigate this, we repeated the same experimental paradigm by carrying out *SLC2A3*-RNAi pregnancies and NTS-RNAi controls to near-term. Our objective was to assess fetal growth, uterine nutrient uptake, placental utilization and transfer to the fetus, as well as assess the responsiveness of the fetal pancreas to glucose and arginine challenges *in vivo* to determine the long-term impact of fetal hypoglycemia during the first-half of gestation. Pregnant ewes underwent surgical catheterization followed by a metabolic study at 133 ± 2 dGA. We observed rescued fetal and pancreatic growth. There was also significantly reduced uterine glucose uptake and placental glucose utilization, along with a tendency for increased uteroplacental amino acid carbon utilization. At baseline, ewes with *SLC2A3*-RNAi pregnancies had significantly reduced uterine arterial IGF1 concentrations, but no differences in glucagon or insulin concentrations. During the metabolic study, umbilical artery insulin concentrations were significantly greater in the *SLC2A3*-RNAi pregnancies during early GSIS, tended to be greater during late GSIS, and were significantly greater again during ASIS. These data demonstrate that the global effect on pancreatic growth and development observed at 75dGA continued into late gestation as altered pancreatic glucose and arginine sensitivity, despite rescued fetal growth. Placental compensatory mechanisms appeared to also rescue fetal growth and umbilical glucose concentrations. The decrease in uteroplacental glucose utilization while increasing amino acid utilization appears to be one major compensatory mechanism aiding in recovering glucose transfer to the fetus. In conclusion, microvillous glucose uptake to the placenta appears to be rate-limiting to fetal growth and development early in gestation, but when *SLC2A3*-RNAi is carried out to near-term, despite rescued fetal growth, the physiology of the entire maternal-placental-fetal unit is still impacted.

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INTRODUCTION

The placenta is an extraordinary transient organ that facilitates fetal development via crosstalk between the maternal and fetal systems, hormone production, exchange of gases and nutrients, and removal of waste, in addition to serving as the immunological barrier between maternal and fetal circulations (Gaccioli & Lager, 2016; Rampersad et al., 2011). Successful fetal growth therefore requires unhindered nutrient availability, which is consequently dependent on maternal diet, uteroplacental blood supply, placental villous development, and the ability of villous trophoblast together with fetoplacental circulation to transport those nutrients (Burton, 2022). The placenta must also accommodate the increasing fetal oxidative demands as gestation progresses, resulting in many adaptations over time, all accomplished while also satisfying its own metabolic and oxidative demands. This also involves rapid expansion in the second and third trimesters, achieved by elevated transfer capacity in addition to increased uptake and metabolism of nutrients (Illsley, 2011; Wright & Sibley, 2011). By virtue of its invaluable roles to the fetus, along with the many demands of its own growth and functionality, the placenta has a considerable influence on the successful development of healthy offspring.

The early development environment has also been shown to have long-lasting effects on fetal growth and post-natal life, with all three compartments (maternal, placental, fetal) having a major influence. This concept is known as fetal programming, or developmental origins of health and disease (DOHaD) and was first brought to light in the 1980s by David Barker and colleagues while investigating associations between geographical distributions of heart disease in the United Kingdom and low birth weight (Barker, 1990; Barker et al., 1989). The relationship between early undernutrition, low birth weight, and diseases such as obesity, diabetes, and hypertension in adulthood offered "…the first clue that the disease originates during intra-uterine development" (Barker, 2007). Since then, DOHaD has evolved into a large field of study within the realm of reproductive physiology, but summarizing that abundant body of literature is not within the scope of this review.

However, one serious complication of human pregnancy that contributes to adult-onset disease due to fetal programming is intrauterine growth restriction (IUGR). IUGR is defined as the failure of a fetus to reach its genetic growth potential, or more clinically, a fetal weight below the 10th percentile for gestational age (Belkacemi et al., 2011; D'Agostin et al., 2023; Gaccioli & Lager, 2016). Another generally agreed-upon clinical diagnosis is when abnormal Doppler waveforms are observed in the umbilical artery or decreased pulsatility is measured in the middle cerebral artery, suggesting abnormalities of fetal circulation and therefore IUGR (Crocker, 2011; Gaccioli & Lager, 2016; Suhag & Berghella, 2013). An accurate measure of worldwide occurrences is still difficult due to lack of international consensus for the definition and diagnosis of IUGR (Gaccioli and Lager, 2016; D'Agnostin et al., 2023). A recent study reported that IUGR affects 10-15% of pregnancies worldwide (Armengaud et al., 2021), while others have reported up to 24% of newborns (Dapkekar et al., 2023). The prevalence varies by country's geographical location and relative affluence, with 5-15% of pregnancies reported in the United States and Europe, and a wider variation in poorer countries (ranging between 10-20% in Latin America to 30-55%

in South Central Asia; Gaccioli and Lager, 2016). IUGR is associated with an increased risk of stillbirth and is second only to prematurity in causing perinatal morbidity and mortality (Gaccioli and Lager, 2016; Dapkekar et al, 2023).

The etiologies of IUGR are idiopathic and numerous but are often categorized by compartment of origin: maternal, placental, or fetal. Briefly, these can include (but are not limited to) extremes in maternal age, undernutrition, maternal disease, smoking, pre-eclampsia, placental infections, impaired uteroplacental blood flow, fetal genetic disease, infection, and multifetation (Suhag and Berghella, 2013; Gaccioli and Lager, 2016). Thanks to much of the research developed by the DOHaD field, we know that IUGR can have long-term consequences on health in adulthood, with up to 63% of adult diabetes, hypertension and heart disease connected to low-birth-weight conditions (Belkacemi et al., 2011). IUGR has also been associated with impaired neuro-developmental progress (Gaccioli & Lager, 2016; Gagnon, 2003).

While the primary cause of IUGR can vary, the most common cause of fetal growth restriction is decreased oxygen and nutrient supply resulting in fetal hypoxia, often due to placental insufficiency (PI). Placental insufficiency progressively impairs placental transport capacity, directly affecting the availability of nutrients to the fetus and therefore restricting the growth of the fetus (Gagnon, 2003). In some cases, continued impaired nutrient transport and hypoxia results in a redistribution of energetic substrates to essential organs, such as the brain and heart, at a detriment to the rest of the body (often referred to as “brain sparing” growth; D’Agostin et al, 2023).

Glucose is one of those critical energetic substrates transported from the maternal circulation to fetal circulation by the placenta. Net transfer of glucose to the fetus is dependent on uteroplacental and fetal-placental blood flow, as well as the availability of glucose transporters (Barry et al., 2008; Widdas, 1952; F. B. P. Wooding et al., 2005). Unsurprisingly, hypoglycemia is therefore the most common nutrient deficiency during IUGR or placental insufficiency (Gagnon, 2003; Barry et al, 2008).

The experiments described in this dissertation focus on the impacts of hypoglycemia due to impaired glucose transport across the microvillous membrane, resulting in a phenotype of fetal IUGR at mid-gestation that appears to have salvaged growth by near term at the detriment of fetal pancreatic function.

CHAPTER I: LITERATURE REVIEW

PLACENTATION

The study of placentation in both humans and animal models has had indispensable contributions to our understanding of compromised pregnancies. Specifically, the use of pregnant sheep as a model for placental and fetal physiology has been used to investigate maternal-placental-fetal interactions for 50+ years, primarily due to the ability to maintain catheterization of maternal and fetal vasculature for repeated blood sampling under non-anesthetized, steady-state conditions *in-vivo* (Barry et al., 2008; Tanner et al., 2022). To appreciate how valuable the pregnant sheep is to understanding compromised pregnancies in humans, we must first delve into the process of placentation and understand where the two species are similar.

Placentation commences in early embryonic development in both humans and sheep. In all eutherian mammals, this begins with contact between the blastocyst-derived placental trophoblast and endometrial tissue, establishing an essential connection between the developing fetus, placenta, and maternal system that will develop continuously across gestation to facilitate fetomaternal exchange of nutrients and establishment of a closely adjoined microcirculatory system (reviewed by Frank, 2017; Johnson et al., 2023). In both species, successive cleavage of a zygote results in the differentiation of pluripotent blastomeres into a blastocyst, which includes the inner cell mass (or embryoblast), and outer, single-layered epithelium called the trophoblast. The embryo, umbilical cord and amnion are all derived from the embryoblast, whereas the fetal membranes and placenta will come from the trophoblast. Before implantation can occur, the blastocyst must then hatch from the zona pellucida (3-4 days post conception in humans, 6-7 in sheep; Bazer et al., 2012; Burton & Jauniaux, 2021). At this point, sheep and humans differ in their implantation strategies, despite both early processes being referred to as the beginning of placentation. The human conceptus truly invades the uterine lining and forms a discoid, hemochorial placenta. The sheep conceptus does not invasively implant, and their placenta is a cotyledonary, synepitheliochorial placenta (Wooding, 1992).

Human placentation

In humans, implantation starts around 6 -7 days post-conception (d.p.c.), beginning with apposition, when the blastocyst aligns itself to invade the uterine endometrium for attachment to the endometrial wall. First, the single layer of trophoblast cells proliferates to become a double-layered trophoblast, with the outermost (maternal facing) layer fusing during primary syncytial fusion to establish areas of multinucleated syncytial trophoblast (reviewed by (Burton & Jauniaux, 2022; Frank, 2017). The remaining unfused trophoblast cells are the uninucleate cytotrophoblast cells that represent a progenitor cell population, which contribute to the continuous proliferation of the syncytial trophoblast. The prelacunar stage follows, during which the syncytial masses fuse to form the primary syncytiotrophoblast, which invades the maternal endometrium and, with accompanying endometrial erosion and regression, results in the conceptus being both encapsulated and sunk deeper into the uterine wall (reviewed by Frank 2017; Burton and Jauniaux, 2022).

During the lacunar period (8 – 13 d.p.c.) fluid-filled spaces referred to as lacunae begin to appear in the syncytial mass. They enlarge and separate into two different layers as well as organize themselves into pillars, known as trabeculae, which represent early anchoring villi. A new type of syncytial trophoblast appears, forming a unilaminar microvillous polarized epithelium that lines the lacunae and represents an early transition from invasive trophoblast to one suited for its future role in transport and absorption. Lacunar formation results in three different layers of trophoblast covering the blastocyst: 1) primary chorionic plate (facing the blastocyst); 2) trabeculae and lacunar system; 3) cytotrophoblastic shell or future basal plate (endometrium facing).

Fetal villi formation begins with continued cytotrophoblast proliferation into the trabeculae and lacunae, creating branches composed of cytotrophoblast and syncytiotrophoblast that represent the primary villi, or precursors of anchoring villi which pass between chorionic and basal plates (12-13 d.p.c reviewed by Frank 2017; Burton and Jauniaux, 2022). Around the same time, the lacunae are first perfused by maternal blood as maternal endometrial vessels are eroded by the trophoblastic shell. Further proliferation results in the branching of primary villi and initiation of the development of the primitive villous trees. It should be noted that these are derived from former trabeculae, which remain continuous with the chorionic plate, as well as the lacunae, which therefore become the intervillous space (Burton & Jauniaux, 2022). During this time, maternal secretions from the now hypersecretory uterine glands called histotroph are delivering nutrients (carbohydrates, lipids, proteins) and growth factors (vascular endothelial growth factor [VEGF], epidermal growth factor [EGF], leukemia inhibitory factor [LIF], and fibroblast growth factor [FGF]) to the developing embryo and placenta. Histotrophic nutrition provides much of the support for the early conceptus prior to establishment of maternoplacental circulation and can be found in the intervillous space as well as the developing basal plate throughout the first trimester (Burton and Jauniaux, 2021).

Secondary villi begin to appear around 15-20 d.p.c. resulting from the invasion of extraembryonic mesenchyme into the primary villi. Hemangioblastic cells from these mesenchymal cells differentiate into villous macrophages, also known as Hofbauer Cells, that secrete angiogenic growth factors, regulate angiogenesis and villous morphogenesis, and later have a vital role in the immunological defense of the fetus (Burton, 2022). Other mesenchymal cells become angiogenic and hematopoietic precursor cells, contributing to sites of local vasculogenesis and hematopoiesis (reviewed by Frank, 2017). Around 20 d.p.c., the appearance of capillaries in the mesenchymal core of secondary villi marks their further development into tertiary villi. Thus, all fetally vascularized villi from this point onward are considered tertiary villi, composed of trophoblastic epithelial layers, mesenchyme, and vascular networks – the major components of the placental barrier. Only transitory, developing villi correspond to primary and secondary villi (reviewed by Frank, 2017; Burton and Jauniaux, 2022). At the same time, extravillous cytotrophoblasts are migrating and invading maternal spiral arteries to begin remodeling.

From this point onward, continued villous growth is essentially completed in two phases. The first phase has stem villi but is predominantly immature intermediate villi (I-IMV) and mesenchymal villi (MES-V), with I-IMV fulfilling the role of fetomaternal exchange. The second phase (15 – 17 weeks of gestation) is dominated by stem villi (which are differentiated from I-IMV), mature intermediate villi (M-IMV) and terminal villi – the latter two resulting from the differentiation of MES-V, which take over the fetomaternal exchange function. To achieve this, the first phase is a continuous cycle of growth based on sprouting of MES-V from I-IMV, creating new MES-V and stem villi. At the same time, fetoplacental vessels undergo both vasculogenesis and branching-angiogenesis to establish a branched capillary bed connected to the allantoic vascular system of the fetus and the umbilical cord. The developing villous tree is rapidly increasing the number, volume, and surface area of the villi. This timeframe is defined by increased blood flow and pressure in the developing vessels, in addition to low oxygen tension, high placental levels of VEGFr (signaling sprouting) and angiopoietin 2 (ANGPT2; signaling vessel destabilization at the edge of the new sprout), with moderate levels of placental growth factor (PIGF). By 6 weeks post-conception, a complete fetal-placental circulation has been established (Burton and Jauniaux, 2022).

The second phase sees the typical villi of the mature villous tree emerging from two prevailing differentiation pathways: IMV-V into stem villi and M-IMV into terminal villi. The terminal villi are the final branches of the villous tree and primary functional units for maternal-fetal exchange. This happens during a rise in intraplacental oxygen tension, increasing VEGF expression, a slow decline in PIGF expression, and a shift from ANGPT2 expression to ANGPT1 expression to promote further non-branching angiogenesis (reviewed by Frank, 2017, Barry and Anthony, 2008).

The reduced width of the intervillous space (in part from the maturation of the villous tree), the intimate association between the terminal villi and maternal and placental blood supply, and erosion and remodeling of maternal spiral arteries all contribute to the fetus effectively gaining control over maternal blood flow in the placenta. Onset of maternal blood flow has architectural impact on the villous trees, influencing their organization into “lobules,” which effectively comprises an individual maternal-fetal exchange unit (reviewed by Frank, 2017; G.J. Burton, 2022; Stanirowski et al., 2021). These have also been referred to as placentomes or cotyledons, and while sharing architectural similarities to placentomes of the sheep placenta, this does not mean the human has a cotyledonary placenta. These lobules are centered around the opening of a maternal spiral artery (from the basal plate), with blood entering the intervillous space, and at least one villous tree originating from the opposing chorionic plate (G.J. Burton, 2022). The majority of the maternal circulatory contributions to the placenta is found within these sometimes-overlapping placentomes (reviewed by Frank, 2017). While the center of the placentomes (as evident by different villous types described above) are where formation, growth and differentiation of villi take place, their periphery represents a more metabolically active zone to facilitate fetomaternal exchange (G.J. Burton, 2022). Finally, a few notes on the changes and adaptations the placenta must undergo to keep up with the increasing oxidative demands of fetal growth as gestation progresses. Terminal villi become more elaborate, increasing the villous surface area and reducing the diffusion distance between maternal and fetal circulation. This is accompanied by an increase in volume but reduction in thickness of the trophoblast, as well as a lengthening of the umbilical cord. All of which facilitate an increase in blood flow to the fetus as well as increased diffusional exchange of nutrients.

Sheep placentation comparison

In sheep, embryos enter the uterus 4 d.p.c., however, elongation of the conceptus is a prerequisite for implantation, which involves apposition and adhesion between the uterine luminal epithelia and developing trophectoderm over a longer timeframe compared to humans (from hatching at day 7 to attachment around day 16 p.c.; Johnson et al., 2023). It is thought to be primarily driven by progesterone and uterine-secreted histotroph. Unlike humans, the sheep conceptus does not invade the endometrial wall, but instead undergoes a semi-invasive type of attachment where it adheres and migrates to the endometrial luminal epithelium (LE) of the uterine caruncles, undergoing interdigitation between the microvilli and endometrial LE, followed by fusion and formation of a multinucleated syncytium in specialized zones that eventually become placental cotyledons. Briefly, this is accomplished by two cell types in the sheep trophoblast: mononucleated trophoblast cells and binucleate trophoblast giant cells (BNCs). BNCs differentiate from mononucleated trophoblast cells and the primary cells that migrate to the endometrial LE to initiate syncytialization, subsequently enlarging into syncytial plaques containing many nuclei (Johnson et al., 2023; F. B. Wooding, 1984). These plaques form the foundation of the cotyledons, whose chorionic villi protrude into the non-glandular endometrial crypts of preformed maternal caruncles, lengthening and branching into an extensive interdigitation by day 40 that results in increased surface area of the highly vascularized, discrete regions called placentomes (90-100 in number; Davenport et al, 2023; Johnson et al, 2024). Placentomes continue to develop across gestation, with continuous elongation and branching of fetal villi into similar villous tree structures as in humans (i.e. stem, intermediate, and terminal) which increases surface area for maximal exchange between fetal and maternal blood supply. As gestation progresses, the interdigitation between caruncles and cotyledons increases. Additionally, the fetal vessels within the villi mature and organize themselves similarly to humans, with stem arteries and veins, intermediate arterioles and venules, and terminal capillaries (Barry and Anthony, 2008).

ENDOCRINE FUNCTIONS OF THE PLACENTA

A major role of the placenta is endocrine function, secreting a variety of factors needed for maternal recognition of pregnancy, modifying maternal physiology to maintain pregnancy, influencing fetal growth and development, and modulating metabolic and immunological changes required for a successful pregnancy (Tal & Taylor, 2021). The supply of hormones provided by the placenta depends on placental metabolism (Barry and Anthony, 2008). Placental-produced hormones can be described in the following categories: steroid hormones, pituitary-like hormones (including growth factors), hypothalamic-like hormones, neuropeptides, placental cytokines, and eicosanoids (reviewed by Penn, 2017). A brief overview of key hormones will be provided for context.

Steroidogenic hormones

The placenta is the primary source of active steroidogenesis during pregnancy in both humans and sheep, playing crucial roles in maintaining pregnancy as well as modifying maternal physiology to support the growing fetus. Estrogens, androgens, and progestins are all critical throughout gestation; their production requires integration of the entire maternal-placental-fetal unit, as the placenta requires maternal and fetal precursors for steroid production (Judge-Golden et al., 2024). Consequently, placental steroid hormone production is characterized by significant aromatase, sulfatase, in addition to 11beta-hydroxysteroid dehydrogenase type 2 activity (Tal and Taylor, 2021). Concentrations of steroid hormones in maternal circulation increase throughout gestation (Penn, 2017). Progesterone and estrogen have been demonstrated to have roles in regulating insulin and glucose homeostasis as well as appetite regulation in many animal models, which may be important in promoting the necessary metabolic changes in the mother for pregnancy maintenance (Napso et al., 2018). Finally, impaired production of steroid hormones by the placenta has been linked to several pregnancy complications in humans, including preeclampsia and gestational diabetes (Napso et al, 2018). Low progesterone impairs embryo development in sheep and cattle (Davenport et al., 2023).

Progesterone plays several roles across gestation for pregnancy maintenance. In early development, progesterone primes the endometrium for implantation and immune tolerance (Judge-Golden et al., 2024). In sheep, progesterone supports conceptus elongation by inducing gene expression and secretion of migration, proliferation and attachment factors from the endometrial glands and luminal epithelium (Davenport et al., 2023; Johnson et al., 2023). It should be noted that placental produced progesterone isn't able to entirely support pregnancy maintenance independent of CL-produced progesterone until after around 35-47 days after ovulation in humans (termed the "luteo-placental shift;" Penn, 2017). In sheep, the CL is necessary to maintain pregnancy until at least day 60 of pregnancy (Al-Gubory et al., 1999). During the rest of pregnancy, progesterone promotes uterine quiescence by suppressing prostaglandin production and uterine contractions (Penn, 2017; Judge-Golden et al., 2024). Progesterone also leads to smooth muscle relaxation, which can affect many parts of the maternal system, but perhaps the most important effect is peripheral vasodilation and subsequent decrease in systemic vascular resistance, which is needed for the pregnancy-associated 50% increase in maternal blood volume (Judge-Golden et al., 2024). The syncytiotrophoblasts of the placenta produce progesterone from maternal cholesterol and then release about 90% of it back to the maternal system, with 10% going to the fetus; therefore, circulating progesterone levels are more reflective of placental steroidogenic function than fetal health (Penn, 2017). Perhaps the most striking difference in the role of progesterone during pregnancy between sheep and humans is that withdrawal of progesterone near term does not help precipitate parturition in humans as it does so precisely in sheep (Judge-Golden et al., 2024; Liggins et al., 1977; Penn, 2017).

The other major steroid hormones produced by the placenta are the estrogens; estrone (E1), estradiol (E2), and estriol (E3). Their production in the placenta is reliant upon both fetal and maternal biologically inactive androgen precursors, which are then converted by placental aromatase (Judge-Golden et al., 2024). Estriol is the major estrogen of pregnancy in humans, with the majority of it secreted towards the maternal system, whereas estrone is preferentially secreted towards the fetus (Penn, 2017). Maternal serum levels of estrogens rise early in pregnancy and peak at term. Estrogens serve numerous roles during pregnancy, mostly influencing maternal physiology via uterine growth, blood flow, contractility, metabolism (and expansion of beta cell mass) and breast development

(Judge-Golden et al., 2024; Napso et al., 2018; Penn, 2017). Estrogens also have trophic effects on fetal tissues, facilitating the maturation of fetal lungs, liver, intestines, and CNS (Judge-Golden et al., 2024). Interestingly, high estrogen levels are not a requirement for pregnancy, as there have been reports of normal pregnancies lacking placental sulfatase or aromatase(Bradshaw & Carr, 1986; Harada, 1993), but there are no reports of pregnancies without any detectable estrogen levels (Penn, 2017).

Pituitary-like hormones

One of the most notable pituitary-like hormones in human pregnancy is human chorionic gonadotropin (hCG), which is synthesized primarily by syncytiotrophoblasts, and is a reliable biochemical marker for pregnancy (showing up in circulation as early as 1 week post conception) and maintains progesterone production by the corpus luteum until the placenta takes over the role (from 6-9 weeks of pregnancy). It has also been demonstrated that hCG regulates placental structure and function, modifies the intrauterine environment to support implantation, and can also regulate differentiation of cytotrophoblasts into syncytiotrophoblasts to amplify hCG production by syncytiotrophoblasts (Penn, 2017). The sheep placenta does not produce chorionic gonadotropins. The anti-luteolytic agent that serves to induce maternal recognition of pregnancy is instead interferon tau, which is synthesized and secreted by the trophectoderm from days 10 – 21 (peaking around day 14) post conception in sheep (Johnson et al., 2023) and helps maintain progesterone production by the corpus luteum (Davenport et al., 2023).

Another important pituitary-like hormone that is also considered part of the growth hormone family is chorionic somatomammotropin (CSH; aka placental lactogen or hCS). It can be first detected in trophoblast tissue in humans within 10 d.p.c. and in maternal serum by the third to fourth week of gestation (Pen, 2017). In ruminants, it is synthesized by the binucleate cells and is detectable in maternal serum by 50 dGA (Anthony et al, 1995). Circulating concentrations of CSH increase over gestation in humans (Penn, 2017) and peak near term (130-139 dGA) in sheep followed by a decrease to term (Kappes et al., 1992). CSH's exact functions are still being studied, despite early discovery and elucidation of its chemical properties and molecular biology in relation to the rest of the growth hormone family (Handwerger, 1991). Recent studies utilizing lentiviral-mediated mediated RNA-interference for *in-vivo* investigation (discussed in depth later in this literature review) of CSH's actions during pregnancy highlight the many impacts that CSH has on maternal, placental, and fetal physiology (Tanner et al., 2022). Briefly, CSH clearly influences global nutrient transfer, uterine and umbilical blood flows, placental growth and metabolism, as well as fetal growth and metabolism.

Other essential growth factors produced by the placenta include insulin-like growth factors (IGFs), which interact closely with growth hormone (GH) and play critical roles in fetal growth and placental development (Penn, 2017; Kaur et al, 2021). GH from the maternal pituitary is the main form of GH present in maternal circulation early in pregnancy, but from around 15-20 weeks until term, increasing levels of placental GH replace it (Tal and Taylor, 2021). IGF1 and IGF2 are present in both human and sheep placentas (Jeckel et al, 2018) and can be found expressed in most tissues during fetal development, but IGF1 is primarily expressed in the syncytiotrophoblast throughout gestation and IGF2 in cytotrophoblasts earlier in gestation (Penn 2017). IGF binding proteins (IGFBPs) are produced in syncytiotrophoblast and binucleate cells and regulate the actions of IGFs by acting as carrier molecules for transport, mediating bioavailability of IGFs, and protecting them from degradation (Han and Carter, 2000). The IGF axis has been studied extensively in many *in-vitro* and *in-vivo* models, and when perturbed, has been implicated in many disease states associated with pregnancy, particularly FGR. An extensive review is not within the scope of this dissertation, but many have been written (Kaur et al., 2021; Lee & Styne, 2017).

Hypothalamic-like releasing/inhibiting hormones

A placenta analog for every known hypothalamic-releasing or inhibiting hormone has been reported (Penn, 2017), including gonadotropin-releasing hormone (GnRH), corticotrophin-releasing hormone (CRH), thyrotropin-releasing hormone (TRH), and regulators of the GH axis such as GH-releasing hormone, somatostatin and ghrelin. The mechanisms of action of placenta specific TRH are not well defined because the thyroid-stimulating function of the placenta appears to be controlled by hCG. Regardless, placental regulation of thyroid hormone transport and metabolism is still considered to be critical for fetal well-being. Regulators of the GH axis (GH-releasing hormone, somatostatin, and ghrelin) have all been localized to human cytotrophoblasts, but their roles in successful pregnancy maintenance have yet to be elucidated (Penn, 2017; Voltolini & Petraglia, 2014).

Two isoforms of GnRH (1 & 2) are produced in the human placenta and are important for early pregnancy maintenance. GnRHR-1 is expressed in the syncytiotrophoblasts, and while a second receptor, GnRHR-2 has been identified, it does not appear to be functional in humans (Penn, 2017). GnRH-1 stimulates secretion of the beta-subunit of hCG; lack of GnRH or GnRH receptor-1 activity can lead to pregnancy failure, presumably due to inadequate hCG production. Concentrations of placental and maternal circulating GnRH are high in early pregnancy and decline later one, further supporting the potential interaction of GnRH with hCG production and maternal recognition of pregnancy (Voltolini and Petraglia, 2014). GnRH 1 & 2 also directly regulate placental steroid release and prostanoid production (Penn, 2017).

Chorionic CRH and urocortins (members of the CRH family) have been shown to stimulate POMC-derived hormones, including ACTH and beta-endorphins in the placenta. CRH has been linked to promoting immune tolerance early in pregnancy, and because concentrations increase with advancing gestation, including a reliable rapid increase at term, CRH levels are viewed as a “placental clock” for timing parturition (Penn, 2017). CRH also stimulates placental oxytocin secretion (Voltolini and Petraglia, 2014). It has been suggested that CRH and urocortins may be valuable biomarkers for detecting pregnancy related disorders, as circulating levels of maternal CRH in hypertensive pregnancies are elevated earlier in pregnancy, and FGR fetuses release CRH into circulation in response to stress (Tal and Taylor, 2021).

Other Peptide Hormones

Some key peptide hormones produced by the placenta include oxytocin, leptin, prolactin, relaxin, and vasoactive peptides such as VEGF (see previous section about villous development), endothelin, angiotensin, and arginine vasopressin.

Oxytocin is produced by the placenta in concentrations approximately five times greater than the posterior pituitary lobe, leading to speculation that the placenta may be the primary source of oxytocin during pregnancy (Voltolini and Petraglia, 2014). It's direct contribution to the mechanisms of parturition remains unclear, and while exogenous infusion can initiate labor, our current understanding is that this is a result of increased uterine sensitivity to oxytocin due to an upregulation of oxytocin receptor concentrations (Voltolini and Petraglia, 2014; Penn, 2017).

The placenta is the primary producer of leptin during pregnancy, synthesizing it in both the cyto- and syncytiotrophoblasts. Its precise roles during pregnancy are not well understood, as it does not appear that the elevated levels of leptin during pregnancy (much higher than the nonpregnant state) correlate with maternal BMI (Penn, 2017; Tal and Taylor, 2021). Additionally, overproduction of leptin by the placenta has been associated with diabetes mellitus and maternal hypertension (Hauguel-de Mouzon et al., 2006).

Relaxin is also produced in the placenta, with receptors localized in syncytiotrophoblasts. It is a potent vasodilator as well as a stimulator of collagenase activity and prostaglandin release, suggesting a role for relaxin in parturition that is not clearly defined, as most circulating relaxin is made by the corpus luteum (Penn, 2017).

The placenta produces several vasoactive peptides (and their respective receptors) such as VEGF, endothelins, angiotensin and arginine vasopressin. Endothelin-1 and endothelin-3 are both produced by the placenta and have potent vasoconstrictor activity on fetal-placental circulation. Angiotension II has been shown to mediate vascular tone in the placental bed. VEGF, as already described, is a major regulator of placental angiogenesis and vascular development (Frank, 2017; Penn, 2017).

Eicosanoids

The placenta produces a variety of eicosanoids that have many autocrine or paracrine roles related to pregnancy. They are primarily inflammatory mediators derived from arachidonic acid. Some are involved in implantation (thromboxane and prostaglandins), while others impact vasoregulation of the fetoplacental unit (prostacyclin and prostaglandin E2) or vasoconstriction of maternal and umbilical circulation (PGF2alpha). Prostanoids have also been proposed to be one of the mechanisms through which CRH and cytokines initiate labor (Penn, 2017).

TRANSPORT OF SUBSTRATES

One of placenta's main roles for facilitating successful fetal growth is transfer of substrates, particularly oxygen and nutrients, which has been demonstrated to function similarly between humans and sheep. Much of our current knowledge of fetal-placental interactions, placental metabolism, and placental transfer capacity comes from *in vivo* studies from large animal models, particularly sheep (Barry and Anthony, 2008). Starting with the 1960s, studies utilizing pregnant sheep have demonstrated the similarities between sheep and humans in terms of uteroplacental oxygen and nutrient use (Meschia et al., 1965; 1980). These observations became some of the first evidence demonstrating that the placenta is not merely a conduit for maternal-fetal exchange, but a highly metabolically active organ (reviewed by Pardi and Cetin, 2006; Barry and Anthony 2008). Since then, many of those early findings were later confirmed by more advanced imaging technologies, and the pregnant sheep continues to contribute a wealth of knowledge to our understanding of maternal-placental-fetal interactions (Tanner et al, 2022).

In both humans and animals, the nutrient transfer capacity of the placenta plays a critical role in fetal growth trajectory and ultimately the ability of the fetus to reach its full growth potential. In both humans and sheep, placental size has been directly correlated with fetal growth. The transport capacity of the placenta is also directly related to its size, structural and vasculature development, blood flow, and transporter abundance. However, the supply of oxygen and nutrients to the fetus is dictated by placental substrate utilization (reviewed by Barry and Anthony, 2008). The major substrates required for fetal growth are oxygen, supplied by simple diffusion, and glucose and amino acids, supplied by transporter-mediated transfer (Barry & Anthony, 2008; Hay et al., 2016). The placenta contains membrane transporter proteins for glucose, lactate (glucose's metabolic product), amino acids, and fatty acids, facilitating their transport to the fetus via concentration gradients (Hay et al., 2016).

Oxygen

Oxygen is transported across the placenta by simple diffusion and is therefore dependent on the rate of blood flow in the uterine and umbilical vessels, blood oxygen carrying capacity and hemoglobin oxygen binding affinity, the surface area of the placenta, the placenta's permeability, and placental oxygen consumption (Barry and Anthony, 2008). However, the major determinant is blood flow, in sheep and humans. As such, alterations in uteroplacental blood flow during pregnancy, and therefore oxygen delivery to the fetus can lead to fetal hypoxia and impaired fetal development (Giaccioli and Lager, 2016). Once again, studies in pregnant sheep provided much of our first understandings of fetal-placental respiratory function, which with the advent of advances in technology (such as Doppler ultrasonography), have been confirmed to be similar in humans. As such, the changes in oxygen tension and placental oxygen consumption across gestation have also been found to be similar in sheep and humans. Meschia et al. (1965) determined that the transplacental oxygen gradient is relatively constant across gestation, but placental uptake of oxygen (and thus rate of transfer) increases with increasing fetal weight. Oxygen tension early in gestation is relatively low within the placenta (pO_2 <10 mmHg at 8 weeks of gestation in humans, similar values have been reported in sheep), but by the end of the first trimester, placental pO_2 is already increasing dramatically to around 60mmHg (humans; Barry and Anthony, 2008). During mid-gestation, the placenta consumes about 80% of the oxygen supplied by the uterus (Bell et al., 1986). Uterine blood flow (i.e. oxygen delivery) increases by three-fold in sheep and two-and-a-half-fold in humans throughout the second half of gestation (reviewed by Barry and Anthony 2008). In late gestation, fetal oxygen consumption remains constant and there is a proportional decrease in oxygen demands by the placenta, but put in the context of weight, the placenta continues to consume significantly more oxygen than the fetus in both sheep and humans (4-5 times more; Bonds et al., 1986; Meschia et al., 1980).

Amino Acids

Transfer of amino acids is more complex than other substrates due to multiple transporter systems, overlapping actions of transport systems, and competitive inhibition between amino acids for transport (Barry and Anthony, 2008; Hayward et al, 2017). Amino acids are an important carbon source for both fetal and placental development. Their availability to the fetus is dependent upon rates of active transport across the placenta, placental consumption, and rates of interconversion (all of which are energy dependent; Barry and Anthony, 2008). Generally, the concentration of amino acids in circulation is higher in the fetus compared to the mother, primarily due to being actively concentrated in the trophoblast before being transported to fetal plasma, which reflects an overall net transfer of amino acids from maternal to fetal circulation (Barry and Anthony, 2008; Hay et al., 2016). The net total amino acid uptake by the fetus can account for up to 30-40% of the combined carbon requirement for oxidative metabolism and deposition of fetal protein, glycogen and fat, in addition to providing 100% of fetal nitrogen requirements (Hay et al., 2016). The many amino acid transporters can be categorized by family, physiological function (i.e. accumulative vs. exchangers; Gaccioli and Lager, 2016), or generally as neutral, cationic, and anionic transport systems (Barry and Anthony, 2008). Transfer of amino acids across the placenta from maternal circulation increases with advancing gestation as the increased protein synthesis and nitrogen balance demands of the growing fetus are met by the placenta increasing its size and amino acid transport capacity (Hay et al., 2016; Hayward et al, 2017).

Lipids

While lipids may not be considered a “major” fetal nutrient for nutritional or energy metabolism, lipids and fatty acids are still transported across the placenta to use in placental and fetal development. Placental transport capacity of fatty acids and lipids varies by species, with hemochorial placentas (i.e. humans) being greater than the epitheliochorial placentas of ruminants (Hay et al., 2016). Many lipid substances found in fetal circulation differ from those taken up by the uteroplacenta, suggesting placental metabolism of lipid substances. Some examples include lipoprotein lipase produced by trophoblast cells that hydrolyze maternal triglycerides into free fatty acids, which can then readily diffuse across the placenta (Hayward et al, 2017). There are also membrane-bound fatty acid transport proteins (FATPs) in the placenta that mediate the uptake of long-chain fatty acids, as well as fatty acid binding proteins (FABPs) that facilitate transport down a transplacental concentration gradient (Gaccioli and Lager, 2016). Generally, the most important factor regulating the flux of lipids into the placenta and their transport and metabolism is the concentration of FFAs and lipids in maternal plasma (Hay et al., 2016). The net flux of nonesterified FAs into the fetus from maternal circulation accounts for the fetal requirement of fatty acids near the end of pregnancy. There appears to be a direct relationship between the permeability of the placenta to lipids and the adiposity of the fetus at term; this demonstrates a key difference between humans and sheep, with human fetuses developing the most body fat at term (15%-18% of their body weight) compared to sheep fetuses having only around 3% body fat at term (Hay et al., 2016). Finally, it has been shown that in humans, there are limited amounts of enzymes and transporters in fetal tissues for delivering fatty acids into the mitochondria, and fatty acid oxidation occurs after birth (even in preterm infants), suggesting a lack of significant fatty acid oxidation in the fetus during gestation (Hay et al., 2016).

GLUCOSE TRANSPORT AND UTILIZATION DURING PREGNANCY

Last but not least is glucose. Glucose is the primary substrate for placental energy generation and fetal oxidative processes (in humans and sheep). The fetus has limited ability for glucose production during gestation, therefore fetal glucose supply depends on transport from the maternal circulation (Barry and Anthony, 2008). Glucose transport during pregnancy is a major theme of the experiments outlined in this dissertation and will therefore have its own section dedicated to comparing glucose transport across the placenta in humans and sheep, its contributions to the development and metabolism of the placenta and fetus, and its homeostasis in the context of the developing fetal pancreas.

Uteroplacental glucose transport and metabolism

Bidirectional transport of glucose in the placenta relies on a facilitated diffusion system of GLUT transporters on both the maternal-facing microvillous membrane and the basolateral, fetal-facing membranes of the placenta (in sheep and humans). In addition to higher glucose concentrations in maternal circulation compared to fetal circulation, an asymmetrical arrangement of transporters between the two opposing sides of the placenta increases the concentration of intrasyncytial glucose. As a result, the glucose gradient driving its transport across the basal membrane is maximized and directs net glucose transport to the fetus (Illsley, 2011; Gaccioli and Lager, 2016). The placenta itself also consumes a high rate of the glucose delivered from the maternal system; placental glucose consumption is significantly higher than estimates for skeletal and cardiac muscle or even whole-body glucose consumption (Illsley, 2011). Uteroplacental glucose metabolism is mostly placentally driven. In humans near term, studies have shown as much as 60-80% of glucose taken up is consumed by the placenta (reviewed by Barry and Anthony, 2008). In sheep studies, placental glucose transport capacity increases eightfold by the second half of gestation (Hay et al., 1991), consuming roughly 80% of glucose provided by the maternal circulation, and continues to consume around 72% of the glucose supply by late gestation (Meschia et al., 1980; Bell et al., 1986; Bell et al., 1999). *In-vivo* studies in the pregnant ewe have also aided in the understanding that placental-to-fetal glucose transfer is more sensitive to changes in fetal glucose concentrations than maternal concentrations, making uteroplacental glucose consumption essentially independent of maternal glucose (Hay et al., 1991 & 2016). Sheep studies also demonstrated that the fetal side of the uteroplacental unit is more permeable to glucose than the maternal side (Illsley, 2011). The glucose transport capacity of the placenta also increases dramatically over gestation. In sheep, transport capacity accounts for a 60% increase in placental glucose transport, with the other 40% being an increase in the transplacental glucose concentration gradient – all likely due to the increased surface area of the growing placenta and therefore greater numbers of GLUT transporters (Hay et al., 2016).

Uterine blood flow also influences nutrient delivery from maternal circulation through the placenta and to fetal circulation, which directly impacts glucose delivery to the fetus. Blood flow to the uteroplacental unit increases across gestation, which increases glucose supply to match the demands of the growing fetus (reviewed by Kalhan, 2017). The glucose concentrations in the maternal, placental, and fetal compartments are determined by the rate at which glucose diffuses in from the maternal supply, and metabolism within the placenta and fetus (Illsley and Baumann, 2020). As such, impaired blood flow to the uteroplacental unit or umbilical vessels can negatively impact the glucose supply to the fetus (similar to pregnancy complications resulting in FGR). An experiment of significantly reduced uterine blood flow to pregnant sheep (less than 300 ml/min/kg of fetus) produced significantly decreased fetal glucose uptake (Wilkening et al., 1985). Single umbilical artery ligation, which reduces umbilical blood flow also reduced fetal glucose uptake (Oh et al., 1975).

The metabolic pathways the placenta uses glucose for also change across gestation. Earlier in pregnancy, almost 75% of glucose is metabolized through glycolysis, 15% through non-triose phosphate pathways (such as glycogen synthesis), and 10%

through the pentose-phosphate pathway (Illsley, 2011). By term, nearly 90% of glucose is metabolized by glycolysis, with the other two accounting for around 5% each (Illsley, 2011).

Another common hallmark of FGR is hypoxia, which has been shown to alter placental glucose metabolism as well. In a study of chronic hypoxia in high-altitude pregnancies, preferential anaerobic glucose consumption by the placenta maintains fetal oxygen delivery at the cost of reduced fetal glucose transfer, resulting in smaller, hypoglycemic and hypoinsulemic fetuses (Zamudio et al., 2010). Other factors that can impact placental glucose transport capacity, such as type-1 diabetes, which has been shown to cause an upregulation of placental glucose transporters (Jones et al., 2007).

Glucose transporters in the placenta

Glucose is transported across cell membranes in the body by facilitative transporters in the GLUT family, which are encoded by SLC2 genes (Mueckler & Thorens, 2013). The GLUT family of human transporters includes 14 GLUT proteins that are made up of ~500 amino acid residues and are categorized into three classes (based on sequence similarity). Class 1 includes GLUTs 1-4 and 14; class 2 includes GLUTs 5, 7, 9, and 11; and class 3 includes GLUTs 6, 8, 10, and 12, as well as HMIT (also known as GLUT13; Mueckler and Thorens, 2013; Illsley and Baumann, 2020). All the GLUT proteins possess 12 transmembrane segments, one N-linked glycosylation site, a large centralized cytoplasmic linker domain, and have topologies with their unique NH_2 and COOH termini located in the cytoplasm (Mueckler & Thorens, 2013; Simmons, 2017; Stanirowski et al., 2021). While GLUT isoforms share the same family, they exhibit discrete differences in their substrate specificity, transport kinetics, expression and tissue distribution. The diverse characteristics of the GLUT transporters highlight the importance of glucose in cellular metabolism and the body's need to provide energetic substrates in amounts adjusted to individual cell requirements with minimized risk of disruption (Stanirowski et al., 2021).

Glucose transport down the concentration gradient from maternal to fetal circulation primarily uses the sodium-independent facilitative transporter proteins GLUT1 (SLC2A1) and GLUT3 (SLC2A3), both of which are found in the human and sheep placentas. GLUT1 is expressed throughout the human placenta, with its highest expression being in the syncytiotrophoblast, but is also found in the cytotrophoblast, endothelial cells, and villous stromal elements (Illsley & Baumann, 2020; Stanirowski et al., 2021). In human placentas, GLUT1 is found at a greater density on the microvillous membrane (MVM) of the syncytiotrophoblast (approximately 3-fold higher by term) compared to the basal membrane (BM), however, BM expression has been shown to increase with advancing gestation while MVM expression remains similar to second trimester concentrations until term (Illsley, 2000; Illsley & Baumann, 2020; Jansson et al., 1993). GLUT3's primary location in the human placenta is on the MVM of the syncytiotrophoblast but has also been found in cytotrophoblasts at term (Janzen et al., 2013). In human placentas, GLUT3 expression has been shown to decrease with advancing gestation, to 48% of the first trimester by the second trimester, and to 34% of the first trimester by the third, suggesting a more important role during early gestation (Brown et al., 2011). A study by Wooding et al. (2005) localized glucose transporters in the sheep placenta, finding that GLUT1 is localized at the maternal-fetal syncytial layer around maternal blood capillaries as well as the basolateral membrane of the trophoblast cells. GLUT3 is located on the apical MVM of the trophoblast cells. Their localization infers a sequential relationship of glucose transport across the placenta. As pregnancy advances in sheep, concentrations of GLUT1 decrease while GLUT3 increases (Barry and Anthony, 2008).

While GLUT-4 has been identified in human syncytiotrophoblast in the first trimester (and at lesser concentrations by the third trimester), it is still unclear whether the placenta is an insulin-regulatable tissue (Illsley & Baumann, 2020; Simmons, 2017). However, reduced or absent expression of insulin-regulatable GLUT-4 near term does fit with observations of a placental insulin receptor early in gestation being down-regulated later in pregnancy. Instead, the primary localization of the insulin receptor near-term is found in fetal endothelium (Illsley and Baumann, 2020). GLUT-8, 9, 10, and 12 have all also been identified in the human placenta (Hayward et al., 2017; Illsley & Baumann, 2020; Stanirowski et al., 2021).

GLUT1

GLUT1, encoded by the *SLC2A1* gene, was one of the first membrane transporters to be purified and the first glucose transporter to be cloned (Baldwin & Lienhard, 1989; Kasahara & Hinkle, 1977; Mueckler & Thorens, 2013). GLUT1 is present ubiquitously in the body and is responsible for transport of its principal substrate, glucose, but is also capable of transporting mannose, galactose, glucosamine, and reduced ascorbate (Mueckler and Thorens, 2013). It is considered the main glucose transporter isoform in the placenta of humans and sheep (Barry & Anthony, 2008; Hayward et al., 2017; Jansson et al., 1993). As mentioned above, in human placentas, GLUT1's highest density is at the MVM, which coupled with the increase in surface area due to folding of the villi in the growing placenta, correlates to an approximately 20-fold higher glucose transport capacity than the BM (Jansson et al., 1993). Intrasyncytiotrophoblast glucose concentrations are likely to be similar to maternal circulation, allowing for both a high rate of placental glucose consumption while still supplying glucose to the fetus at a concentration not far behind that of maternal concentrations and consequently keeping up with fetal demand for growth (~1mmol/L reported by Jansson et al., 1993). Taken with the asymmetric distribution of GLUT1 in the syncytiotrophoblast, GLUT1 glucose transport at the basal membrane has therefore been suggested to be the rate-limiting step in transplacental glucose transfer to the fetus (Jansson et al., 1993; Barry and Anthony, 2008; Illsley and Baumann, 2020).

The amino acid sequence of GLUT1 is highly conserved across the mammalian species it has been studied in, with 97-98% identity between humans and rats, mice, rabbits, sheep, and pigs (Currie et al, 1997; Simmons, 2017). Mouse models of homozygous GLUT1 gene deletion (*Slc2a1*$^{-/-}$) are embryonic lethal, with the conceptus not surviving past embryonic day 14 (Wang et al., 2006). Heterozygous deletions (*Slc2a1*$^{+/-}$) are viable, but with a similar phenotype to human GLUT1 deficiency syndrome, including developmental delays, infantile seizures, microcephaly and decreased brain glucose uptake (Wang et al., 2006). Decrease GLUT1 expression also impacts the pre-implantation embryo, with reduced glucose transport and high incidence of apoptosis at the blastocyst stage (Chi et al., 2000).

Placental expression of GLUT1 has also been investigated in relation to FGR in humans and animal models, with conflicting results. In human placentas from normal and IUGR pregnancies, no differences in GLUT1 expression have been observed when comparing the MVM and BM (Jansson et al., 1993; Jansson & Powell, 2006), or when compared by considering the basal plate vs. the chorionic surface (Janzen et al., 2013). In human-term placental trophoblast cells exposed to glucocorticoids, and in rats given a single intraperitoneal injection of glucocorticoids (i.e. triamcinolone), GLUT1 was significantly downregulated (Hahn et al., 1999). Pregnant ewes that were nutrient restricted (50% NRC requirements) from early to mid-gestation had significantly reduced placental and fetal weights with increased GLUT1 mRNA and protein levels, but after re-alimentation from mid to late-gestation, the differences were lost (Ma et al., 2011). A primate model of maternal nutrient restriction during pregnancy (baboons fed 70% of control diets) demonstrated reduced fetal weights and GLUT1 MVM protein expression (Kavitha et al., 2014).

While a common characteristic of FGR is impaired oxygen and glucose transport to the fetus, expression of GLUT1 in these states has also yielded contrasting results. In pregnant sheep exposed to chronic maternal glucose and insulin infusions, GLUT1 expression increased during hypoglycemia and decreased during hyperglycemia (Das et al., 1998). The same group investigated uterine artery ligation-induced FGR in rat models, finding that reduced fetal glucose and insulin concentrations, along with smaller fetuses and placentas had a 50% decline in placental GLUT1 protein. A study measuring GLUT1 expression in placentas from high-altitude women (>2700m) observed smaller fetuses with lower circulating glucose concentrations and fetal glucose consumption, as well as significantly reduced GLUT1 protein levels in the BM of the syncytiotrophoblast (Zamudio et al., 2010).

GLUT3

Another important glucose transporter found in the placenta is GLUT3, the third glucose transporter to be cloned, and first reported in the human placenta in 1988 by Kayano et al. It is encoded by the *SLC2A3* gene and in addition to glucose, transports galactose, mannose, and xylose (Mueckler and Thorens, 2013). It has a much higher affinity for glucose than GLUT1 (Km = approximately 1-2mM) and higher maximum transport capacity than the other class 1 GLUT proteins (at least fivefold greater than GLUT1 and -4, Mueckler & Thorens, 2013; Simpson et al., 2008), which has been shown to give GLUT3 the ability to effectively transfer energetic substrates into cells even in conditions of glucose deprivation (Stanirowski et al., 2021). These characteristics are why GLUT3 is historically associated with neuronal transport and was originally designated as "neuronal GLUT" (Simpson et al., 2008). Its location on the MVM in the human placenta and higher expression earlier in pregnancy (Brown et al., 2011; Simpson et al., 2008) has led to the belief that GLUT3 protein may be more important for cells with high energy demand and intensive metabolism, such as proliferating trophoblast cells during early pregnancy—i.e. by securing an energy supply during conditions of low glucose and oxygen concentrations due to insufficiently developed utero-placental circulation (Stanirowski et al., 2021).

Other studies have shown support for the notion of GLUT3's essential role in early placental and embryonic development. One study using BeWo cells demonstrated the stimulating effects of hypoxia on GLUT3 expression (Baumann et al., 2007). Pantaleon et al. (1997) localized GLUT3 in the apical membrane of the trophectoderm in the compact morula, demonstrating its necessity for blastocyst formation when cultured with GLUT3 antisense oligonucleotides, which resulted in significantly reduced number of embryos formed from blastocysts. Another study found decreased expression of GLUT3 mRNA and protein in blastocysts from mice with diabetes mellitus, demonstrating that high maternal glucose levels is unfavorable to the developing embryo, but a certain threshold of glucose is still required for embryonic growth and development (Moley et al., 1998). A homozygous deletion of GLUT3 in mice (*Slc2a3*$^{-/-}$) resulted in embryonic loss at day 8.5 (Ganguly et al., 2007) or severe disorganization and growth retardation at day 7.5 followed by complete loss of embryos at day 12.5 in another study (Schmidt et al., 2009). Heterozygous blastocysts (Slc2a3$^{-/+}$) survived post-implantation as pups with normal placental weights but reduced transplacental glucose transport and significant fetal growth restriction (Ganguly et al., 2007). The heterozygotes also had abnormal localization of both GLUT3 and GLUT1, suggesting that these transporters may need different timing and location of expression for appropriate glucose uptake and development. These studies highlight GLUT3's critical function in the placenta, indirectly affecting fetal growth.

In human placental samples collected at full term affected by idiopathic IUGR, GLUT3 protein expression on the maternal aspect of the placenta was increased compared to normal pregnancies. The increase was also associated with an increase in HIF-1α

(hypoxia-inducible factor 1α), further implicating hypoxia's potential role in upregulating GLUT3 expression (Janzen et al., 2013). The authors suggest that this increase in GLUT3 expression in the late-term IUGR placenta may lead to increased glucose consumption by the placenta and therefore reduced transplacental glucose transport capacity to the fetus. A more recent study proposed a potential mechanism for HIF-1α's regulation of GLUT3 expression, finding a noncoding intergenic co-induced transcript (NICI), which is regulated by hypoxia via HIF-1 promoter binding, to be co-expressed with GLUT3 *in vitro* (Lauer et al., 2020). Mutations of the NICI resulted in reduced glucose consumption and cell proliferation under hypoxic conditions without impacting expression of GLUT1.

Experimental models of placental insufficiency, maternal undernutrition and abnormal hormone secretion implicate altered expression of GLUT3 in the placenta as a possible mechanism of *in utero* FGR. Das et al. (1998) investigated the effects of maternal hyper- and hypoglycemia on physiological regulation of GLUT3 in pregnant sheep and found a time-dependent and tissue-specific effect of circulating glucose on fetal GLUT1 and GLUT3 protein expression. During hyperglycemia, GLUT1 protein expression in fetal tissues decreased earlier than GLUT3, which the authors suggest may lead to a reduction in the total placental glucose transporter pool and therefore potentially act as a protective mechanism against glucose toxicity for the fetus. In response to maternal hypoglycemia, no significant changes were observed in GLUT3 protein expression, but GLUT1 protein expression was decreased along with reductions in uterine, uteroplacental and fetal net glucose uptake rates. Therefore, GLUT3's relative contribution to total glucose transport was increased. Coupled with GLUT3's higher affinity for glucose and localization on the apical membrane of the sheep trophoblast, GLUT3 would likely be ensuring glucose supply to the fetus. Our lab recently established a model of GLUT3 deficiency (*SLC2A3*-RNAi) in the sheep placenta at mid-gestation (75 days gestational age) that resulted in a 38% reduction in GLUT3 protein in the placenta and fetuses that were smaller with significantly reduced umbilical concentrations of glucose, insulin, and glucagon (Lynch et al., 2022). This model is the basis for the experiments outlined in this dissertation and will be discussed in further detail in subsequent sections.

Fetal glucose uptake & utilization

Fetal glucose utilization near term is similar in sheep and humans (5-7 mg/min/kg), which is about half of the values found at mid-gestation in fetal sheep – the time of the greatest rate of fetal growth and protein turnover, implicating another important role of glucose as a major energy supply for protein synthesis required for growth (Hay et al., 2016). As such, fetal growth restriction is directly related to hypoglycemia. Glucose tracer studies in pregnant sheep allow us to calculate fetal glucose utilization and production, where glucose utilization = exogenous glucose uptake + endogenous glucose production (Battaglia and Meschia, 1978). Nearly all fetal glucose comes from the exogenous, maternal supply of glucose, making the net uptake of glucose by the fetus from the placenta equal to the fetal glucose utilization rate. Thus, under normal conditions, there is very little (less than 4% reported in some studies; Battaglia and Meschia, 1978) to no evidence (Illsley, 2011; Kalhan, 2017) for fetal glucose production during pregnancy. Although the potential for gluconeogenesis exists due to the presence of key enzymes in the liver (i.e. pyruvate carboxylase, PEPCK, and fructose diphosphatase), fetal glucose production only develops during periods of sustained fetal hypoglycemia and hypoinsulinemia, with most evidence occurring only in late gestation (Illsley, 2011; Kalhan, 2017). Studies modulating glucose concentration with insulin infusions in pregnant sheep show variable effects on fetal glucose utilization, including stimulus of compensatory fetal glucose production. During insulin infusion to produce acute maternal hypoglycemia (2 hours), no compensatory fetal glucose production was observed, indicating a reduction in fetal glucose utilization (Anand et al., 1980). During prolonged maternal hypoglycemia (5-7 days), fetal compensatory glucose production was observed (Hay et al., 1984). Chronic hypoglycemia because of a 3-week insulin infusion also resulted in compensatory fetal glucose production to maintain utilization (Digiacomo & Hay, 1989).

Fetal glucose uptake and utilization are also impacted by other factors such as hormones (insulin, glucocorticoids), growth factors (IGFs), cytokines, hypoxia, and oxidative stress, but more extensive research is still needed (Stanirowski et al., 2021). Briefly, fetal thyroid hormone can indirectly increase fetal glucose utilization by increasing fetal metabolic rate, and therefore oxygen consumption, more dramatically in the fetal sheep than human (Limesand & Davis, 2018). Fetal plasma cortisol concentrations, which increase during late gestation, may also enhance the glucogenic capacity of the fetus and contribute to endogenous glucose production seen in normal fetuses near-term (Hay et al., 2016). Many studies in humans and other animal models have investigated effects of fetal adaptations to maternal stress during pregnancy, showing impacts on the fetal hypothalamic-pituitary-adrenal axis, which has indirect impacts on fetal steroids

(Limesand and Davis, 2018). The role of fetal glucagon throughout pregnancy is still being investigated, but when infused into the fetal sheep can stimulate fetal glucogenesis (Limesand and Davis, 2018) while chronic fetal hyperglucagonemia can result in a FGR phenotype (Cilvik et al., 2021). Glucagon is also elevated in FGR pregnancies in both humans and sheep (Cilvik et al., 2021; Hubinont et al., 1991; Limesand et al., 2006).

Insulin and IGFs during pregnancy

A major player in fetal growth, glucose uptake and utilization is insulin, produced by the beta cells of the fetal pancreas. Insulin's primary actions occur through binding its various receptors, the primary one being insulin receptor (IR). Insulin receptor localization during gestation changes, suggesting a shift in the regulation of insulin's effects from mother to fetus across gestation. During the first trimester, the majority of IR is expressed on the MVM of the syncytiotrophoblast (i.e. facing maternal circulation), with expression shifting to the placental endothelium (i.e. facing fetal circulation) by term (Hiden et al., 2009; Jones et al., 2007). A more in-depth discussion of fetal pancreatic development and the maturation of insulin action follows later in this literature review. Glucose-stimulated fetal insulin secretion has been documented in third-trimester human fetuses and in the second half of gestation in fetal sheep, where insulin concentrations increase fivefold (Hay et al., 2016). In FGR, fetal insulin levels and placental insulin receptor numbers are reduced, while maternal plasma insulin concentrations are unaltered (Jones et al., 2007; Potau & Riudor, 1979).

Glucose and insulin clamp studies in fetal sheep have demonstrated that fetal plasma glucose and insulin concentrations act independently on glucose utilization and oxidation. Insulin secretion can be modified by the degree of changes in fetal glucose concentrations, such that sustained hyperglycemia decreases fetal insulin secretion, while pulsatile hyperglycemia increases fetal insulin secretion (reviewed by Hay et al., 2016).

In a study examining fetal glucose kinetics independently of insulin, Bloch et al. (1988) performed acute somatostatin infusions, which reduced plasma insulin and glucose concentrations without affecting fetal glucose utilization and umbilical uptake. Exogenous infusion of glucose did not produce an insulin response, but fetal glucose utilization still increased. Chronic withdrawal of insulin via streptozocin injection into fetuses results in fetal hypoinsulinemia, hyperglycemia and reduced fetal glucose utilization and oxidation of glucose carbon (Digiacomo & Hay, 1989; Hay & Meznarich, 1988). When insulin is directly infused into fetal circulation, there is increased fetal glucose utilization (in both sheep and rat models; reviewed by Kalhan, 2017).

The insulin-like growth factor system consists of two ligands (IGF1 and IGF2), four receptors (IGF1R, IGF2R, hybrid-A, and hybrid-B), and six binding proteins (IGFBP1 – IGFBP6; Chard, 1994). An overlap in binding affinity exists between insulin, IGFs and their receptors. While insulin and IGF1 exclusively bind to their own receptors (IR and IGF1R), IGF2 can bind to IGF1R, IGF2R and an IR isoform (IR-11; Hiden et al., 2009). This reciprocal interaction between receptors makes it difficult to consider effects the effects of insulin without considering IGFs. IGF1 and IGF2 mediate a variety of metabolic and mitogenic effects that promote fetal and placental growth and are synthesized in both the placenta and fetus (Hiden et al., 2009). IGF1 is present in the syncytiotrophoblast and cytotrophoblast across gestation, whereas IGF2 is not found in the syncytiotrophoblasts. Both have also been found in most fetal tissues, with IGF2 mRNA highly expressed in the liver, adrenal glands and skeletal muscle and IGF1 mRNA in the placenta and stomach (Han et al., 1988). In fetal sheep, IGF2 is highest earlier in gestation and decreases over time (Delhanty & Han, 1993), which supports IGF2's role as the primary growth factor in embryonic growth and IGF1's greater involvement in later fetal development (Gluckman and Pinal, 2003). While IGF1 and -2 are both found in fetal circulation early in gestation, by late gestation IGF2 concentrations are 3-10 fold higher than IGF1 in humans, sheep and many other species studied (Daughaday et al., 1982; Gluckman & Pinal, 2003; Owens et al., 1994). Glucose also regulates transcription of both IGF1 and IGF2 and therefore their plasma concentrations (Hay et al., 2016; Oliver et al., 1993). IGF1 and IGFBP3 levels at term are also directly correlated to birth weight in healthy and diseased states (Chard, 1994; Hiden et al., 2009). Numerous studies in pregnant sheep indicate IGF1 indirectly enhances glucose's capacity to promote fetal nitrogen balance and growth, including infusion of IGF1 into late gestation fetal sheep promoting both glucose and amino acid utilization (reviewed by Hay et al., 2016). In human FGR pregnancies, maternal serum IGF1 and –II levels were reduced, with increased circulating levels of IGFBP-1 (Holmes et al., 1997; Jones et al., 2007). In human and sheep studies of FGR, circulating concentrations of fetal insulin and IGF1 are reduced while IGFBP's are increased (reviewed by Chen & Jansson, 2017; Gluckman & Pinal, 2003), with IGFBP-1 in humans being most sensitive to insults to fetal nutrition (Chard, 1994). A uterine artery ligation model of FGR in rats exhibits reduced placental expression of IGF1R along with decreased placental glucose and amino acid transport (Reid et al., 2002).

Other energetic substrates utilized by the fetus

Glucose utilization by the fetus cannot account for all fetal oxidative metabolism. Calculation of the fetal glucose and O_2 quotient for fetal humans (0.8; Cetin et al., 2020) and sheep (approximately 0.6, but reducing with advancing gestation; Boyd et al., 1973) suggests that maternally acquired glucose is not sufficient for the oxidative metabolism of the fetus. Therefore, other energetic substrates are being used—such as lactate and amino acids (Hay et al., 2016; Kalhan, 2017).

While it is hard to obtain accurate measures of lactate in humans that reflects steady state fetal metabolism (samples are also often confounded by effect of labor), studies in pregnant sheep can be performed under non-stressed, steady-state conditions for investigating lactate concentrations. Lactate is a significant carbon source for the fetus, second only to glucose, and accounts for 25% of oxygen consumption (or 0.25 metabolic quotient; Burd et al., 1975; Char & Creasy, 1976). The placenta is the net producer of lactate for both fetal and maternal circulation. Fetal concentrations of lactate are higher than maternal, perhaps contributing to fetal oxidative and nonoxidative processes, such as glycogen synthesis or gluconeogenesis in the fetal liver (Battaglia and Meschia, 1978; Kalhan, 2017).

The fetal liver has enzymes required for synthesis and breakdown of glycogen. Studies have shown that glucose may not be the precursor of hepatic glycogen, and that perhaps lactate, pyruvate and alanine derived from peripheral glucose are serving as precursors (reviewed by Kalhan, 2017). A study in late gestation catheterized sheep fetuses showed that glycogenesis from glucose is partly "indirectly" from gluconeogenesis and that lactate may be an important glycogenic precursor, which fits with the high turnover rates of lactate in the sheep fetus (Kalhan, 2017; Levitsky et al., 1988). Glycogen synthesis is generally a low steady rate of accumulation in humans and sheep (presumably due to the larger, slow growing nature of their fetuses compared to other species; Hay et al., 2016).

As previously mentioned, amino acids are actively transported across the placenta. They also make up a large source of carbon for fetal growth and metabolism, can be used as substrates for glucose? and urea synthesis, as well as an energetic substrate for placental function (Kalhan, 2017). One study estimated that up to 25% of total fetal oxygen consumption in sheep fetuses is catabolism of amino acids (Gresham et al., 1972). In humans, amino acids are catabolized for urea synthesis, and one study using stable-isotope tracer methods found that endogenous alanine synthesis (a major glucogenic precursor & source of nitrogen for urea nitrogen) accounts for approximately 44% of alanine flux in the fetus at term (Gilfillan et al., 1985). This also suggests utilization of amino acids for energy metabolism in the fetus (Kalhan, 2017).

FETAL PANCREAS

Fetal organ development is dependent on plasma glucose concentrations, but later in gestation some organs (skeletal muscle, heart, liver) develop insulin sensitivity as fetal plasma insulin concentrations rise and increase fetal glucose utilization (Hay et al., 2016). Insulin produced by the fetus promotes systemic growth and therefore, fetal beta-cells link fetal nutrient supply with fetal nutrient metabolism and signal for growth (Boehmer et al., 2017). Successful fetal pancreatic development is critical for appropriate glucose homeostasis, with disruptions leading to metabolic disease into adult (Limesand & Davis, 2018).

Based on the current body of literature, fetal sheep pancreatic development mimics humans in the overlapping developmental transition periods of the pancreas and islets as well as timing of insulin and arginine responsiveness (Limesand and Davis, 2018). Pancreatic development can be separated into three time periods: the first and second (or primary and secondary) transition periods, followed by the third stage of maturation which extends postnatally (Gittes, 2009; Pin & Fenech, 2020).

The primary transition period begins with the foregut endoderm giving rise to the dorsal and ventral pancreatic buds between gestational day 26-31 in humans (Mehta et al., 2022) and before 24 days in sheep (Boehmer et al., 2017). The two buds fuse by week 6 in humans, and the majority of the pancreas is then derived from the dorsal bud, while the ventral bud becomes part of the pancreas head (Mehta et al., 2022). During the primary transition, some cells maintain their multipotency (multipotent pancreatic progenitor cells; MPCs), the number of which appears to dictate final pancreas size (Pin and Fenech, 2020). Other MPCs will eventually give rise to endocrine, acinar and duct cells. Glucagon containing alpha-granules are first seen during this time, reflecting high glucagon levels early in gestation that decline with pancreatic growth (Gittes, 2009).

The secondary transition period encompasses a broader period during gestation (approximately 20% of gestation to term) in humans and sheep compared to rats (E15-22), but is defined by presence of mature secretory products (such as zymogen granules), isletogenesis, and endocrine cell proliferation (Boehmer et al., 2017). At this point, the majority of cells will have specified into endocrine, acinar, or duct cells, with only a small population of MPCs remaining (Pin and Fenech, 2020). This transition begins with significant expansion and branching of the pancreatic bud, development of individual islet cells from the pancreatic epithelium, and expression of hormones and digestive enzymes. In humans, beta-cell proliferation begins around 8 weeks of gestation and continues throughout gestation. Islets begin forming around 11 weeks (Limesand and Davis, 2018). Human fetal beta-cells begin synthesizing and secreting insulin in addition to becoming responsive to glucose around mid-gestation. In sheep, beta-cells begin responding to glucose and arginine around mid-gestation as well (Limesand and Davis, 2018). Glucagon, which opposes insulin action and promotes gluconeogenesis in the liver, is detected in human fetal pancreases around 6 weeks and is secreted into fetal circulation by 14 weeks (Limesand and Davis, 2018; Cilvik et al., 2021)

From the secondary transition until birth and postnatally, the islets of the pancreas proliferate and mature, comprising the glucagon secreting alpha-cells, insulin secreting beta-cells, somatostatin secreting delta-cells, ghrelin secreting epsilon-cells, and pancreatic polypeptide cells (Pin and Fenech, 2020). The islets make up only 1-2% of the pancreas, with the rest being predominantly acinar and duct cells (exocrine pancreas; Mehta et al., 2022). The exocrine pancreas is responsible for secretion of digestive enzymes and the duct system that delivers them. These include: amylase, pancreatic triglyceride lipase, colipase, trypsinogen, chymotrypsinogen, carboxypeptidase A1 and A2, and elastase (Mehta et al, 2022).

Pancreatic development and function (particularly beta-cells) are closely associated with glucose and insulin homeostasis and are therefore often impacted by pregnancy complications such as FGR. Nutrient restriction and chronic hypoglycemia (seen in fetuses from placental insufficiency and FGR) has been shown to reduce beta-cell mass and insulin secretion (Limesand et al., 2006; Hay et al., 2016). Beta-cell development may also be delayed (Holemans et al., 2003). A study in fetal sheep investigated beta-cell responsiveness to hypoglycemia for 14 days (H group), followed by a 5 day euglycemic recovery period (R group; Limesand and Hay, 2002). Hypoglycemia reduced fetal insulin concentrations, which were recovered during the recovery period. However, an effect of programming was found on the pancreas, with impaired insulin secretion responsiveness to glucose and arginine. Pancreatic islets from FGR fetal sheep have deficient glucose metabolism and insulin secretion due to impaired glucose-stimulated glucose oxidation rate, insulin biosynthesis and insulin content, representative of beta-cell dysfunction (Limesand et al., 2006). These studies highlight the pancreatic beta-cells as a potential target for nutritional adaptations *in utero*, with complications that could persist into adulthood.

In the absence of a pancreas (pancreatectomy), chronic decreases in fetal plasma insulin result in increased fetal plasma glucose concentrations and decreased glucose utilization, leading to reduced peripheral tissue insulin sensitivity and glucose utilization capacity (Hay et al., 2016). However, when pancreatectomized fetal lambs are supplemented with exogenous insulin, fetal growth is rescued (Fowden et al., 1989). In another study of pancreatic agenesis, not only is fetal insulin reduced, but placental weight was reduced (Hiden et al., 2009).

Important genes in pancreatic development

Several transcription factors and cell signaling molecules are associated with successful development of the fetal pancreas, though as our knowledge of pancreatic development expands, others have been discovered. A brief overview of a few essential ones is provided here, thanks in part to reviews by Pin and Fenech (2020) and Mehta et al. (2022).

Early pancreatic tissue growth, especially acinar cells, requires involvement of *WNT/ CTNNB1*. Its inhibition allows for pancreatic (and liver) development, but later activation is needed for cellular growth. Overactive *WNT* signaling has been associated with pancreatic agenesis or hypoplasia, affecting both endocrine and exocrine development (Gittes, 2009). Around the same timeframe, signaling from the notochord decreases activity of Sonic Hedgehog (*SHH*), allowing for future expression of pancreas-duodenum homeobox 1 (*PDX1;* aka insulin promoter factor 1). *SHH* mutants have increased pancreatic mass and endocrine cell numbers, which may lead to congenital pancreatic malformations and glucose intolerance (Hebrok et al., 2000). *PDX1* is essential for early pancreas formation and is found in MPCs, duct and endocrine cells. Mutations in

PDX1 have been associated with MODY (maturity onset diabetes of the young; Fujitani, 2017), and when removed in mice by targeted mutagenesis, results in embryos that completely lack a pancreas (Slack, 1995) . Genes involved with defining developing cell types in the pancreas include forkhead box protein A2 (*FOXA2*), SRY-related homeobox 9 (*SOX9*), cytokeratin 19 (*CK19*), and hepatocyte nuclear factor 1b (*HNF1B)*, all found in ductal cells. Mutations in *HNF1B* have also been linked to MODY (Mehta et al., 2022). Transcription factor *GATA4* is involved in early dorsal and ventral pancreatic bud formation, then later in acinar cells. The transcription factors *PDX1*, *HNF1B*, *PTF1A,* and *SOX9* have all been implicated in pancreatic malformation and agenesis (Mehta et al., 2022). Notch signaling is associated with specification in pancreas, particularly in promoting *PTF1A* function and repressing neurogenin-3 (*NEUROG3*) expression, both of which are key transcriptional regulators of exocrine and endocrine islet cell differentiation (respectively; Gittes, 2009; Pin and Fenech, 2020; Mehta et al., 2022). Hepatocyte growth factor (*HGF*) signaling has also been found to support beta-cell formation (Gittes, 2009).

IN-VIVO ASSESSMENT OF MATERNAL, PLACENTAL, AND FETAL METABOLISM

As mentioned in previous sections, sheep models of FGR have contributed an immense amount of knowledge to our understanding of human FGR pregnancies. One of the driving forces behind the successful and robust assessment of maternal-placental-fetal interactions has been the ability to take repeated blood samples from both maternal and fetal sides of the placenta due to the ability to surgically place catheters in pregnant sheep (reviewed in Tanner et al., 2022). This allows for repetitive and simultaneous samples of both maternal and fetal arterial and venous blood from animals under non-stressed, non-anesthetized pregnancies. Early studies using these methods aided in our understanding of the placenta as a highly metabolic organ, instead of a mere conduit of nutrient delivery (Battaglia & Meschia, 1978; Meschia et al., 1965). More recently, the method used for investigating maternal-fetal-placental interactions and metabolism is the transplacental diffusion technique, which, coupled with applying the Fick principle, allows for the calculation of specific substrate uptakes (reviewed by Tanner et al., 2022). Finally, recent developments in applying *in-vivo* RNA interference to produce placental-specific gene deficiency models in pregnant sheep now allow us to have a more direct assessment of "cause and effect" relationships.

In-vivo assessment of placental function in pregnant sheep under non-stressed, non-anesthetized conditions applies a technique of surgically placing indwelling catheters, followed by infusion of tracer molecules until steady states concentrations are achieved – known as the transplacental diffusion technique (Battaglia & Meschia, 1978, 1988; Meschia et al., 1965, 1980). While the complexity of the surgical preparation varies across studies, the technique has also been improved over the years, with more catheters being used for more expansive metabolic studies. The minimal amount for measuring uterine and umbilical blood flows includes placement in the umbilical vein, fetal descending aorta (a proxy for umbilical artery), the uterine vein, and the maternal femoral artery (a proxy for uterine artery), along with an additional catheter into the fetal femoral vein for tracer infusion (Tanner et al., 2022). The tracer molecule must be freely diffusible but metabolized or produced within the exchanger, therefore making the transplacental diffusion rate blood flow dependent and easily calculated by the difference between infusion and escape rates of the tracer (Battaglia & Meschia, 1986; Tanner et al., 2022). The tracer is infused into the fetal femoral vein and mixes with other venous blood in the atria and ventricles of the fetal heart, resulting in the highest concentrations of tracer molecules in the umbilical artery, followed by the umbilical vein, uterine vein, and uterine artery, respectively (reviewed by Tanner et al., 2022).

The transplacental diffusion technique also employs the Fick Principle to calculate blood flow and specific substrate uptake rates. It is considered the gold standard in research for investigating placental and fetal physiology because it addresses both blood flow and nutrient transport. The Fick Principle is defined as the quantity of a substrate entering an organ (via arterial blood) equals the quantity of the substrate's extraction from the blood by the organ (i.e. for metabolism) plus the quantity of the substrate leaving the organ (via venous blood). To calculate the uptake rate of a given substrate by the uterus (uteroplacental unit) or umbilical circulation (the fetus), rate of blood flow is multiplied by the arteriovenous differences of the substrate. Calculation of uteroplacental utilization is therefore the difference between uterine and umbilical uptakes. Plasma concentrations of glucose must be converted to whole blood concentrations before calculating uptakes to account for rapid erythrocyte uptake and utilization, whereas amino acids and lactate concentrations do not need adjustments. Another useful calculation is fetal nutrient:oxygen quotients, which represent the ratio of fetal substrate uptake rates to fetal oxygen consumption rates (Meschia et al., 1980; Battaglia and Meschia, 1986; Cilvik et al., 2021; reviewed by Tanner et al., 2022). Table 1.1 summarizes these calculations.

Table 1.1 Calculations for blood flow, nutrient uptake, utilization and quotients

Blood Flow ($^3H_20$ tracer)	
R_{inf} $^3H_20$ (dpm/min)	Pump rate x [infusate]
$R_{acc(f)}$ (dpm/min)	α_{pl} slope * (0.8 * fetal weight)
$R_{acc(m)}$ (dpm/min)	Racc(f) + [αpl slope * 0.8(uterine weight)]
Umbilical Blood Flow (UBF; mL/min)	$(R_{inf} – R_{acc(f)}) / ([^3H_2O]_{\alpha(WB)} - [^3H_2O]_{\gamma(WB)})$
Umbilical Plasma Flow (UPF; mL/min)	$UBF * [1 – Hct_{f(avg)}]$
Uterine Blood Flow (UtBF; mL/min)	$(R_{inf} – R_{acc(m)}) / ([^3H_2O]_{V(WB)} - [^3H_2O]_{A(WB)})$
Uterine Plasma Flow (UtPF; mL/min)	$UtBF * [1 – Hct_{m(avg)}]$
Nutrient uptakes and utilization (Fick principle)	
Umbilical Oxygen Uptake (fetal oxygen utilization; UOU; mmol/min)	$UBF \ast ([O_2]_{\gamma(WB)} - [O_2]_{\alpha(WB)})$
Uterine Oxygen Uptake (UtOU; mmol/min)	$UtBF \ast ([O_2]_{A(WB)} - [O_2]_{V(WB)})$
Uteroplacental Oxygen Utilization (mmol/min)	UtOU – UOU
Plasma to WB Glucose Conversion	$[G]_{pl} \ast [1 - (0.24 \ast Hct)] - (3.3 \ast Hct)$
Umbilical Glucose Uptake (UGU; μmol/min)	$UBF \ast ([G]_{\gamma(WB)} - [G]_{\alpha(WB))}$
Uterine Glucose Uptake (UtGU; μmol/min)	$UtBF \ast ([G]_{A(WB}) - [G]_{V(WB)})$
Uteroplacental Glucose Utilization (μmol/min)	UtGU − UGU
Umbilical Lactate Uptake (μmol/min; ULU)	$UBF \ast ([L]_{\gamma(pl)} - [L]_{\alpha(pl)})$
Uterine Lactate Secretion (μmol/min; UtLS)	$UtBF \ast ([L]_{V(pl)} - [L]_{A(pl)})$
Uteroplacental Lactate Production (μmol/min)	ULU + UtLS
Umbilical AA Uptake (μmol/min; UAAU)	$UPF \ast ([AA]_{\gamma(pl)} - [AA]_{\alpha(pl)})$
Uterine AA Uptake (μmol/min; UtAAU	$UtPF \ast ([AA]_{A(pl)} - [AA]_{V(pl)})$
Umbilical AA Carbon Uptake (μmol/min; UCU)	(#AA carbons) ∗ UAAU
Uterine AA Carbon Uptake (μmol/min; UtCU)	(#AA carbons) ∗ UtAAU
Umbilical AA Nitrogen Uptake (μmol/min; UNU)	(#AA nitrogens) ∗ UAAU
Uterine AA Nitrogen Uptake (μmol/min; (UtNU)	(#AA nitrogens) ∗ UtAAU
Fetal nutrient:oxygen quotient	
Glucose:oxygen quotient (G:O)	6 × ([G]γ (WB) – [G]α(WB))/([O2]γ (WB) – [O2]α(WB))
Lactate:oxygen quotient (L:O)	3 × ([L]γ (pl) – [L]α(pl))/([O2]γ (WB) – [O2]α(WB))
Amino acid:oxygen quotient (AA:O)	Q × ([AA]γ (pl) – [AA]α(pl))/([O2]γ (WB) – [O2]α(WB))
Total nutrient:oxygen quotient	G:O quotient + L:O quotient + Total AA:O quotient

Metabolic studies

Another advantage to the chronically catheterized sheep is that after steady-state baseline assessments of metabolism have been done, it is easy to clamp maternal or fetal nutrient concentrations at varying concentrations. The focus of this dissertation is placental glucose transfer, therefore, the methodology of investigating fetal insulin response to fetal hyperglycemia and arginine infusions will be discussed. These protocols have been briefly discussed in Tanner et al. (2022) and were used in studies outlined by Cilvik et al. (2021). The metabolic study begins with blood samples from all four catheters (umbilical artery and vein, α, γ, respectively, and uterine artery and vein, A ,V respectively). As discussed in the previous section, continuous infusion of ^3H_2O is then started to measure uterine and umbilical blood flow via the transplacental diffusion technique. Once a baseline, steady state infusion is achieved, blood samples (usually every 15 minutes for a total of four samples) are taken representative of “baseline.” To produce a fetal hyperglycemic clamp, dextrose is infused and titrated for each fetus individually (by taking repeated blood samples) to create a steady state of fetal hyperglycemia, which is referred to as “Glucose Stimulated Insulin Secretion (GSIS).” Blood samples (usually four every 15 minutes) are taken during the GSIS period. To assess maximal fetal insulin production during this GSIS state, an arginine bolus is then administered to produce “Argine Stimulated Insulin Secretion (ASIS)”, where blood samples are taken continuously to capture, ideally, a spike in insulin secretion. In the case of the study outlined in chapter 3 of this dissertation, the metabolic study concludes with termination and tissue collection (see also Figure 3.1). Combinations of these methods in chronically catheterized pregnant sheep have been paramount to much of our knowledge of the transfer and uptake of various nutrients, and much of the knowledge discussed earlier in this literature review).

Approaches to modifying placental nutrient transfer

Assessing the function of specific genes in the placenta has been difficult, as classical methods for gene ablation and whole genome editing using CRISPR-Cas9 methods, are not currently feasible for organ/tissue-specific studies in sheep. However, one method that has been used recently to successfully assess gene function in the ruminant placenta is lentiviral-mediated RNA interference (RNAi), produced by viral infection of the trophectoderm layer of hatched blastocysts using a lentivirus expressing short-hairpin RNA (shRNA). This was first reported by Purcell et al (2009) to produce proline-rich 15 (PRR15) RNAi pregnancies in sheep, demonstrating the requirement of PRR15 for conceptus elongation.

This method takes advantage of the natural processes of how endogenous micro-RNAs (miRNAs) are generated, processed, and function. Briefly, when shRNAs are introduced into cells (in this case, via lentiviral vectors), they are processed in the same way as miRNAs, negatively regulating gene expression. The shRNA synthesized in the nucleus of the infected cell, leads to either targeted mRNA degradation or mRNA translational repression (Anthony et al., 2010; Paddison et al., 2002). This ultimately results in a deficiency of the target gene product, leading to reduced protein expression. There are two major advantages of this approach. First, the ability to target specifically the trophectoderm cells of the blastocyst, leaving out the inner cell mass, since lentiviral vector used is incapable of replicating within the host cell. The second is stable integration into the host genome, creating a stable, placenta-specific expression of shRNA throughout gestation (reviewed by Tanner et al., 2022). Variations of this method have been successfully employed to investigate the roles of specific genes in the placenta by our lab, resulting in the combination of lentiviral-mediated RNAi with *in-vivo* assessment of placental function (Baker et al., 2016; Jeckel et al., 2018; Tanner et al, 2021 a & b, 2024).

Most recently, this approach has been used to generate *SLC2A3*-deficient pregnancies through mid-gestation in sheep (75 days of gestational age, dGA; Lynch et al, 2022). In this study, a phenotype of FGR was observed, where fetuses were smaller, hypoglycemic, hypoinsulemic and hypoglucagonemic. Umbilical artery concentrations of IGF1 were not impacted, but CSH was increased 70%. Fetal liver weights were not different, but pancreases were significantly smaller (23%). While placental weight was less, the difference did not reach statistical significance. This study was the impetus for the two studies outlined in the subsequent chapters of this dissertation.

SUMMARY

As previously discussed, the fetus must rely on glucose delivered from the maternal system via glucose transfer mediated by *SLC2A1* and *SLC2A3*. Investigation of a deficiency in placental uptake and transfer of glucose by *SLC2A3* -RNAi revealed negative consequences on fetal development at mid-gestation, with impacts on fetal pancreatic growth. Therefore, our objectives for the following two studies are to further investigate the impact of *SLC2A3*-RNAi on: 1) the transcriptome of the fetal pancreas from pregnancies at mid-gestation, and 2) impacts on maternal, placental, and fetal metabolism near-term. We hypothesize that the effects of *SLC2A3*-deficiency observed at mid-gestation will have transcriptomic consequences on the fetal pancreas influencing both growth and function. Altered trajectories of pancreatic growth and function by mid-gestation could potentially have negative consequences for not only fetal development and glucose homeostasis during the remainder of pregnancy, but also post-natal influences. After carrying out *SLC2A3*-RNAi pregnancies to near-term, we hypothesize the impaired fetal growth may persist at the detriment of fetal development and metabolism, particularly glucose homeostasis, as well as potentially influencing placental and maternal metabolism.

CHAPTER II: FETAL HYPOGLYCEMIA INDUCED BY PLACENTAL SLC2A3 RNA INTERFERENCE ALTERS FETAL PANCREAS DEVELOPMENT AND TRANSCRIPTOME AT MID-GESTATION

INTRODUCTION

Functional placental insufficiency is a major cause of intrauterine growth restriction (IUGR), which commonly results in hypoglycemic fetuses due to reduced placental transfer of glucose (Limesand et al., 2006, 2007; Marconi & Paolini, 2008). Sheep models of IUGR have been able to recapitulate many of the same challenges that human pregnancies with IUGR experience, particularly fetal hypoglycemia (Limesand et al., 2006, 2007; Tanner et al., 2021a). Glucose, the primary energy substrate for fetal and placental oxidative processes and growth, is not produced endogenously until near term and therefore relies on transfer from the maternal circulation. In sheep, this is accomplished through a maternal-to-fetal glucose concentration gradient and facilitative glucose transporter proteins on both maternal-facing apical microvillus SLC2A3 (GLUT3) and fetal-facing basal trophoblast membranes SLC2A1 (GLUT1; (F. B. P. Wooding et al., 2005). The distinct localization of these two glucose transporters makes the sheep placenta an ideal model for studying the relative importance of apical versus basolateral glucose transport.

The transport of glucose from the maternal circulation plays a crucial role across different stages of pancreatic development. During the primary transition period (before 24 days gestational age [dGA] in sheep; 25-26 dGA in humans), glucose is needed for early organogenesis, endocrine cell development (including early insulin expression), and proliferation of multipotent progenitor cells, which have been shown to directly contribute to the mature size of the pancreas (Limesand & Hay, 2002).

During the second transition period (approximately 20% of gestation to term) is when isletogenesis, branching, and full differentiation of β- and α- cells occur, including the start of β-cell responsiveness to glucose (Limesand & Hay, 2002). All of these are essential processes to prepare the fetus for regulation of glucose metabolism later in pregnancy and into adult life. Perturbed glucose transport across gestation can therefore result in com-pounding insults to pancreatic growth and function. Studies of IUGR's impacts on the fetal pancreas in multiple species have demonstrated inappropriate insulin response to glucose, likely due to pancreatic insufficiency from β-cell deficiency (Limesand et al., 2006) and global reduction in pancreatic endocrine tissue mass {Citation} (9, 10).

Our laboratory has previously published a sheep model of diminished placental SLC2A3 glucose transport at mid-gestation (75 dGA) using in-vivo lentiviral-mediated RNA interference (RNAi; Lynch et al., 2022), which resulted in smaller, hypoglycemic fetuses with reduced umbilical artery insulin and glucagon concentrations, in addition to diminished pancreas weights. These findings lead us to subject RNA derived from fetal pancreases from both SLC2A3-RNAi and NTS-RNAi pregnancies to real-time quantitative PCR (qPCR) and transcriptomic analysis to further elucidate the impact of placental SLC2A3 deficiency on fetal metabolism. We hypothesized that the impact of hypoglycemia on the fetal pancreas will have a global impact on genes related to growth and function as well as a direct impact on mRNA expression of glucagon and insulin.

MATERIALS AND METHODS

All procedures conducted with animals were approved by the Colorado State University Institutional Animal Care and Use Committee (Protocol 1483), as well as the Institutional Biosafety Committee (17-039B).

Generation of Lentivirus and SLC2A3 RNAi Pregnancies

Lentiviral generation and titering of *SLC2A3*-RNAi and NTS-RNAi are as described previously (Jeckel et al., 2018) with the shRNA sequences for SLC2A3-RNAi and NTS-RNAi constructs previously reported (Lynch et al., 2022). Animal management, estrus synchronization, embryo transfers, and generation of SLC2A3-RNAi and NTS-RNAi pregnancies were done as previously described (Baker et al., 2016; Jeckel et al., 2018; Lynch et al., 2022; Tanner et al., 2021). In summary, all ewes (Dorper breed composition) were group housed in pens at the Colorado State University Animal Reproduction and Biotechnology Laboratory, and were provided access to hay, trace minerals, and water to meet or slightly exceed their National Research Council (*Nutrient Requirements of Small Ruminants*, 2007) requirements. Lentiviral infection of day 9 hatched sheep blastocysts was used to stably integrate and express shRNA targeting SLC2A3 mRNA or a non-targeting sequence in the trophectoderm. Tissues from six NTS-RNAi (5 males and 1 female) and six SLC2A3-RNAi (3 males and 3 females) pregnancies were collected during terminal surgeries at 75 dGA (Lynch et al., 2022).

RNA Isolation

Frozen fetal pancreases were pulverized, and RNA was isolated using RNeasy Mini Kit (QIAGEN, Hilden, Germany) according to manufacturer's instructions. RNA concentration was quantified using the BioTek Synergy 2 Microplate Reader (BioTek, Winooski, VT, USA). RNA quality was measured by the 260/280 nm absorbance ratio, and RNA 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, with quality measured by the 260/280 absorbance ratio. An equal mass of cDNA (10 ng/μL) was used for each sample in the quantitative real-time PCR (qPCR) reaction. qPCR was performed using the CFX384 Real-Time System (BioRad). Forward and reverse primers for qPCR were designed using NCBI's Primer-BLAST tool (Ye et al., 2012) to amplify an intron-spanning product. Primer sequences and amplicon size are summarized in Table 2.1. Standard curves were generated as described previously(Jeckel et al., 2018). Briefly, a PCR product for each gene was generated using cDNA from pooled 75 dGA fetal pancreases 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 $1x10^{-5}$ pg 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; Jeckel et al., 2018). Normalized values were compared by unpaired Student's T-test, with $p \leq 0.05$ considered significant and a trend at $p \leq 0.10$.

Table 2.1 Primers and product sizes for cDNA used in qPCR

cDNA	Forward Primer (5'-3')	Reverse Primer (5'-3')	Product, bp
RPS15	ATCATTCTGCCCGAGATGGTG	TGCTTGACGGGCTTGTAGGTG	134
INS	GAGAGCGCGGCTTCTTCTAC	CGGGGCAGGTCTAGTTACAG	198
GCG	ACTCACAGGGCACATTCACC	CGGCGGAGTTCTTCAACGAT	274

RNA Sequencing, Alignments, and Analysis:

Assessment of the RNA samples integrity and quality, library generation and RNA-Seq (2x150 cycles, 80 million paired-end reads/sample; NovSeq6000, Illumina, San Diego, CA, USA) was conducted by the Genomics Shared Resource Core Facility, University of Colorado Anschutz Medical Campus (Aurora, CO). The sequencing data from this study have been deposited in NCBI's Gene Expression Omnibus (Edgar et al., 2002) and are accessible through GEO Series accession number GSE261932 (https://www.ncbi.nlm.nih.gov/geo/query/acc.cgi?acc=GSE261932).

RNA integrity was assessed by Agilent TapeStation (Agilent Technologies, Inc., Boulder, CO) and all samples had a minimum RNA integrity number (RIN) of ≥ 9.0. RNAseq analysis was performed on the Illumina NovaSEQ6000 platform (100,000,000 paired end reads) and FASTQ files were generated for each sample. Data were assessed for quality with FastQC tool version 0.11.9 (Babraham Bioinformatics), as well as with QIAGEN CLC Genomics Workbench software, version 21 (www. qiagenbioinformat-ics.com). CLC

Genomics workbench was then used for trimming of raw sequencing reads based on quality score (Q-score > 30), read length (≥ 15 nucleotides), and removal of adapter sequences. Raw sequence reads were mapped to the reference ovine genome (Ovis aries, Oar.ra_1.0, Baylor College of Medicine) with default software parameters applied. Expression data was normalized using the trimmed mean of M-values (TMM) normalization method (Robinson & Oshlack, 2010) and presented as fragments per kilobase of transcript per million mapped reads (FPKM).

Differential expression analysis for n=4 sample per group was performed using the CLC Genomics Workbench Differential Expression tool as well as the DESeq2 package (Love et al., 2014) in R studio (RStudio Team (2022), RStudio: Integrated Development for R. RStudio, PBC, Boston, MA URL http://www.rstudio.com/) for comparison. Thresholds for differentially expressed genes were a false discovery rate of $Q \leq 0.1$ and absolute fold-change of >1.5. Functional annotation of DEGs was performed using the Database for Annotation, Visualization, and Integration Discovery (DAVID, https://david.ncifcrf.gov/). Pathways were determined from the KEGG database (Ogata et al., 1999) and biological processes from GOTERM_BP_DIRECT annotation sets. Interaction networks were constructed with the Cytoscape software (Shannon et al., 2003). Finally, additional insight into altered networks was obtained by submitting the same list of DEGs to QIAGEN Ingenuity Pathway Analysis (Krämer et al., 2014) QIAGEN Inc., https://digitalinsights.qiagen.com/IPA). The threshold for considering a pathway significant was $p \leq 0.05$.

Quantitative Real-Time PCR:

Fetal pancreas insulin mRNA concentrations (Figure 2.1) were 67.6% lower in SLC2A3- RNAi pregnancies ($p \leq 0.05$), whereas there was a 40.4% difference in glucagon mRNA, although this was not statistically different.

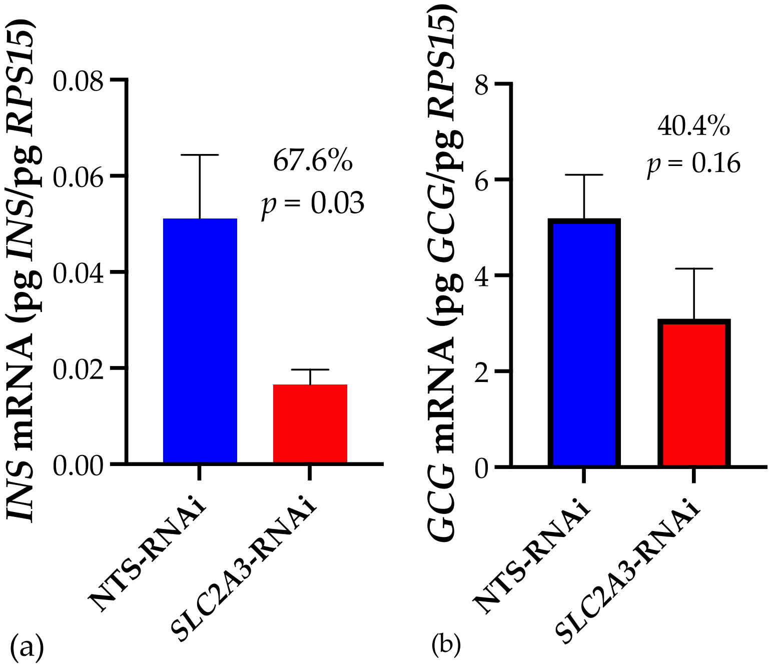

Figure 2.1 Impact of SLC2A3-RNAi on fetal pancreatic concentrations of (a) INS and (b) GCG, mRNA. Data are shown as means ± SEM. NTS, non-targeting sequence; RNAi, RNA interference

Transcriptomic Analysis:

A total of n= 4 samples per group were used for transcriptomic analysis. Approximately 157 million reads were sequenced from both groups. After adapter trimming and quality filtering, 100% of reads were retained. An average of 98.2% of the quality-controlled (QC) reads were mapped to the sheep reference genome. A summary of the total number of reads and the mapping results for each sample is presented in Table 2.1. Principal component analysis (PCA) and a hierarchical heat map (Figure 2.2) revealed samples from the two treatments grouped into two distinct clusters. A total of 771 genes met our thresholds of a false discovery rate (FDR) of Q ≤ 0.1 and absolute fold change (FC) of >1.5, with 714 being upregulated and 57 being downregulated, which is illustrated in the volcano plot in Figure 2.3. Notable DEGs are featured in Table 2.2. Due to few downregulated genes, gene ontological (GO) enrichment analysis revealed predominantly pathways affected by gene upregulation.

Table 2.2. Summary of sequence reads mapped to sheep reference genome.

Group	Sample I.D.	Raw Reads	Quality Control Reads	Mapping Rate (%)
SLC2A3- RNAi	2_472	201264985.3	185063154	98.26
	3_472	175336416.6	160783494	98.06
	5_472	190077663.5	175232598	98.24
	6_472	192195658	176704688	98.21
NTS-RNAi	7_SC	169767017.4	155846122	98.27
	8_SC	179660289.9	164874248	98.11
	11_SC	129489997.8	118975410	98.22
	12_SC	162587201.6	149141240	98.19

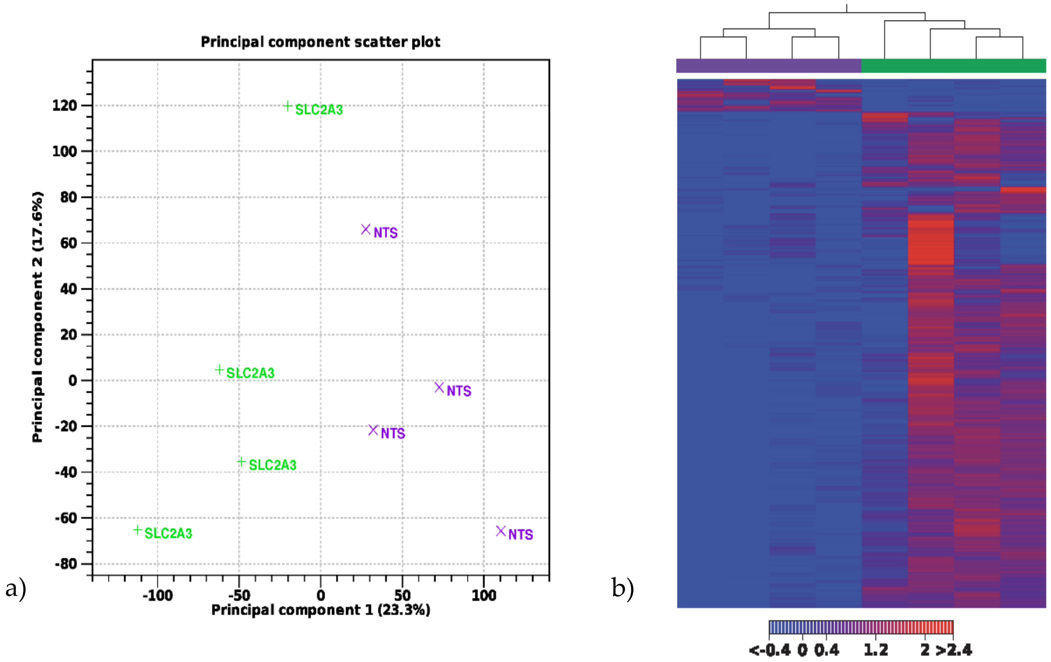

Figure 2.2. (a) Principal component analysis and (b) hierarchical heat map. Samples in purple denote NTS-RNAi, while green denotes *SLC2A3*-RNAi.

Biological processes (BP) identified by GOTERM_BP_DIRECT database included pathways associated with multiple metabolic processes, gluconeogenesis, digestion, and negative regulation of cell proliferation (Table 2.3). Twelve (17%) genes contributing to biological processes overlapped with more than one biological process.

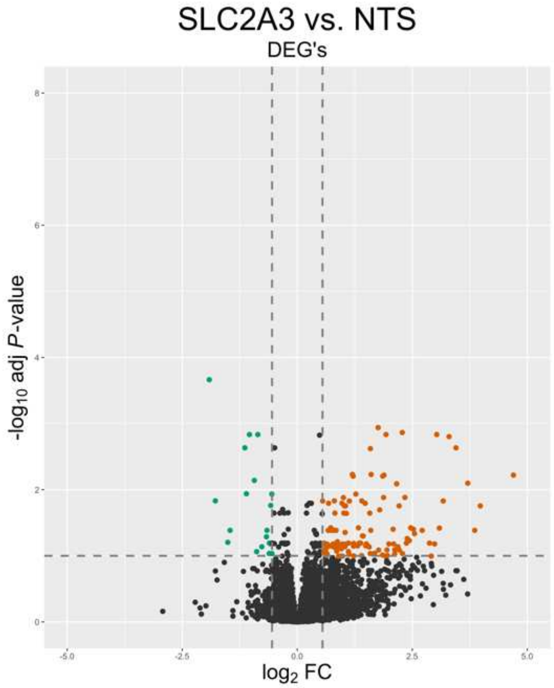

Figure 2.3. Volcano plot displaying differentially expressed genes (DEGs). Green denotes downregulated and red denotes upregulated.

Table 2.3. Notable Differentially Expressed Genes (DEGs).

Gene	Name	Category	Log2FC	FDR
CLPS	Colipase	Fat digestion and absorption	-0.80984	0.000412
CCK	Cholecystokinin	Pancreatic secretion	2.85173	2.48E-13
SHH	Sonic Hedgehog Signaling Molecule	Cellular development/ growth and proliferation; tissue development	1.39917	0.023658
PLCB3	Phospholipase C Beta 3	IP3-DAG signaling	1.17916	4.17E-12
SLC26A3	Solute Carrier Family 26 Member 3	Ion transport; pancreatic secretion	2.57893	4.02E-09
SLC27A2	Solute Carrier Family 27	PPAR signaling; fatty acid transport	2.60986	3.77E-10
SLC27A4	Solute Carrier Family 27	PPAR signaling; fatty acid transport	0.8981	0.0002589
TDO2	Tryptophan 2,3-dioxygenase	Tryptophan metabolism	4.23593	0.0088385
HAA0	Hydroxyacid Oxidase	Tryptophan metabolism; biosynthesis of cofactors	3.13823	7.24E-13
KYNU	Kynureninase	Tryptophan metabolism; biosynthesis of cofactors	2.62505	1.02E-07
HNF4A	Hepatocyte Nuclear Factor 4 Alpha	Negative regulation of cell proliferation	0.58768	0.0075142
ST18	ST18 C2H2C-type zinc finger transcription factor	Negative regulation of cell proliferation; apoptosis	-0.59897	0.0045875
HS3ST1	Heparan sulfate-glucosamine 3-sulfotransferase 1	Cell signaling; growth factor binding; tissue development	0.88156	0.0742444
CREB3L3	cAMP Responsive Element Binding Protein 3 Like 3	Glucagon signaling; insulin resistance; pancreatic secretion	1.3806	8.09E-12
FBP1	Fructose-bisphosphatase 1	Glucagon signaling; carbon metabolism; glycolysis / gluconeogenesis	0.6707	0.0013089
LDHA	Lactate dehydrogenase A	Glucagon signaling; glycolysis/gluconeogenesis	0.73	0.00382388
PCK1; PCK2	Phosphoenolpyruvate carboxykinase 1; 2	Glucagon signaling; insulin resistance; glycolysis / gluconeogenesis; PPAR signaling	2.2652; 1.4892	1.5541E-07; 2.31E-18
G6PC2	Islet-specific glu-cose-6-phosphatase catalytic subunit 2	Glucagon signaling; insulin resistance; glycolysis / gluconeogenesis; carbohydrate digestion	-0.6281	0.02096731

Table 2.4. GOTERM_BP_DIRECT Biological Processes.

Term	Count	*p*-Value	Genes	Fold Enrichment	FDR
Carbohydrate metabolic process	10	0.00353	*LDHA, MGAM, GNPDA1, SI, SLC3A1, SLC3A2, NPL, RENBP, FBP1, GLB1L3*	3.26251	0.44330
Cholesterol homeostasis	10	0.00003	*EHD1, ABCG8, ABCG5, DGAT2, LIPC, SOAT2, HNF4A, APOA2, ANGPTL3, ABCB11*	6.24377	0.03353
Negative regulation of cell proliferation	10	0.08406	*SLC9A3R1, HNF4A, CLDN19, PTK2B, PTH1R, PODN, ST18, SKAP2, ENPP7, BMP5*	1.88614	0.99922
Cell surface receptor signaling pathway	6	0.05824	*VIPR1, ADGRG7, EDN3, GLP2R, PTH1R, F2*	2.85899	0.99922
Lipid catabolic process	6	0.05054	*PLCB3, LIPC, PLA2G12B, CLPS, NEU1, PLBD1*	2.97648	0.99922
Cholesterol metabolic process	5	0.01663	*CUBN, LIPC, SOAT2, APOA2, ANGPTL3*	5.02971	0.97370
Fatty acid metabolic process	5	0.01510	*NAAA, CYP1A1, ANGPTL3, HACL1, ABCB11*	5.17341	0.92632
Gluconeogenesis	4	0.00888	*G6PC2, PCK1, FBP1, PCK2*	9.05347	0.71451
Phospholipid homeostasis	4	0.00379	*HNF4A, ANGPTL3, ITGB6, ABCB11*	12.07130	0.44330
Cell-cell signaling	3	0.08660	*SHH, GJB4, IHH*	6.03565	0.99922
Digestion	3	0.04159	*CLPS, CYM, AKR1D1*	9.05347	0.99922
Fatty acid transport	3	0.01449	*SLC27A6, SLC27A2, SLC27A4*	15.52024	0.92632

The top pathways identified by KEGG analysis of DEGs were predominantly related to metabolism (96 genes). Figure 2.4 presents the specific metabolic pathways identified by KEGG pathway analysis, excluding the overall metabolism category.

The interaction networks of DEGs involved in highly enriched pathways are presented in Figure 2.5, which demonstrates the interaction between genes from the different enriched pathways and their relation to the large number of genes contributing to the metabolic pathways term. Of the 245 genes contributing to the interaction networks in Figure 2.5, 37% (91 genes) had overlapping contributions to more than one enriched pathway.

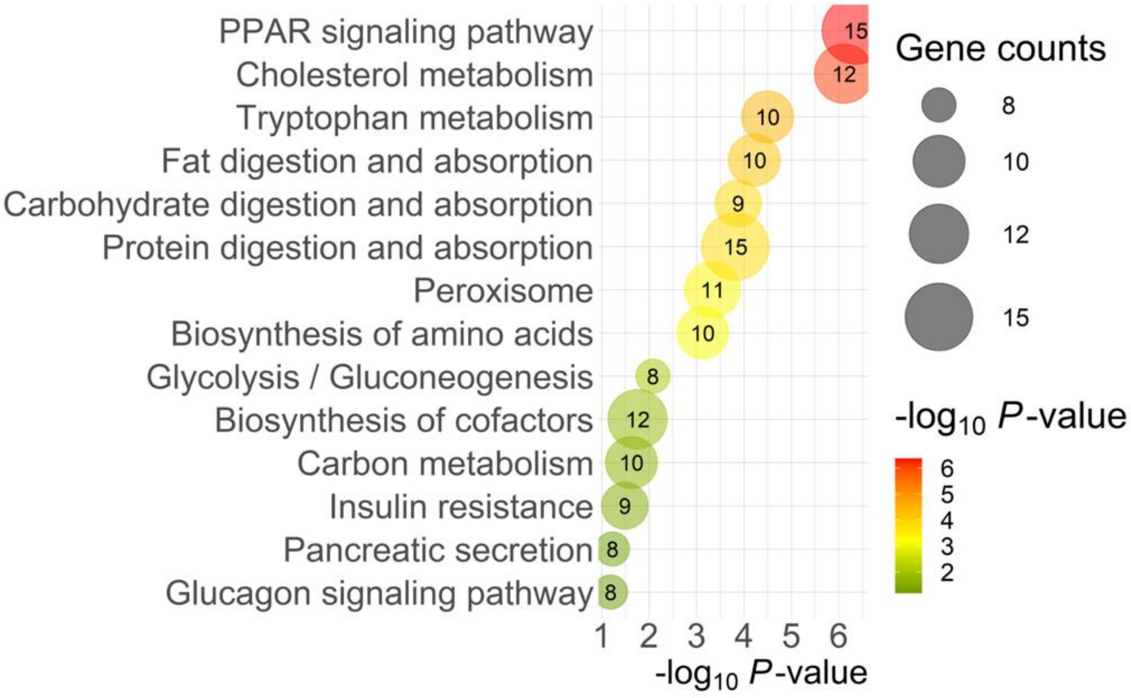

Figure 2.4. Top KEGG pathways from DAVID analysis. The color and size of each bubble represent the p-value and number of gene counts.

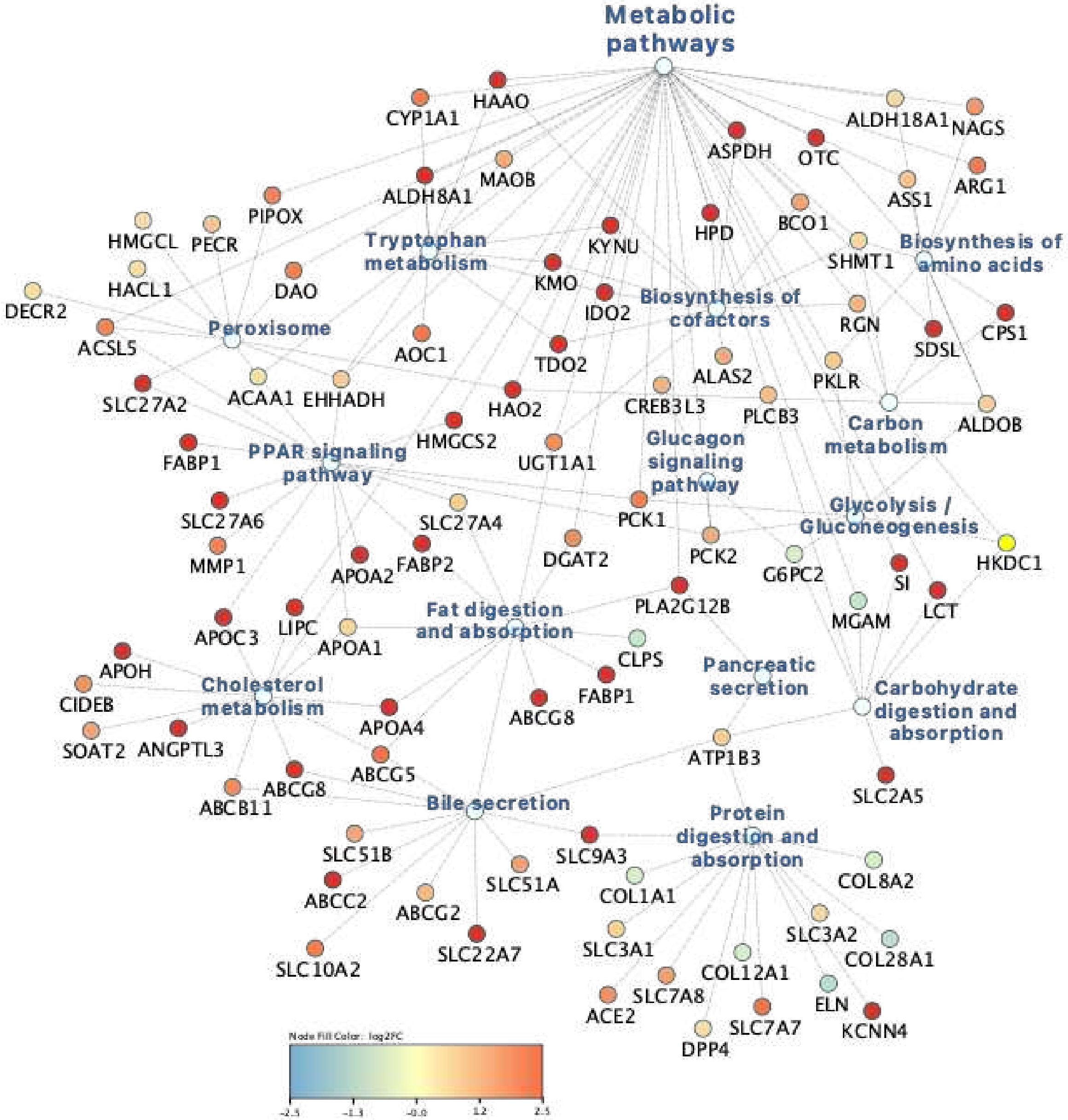

Figure 2.5. Cytoscape interactive network of DEGs involved in highly enriched pathways related to metabolism.

DISCUSSION

Glucose is the primary energy substrate for fetal and placental development and growth (Hay, 2006). Its delivery to the fetus is reliant upon the maternal-to-fetal concentration gradient enabled by facilitative glucose transporters. In sheep, the locations of SLC2A3 and SLC2A1 on the trophoblast membranes create an ideal model for investigating the relative importance of apical (SLC2A3) compared to basolateral (SLC2A1) glucose transport (F. B. P. Wooding et al., 2005). The importance of placental glucose uptake, transport and metabolism is highlighted in IUGR pregnancies, in which the degree of fetal hypoglycemia correlates with the severity of IUGR (Economides & Nicolaides, 1989; Marconi & Paolini, 2008). Glucose availability also plays a crucial role to different stages of pancreatic development. During the primary transition period, glucose is essential for organogenesis, endocrine cell development and proliferation of multipotent progenitor cells. The second transition period sees the differentiation of β- and α- cells and the beginning of β-cell responsiveness to glucose, setting the stage for regulation of glucose metabolism later into pregnancy and postnatal life (Boehmer et al., 2017; Dassaye et al., 2016).

We previously established (Lynch et al., 2022) that diminished placental SLC2A3 concentrations during the first half of gestation results in smaller, hypoglycemic fetuses, that exhibit reduced umbilical artery insulin and glucagon concentrations, and smaller pancreases. This led us to hypothesize that those pancreases were enduring a global effect of hypoglycemia on development and growth, not simply impaired β-cell function. Our current results of 67.6% reduction in insulin mRNA and 40.4% reduction in glucagon mRNA in SLC2A3-RNAi fetal pancreases (Figure 2.1), align with the reported reductions in those hormones in umbilical arterial blood (Lynch et al., 2022). Reduced insulin secretion, smaller pancreases, and pancreatic dysfunction have all been shown to be hallmarks of the IUGR fetus (Boehmer et al., 2017). A sheep model of near-term IUGR demonstrated impaired insulin secretion as a result of significantly reduced insulin content of the fetal pancreases, with deficient insulin storage and/or biosynthesis, in addition to decreased β-cell mass (Limesand et al., 2006). Other sheep models demonstrate hypoglycemic fetuses' insulin secretion is diminished despite similar numbers of β-cells, that did not fully recover even when glucose was returned to normal concentrations, revealing hypoglycemic programming of fetal β-cell function (Limesand & Hay, 2002). Chronic hypoglycemia in similar studies have bolstered this β-cell programming effect in addition to decreased β-cell mass and impaired glucose-stimulated insulin secretion (GSIS; (Boehmer et al., 2017). Clearly the smaller pancreases observed in the hypoglycemic, SLC2A3-RNAi fetuses could have smaller β-cell mass and therefore ability to secrete insulin at sufficient levels.

Therefore, we conducted a transcriptomic analysis of fetal pancreases from SLC2A3- and NTS- RNAi pregnancies to further investigate how fetal hypoglycemia during the first half of gestation contributed to impaired pancreatic growth and function. Our findings demonstrate impacts on pathways related to the critical transition periods of pancreatic development during early to mid-gestation, including both organogenesis and pancreatic function, and altered pancreatic metabolism, with potential long-term impacts on glucose homeostasis. In the context of early pregnancy and pancreatic organogenesis, our analysis revealed several pathways associated with cellular signaling and growth. SHH, an important ligand in hedgehog signaling, was significantly upregulated (log2FC = 1.399). Hedgehog signaling regulates growth, differentiation, and function in many organs in both the fetus and adult, however, in the pancreas, SHH's role in pancreatic development is more complex. Studies from mice have demonstrated that increased hedgehog signaling antagonizes organogenesis, and SHH-specific inhibition is a prerequisite for pancreas formation, with other hedgehog ligands (i.e. IHH) likely promoting pancreatic formation (Gittes, 2009; Hebrok et al., 2000). Upregulation of SHH in the SLC2A3-RNAi fetal pancreas fits with these studies in mice and could help explain the reduced pancreas size and initial organogenesis during fetal hypoglycemia (Lynch et al., 2022).

The hepatocyte nuclear factor (HNF) family of transcription factors have been implicated in the early stages of organ formation, including interacting with crucial pancreatic transcription factors such as PDX1, a marker of pancreatic progenitor cells critical to β-cell determination (Dassaye et al., 2016; Mohan et al., 2018; Shih et al., 2013)Hepatocyte Nuclear Factor 4 Alpha (HNF4α) was differentially expressed (log2FC = -0.599) and in analysis of biological processes, contributed to pathways associated with negative regulation of cell proliferation, phospholipid, and cholesterol homeostasis. A study investigating transcriptional regulatory networks of pancreatic islets found that HNF4α contributes to a large fraction of pancreatic islet transcriptomes by binding directly to nearly half of the actively transcribed genes (Odom et al., 2004). HNF4α's large influence on organogenesis and the development of functional β-cells has also been shown in many mouse studies. One such study, by Chen et al. (W. S. Chen et al., 1994), demonstrated that homozygous germline ablation of HNF4α is embryonic lethal, even before pancreas development. However, β-cell specific ablation of HNF4α resulted in hyperinsulinemia in utero and reduced blood glucose levels at birth (Maestro et al., 2007). Additionally, mutation of HNF4α in people is associated with MODY (maturity onset diabetes of the young; Dassaye et al., 2016; Maestro et al., 2007; Odom et al., 2004), further demonstrating its crucial contribution to glucose homeostasis. These studies highlight the broad influence of HNF4α on the pancreatic transcriptome and could explain why changes in its expression, specifically the downregulation seen in our study, could have such a large impact on both growth and function of the fetal pancreas.

The secondary transition period of the fetal pancreas overlaps with the first transition period in both sheep and humans and occurs over a broad period from around 20% of gestation to term (Boehmer et al., 2017; Peterson et al., 2021). It is characterized by growth, branching, and differentiation into the different pancreatic cell lineages that coincide with an increase in endocrine and exocrine gene expression (Dassaye et al., 2016; Gittes, 2009). In our model, many genes and pathways specifically associated with pancreatic function were affected, in addition to metabolism and delivery of energetic substrates. KEGG pathways analysis revealed several genes contributing to pancreatic secretion: PLCB3, PLA2G12B, CLCA1, ATP1B3, ITPR3, CCK, ATP2B1, SLC26A3, some of which overlap with other biological processes were identified. For example, PLCB3 and

PLA2G12B both contribute to lipid catabolic processes, demonstrating the complex interactions between intracellular signaling and pancreatic function. Arguably the most notable DEG in this list is CCK, which was up-regulated (log2FC = 2.852). CCK acts on numerous tissues in the body but is primarily involved in metabolism and digestion. It is best known for stimulation of exocrine pancreas enzyme secretion but has also been shown to stimulate adaptive pancreatic growth in neonatal and adult rodent models (Williams, 2019). Little research exists on the role of CCK during pancreatic development, especially in sheep, however, studies in mice have shown expression of CCK as a marker of endocrine cell lineage early on, as well as expression in acinar and α-cells in later stages (Liu et al., 2001). A study utilizing human pancreatic tissues reported that CCK-B/Gastrin receptors mediate autocrine effects of gastrin on the developing islets in the fetal pancreas, implicating an important role for CCK in the regulation of glucose homeostasis (Saillan-Barreau et al., 1999).

Another pathway highlighted by KEGG analysis related to intercellular signaling was Peroxisome Proliferator-Activated Receptor (PPAR) signaling. PPAR signaling is involved in lipid homeostasis during development throughout the body, and disruption of PPARs has been linked to metabolic diseases, such as lipid accumulation in the pancreatic β cells with implications for diabetes (Venezia et al., 2021). In zebrafish development, disruption of PPAR signaling results in ligand-specific impaired endocrine and exocrine pancreas development and disrupted gene expression of key developmental genes for the pancreas (i.e. PDX1; Venezia et al., 2021). Additionally, PPARβ/δ knockout mice have impaired insulin secretion (Iglesias et al., 2012). In our model, multiple genes related to peroxisomes and PPAR signaling were upregulated (Figure 2.4), including some overlapping contributions from ACAA1 (Acetyl-CoA acyltransferase 1), EHHADH (enoyl-CoA hydratase and 3-hydroxyacyl CoA dehydrogenase), and SLC27A2. ACAA1 and EHHADH are both involved in β-oxidation throughout the body and are likely contributing to lipid metabolism within the pancreas, in concert with SLC27A2's role in fatty acid transport. This further demonstrates the complexity and interconnectedness of intercellular signaling and substrate transport with pancreatic growth, metabolism, and function.

Many biological processes (Table 2.2) related to lipid metabolism were affected, including phospholipid homeostasis, lipid catabolic processes, fatty acid metabolic processes, and transport. KEGG pathways analysis (Figure 4) also revealed many genes contributing to fat digestion and absorption pathways. Free fatty acids (FFA's) in particular have complex actions in the pancreas, but nevertheless are important regulators in glucose metabolism and insulin release, and the activation of their receptors directly impacts pancreas secretion (Zhao, 2022). They can also stimulate the release of CCK from enteroendocrine cells, which in turn induces pancreatic enzyme secretion. While short-term exposure to FFA's, specifically long-chain fatty acids, results in insulin release from β-cells, it has been well documented that chronic exposure and/or lipotoxicity results in desensitization, impaired GSIS, insulin resistance, and over the long-term, β-cell damage (Cen et al., 2016; Haber et al., 2003; Ježek et al., 2018; Zhao, 2022).

Fatty acids are also oxidized by the TCA cycle within the mitochondria, yielding ATP, which can contribute to increased ATP concentrations, plasma membrane depolarization via ATP-sensitive K+ channels (KATP), and eventual Ca2+ dependent exocytosis of insulin (Acharya et al., 2023; Best et al., 2011; Ježek et al., 2018). Pancreases from SLC2A3-RNAi fetuses had increased expression of members of the solute carrier family 27 (SLC27A2, SLC27A4, SLC27A6), all of which are long-chain fatty acid transport transmembrane proteins (Acharya et al., 2023). Upregulation of these transporters could be a compensation of the hypoglycemic fetal pancreas to increase intake of fatty acids for use as an energetic substrate and also an attempt to maintain insulin responsiveness, albeit at lower levels than their NTS-RNAi counterparts. FFA uptake, storage, and oxidation can also be regulated by binding to PPARs within the cell (Chueire & Muscelli, 2020). PPAR signaling also maintains lipid metabolic homeostasis during cold stress with reduced plasma glucose concentrations in fish (Wu et al., 2023).

In our analysis of biological pathways, the term “negative regulation of cell proliferation,” contains one of the few downregulated genes: ST18. While not much is known about ST18’s role in the body and particularly pancreatic development, Henry et al. (2014) investigated ST18’s expression in the developing mouse pancreas, demonstrating its expression specific to endocrine cells. They also observed increased expression in the presence of cytotoxic levels of free fatty acids, implicating ST18’s role as a transcriptional regulator of lipotoxicity and cytokine-induced β-cell apoptosis, and therefore impaired insulin secretion. It is possible that the downregulation ST18 coupled with increased expression of pathways related to lipid metabolism and fatty acid metabolic processes seen in our data demonstrate a shift in energetic substrates from glucose to lipids is just beginning by mid-gestation and has not reached the lipotoxic levels that Henry et al.'s (2014) study achieved in their goal of emulating the environment of type 2 diabetes. Regardless, while there remains a large knowledge gap in the biological function of ST18, it appears to have a clear role in β-cell mass and function.

Finally, KEGG pathways analysis also revealed pathways related directly to pancreatic function, namely: pancreatic secretion, insulin resistance, glycolysis, gluconeogenesis, and glucagon signaling pathway. Many of the DEGs involved overlapped in their contributions to those pathways, making it difficult to interpret which processes were most impacted, however, the genes involved demonstrate a clear impact on the functionality of the pancreas. Many of these encode essential regulatory enzymes in gluconeogenesis. For example, PCK1 is the cytosolic form of phosphoenolpyruvate carboxykinase and PCK2 the mitochondrial form; both catalyze the formation of phosphoenolpyruvate from oxaloacetate, an essential step in gluconeogenesis. FBP1 catalyzes the hydrolysis of fructose 1,6-bisphosphate to fructose 6-phosphate. PCK1, PCK2, and FBP1, when deficient, have all been associated with hypoglycemia (Wesolowski & Hay, 2016), and may have been upregulated as a compensatory mechanism.

One of the few genes that were downregulated includes G6PC2, which is involved in the final step of gluconeogenesis. G6PC2 (glucose-6-phosphatase catalytic subunit 2) is islet-specific, and studies of G6PC2-/- mice confirm its essential role in glycolytic flux and sensitivity of GSIS. Isolated islets from G6PC2-/- mice exhibit a left shift in GSIS, such that under fasting conditions, insulin levels are the same as wild-type mice, infer-ring G6PC2's potential role in protecting against hypoglycemia under stressed conditions (Bosma et al., 2020). A more recent study utilizing the same model also implicates G6PC2 as a negative regulator of oxidative metabolism in the TCA cycle (Rahim et al., 2022). In our model, G6PC2 was downregulated in response to hypoglycemia and could contribute to potentially altered GSIS response in our fetuses.

Fetal hypoglycemia during the first half of gestation, generated by placenta-specific SLC2A3-RNAi (Lynch et al., 2022), altered the fetal pancreas transcriptome in multiple aspects. Many interconnected pathways contributing to pancreatic function and glucose metabolism were affected by SLC2A3-RNAi, demonstrating a multifaceted and broad impact of hypoglycemia on the fetal pancreas that is challenging to interpret. However, the smaller size of the SLC2A3-RNAi pancreases is impacting fetal metabolism, as evidenced by reduced expression of INS and GCG mRNA, and reduced umbilical concentrations of INS and GCG (Lynch et al., 2022). Our data implicate altered pathways directly related to both pancreatic transition periods needed for growth and metabolism as well as pancreatic function. The upregulation of genes involved with fatty acid and lipid metabolism may suggest fatty acid utilization as compensatory energy sources for development and metabolism by the pancreas in early fetal life.

CONCLUSIONS

The results of our transcriptomic analysis indicate that fetal hypoglycemia had a generalized impact on pancreas growth and development, identifying pathways involved in growth and metabolism by the pancreas, spanning both transition periods. Of note, are altered expression of genes involved in carbohydrate and fatty acid metabolism, suggesting a potential compensatory shift towards a greater utilization of fatty acids for metabolism. If a true compensatory shift in substrate utilization occurred, this could have long-term impacts on pancreas function. The true impact of the altered fetal pancreas transcriptome at mid-gestation, as the result of SLC2A3-RNAi induced fetal hypoglycemia, awaits thorough physiological assessment later in gestation or postnatally.

CHAPTER III: *IN-VIVO* PHYSIOLOGICAL RAMIFICATIONS OF PLACENTAL SLC2A3-RNAi THROUGHOUT GESTATION IN SHEEP

INTRODUCTION

The primary energy substrate for fetal oxidative processes and growth is glucose. Due to the limited ability of the fetus to produce glucose in utero until near-term, fetal glucose supply is dependent on transfer down the maternal-to-fetal glucose gradient, mediated by members of the SLC2A gene family of facilitative transporters (Barry and Anthony, 2008). In the human placenta, SLC2A1 is the most abundant transporter, is localized on both the microvillous (apical) and basal membranes of the syncytiotrophoblast (Jansson et al., 1993; Hauguel-de Mouzon et al., 1994), and increases as gestation progresses. By contrast, SLC2A3 is specific to the microvillous membrane and is most abundant during the first-half of gestation (Brown et al., 2011). While SLC2A1 is more abundant than SLC2A3, SLC2A3 has approximately a 5-fold greater affinity and glucose transporter capacity than SLC2A1 (Simpson et al., 2008). Like the human, in the sheep placenta SLC2A1 is the most abundant transporter and also increases with gestational age in sheep placenta (Ehrhardt and Bell, 1997), whereas SLC2A3 is more abundant during early pregnancy and is localized to the microvillous membrane (Wooding et al., 2005). The one aspect of the placental SLC2A1 and SLC2A3 relationship that differs between humans and sheep is that SLC2A1 appears to be localized solely to the basolateral membranes of the trophoblast in sheep (Wooding et al., 2005).

Functional placental insufficiency is a major cause of fetal growth restriction (FGR), often resulting in hypoglycemic fetuses due to reduced placental transfer of glucose (Limesand et al., 2007; Marconi & Paolini, 2008). However, studies assessing pre- or near-term FGR placentas did not find a deficiency in glucose transporters to be the cause of fetal hypoglycemia (Jansson et al., 1993; Janzen et al., 2013). One aspect of placental glucose transfer that may or may not be impacted by glucose transporter abundance is glucose metabolism by the placenta. The placenta is a highly metabolic organ in itself, and when measured under steady-state conditions in sheep, the placenta utilizes 80% of the glucose taken up by the uterus at mid-gestation (Bell et al., 1986), and 72% of uterine glucose uptake near-term (Meschia et al., 1980). The apparent lack of a placental glucose transporter deficiency in human FGR placenta (Jansson et al., 1993; Janzen et al., 2013), could suggest a shift in placental glucose utilization with FGR. However, there is evidence both supporting (Challis et al., 2000) and refuting (Magnusson et al., 2004) this concept, although this cannot be readily addressed in vivo during human pregnancy.

With the distinct separation of where SLC2A1 (basolateral) and SLC2A3 (apical) reside in the sheep trophoblast (Wooding et al., 2005), we reasoned that creating a deficiency in SLC2A3 would impact both placental utilization and transfer of glucose to the fetus. To that end, we recently reported the impact of lentiviral-mediated RNA interference (RNAi) of SLC2A3 during the first-half of gestation in sheep (Lynch et al., 2022). At mid-gestation (75 days of gestation; 75 dGA), the fetuses were smaller, hypoglycemic, and had reduced umbilical artery insulin and glucagon concentrations. Furthermore, the fetal pancreas was significantly smaller, and exhibited an altered transcriptome, suggesting a shift in substrate utilization (Kennedy et al., 2024). Since SLC2A3 is more abundant during the first-half of gestation (Ehrhardt and Bell, 1997; Brown et al., 2011), it is possible that the placenta can compensate for a deficiency in SLC2A3 during the second-half of gestation in order to maintain or recover fetal growth and development. Therefore, it was our objective to assess the ramifications of SLC2A3 RNAi on fetal growth, uterine nutrient uptake, placental utilization and transfer to the fetus near-term, as well as assess the responsiveness of the fetal pancreas to glucose and arginine challenge in vivo to determine the long-term impact of fetal hypoglycemia during the first-half of gestation.

MATERIALS AND METHODS

All procedures conducted with animals were approved by the Colorado State University Institutional Animal Care and Use Committee (protocols #1576 and #3506), and the Institutional Biosafety Committee (17-039B and 18-029B). Metabolic study experiments were conducted at the Perinatal Research Center, University of Colorado School of Medicine, in compliance with the Institutional Animal Care and Use Committee (Protocol #00714) and the ARRIVE 2.0 guidelines (Sert et al., 2020).

Dorper breed composition ewes were group housed in pens at the Colorado State University Animal Reproduction and Biotechnology Laboratory, and were provided access to hay, trace minerals, and water to meet or slightly exceed their National Research Council (Nutrient Requirements of Small Ruminants, 2007) requirements.

Generation of Lentivirus and RNAi pregnancies

Lentiviral generation and tittering of *SLC2A3*-RNAi and NTS-RNAi virus were performed as previously described (Baker et al., 2016; Jeckel et al., 2018), using the shRNA sequences from Lynch et al (2022).

Animal management, estrus synchronization and embryo transfers were performed as previously described (Baker et al., 2016; Lynch et al., 2022; Tanner et al., 2021a). Briefly, after estrus synchronization and subsequent breeding, at 9 days post-conception (d.p.c.), donor ewes were euthanized and uteri were harvested and flushed for collection of hatched, expanded blastocysts. Single blastocysts were infected with 150,000 transducing units of either NTS-RNAi or *SLC2A3*-RNAi virus, as previously described (Baker et al., 2016; Tanner et al., 2021a,b, 2024). Following a minimum of 4 hr incubation, single blastocysts were surgically transferred into the uterine horn ipsilateral to the corpus luteum of a synchronized recipient ewe. Recipient ewes were monitored daily for return to standing estrus or confirmed pregnant at 50, 70, and 90 days gestational age (dGA) by ultrasonography (Mindray Medical Equipment, Mahway, NJ, USA) as previously described (Tanner et al., 2021a,b, 2024). After 100 days of gestation, pregnant recipients were transferred to the University of Colorado Anschutz Medical Campus, Perinatal Research Center (Aurora, CO).

Animal preparation

To assess the metabolic impacts of *SLC2A3*-RNAi on glucose transport by the placenta, we used an established experimental model for steady-state investigation of blood flow, nutrient uptakes and utilization, and hormone secretion in chronically catheterized late gestation fetal lambs (Barry et al., 2008; Cilvik et al., 2021; Meschia et al., 1965; Rozance et al., 2006; Tanner et al., 2022). All pregnant ewes were kept in individual carts with 12 hr light/12 hr dark cycle and given *ad libitum* access to alfalfa pellets (Standlee Hay, Kimberly, ID, USA), water, and mineral supplements throughout the study. Feed and water intake were monitored and recorded daily.

After fasting for 24 hrs, and without access to water for 12 hr, animals were anesthetized at 126 ± 2 dGA and surgeries were performed to place indwelling catheters into the descending fetal abdominal aorta, femoral vein, umbilical vein, and maternal femoral vein and artery as previously described (Cilvik et al., 2021; Tanner et al., 2021a, 2022). Briefly, the gravid uterus was exteriorized via midventral laparotomy and fetus exposed through a small (~6cm) incision in the uterine wall. Fetuses were instrumented with indwelling catheters in the descending aorta (a proxy for umbilical artery blood), femoral vein, and umbilical vein. Maternal catheters included the femoral artery (a proxy for uterine artery blood), femoral vein, and uterine vein. All catheters were tunneled subcutaneously to the paralumbar fossa, exteriorized into a pouch, and maintained with 5% heparinized saline flushes. Before the metabolic study, animals were allowed to recover for approximately seven days.

<u>*Metabolic Study and Blood Flow Calculations*</u>

The overall study design is depicted in Figure 3.1 and was performed at 133 ± 2 dGA, based off previously published protocols (Cilvik et al., 2021; Gadhia et al., 2013; Limesand et al., 2006; Tanner et al., 2024). Uterine and umbilical blood flows were determined by the steady-state $^3H_20$ transplacental diffusion technique. Before any infusions, the study began with samples (draw 0) collected from maternal femoral artery (A), uterine vein (V), umbilical vein (γ), and fetal descending aorta (α) simultaneously. Then, $^3H_20$ was infused (Figure 3.1: Metabolic Tracer Infusions) into the fetal femoral vein and once an isotopic steady state was reached by continuous infusion at 3 ml/hr (15 µCi/min) for approximately 90 mins, four blood samples (draws 1-4, representing steady-state baseline) were simultaneously collected every 20 minutes. During this period, fetuses also received an isovolumic transfusion of maternal blood (Figure 3.1: Fetal Blood Tx) to replace fetal blood lost from sampling and prevent hypovolemia and acute anemia (Cilvik et al., 2021). Immediately following the fourth baseline steady-state blood draw, the fetuses were subjected to a square-wave hyperglycemic clamp to determine insulin secretion in response to glucose (Figure 3.1: GSIS) by administration of a 2.5 ml bolus of 33% dextrose, followed by continuous infusion (approximately 5 ml/hr) to maintain fetal arterial plasma concentrations of glucose at 2.2-2.8 mmol/L. Umbilical arterial blood samples were collected at 5, 10, 15, 20, 30, 45, 60, 75, 90, and 105 mins following the initial dextrose bolus, with the final four samples representing the steady-state GSIS period. After the 105 min hyperglycemic sample, a bolus of arginine (0.5 mmol/kg estimated fetal weight in 5 ml 0.4M sodium acetate and 0.9% sodium chloride) was infused over 4 mins (75 ml/hr) followed by 2ml of saline, to determine glucose-potentiated arginine-stimulated insulin secretion (Figure 3.1: ASIS). Umbilical arterial blood samples were collected at 115, 120, 130 and 140 mins, representing the ASIS period. Finally, the metabolic study was concluded at approximately 170 mins with cessation of infusions and subsequent necropsy.

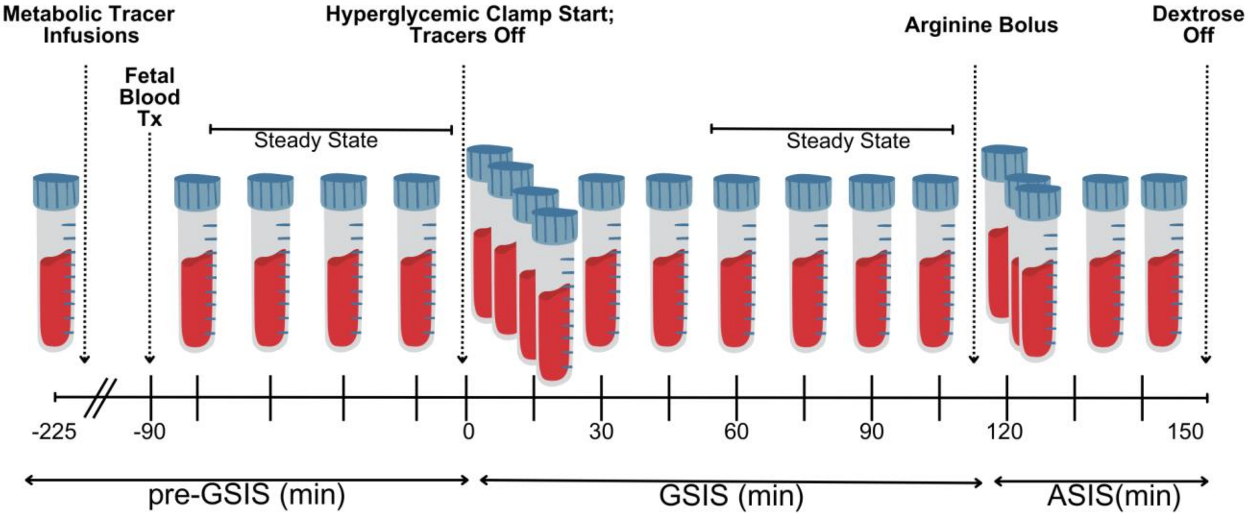

Figure 3.1. Blood flow and metabolic study protocol

Tissue Collection

Euthanasia and tissue collection was performed as previously described (Cilvik et al., 2021; Tanner et al., 2024). Briefly, ewes and fetuses were euthanized by a lethal intravenous dose of sodium pentobarbital. Fetal and uteroplacental weights were recorded. Placentomes were dissected from the endometrium and weighed ("placental weight"), then separated into fetal (cotyledonary) and maternal (caruncular) components before being snap-frozen in liquid nitrogen and stored at -80ºC. Excess fluid was removed from fetal membranes and weighed. The uterus was also weighed after removal of all placentomes, fluid and fetal membranes. Fetal sex and measurements of growth were recorded, which included crown-rump and lower limb lengths. Fetal organs were carefully dissected and weighed, then snap-frozen in liquid nitrogen and stored at -80ºC.

<u>Biochemical Analysis of Blood Samples</u>

Biochemical assays were performed as previously described (Cilvik et al., 2021). Whole blood collected in heparin-coated syringes was immediately analyzed for P_{CO2}, P_{O2}, bicarbonate, pH, hematocrit and S_{O2} , with the ABL 825 Blood Gas Analyzer (Radiometer America, Brea, CA, USA). Oxygen content of the blood was calculated by the ABL 825 Blood Gas Analyzer. Whole blood collected in EDTA-coated syringes was centrifuged at 14,000 g for 3 min at 4°C to isolate plasma for further analysis. Glucose and lactate were immediately measured with the YSI 2900D Biochemistry Analyzer (Yellow Springs Instruments, Inc., Yellow Springs, OH, USA), while the remaining plasma was stored at −80°C for hormone and amino acid analysis. Plasma amino acid concentrations were measured using a Dionex TM ICS 5000+ high pressure ion chromatograph with a Pickering PCX Pinnacle 120-4 channel variable wavelength detector (Thermo Electron North America, LLC). Plasma insulin, insulin-like growth factor 1 (IGF1) and glucagon concentrations were measured using enzyme-linked immunosorbent assays (ALPCO Immunoassays 80-INSOV-E01, 22-IGFHU-E01 and 48-GLUHU-E0,1respectively), as previously described (Cilvik et al., 2021, Lynch et al., 2022, Tanner et al., 2024).

Study Calculations

All calculations for blood flow, nutrient uptake and utilization rates (using the Fick principle), nutrient fluxes and nutrient: oxygen quotients have been previously published in detail (Cilvik et al., 2021; Tanner et al., 2022). Uterine, umbilical, and uteroplacental uptakes of oxygen, glucose, lactate, and amino acids were calculated based on the transplacental diffusion technique (Meschia et al., 1980) and reported as an average of four blood draws. All other calculations have been previously described extensively (Cilvik et al., 2021; Tanner et al., 2021a,b, 2024).

mRNA Analysis

RNA was extracted from approximately 200 mg of pulverized cotyledonary tissue as previously described (Cilvik et al., 2021). Concentration was measured using a Nanodrop spectrophotometer. Total RNA (2 μg) was reverse transcribed using the SuperScript III First-Strand Synthesis SuperMix (Invitrogen, Waltham, MA), and cDNA was diluted 1:10 with sterile water. Quantitative PCR assays for *eNOS*, *IGF1*, *IGF2* (insulin-like growth factors 1 and 2), *IGF1R*, *IGF2R* (insulin-like growth factor type 1 and type 2 receptor), *IGFBP1*, *IGFBP2* and *IGFBP3* (insulin-like growth factor binding proteins 1, 2 and 3) were performed using primers developed for sheep sequences (Table 3.1) as previously described (Cilvik et al., 2021). A melt-curve analysis was examined to ensure a single peak and correct amplification. Samples were analyzed in triplicate, and the standard curve method of relative quantification was used (Wong & Medrano, 2005). The concentration of specific mRNA of interest were normalized to the mean of reference mRNA (glyceraldehyde 3-

phosphate dehydrogenase, GAPDH; ribosomal protein 32, RPL32; ribosomal protein 41, RPL41) and then fold-change relative to control samples was calculated. The quantitative real-time qPCR experiments and analysis were performed according to the Minimum Information for Publication of Quantitative Real-Time PCR Experiments guidelines (Bustin et al., 2009). Primer sequences for mRNA of interest and reference mRNA can be found in Table 3.1.

Table 3.1. Primer sequences

mRNA	Forward primer	Reverse primer
NOS3	AGATCCGCTTCAACAGTGTCTCCT	CCAGTCCAAACACACAGAACCTGA
GAPDH	TGGAGGGACTTATGACCACTG	TAGAAGCAGGGATGATGTTCT
IGF1	GAGACCCTCTGCGGGGCTGA	CTGCTCGAGCCGTACCCCGT
IGF2	TGTGGGGACCGCGGCTTCTA	CAGGGCCAGGTCGCAGCTTC
IGF1R	TGTCCTGACATGCTGTTTGAGCTG	CCAGGAACGAGGGCCGCATC
IGF2R	ACCAGTTACGCCTGCCCGGA	TCGGGACCGCCCTCGGATTT
IGFBP1	TGATGACCGAGTCCAGTGAG	GTCCAGCGAAGTCTCACAC
IGFBP2	ACCTTGGCCTGGAGGAGCCC	TCCAGGGGACCCCGCTCATC
IGFBP3	TCATGCCAAGGACAGCCAGCG	CCTCCATTTCCCGGCGGCAG
RPL32	AATCAAGCGGAACTGGCG	GGCATTGGGATTGGTGATT
RPL41	TATGAGCAAGTGGACCAGCA	TTCACGTAGGGCTTGAGCTT

Statistical Analysis

We successfully generated and got seven *SLC2A3*-RNAi and seven NTS-RNAi pregnancies to the point of surgical catheterization. Unfortunately, there was a post-surgical fetal demise from each group, leaving a total of n = 6 pregnancies per group which were included in this analysis. Unexpectedly, fetal sex was unevenly distributed, with two female fetuses in the NTS-RNAi group and one male in the *SLC2A3*-RNAi group. Therefore, fetal sex was not included in statistical analyses.

Statistical analysis was performed using GraphPad Prism (Version 10.2). Fetal and organ weights and measurements collected at necropsy, cotyledonary mRNA concentrations, and baseline hormone concentrations, blood flows and nutrient uptakes were compared using unpaired Student's t-tests. A two-way ANOVA was used to examine differences among metabolic study periods, including terms for period and treatment. The study periods included baseline (-60 to -15 mins), early GSIS (5, 10, 15, 20, 30 mins), late GSIS (60, 75, 90, 105 mis), and ASIS (115, 120, 130, 140 mins). When the treatment–time interactions of the overall ANOVA had a p-value ≤ 0.10, post hoc test comparisons were made using Fisher's least squares difference. Results are expressed as means ± standard error of the mean (SEM). P-values ≤ 0.05 were considered to be statistically significant and p-values ≤ 0.10 were considered as statistical trends.

RESULTS

Fetal and placental measures

Necropsy was performed following the metabolic study period at 133 ± 2 dGA. Fetal measurements are summarized in Table 3.2. Fetal body weight (FW; Figure 3.2a) and organ weights were not different. Table 3.3. summarizes uteroplacental measurements obtained at necropsy. There were no differences between treatment groups in total placentome weight and number. However, there was a tendency for uterine weight:FW to be higher in the *SLC2A3*-RNAi group compared to controls (0.18 vs 0.16, *P* = 0.07). While total fetal membrane weights did not significantly differ, when normalized to fetal weight (FM:FW), there was a tendency for the *SLC2A3*-RNAi group to be smaller (0.02 vs. 0.14, *P* = 0.07). Uteroplacental weight, which was calculated as the total weights of placentomes, the uterus and fetal membranes, was not different between groups. Placenta weight was also not different (Figure 3.2b), however, when uteroplacental weight was normalized to fetal weight (uteroplacental weight:FW), the *SLC2A3*-RNAi group was 25% greater than the NTS-RNAi controls (*P* = 0.04; Figure 3.2c).

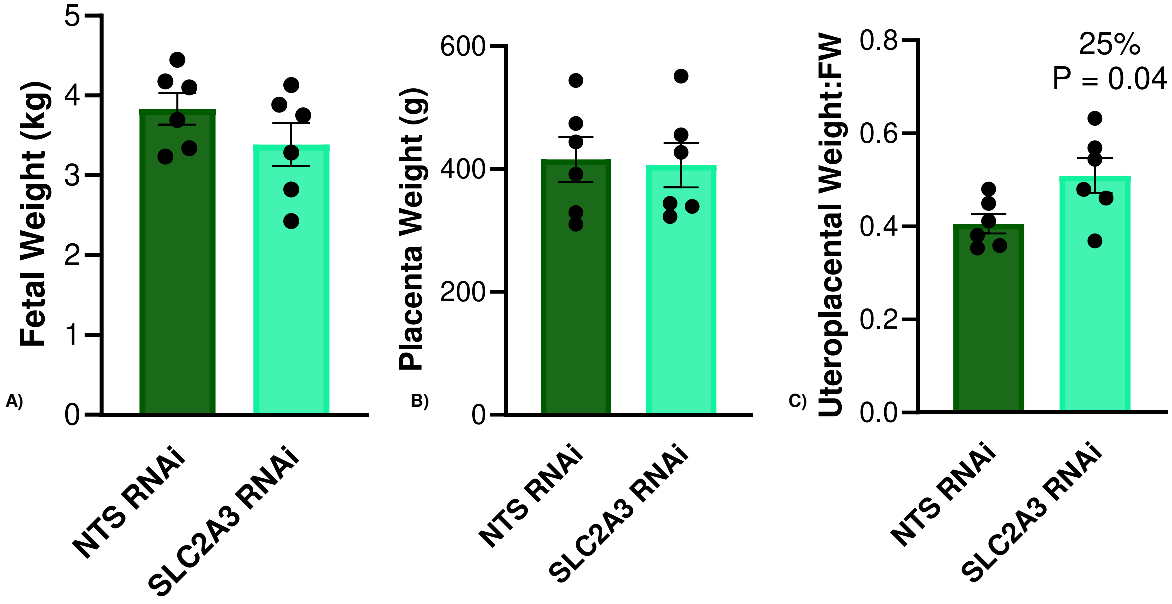

Figure 3.2. Impact of *SLC2A3*-RNAi on fetal weight (FW; A); placenta weight (B); uteroplacental weight:fetal weight (C). Data are shown as means ± SEM. NTS = non-targeting sequence; RNAi = RNA interference.

Table 3.2. Fetal measurements obtained at necropsy

	NTS-RNAi			***SLC2A3*-RNAi**			***p*-value**
Crown-rump length, cm	47.97	±	0.82	47.02	±	0.61	0.37
Ponderal index	3.46	±	0.09	3.26	±	0.25	0.46
Lower leg length, cm	32.05	±	0.63	31.37	±	0.66	0.47
Pancreas, g	3.45		0.32	3.30		0.30	0.74
Liver, g	114.49	±	12.33	106.12	±	9.27	0.60
L liver lobe, g	36.94	±	5.21	31.86	±	3.25	0.43
R liver lobe, g	78.34	±	7.51	74.53	±	6.48	0.71
Kidneys, g	19.43	±	0.93	18.45	±	1.14	0.52
Perirenal adipose tissue (PRAT), g	11.47	±	0.87	10.09	±	0.90	0.30
Spleen, g	7.96	±	1.31	5.73	±	0.45	0.14
Adrenal glands, g	0.41	±	0.05	0.36	±	0.02	0.27
Brain, g	51.26	±	1.70	49.31	±	0.96	0.34
Brain:liver	0.47	±	0.05	0.48	±	0.03	0.92
Lungs, g	132.37	±	11.10	127.59	±	7.13	0.72
Heart, g	24.16	±	2.01	23.08	±	1.84	0.70
Left ventricle, g	6.50	±	0.68	6.14	±	0.46	0.67
Right venticle, g	4.14	±	0.16	4.14	±	0.22	1.00
Biceps femoris (BF), g	18.60	±	1.32	16.19	±	0.95	0.17
Flexor digitorum superficialis (FDS), g	2.78	±	0.20	2.50	±	0.22	0.36
Tibialis Anterior muscle, g	3.53	±	0.31	3.04	±	0.23	0.23

Data are shown as means ± SEM. NTS = non-targeting sequence; RNAi = RNA interference.

Table 3.3. Uteroplacental measurements obtained at necropsy

	NTS-RNAi			***SLC2A3*-RNAi**			***p*-value**
Total placentome wt, g	415.69	±	36.47	406.63	±	36.18	0.86
Total placentomes	75.33	±	5.43	74.33	±	4.21	0.89
Total uterus wt, g	613.27	±	42.05	615.13	±	37.61	0.97
Uterus:FW	0.16	±	0.01	0.18	±	0.01	0.07
Total fetal membrane (FM) wt, g	519.40	±	44.39	660.62	±	69.31	0.12
FM:FW	0.14	±	0.20	0.02	±	0.03	0.07
Uteroplacental weight, g	1548.36	±	86.98	1682.38	±	86.98	0.30

Data are shown as means ± SEM. NTS = non-targeting sequence; RNAi = RNA interference.

Placental mRNA concentration

Cotyledonary tissues harvested at necropsy were assessed for *NOS3*, *IGF*, *IGFBP* and *IGFR* mRNA concentrations. Concentrations are expressed as a fold change from the NTS-RNAi mRNA and are presented in Table 3.4. No differences were seen in mRNA concentrations of the IGF binding proteins (*IGFBP1-3*). *NOS3* mRNA was significantly increased, and although *IGF1* was not different, *IGF2*, as well as *IGF1R* and *IGF2R,* all tended to have increased mRNA concentrations compared to the NTS-RNAi group.

Table 3.4. Placental mRNA concentrations expressed as a fold change relative to NTS-RNAi mRNA concentrations.

mRNA	**NTS-RNAi**			***SLC2A3*-RNAi**			***p*-value**
NOS3	1.00	±	0.087	1.54	±	0.131	0.023
IGFBP1	1.00	±	0.540	1.44	±	0.531	0.193
IGFBP2	1.00	±	0.104	1.03	±	0.081	0.849
IGFBP3	1.00	±	0.169	1.29	±	0.152	0.323
IGF1	1.00	±	0.089	1.26	±	0.127	0.290
IGF2	1.00	±	0.065	1.48	±	0.123	0.080
IGF1R	1.00	±	0.077	1.28	±	0.076	0.080
IGF2R	1.00	±	0.043	1.42	±	0.139	0.102

Values are expressed as mean fold change relative to NTS-RNAi (± SEM).

Blood flow calculations and nutrient uptakes during baseline

During the baseline period of the metabolic study (prior to GSIS and ASIS periods), there was no statistical difference in uterine or umbilical blood flow rate (Figure 3.3) between treatment groups. Additionally, there no differences in uterine blood flow relative to uterine weight (2570.0 ± 296.2 vs. 2826.7 ± 300.0 ml/min/kg uterus) or umbilical blood flow relative to fetal weight (161.3 ± 17.0 vs. 140.3 ± 13.6 ml/min/kg fetus).

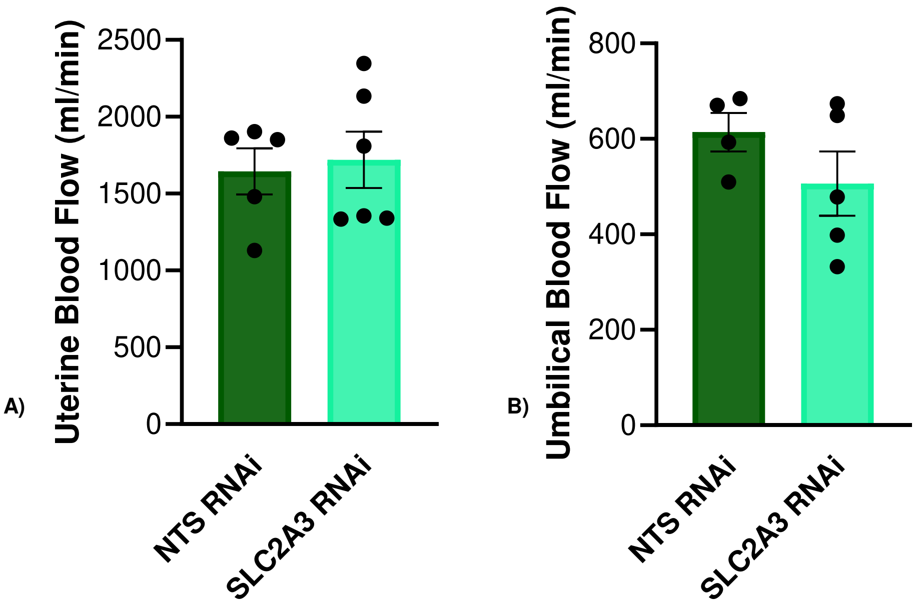

Figure 3.3. Uterine (A) and umbilical (B) blood flow rates determined during the baseline period. Data are shown as means ± SEM. NTS = non-targeting sequence; RNAi = RNA interference.

Glucose concentrations during the baseline period were lower in both the uterine artery (3.71 ± 0.113 vs. 3.35 ± 0.062 mmol/L; $P<0.05$) and uterine vein (3.47 ± 0.118 vs. 3.18 ± 0.059 mmol/L; $P<0.10$), and the uterine artery-to-vein glucose gradient (0.246 ± 0.035 vs. 0.173 ± 0.015 mmol/L; $P=0.10$) was 30% lower in *SLC2A3*-RNAi pregnancies. Glucose uptake by the uterus and fetus, as well as placental glucose utilization were calculated and are presented in Figure 3.4. In the *SLC2A3*-RNAi pregnancies, uterine glucose uptake was reduced 29% ($P< 0.05$), whereas umbilical glucose uptake was 18% lower, which was not statistically significant ($P =0.14$). Uteroplacental glucose utilization (Figure 3.4c) was also reduced ($P=0.07$) 36% in the *SLC2A3*-RNAi group. When normalized on tissue weight, glucose uptake by the uterus (546.7 ± 49.80 vs. 425.7 ± 28.95 μmol/min/kg uterus) was still 22% lower (*P* =0.07) in *SLC2A3*-RNAi pregnancies, uteroplacental glucose utilization was 26% lower (*P*<0.05; 621.3 ± 45.67 vs. 461.6 ± 110.5 μmol/min/kg placenta), but umbilical glucose uptake was not different (26.60 ± 3.13 vs. 23.60 ± 4.99 μmol/min/kg fetus; *P*=0.50). Oxygen and lactate uptakes and utilization were also calculated, and are reported in tables 3.5 and 3.6, respectively; no differences between treatment groups were observed.

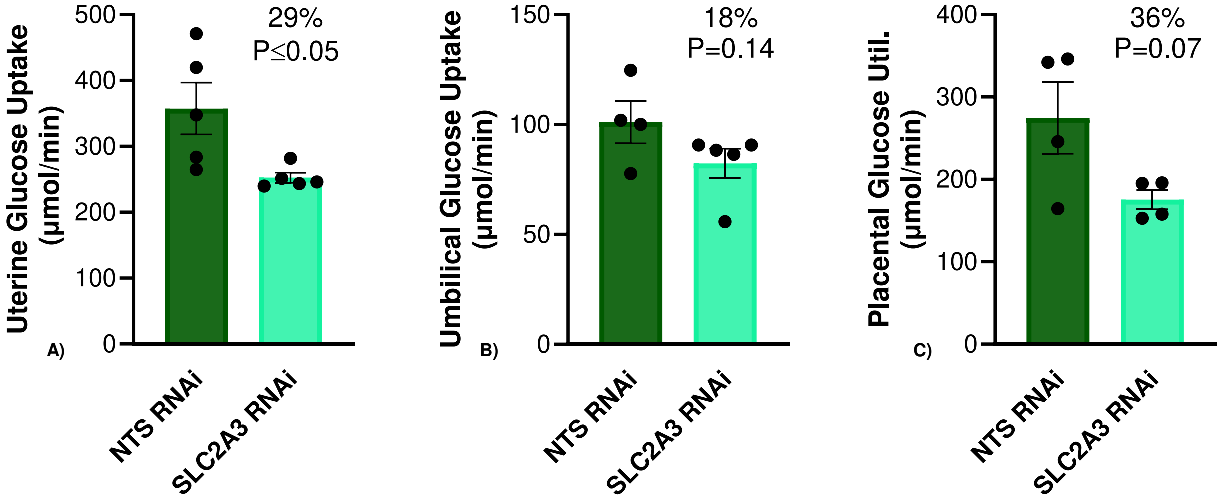

Figure 3.4. Uterine (A) and umbilical (B) glucose uptake (umol/min); placental (C) glucose utilization during the baseline period. Data are shown as means ± SEM. NTS = non-targeting sequence; RNAi = RNA interference.

At baseline, uterine arterial plasma samples were analyzed for insulin, glucagon, and IGF1 (Figure 3.5). Of these, only IGF1 was impacted, with *SLC2A3*-RNAi pregnancies exhibiting 20% lower IGF1 concentrations (*P* = 0.06). When the same hormones were assessed in the umbilical artery, concentrations of insulin, glucagon, and IGF1 were not impacted in *SLC2A3*-RNAi pregnancies during the baseline period (Figure 3.6).

Table 3.X. Oxygen uptakes and utilization

	NTS-RNAi			***SLC2A3-RNAi***			***p-value***
Fetal Oxygen utilization (mmol/min)	1.145	±	0.123	1.109	±	0.037	0.761
Fetal Oxygen utilization per kg (mmol/min/kg fetus)	0.301	±	0.037	0.314	±	0.018	0.743
Uterine O2 uptake (mmol/min)	2.085	±	0.168	1.996	±	0.155	0.706
Uterine O2 uptake per kg (mmol/min/kg uterus)	3.206	±	0.223	3.272	±	0.247	0.850
Uteroplacental O2 utilization (mmol/min)	0.990	±	0.187	1.007	±	0.220	0.942
Uteroplacental O2 utilization (mmol/min/kg placenta)	2.282	±	0.444	2.373	±	0.459	0.840

Table 3.X. Lactate uptakes, production and utilization

	NTS-RNAi			*SLC2A3*-RNAi			*p*-value
Lactate uptake from placenta (umol/min)	103.292	±	13.540	93.997	±	20.471	0.630
Lactate uptake from placenta per kg fetus (umol/kg fetus/min)	26.607	±	2.661	26.234	±	5.423	0.928
Placental lactate export to uterus (umol/min)	117.092	±	3.324	100.859	±	10.234	0.199
Relative uterine lactate export per kg (umol/min/kg uterus)	181.287	±	8.668	164.548	±	13.933	0.358
Placental export per kg (umol/min/kg placenta)	276.268	±	25.677	255.984	±	34.098	0.658
Total placental lactate production (umole/min)	220.194	±	13.205	195.210	±	41.741	0.431
Total placental lactate production per kg (umole/min/kg placenta)	515.990	±	33.388	483.880	±	116.411	0.759

Table 3.6. Lactate uptakes, production and utilization

	NTS-RNAi			***SLC2A3*-RNAi**			***p*-value**
Lactate uptake from placenta (umol/min)	103.292	±	13.540	93.997	±	20.471	0.630
Lactate uptake from placenta per kg fetus (umol/kg fetus/min)	26.607	±	2.661	26.234	±	5.423	0.928
Placental lactate export to uterus (umol/min)	117.092	±	3.324	100.859	±	10.234	0.199
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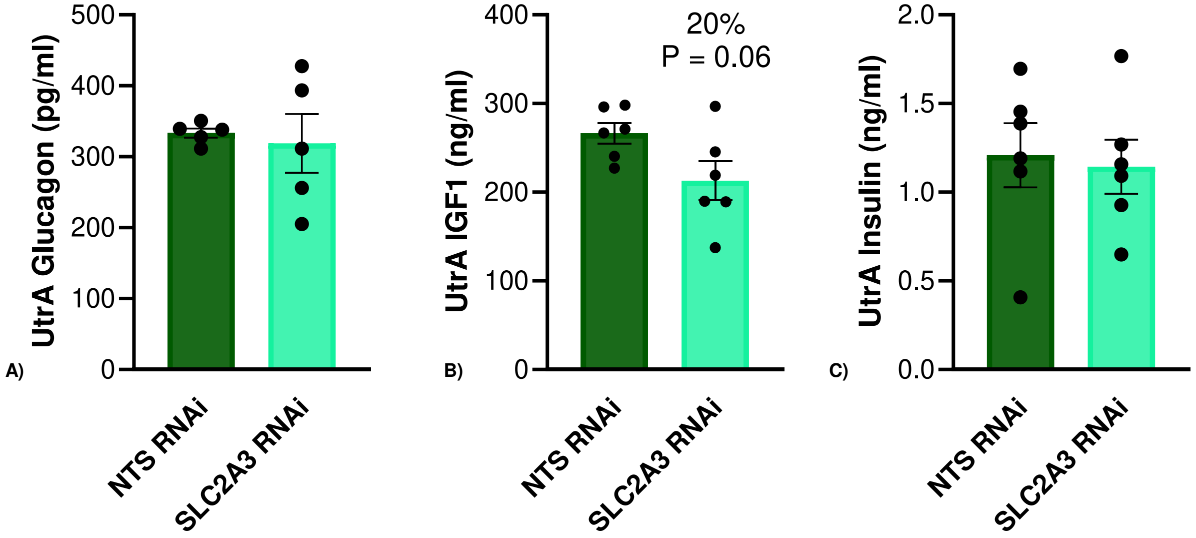

Figure 3.5. Baseline uterine artery concentrations Data are shown as means ± SEM. NTS = non-targeting sequence; RNAi = RNA interference.

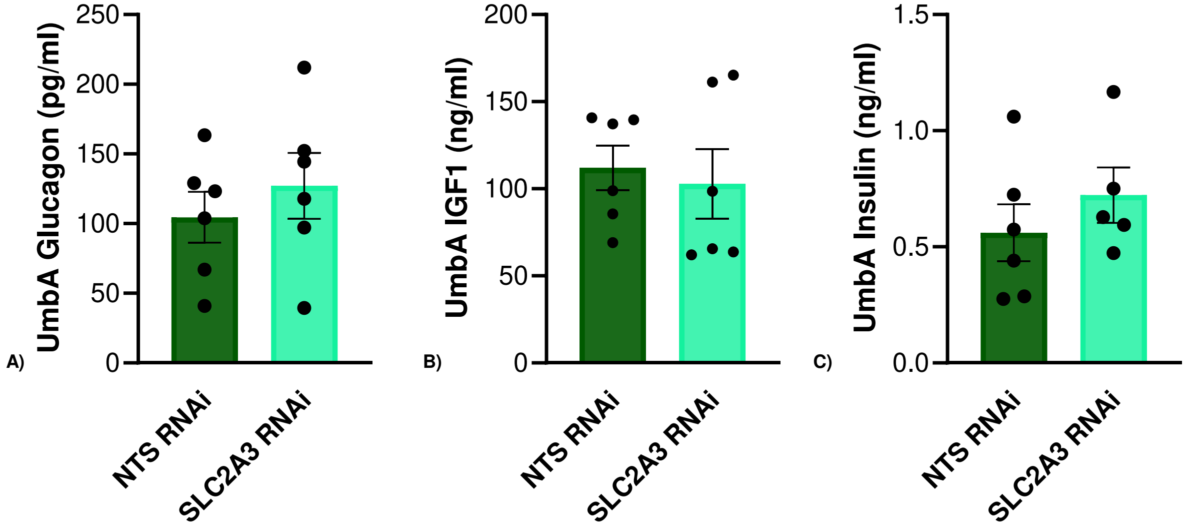

Figure 3.6. Baseline umbilical artery concentrations of of glucagon (A), IGF1 (B), and insulin (C). Data are shown as means ± SEM. NTS = non-targeting sequence; RNAi = RNA interference.

Amino acids

During the baseline period, plasma concentrations of 23 amino acids were measured in uterine and umbilical arteries and veins. While the majority of the amino acids measured in the uterine artery and vein were not different between treatment groups, glutamate was significantly ($P<0.05$) reduced in both the uterine artery and vein, whereas citrulline and lysine tended to be increased in both vessels (Table 3.7). Within the umbilical artery and umbilical vein, plasma concentrations of the amino acids did not statistically differ between the two groups (Table 3.8).

Uterine and umbilical uptakes as well as uteroplacental utilization of amino acids were also calculated. Uterine uptake, per kg of uterus, of taurine, threonine, serine, alanine, isoleucine and leucine all tended ($P\leq0.10$) to be greater in the *SLC2A3*-RNAi pregnancies compared to the NTS-RNAi controls (Table 3.9). By contrast, umbilical uptake of amino acids, per kg of fetus, did not significantly differ (Table 3.10). The most notable changes were found in uteroplacental utilization of amino acids (Table 3.11). Threonine, glutamine, serine, proline, glycine, citrulline, isoleucine, and leucine all tended ($P\leq0.10$) to be increased in samples from *SLC2A3*-RNAi pregnancies. In contrast to what was seen with uteroplacental utilization of glucose, the sum of amino acid carbons utilized by the uteroplacental unit tended ($P=0.09$) to be increased.

Table 3.7. Uterine plasma amino acid concentrations during baseline

	NTS-RNAi Uterine Artery (n=6)	***SLC2A3*-RNAi Uterine Artery** (n=6)	***p*-value**	**NTS-RNAi Uterine Vein** (n=6)	***SLC2A3*-RNAi Uterine Vein** (n=6)	***p*-value**
TAU	67.46 ± 6.08	60.22 ± 10.74	0.57	69.29 ± 6.16	60.04 ± 11.07	0.48
ASP	9.40 ± 0.80	10.08 ± 1.78	0.74	10.22 ± 0.94	11.21 ± 1.89	0.65
THR	120.31 ± 16.19	166.29 ± 24.75	0.15	114.17 ± 15.79	157.40 ± 23.90	0.16
SER	63.86 ± 8.20	77.35 ± 8.71	0.29	57.83 ± 7.59	68.34 ± 7.45	0.35
ASN	38.09 ± 3.72	50.82 ± 6.65	0.13	35.03 ± 3.54	47.03 ± 5.74	0.11
GLU	80.57 ± 5.67	63.25 ± 5.58	0.05	82.73 ± 5.50	64.50 ± 5.45	0.04
GLN	269.19 ± 14.11	263.13 ± 18.43	0.80	254.34 ± 14.09	245.06 ± 16.73	0.68
PRO	84.70 ± 7.50	103.11 ± 11.91	0.22	78.74 ± 7.29	94.66 ± 10.42	0.24
GLY	301.51 ± 27.37	341.77 ± 30.52	0.35	307.61 ± 27.99	341.26 ± 28.68	0.42
ALA	115.90 ± 9.28	130.10 ± 11.94	0.37	110.18 ± 8.57	122.23 ± 10.76	0.40
CIT	197.38 ± 28.90	273.52 ± 30.89	0.10	191.79 ± 29.53	264.53 ± 30.59	0.12
VAL	226.72 ± 28.28	261.21 ± 25.54	0.39	210.64 ± 28.38	242.64 ± 23.08	0.40
CYS	36.63 ± 1.88	35.13 ± 4.65	0.77	35.78 ± 2.26	34.64 ± 4.69	0.83
MET	34.38 ± 2.74	38.51 ± 2.34	0.28	31.51 ± 2.98	36.47 ± 2.84	0.26
ILEU	125.44 ± 11.58	134.60 ± 10.42	0.57	114.83 ± 11.41	123.38 ± 9.63	0.58
LEU	152.62 ± 14.51	166.41 ± 15.75	0.53	138.72 ± 14.35	151.60 ± 14.91	0.55
TYR	70.46 ± 5.73	72.08 ± 7.45	0.87	67.23 ± 6.08	68.32 ± 7.89	0.91
PHE	54.03 ± 3.96	59.67 ± 5.80	0.44	50.24 ± 4.29	56.38 ± 5.80	0.41
TRP	42.31 ± 5.35	41.50 ± 4.49	0.91	38.73 ± 4.47	38.71 ± 4.43	1.00
ORN	88.83 ± 16.32	112.16 ± 11.14	0.26	82.00 ± 14.72	107.94 ± 12.61	0.21
LYS	154.01 ± 10.08	186.22 ± 12.90	0.08	148.06 ± 9.44	178.36 ± 13.72	0.10
HIS	52.24 ± 3.94	54.74 ± 4.73	0.69	50.02 ± 3.75	49.79 ± 3.32	0.96
ARG	224.91 ± 21.66	214.61 ± 17.92	0.72	214.81 ± 20.90	209.01 ± 21.64	0.85

Ala, alanine; Arg, arginine; Asn, Asparagine; Asp, aspartate; Cit, citrulline; Cys, cystine; Gln,glutamine; Glu, glutamate; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Orn, ornithine; Phe, phenylalanine; Pro, proline; Ser, serine; Tau, taurine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine

Table 3.8. Umbilical plasma amino acid concentrations during baseline

	NTS-RNAi Umbilical Artery (n=6)			***SLC2A3*-RNAi Umbilical Artery** (n=6)			***p*-value**	**NTS-RNAi Umbilical Vein** (n=6)			**SLC2A3-RNAi Umbilical Vein** (n=5)			***p*-value**
TAU	42.26	±	6.08	39.85	±	13.24	0.89	42.39	±	6.16	46.91	±	14.09	0.80
ASP	28.14	±	0.80	29.16	±	2.83	0.75	28.05	±	0.94	29.24	±	2.97	0.73
THR	399.47	±	16.19	437.11	±	61.72	0.71	422.11	±	15.79	504.72	±	48.54	0.42
SER	530.86	±	8.20	527.73	±	50.36	0.96	525.94	±	7.59	507.06	±	54.41	0.79
ASN	41.82	±	3.72	43.34	±	6.06	0.82	52.55	±	3.54	62.17	±	4.09	0.18
GLU	30.49	±	5.67	35.44	±	4.32	0.35	9.55	±	5.50	10.46	±	1.23	0.63
GLN	443.21	±	14.11	430.19	±	38.18	0.79	513.91	±	14.09	522.33	±	35.73	0.86
PRO	147.70	±	7.50	144.10	±	10.04	0.83	168.11	±	7.29	170.36	±	7.37	0.90
GLY	329.95	±	27.37	295.21	±	21.47	0.30	360.70	±	27.99	310.74	±	16.45	0.11
ALA	267.40	±	9.28	287.31	±	23.38	0.63	293.27	±	8.57	329.35	±	26.51	0.39
CIT	192.88	±	28.90	205.94	±	23.40	0.72	197.30	±	29.53	207.81	±	29.76	0.80
VAL	377.13	±	28.28	440.54	±	33.27	0.16	414.04	±	28.38	490.01	±	48.48	0.18
CYS	14.41	±	1.88	13.71	±	0.68	0.76	15.43	±	2.26	16.26	±	0.62	0.75
MET	90.97	±	2.74	84.27	±	5.00	0.68	99.45	±	2.98	95.90	±	6.69	0.84
ILEU	95.63	±	11.58	110.29	±	8.31	0.24	118.77	±	11.41	140.25	±	13.35	0.18
LEU	161.02	±	14.51	173.20	±	16.73	0.59	197.20	±	14.35	228.53	±	20.41	0.23
TYR	107.93	±	5.73	102.78	±	14.03	0.73	119.65	±	6.08	129.15	±	10.23	0.36
PHE	95.37	±	3.96	103.52	±	13.40	0.58	109.07	±	4.29	125.57	±	15.64	0.30
TRP	42.17	±	5.35	46.26	±	5.49	0.54	46.44	±	4.47	53.83	±	5.37	0.29
ORN	83.97	±	16.32	92.77	±	7.06	0.35	85.70	±	14.72	98.25	±	5.72	0.18
LYS	109.14	±	10.08	105.80	±	12.47	0.83	134.25	±	9.44	140.61	±	13.64	0.70
HIS	47.16	±	3.94	46.27	±	6.81	0.91	54.11	±	3.75	60.67	±	4.33	0.34
ARG	162.88	±	21.66	146.57	±	13.68	0.34	181.73	±	20.90	170.87	±	16.07	0.58

Ala, alanine; Arg, arginine; Asn, Asparagine; Asp, aspartate; Cit, citrulline; Cys, cystine; Gln,glutamine; Glu, glutamate; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Orn, ornithine; Phe, phenylalanine; Pro, proline; Ser, serine; Tau, taurine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine

Table 3.9. Uterine uptake per kg uterus (umole/min/kg uterus)

	NTS-RNAi (n=5)			***SLC2A3*-RNAi** (n=6)			***p*-value**
TAU	-4.05	±	1.50	1.00	±	1.67	0.06
ASP	-1.92	±	0.73	-2.24	±	0.95	0.80
THR	7.50	±	1.69	18.20	±	4.81	0.09
SER	8.03	±	1.15	18.96	±	4.53	0.06
ASN	4.07	±	0.60	7.63	±	2.96	0.31
GLU	-3.88	±	1.21	-2.02	±	1.53	0.38
GLN	21.71	±	1.71	36.47	±	9.14	0.18
PRO	8.23	±	2.86	17.85	±	5.64	0.19
GLY	-12.30	±	5.17	2.13	±	7.00	0.14
ALA	6.98	±	2.06	15.77	±	3.92	0.10
CIT	7.16	±	1.36	18.26	±	5.96	0.13
VAL	21.75	±	2.87	38.05	±	8.30	0.12
CYS	0.41	±	0.83	0.83	±	2.50	0.89
MET	3.08	±	0.72	4.21	±	1.88	0.62
ILEU	13.40	±	1.26	22.95	±	4.09	0.07
LEU	18.38	±	2.17	29.89	±	4.88	0.08
TYR	2.81	±	2.07	6.97	±	3.63	0.37
PHE	5.27	±	2.21	6.54	±	1.98	0.68
TRP	5.44	±	2.15	5.99	±	1.75	0.84
ORN	8.70	±	3.14	8.89	±	9.36	0.99
LYS	7.80	±	1.91	15.69	±	4.94	0.20
HIS	3.71	±	0.87	10.05	±	4.01	0.19
ARG	16.50	±	4.68	11.28	±	6.89	0.56

Ala, alanine; Arg, arginine; Asn, Asparagine; Asp, aspartate; Cit, citrulline; Cys, cystine; Gln,glutamine; Glu, glutamate; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Orn, ornithine; Phe, phenylalanine; Pro, proline; Ser, serine; Tau, taurine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine

Table 3.10. Umbilical uptake per kg fetus(umole/min/kg fetus)

	NTS-RNAi (n=4)			***SLC2A3*-RNAi** (n=5)			***p*-value**
TAU	0.03	±	0.04	0.09	±	0.13	0.74
ASP	0.05	±	0.18	0.03	±	0.12	0.94
THR	1.95	±	0.77	2.01	±	0.53	0.95
SER	-0.49	±	0.63	-0.80	±	0.51	0.71
ASN	1.03	±	0.26	1.43	±	0.35	0.41
GLU	-2.53	±	0.30	-2.30	±	0.33	0.64
GLN	6.78	±	1.48	6.74	±	0.61	0.98
PRO	1.88	±	0.23	1.68	±	0.33	0.66
GLY	3.14	±	0.79	3.18	±	0.45	0.97
ALA	2.58	±	1.01	2.79	±	0.26	0.83
CIT	0.56	±	0.36	0.37	±	0.29	0.69
VAL	4.02	±	1.13	4.20	±	0.78	0.89
CYS	0.06	±	0.11	0.22	±	0.10	0.30
MET	0.89	±	0.31	1.09	±	0.19	0.57
ILEU	2.52	±	0.47	2.51	±	0.34	0.99
LEU	4.10	±	0.79	4.03	±	0.39	0.94
TYR	1.20	±	0.26	1.36	±	0.13	0.57
PHE	1.41	±	0.25	1.50	±	0.19	0.77
TRP	0.36	±	0.10	0.40	±	0.06	0.80
ORN	0.22	±	0.18	0.36	±	0.28	0.72
LYS	2.54	±	0.70	2.65	±	0.31	0.87
HIS	0.84	±	0.21	0.80	±	0.13	0.87
ARG	1.90	±	0.49	2.00	±	0.13	0.84

Ala, alanine; Arg, arginine; Asn, Asparagine; Asp, aspartate; Cit, citrulline; Cys, cystine; Gln,glutamine; Glu, glutamate; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Orn, ornithine; Phe, phenylalanine; Pro, proline; Ser, serine; Tau, taurine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine

Table 3.11. Uteroplacental uptake per kg placenta (umole/min/kg placenta)

	NTS-RNAi (n=4)			***SLC2A3*-RNAi** (n=5)			***p*-value**
TAU	-5.76	±	3.25	0.44	±	2.88	0.20
ASP	-4.06	±	2.08	-3.28	±	1.08	0.73
THR	-4.12	±	5.50	15.82	±	7.68	0.09
SER	16.84	±	4.08	38.71	±	9.75	0.10
ASN	-3.51	±	1.44	1.76	±	3.88	0.29
GLU	16.12	±	0.17	17.41	±	4.79	0.82
GLN	-28.56	±	11.85	7.66	±	12.27	0.08
PRO	-7.84	±	1.65	14.58	±	8.67	0.06
GLY	-50.07	±	14.17	-16.10	±	9.65	0.08
ALA	-12.55	±	12.38	2.93	±	5.65	0.26
CIT	3.72	±	4.06	31.46	±	11.22	0.07
VAL	-1.15	±	12.09	27.68	±	12.90	0.15
CYS	-0.83	±	2.12	-0.78	±	4.85	0.99
MET	-4.03	±	2.52	-4.20	±	3.57	0.97
ILEU	-2.68	±	6.02	14.57	±	6.40	0.10
LEU	-8.05	±	10.12	13.08	±	6.26	0.10
TYR	-9.63	±	4.14	-0.80	±	5.81	0.28
PHE	-8.03	±	2.77	-2.69	±	2.83	0.23
TRP	3.77	±	4.03	5.52	±	3.14	0.74
ORN	15.70	±	4.10	5.94	±	20.71	0.69
LYS	-9.69	±	5.41	4.37	±	5.71	0.12
HIS	-0.89	±	1.70	13.32	±	7.64	0.15
ARG	9.72	±	9.38	4.12	±	12.62	0.74

Ala, alanine; Arg, arginine; Asn, Asparagine; Asp, aspartate; Cit, citrulline; Cys, cystine; Gln,glutamine; Glu, glutamate; Gly, glycine; His, histidine; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Orn, ornithine; Phe, phenylalanine; Pro, proline; Ser, serine; Tau, taurine; Thr, threonine; Trp, tryptophan; Tyr, tyrosine; Val, valine

Metabolic study

During the metabolic study, the data was analyzed by period, first by comparing means of glucose and insulin during baseline, GSIS and ASIS. Then, to better understand the differences between individual timepoints during GSIS, data collected during the GSIS period was split into “early GSIS,” representing 5, 10, 15, 20, and 30 mins, and “late GSIS,” representing 60, 75, 90, 105 mins.

First we compared the means of umbilical artery plasma glucose and insulin across all periods: baseline, early-GSIS, late-GSIS, and ASIS (Figure 3.7a). There was only a main effect of period for glucose concentrations, with baseline glucose concentrations being significantly smaller than those during fetal hyperglycemic clamp (GSIS and ASIS). For umbilical artery plasma insulin concentrations (Figure 3.7b), there was a significant interaction of treatment x period, such that Fisher's least significant difference was applied to examine individual periods. As evidenced in (Figure 3.7b), there was no effect of treatment during baseline, however were significantly higher than the NTS-RNAi controls during early-GSIS and tended to be higher during late-GSIS (P =0 .018 and P = 0.082, respectively). During the ASIS period, *SLC2A3*-RNAi insulin concentrations were significantly higher than the NTS-RNAi group (56% increase, P <0.01).

To better understand the insulin response of the fetal pancreas during GSIS, umbilical arterial plasma insulin concentrations were assessed at individual time points during baseline and GSIS (Figure 3.8). During early-GSIS, umbilical insulin was significantly higher in the *SLC2A3*-RNAi pregnancies compared to the NTS-RNAi at 5 mins and 20 mins (P<0.05). At 10 and 15 mins during early-GSIS, *SLC2A3*-RNAi insulin concentrations tended to be greater than the NTS-RNAi group (P = 0.07 and P = 0.06, respectively). During the first four timepoints of late-GSIS, there were no differences between treatment groups, but by 105 mins the mean insulin concentration of the *SLC2A3*-RNAi pregnancies were significantly greater than the NTS-RNAi group (*P*<0.01).

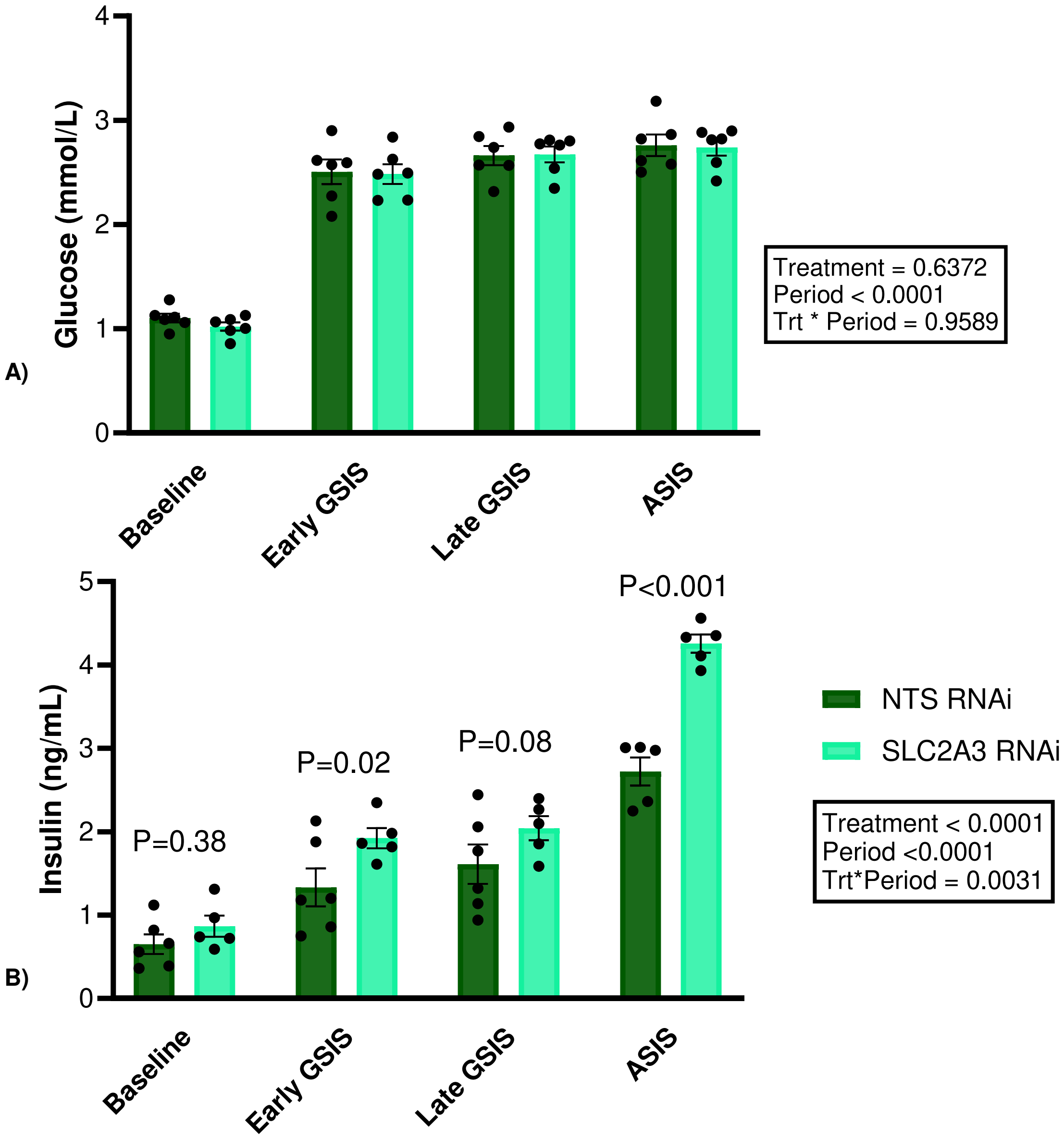

Figure 3.7. Umbilical artery plasma glucose(A) and insulin (B) concentrations during baseline, early and late GSIS, and ASIS periods.

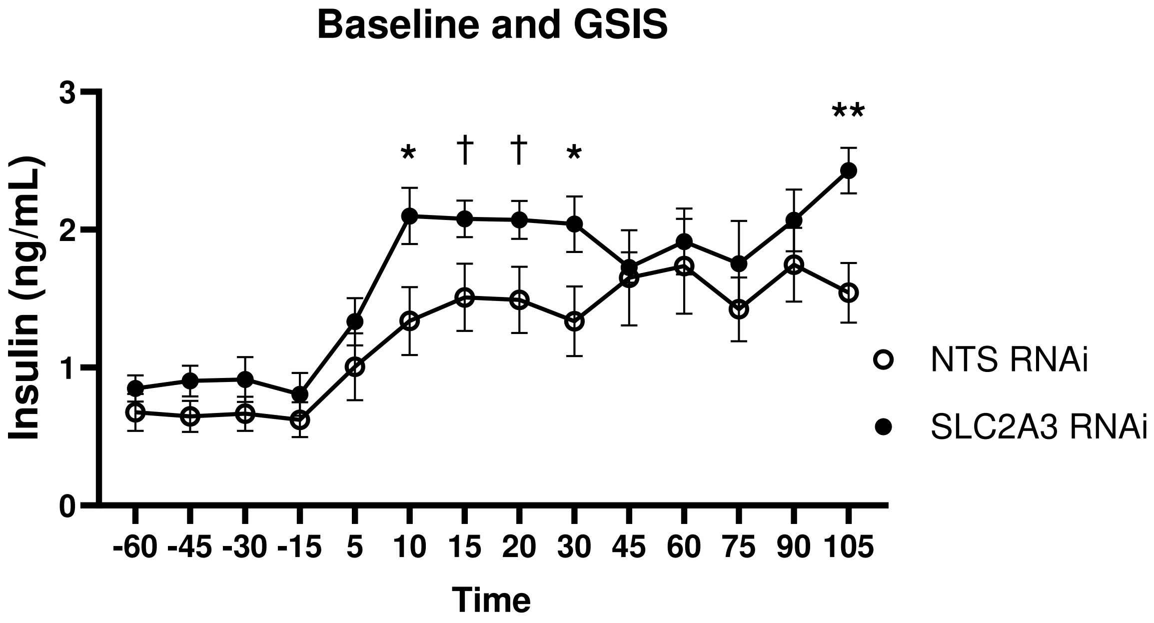

Figure 3.8. Baseline and GSIS mean umbilical artery plasma insulin concentrations.
*† = P<0.10, * = P<0.05, ** = P< 0.01*

DISCUSSION

An adequate supply of glucose to the fetus is critical for fetal oxidative processes and growth. Glucose availability to the fetus depends upon placental uptake and transfer, which is mediated by facilitative glucose transporters, primarily SLC2A1 and SLC2A3 (Barry & Anthony, 2008; Wooding et al., 2005). The importance of placental glucose transport to the fetus is illustrated by the magnitude of fetal hypoglycemia being correlated with FGR (Economides & Nicolaides, 1989; Marconi & Paolini, 2008). Additionally, the placenta is a highly metabolic organ in itself, utilizing 80 and 72% of uterine-supplied glucose at mid- and late-gestation, respectively, to maintain placental function, which also directly impacts the maternal-fetal glucose gradient (Bell et al., 1986; Meschia et al., 1980). As mentioned in the introduction, the distinct separation of where SLC2A1 (basolateral) and SLC2A3 (apical) are located in the sheep trophoblast (Wooding et al., 2005) offers the ability to compare basolateral vs. apical placental glucose transport.

We have previously reported that at mid-gestation (75 d GA), lentiviral-mediated RNA interference (RNAi) of SLC2A3 resulted in fetuses that were smaller, hypoglycemic, and had reduced umbilical artery insulin and glucagon concentrations (Lynch et al., 2022). Their pancreases were also significantly smaller and exhibited an altered transcriptome, suggesting a shift in substrate utilization (Kennedy et al., 2024). Under those same experimental parameters in the current study, by near-term (133 ± 2 dGA) fetuses had rescued growth and umbilical glucose concentrations. Their pancreases were no longer smaller compared to the NTS-RNAi controls, but were more sensitive to glucose and exceptionally sensitive to arginine. Similarly to fetuses from 75 dGA, the placental IGF axis was also impacted. Placental and maternal physiology were also affected, with reductions in both placental glucose utilization and uterine glucose uptake.

The results of these experiments expand upon our earlier work by studying pregnancies near-term under the same experimental paradigm previously investigated at mid-gestation. Our findings highlight the importance of SLC2A3's role in microvillous trophoblast glucose transport and demonstrate that impaired placental glucose uptake at the maternal-fetal interface throughout gestation impacts uterine nutrient uptake, placental glucose and amino acid utilization and transfer to fetus. The phenotype observed at 75 dGA impacted the near-term phenotype by inducing a compensatory response in the placenta, in addition to impacting the responsiveness of the fetal pancreas to glucose and arginine challenges in vivo.

At necropsy, fetal body weight and measurements of fetal growth did not differ, which is in contrast to data obtained at 75 dGA, where fetal weight tended to be smaller and head circumference, femur and tibia length were all significantly smaller (Lynch et al., 2022). Considering the 75 dGA data in the context of the numerous studies of FGR in both humans and animal models, where fetal growth is restricted to due to impaired nutrient delivery by the placenta (Barry et al., 2008; Gagnon, 2003), we anticipated fetal growth restriction would continue throughout gestation in our SLC2A3-RNAi pregnancies.

While placental weight and uteroplacental weight did not differ between treatment groups, when uteroplacental weight was normalized to fetal weight (Figure 3.2c), the SLC2A3-RNAi pregnancies were 25% greater than the NTS-RNAi controls (P = 0.04). Within the placenta, mRNA for NOS3 was significantly increased in the SLC2A3-RNAi pregnancies (Table 3.4). It is well established that NOS3 is an important regulator of placental angiogenesis and vascular development; Nos3-/- knockout mice are characterized by FGR and placental dysfunction (Wilson et al., 2022). IGF2 is thought to control the recruitment of maternal blood supply to the placenta (Roberts et al., 2008), and in our samples, IGF2 mRNA was also increased (to be discussed further). The increase in mRNA of both of these genes may represent increasing vascularity in the placenta to maintain placental growth and function. Placental members of the insulin-like growth factor axis were also affected, with IGF2, IGF1R, and IGF2R mRNA concentrations all tending to be increased compared to the NTS-RNAi pregnancies, all three of which were also significantly increased in the SLC2A3-RNAi group at 75 dGA (Lynch et al., 2022). Sheep models of FGR have reported altered expression of IGFs in response to FGR, with placental IGF2 mRNA increasing (de Vrijer et al., 2006). When pregnant guinea pigs were chronically infused with IGF2, placental and fetal weights as well as fetal uptake of glucose were increased (Sferruzzi-Perri et al., 2006, 2007). The upregulation of these mRNA near-term may be a continuation of compensatory mechanisms that began at mid-gestation to stimulate placental growth and increase the total surface area for nutrient exchange. If we consider the concept of the placenta functioning as a nutrient sensor (Jansson & Powell, 2006) that can modify its receptor activity in response to altered substrate levels (i.e. adaptive regulation; Jones et al., 2007), it is highly possible that the increase in IGF2 and both IGF receptors' mRNA could have been driven by the fetal hypoglycemia seen at mid-gestation. One way the placenta is believed to sense nutrients is through the mammalian target of rapamycin (mTOR) pathway, which is highly expressed in the syncytiotrophoblast and downregulated in placentas of human fetuses with restricted growth (Jansson & Powell, 2006; Roos et al., 2009). Perhaps the mTOR pathway is stimulating increased mRNA concentrations of IGF2, IGF1R, and IGF2R to induce compensatory placental and fetal growth, resulting in no differences in their weights near term.

During the baseline period of the metabolic study, although there was no differences in uterine blood flow (Figure 3.3), glucose concentrations were different in both uterine vessels (Figure 3.4a). At mid-gestation, uterine artery and vein glucose concentrations were 20-25% reduced (Lynch et al., 2022) and were associated with significant reductions in uterine artery IGF1. In the present study, baseline maternal glucose concentration was 10% lower ($P<0.05$), and uterine artery IGF1 concentration was also 20% lower (Figure 3.5b). It appears that the depressed uterine glucose and IGF1 from 75 dGA persisted to near-term. While the the direct impact of reduced placental MVM glucose transport on maternal physiology is unclear, it is likely due to altered placental function that could involve maternal glucagon levels, which were depressed at 75 dGA (Lynch et al., 2022). Unfortunately, there is a deficit of information about maternal glucagon concentrations during FGR in both human and animal model studies, so the direct causes of the observed impacts on maternal physiology are still undefined.

The most notable difference from the 75 dGA phenotype, was the lack of fetal hypoglycemia near-term, even in the face of significantly reduced uterine uptake of glucose (Figure 3.4). The SLC2A3-RNAi experimental paradigm was designed to reduce microvillous membrane uptake of glucose, which we were successful in doing at both 75 dGA (evidenced by fetal hypoglycemia) and near-term (direct assessment of uterine uptake). However, it appears that that the reduction in uterine glucose uptake near-term was offset by a 36% reduction in uteroplacental glucose utilization in the SLC2A3-RNAi pregnancies (Figure 3.4), allowing for near normal umbilical glucose uptake. Given the lack of difference in fetal weight and umbilical glucose concentrations near-term, when both were significantly reduced at mid-gestation, attenuated uteroplacental glucose utilization may be another compensatory mechanism that began with the mid-gestation phenotype. The placenta appears to be downregulating its own glucose utilization in favor of transporting more glucose to a fetus that was hypoglycemic at mid-gestation, resulting in rescued fetal growth and fetal glucose concentrations by near-term.

Studies of late gestation fetal growth restriction in both humans and sheep offer contrasting observations of the placental response, despite similarities in having smaller placentas and hypoglycemic fetuses. One group has reported fetally-driven increases in the transplacental glucose gradient in human FGR pregnancies (Marconi et al., 1996; Marconi & Paolini, 2008), which has been supported by similar data in sheep (Thureen et al., 1992). Another model of FGR in sheep reported the opposite effect on the placenta: reduced absolute placental transport capacity which when normalized to weight was not different, suggesting the limitation of glucose transfer is the smaller placenta (Wallace et al., 2003). In the current study, the transplacental glucose gradient tended to be reduced (10-12%), but this is overshadowed by the fact that the placenta is significantly reducing its own glucose utilization, contributing to recovered fetal weight (i.e. no FGR) and placental mass.

Amino acid concentrations were also assessed at baseline in the uterine and umbilical arteries and veins. At mid-gestation, the only amino acid that differed was arginine, which was significantly reduced in both umbilical vessels (Lynch et al., 2022). Near-term, no differences were found in umbilical concentrations or uptakes of amino acids (Table 3.8). However, uterine uptakes (per kg of uterus) of taurine, threonine, serine, alanine, isoleucine and leucine all tended to be greater in the SLC2A3-RNAi pregnancies (Table 3.7). Many studies of FGR have observed reduced umbilical concentrations of amino acids and increased concentrations of some essential amino acids in maternal circulation (Jones et al., 2007). Perhaps the increased uterine uptakes of those amino acids are tied to the decrease in uterine glucose concentrations as part of the compensatory response to the mid-gestation FGR phenotype. However, the most notable changes in amino acids found at near-term were increased uteroplacental amino acid utilization (Table 3.11). In addition to increased utilization of threonine, glutamine, serine, proline, glycine, citrulline, isoleucine, and leucine, the sum of amino acid carbons utilized by the placenta also tended to be increased. If we consider the “placenta as a nutrient sensor” model, fetal demand could have been the driving force behind modifying placental amino acid transport capacity (Jansson & Powell, 2006).

Increased utilization of branched-chain amino acids (BCCAs) such as leucine, isoleucine, and glutamine provide direct evidence of increased amino acid oxidation for energy production within the placenta. During normal gestation, the human and sheep placenta both have high activity of BCAA transferases, suggesting significant metabolism of BCAAs into their corresponding keto acids, which are then further processed for energy production (Cetin, 2001).

There is also a growing body of research demonstrating the relationship between amino acid concentrations during gestation and mTOR signaling, which impacts protein synthesis in the placenta and amino acid transport into fetal circulation (Hussain et al., 2020; Jansson & Powell, 2006; G. Wu et al., 2010). As mentioned earlier, mTOR signaling could be part of an “intrinsic” placental nutrient sensing mechanism regulating placental transport of amino acids impacted in our model, such as leucine and glutamine (Jansson & Powell, 2006; Jones et al., 2007).

Our data appears to be in contrast to observations of placental amino acid flux in FGR pregnancies, such as in a sheep model of FGR which exhibited significantly reduced transplacental leucine flux (Marconi & Paolini, 2008). Instead, the observed increase in glutamine and leucine utilization by the placenta provides more support for the compensatory response of the placenta to rescue fetal growth that began with the mid-gestation phenotype.

Another study observed that insulin and IGF1 increase placental amino acid transport by altering mTOR signaling (Roos et al., 2009). Our studies exhibited increased placental IGF2, IGF1R and IGF2R mRNA seen at mid-gestation that continued to near-term, which could be interacting with mTOR signaling in the placenta to increase amino acid utilization in a similar fashion to the relationship observed by Roos et al. (2009).

In summary, the placental metabolism of amino acids directly impacts the flux of amino acids to the fetus, and our data indicates that the placenta is utilizing more amino acids, while at the same time utilizing less glucose. This results in no differences in total carbon utilization by the placenta, as the reduction in glucose is balanced out by the increase in amino acid utilization. This could be the first evidence of inducing a placental shift in substrate utilization to provide glucose to the fetus while increasing amino acid oxidation for its own energy production, uniquely contrasting much of the physiology reported in FGR studies. At 75 dGA, fetal pancreas weights were 23% smaller (Lynch et al., 2022) and had altered metabolic transcriptomes suggesting a shift in energetic substrate utilization (Kennedy et al., 2024), but by near-term pancreas weights were no longer different. Therefore, it is possible that either the shift in energetic substrates by the pancreas that had begun by mid-gestation was successful in rescuing pancreatic growth, or another compensatory mechanism provided enough nutrients to support continued pancreatic growth as gestation progressed.

While the fetal pancreas recovered in size between mid-gestation and near-term, the metabolic response of the fetal pancreas to hyperglycemia (GSIS) and an arginine bolus (ASIS) provides more insight into the altered metabolism of the fetus and pancreatic function: we observed significant impacts on the responsiveness of the fetal pancreas to both glucose and arginine challenges in vivo. A significant interaction of treatment by period was observed for umbilical artery plasma insulin concentrations, with the SLC2A3-RNAi pregnancies' insulin concentrations being significantly higher than NTS-RNAi controls during the early-GSIS period and tended to be higher in late-GSIS (Figure 3.7). When we look at individual timepoints during GSIS (Figure 3.8), the difference in glucose sensitivity in the SLC2A3-RNAi group becomes even more apparent. Right at the onset (5 mins) of early-GSIS, they have significantly higher insulin concentrations compared to the NTS-RNAi controls, which trends higher until it reaches significance again at 20 mins. The SLC2A3-RNAi group then had significantly greater insulin concentrations during the late-GSIS period at the 105 min timepoint. The significant differences in insulin concentrations during early GSIS followed by significance again at the last timepoint during late-GSIS could be reflective of different processes in the development of the fetal pancreas' response. It is difficult to study the development of insulin secretion in humans, however, we do have some insights from studies in fetal sheep. One such study by Aldoretta and colleagues (1998) investigating the maturation of glucose-stimulated insulin secretion in fetal sheep points out that the maturation of insulin secretion by the fetus could be related just as much to β-cell mass and/or development as it is to insulin synthesis and mechanisms of insulin secretion. Transcriptomics of the smaller pancreases from mid-gestation certainly points to potentially altered pancreatic function and β-cell activity, as we saw shifts in pathways related to altered metabolic substrate utilization and pancreatic secretion, to name a few (Kennedy et al., 2024).

Following the arginine bolus for the ASIS period, umbilical insulin concentrations were significantly higher in the SLC2A3-RNAi group again (56% increase). After midgestation, fetal sheep develop a significant increase in arginine-induced insulin secretion with advancing gestational age (Aldoretta et al., 1998). The difference in umbilical arterial arginine concentrations at mid-gestation (Lynch et al., 2022) could have been one of the early signs of the fetal pancreas developing an inappropriate insulin response in the face of hypoglycemia. This manifested as higher sensitivity to arginine later in gestation when the fetal pancreas would normally have reached term levels of insulin responsiveness.

These data demonstrates that the overall effect of SLC2A3-RNAi induced fetal hypoglycemia on pancreas development and growth that we observed at 75 dGA in both the physiological data (Lynch et al., 2022) and fetal pancreatic transcriptome (Kennedy et al., 2024) altered fetal pancreas glucose and arginine sensitivity near-term. Both glucose and arginine-stimulated insulin secretion at mid-gestation are only at about 20% of the rate at near-term (Aldoretta et al., 1998). Therefore, it is entirely possible that those fetal pancreases were already predisposed to dysfunctional insulin responsiveness by mid-gestation, with the severity of the impacts being more apparent by the time the metabolic study was performed at near-term.

In conclusion, this study, along with the knowledge of the previous two studies of SLC2A3-RNAi pregnancies at mid-gestation (Kennedy et al., 2024; Lynch et al., 2022) demonstrate that microvillous glucose uptake by the placenta can be rate-limiting to fetal growth and development early in gestation. Concentrations of maternal IGF-1 and glucose were reduced at mid-gestation, and persisted to near-term, when the uterus also exhibited reduced glucose uptake. While the exact mechanisms of the impacts on maternal physiology are difficult to explain with the data at hand, it is still clear that maternal physiology was impacted in some way by SLC2A3-RNAi induced alterations in placental function. In the fetus, despite evidence of recovered glucose delivery by the placenta, normal pancreatic function had not been established by near-term. When those fetuses were challenged during the GSIS and ASIS periods, their pancreases had altered insulin responses, with significantly more sensitivity to arginine. However, the most striking aspect of the near-term phenotype was the placenta seemingly driving its own adaptations to reduced maternal glucose uptake and the smaller fetal growth at mid-gestation, acting as a mediator between the maternal and fetal compartments. The placenta reduced its own glucose utilization to support fetal growth while increasing amino acid utilization, presumably for its own metabolic needs. This placental compensation is happening despite no differences in blood flow and in the face of continued reduced uterine glucose uptake. This data therefore provides evidence of an induced placental compensatory response, offering support for the role of the placenta as a nutrient sensor. Finally, this research also demonstrates the utility of combining RNAi in a large animal model with steady-state metabolic assessment of whole-body physiology to understand the integration of the maternal, placental, and fetal compartments.

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