CONSEQUENCES OF MATERNAL NUTRIENT RESTRICTION ON OVINE PLACENTAL DEVELOPMENT

A Dissertation

by

ASHLEY BROOKE KEITH

Submitted to the Office of Graduate and Professional Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Chair of Committee,	M. Carey Satterfield
Co-Chair of Committee,	Kathrin A. Dunlap
Committee Members,	Fuller W. Bazer
	Guoyao Wu
	Michael C. Golding
Head of Department,	H. Russell Cross

August 2015

Major Subject: Physiology of Reproduction

ABSTRACT

Maternal nutrient intake and partitioning, uteroplacental blood flow, nutrient transporter activity, and fetoplacental metabolism mediate nutrient delivery to the fetus. Inadequate delivery of nutrients results in intrauterine growth restriction (IUGR), a leading cause of neonatal morbidity and mortality. The present studies exploited natural population variance in nutrient-restricted (NR) ewes to identify subpopulations of IUGR and non-IUGR fetuses as subjects for research to elucidate adaptive mechanisms of fetal-placental development.

Singleton pregnancies were generated by embryo transfer and assigned to receive either 50% (n=24) or 100% (n=7) of the National Research Council's (NRC) recommended dietary intake from Day 35 to Day 125 of gestation, at which time ewes were necropsied. Maternal weight did not correlate with fetal weight; therefore, differences in development of the six heaviest (NR non-IUGR) and six lightest (NR IUGR) fetuses from NR ewes, as well as the seven fetuses from control ewes were compared. Mean weights of NR IUGR fetuses (2.8±0.1 kg) were lower ($P<0.05$) than for control (4.0±0.1 kg) and NR non-IUGR (4.1±0.1 kg) fetuses.

The first study investigated potential mechanisms regulating nutrient availability for fetuses. Results indicated that normal fetal growth in a subpopulation of NR ewes is associated with enhanced delivery of a number of amino acids and their metabolites into the fetal circulation, which may at least partially result from up-regulation of expression of amino acid transporter mRNAs in the placentome. The second study elucidated

potential physiological mechanisms regulating placental growth and development in ewes having NR IUGR and NR non-IUGR fetuses. Results suggest that placentome morphology and angiogenic growth factor expression varies in response to maternal nutritional challenge during pregnancy and may play critical roles in regulating fetal growth. The third study was conducted to capitalize on natural population variance in NR ewes to identify novel factors regulating placental growth and function. Results suggest that enhanced fetal growth in NR non-IUGR pregnancies is associated with an altered expression of genes related to immune response and function in the placentome. Collectively, results of these studies suggest that enhanced fetal growth in a subset of NR ewes is associated with enhanced expression of select nutrient transporters and angiogenic factors, increased nutrient availability to the fetus, altered placentome morphology, and an altered immune response within the placentomes of those ewes.

DEDICATION

To the late Brook Isbell

I am forever grateful that Papaw instilled in me his love of livestock, a little of his knowledge, his wisdom, and his stubborn determination to always achieve goals.

Commit to the LORD whatever you do, and your plans will succeed. – Proverbs 16:3

ACKNOWLEDGEMENTS

A simple “thank you” barely begins to express my gratitude to everyone in my personal and professional lives who have helped me achieve my goals. First of all, to my parents, your endless encouragement and love always gave me the extra bit of drive to keep going. Thank you for never failing to listen to me vent, cry, and ramble on with excitement. To all of my brothers, the few words of support, the infinite sarcasm, and the never-ending laughter always brightened even my grumpiest moods. I am blessed to have an enormous family filled with grandparents, aunts, uncles, and cousins that constantly offered abounding support, faith, advice, and love.

I cannot imagine my life without the support of my friends and colleagues. You never know how truly blessed you are until you have friends drop everything in their life, no matter where they are, to be by your side when you need them most. Thank all of you for the smiles, laughter, shoulders to cry on, and knowing how to take my mind off of whatever was worrying me. To all of graduate and undergraduate students I have had the opportunity to work with over the years, thank you for making life as a graduate student entertaining and fun, especially when none of us wanted to be in the office, lab, or at the farm.

I would like to thank my committee members, Drs. Fuller Bazer, Guoyao Wu, and Michael Golding for their guidance, support, and knowledge. Your knowledge and guidance continually pushed me to challenge myself and be more successful. Thank you to all of my professors and mentors, especially Drs. Ron Randel, Tom Welsh, and David

Forrest, for always taking the time to listen to whatever I needed to discuss, offering continual words of advice and encouragement, and guiding me to so many wonderful opportunities.

Lastly, I owe an enormous thank you and immense gratitude to the co-chairs of my committee, Drs. Carey Satterfield and Kathrin Dunlap. It is truly an understatement to say I would not be here without you. Dr. Satterfield, thank you first of all for taking a chance at the very beginning of your faculty career and letting me begin my research experience as an undergraduate in your lab. Thank you for all of the times your procrastination and disorder drove me insane, but taught me to relax just a little bit. Dr. Dunlap, thank you for all of the chocolate, hugs, jokes, and kind threats that kept me seated in my chair during necropsies, but that never allowed me to quit moving forward. To both of you, thank you for not letting me simply settle and for giving me this opportunity. I cannot thank you enough for all of your help and support in all aspects of my life. Thank you!!

TABLE OF CONTENTS

Page

Page

LIST OF FIGURES

Figure Page

LIST OF TABLES

CHAPTER I

INTRODUCTION

Delivery of nutrients from the maternal circulation to the fetus is regulated by a multidimensional relationship involving maternal nutrient intake and partitioning, uteroplacental blood flow, expression and activity of nutrient transporters, and placental metabolism. Perturbation of any of these components may result in inadequate nutrient delivery to the conceptus (fetus and placenta) and potentially lead to intrauterine growth restriction (IUGR) of the fetus. Undernourishment during gestation is a global problem affecting numerous livestock species, such as cattle, sheep, and pigs, and may induce disruptions in the mechanisms regulating fetal nutrient availability [1, 2]. Subsequently, IUGR is a leading cause of neonatal morbidity and mortality in livestock species, as well as humans [3, 4].

Growth, development, and other characteristics of reproductive efficiency in livestock significantly affect the profitability of an operation. Interestingly, the *in utero* environment has been linked to postnatal growth and development [5-12]. Moreover, a correlation has been observed between a suboptimal uterine environment during gestation and an increased incidence of metabolic diseases in the adult [5, 13-16]. Thus, optimal fetal growth is imperative as it is a prerequisite for reproductive efficiency as well as lifelong health and productivity in livestock.

Over the past decades there has been an increased demand for sheep production worldwide, yet there has been a dramatic decline in the sheep population [17].

Therefore, a smaller population of sheep must meet the increased demand for products such as lamb and mutton. Notably, in some regions throughout China, the Middle East, North Africa, and India, lamb and mutton serve as the primary source of animal protein. With a vast majority of the world's sheep being produced in arid climates, nutrition during gestation is often limited. As discussed throughout this review, limited nutrient intake during gestation is a key contributor to neonatal death losses and can profoundly impact postnatal growth and development of the offspring. Based on the ewe's global significance, understanding the effects of nutrition during gestation, interactions between genes and the uterine environment that may affect the outcome of pregnancy, along with the various metabolic disturbances that may alter fetal growth and programming for postnatal life is of critical importance to ensure efficient production of animal protein to feed the world [1, 4, 18-22]. Additionally, studies in the undernourished sheep can be utilized to elucidate mechanisms whereby insufficient placental growth and nutrient transfer can alter economic factors of meat animal production, such as neonatal death loss, health and susceptibility to disease, average daily gains, and maturity at market weight [23]. Sheep models for investigating the impact of maternal nutrition on placental and fetal growth and development are also applicable to human medicine, as they may serve as a source of information pertaining to the impact of placental and fetal growth on pregnancy and health status of individuals throughout life [23].

It is well known that placental development occurs predominately during the first two-thirds of gestation, while rapid fetal growth and development occurs primarily during the latter third. The large increase in trans-placental exchange needed for

extensive fetal growth in the last third of gestation is possible only through vascular growth and the expression of nutrient transporters that occurs during early placental development [24, 25]. Consequently, studies involving various experimental paradigms resulting in IUGR fetuses have shown fetal growth retardation to be highly correlated with restricted placental growth [25, 26]. In fact, placental insufficiency is the predominant cause of IUGR [27].

Angiogenesis, in both the maternal and fetal placental tissues, is a vital part of placental development [28]. The primary role of the placenta is to exchange nutrients, wastes, and gases between the dam and fetus [25, 28, 29]. Importantly, vascular endothelial growth factor (VEGF), basic fibroblast growth factor (bFGF or FGF2), the angiopoietin family (ANG), and all respective receptors are recognized as the major factors regulating placental angiogenesis [26].

Nutrient delivery to the fetus is also dependent, in part, on expression of nutrient transporters and their function in the placental vasculature. Numerous environmental factors, such as, under- and over-nutrition, hypoxia, heat stress, and hormone exposure regulate activities of both glucose and amino acid transporters in the placenta [29-36]. A plethora of nutrient transporters are known to be expressed in the cotyledonary (fetal) placenta of sheep to aid in nutrient delivery to the fetus [37]. Moreover, a reduction in amino acid availability in placental fluids and fetal plasma occurs as a result of global nutrient restriction during gestation [38, 39]. Accordingly, a majority of these amino acids have critical roles in placental and fetal development. For example, glutamine, arginine, serine, alanine, and leucine induce cell proliferation [1, 40]. Metabolism of

glutamine provides reducing equivalents, which may serve as an energy source for the conceptus [40-42]. Finally citrulline and arginine serve as precursors for polyamine and nitric oxide (NO; a potent vasodilator) synthesis, which promote placental development and function [4, 40].

Although work has been done to define models of maternal nutrient restriction and its effects on fetal development, the biochemical cause(s) for nutritional programming, along with its long-term consequences, have not been fully elucidated. A simple and inexpensive solution to reverse the effects of IUGR on a fetus would be immensely beneficial to both the livestock industry and human pregnancies. Likewise, a biomarker to indicate placental and fetal growth restriction would be exceedingly advantageous for the adoption of early intervention strategies.

CHAPTER II

LITERATURE REVIEW

Development and Function of the Ovine Placenta

The mammalian placenta serves a multitude of tasks that work to ensure proper fetal development during gestation. As an organ with a high degree of plasticity, the placenta undergoes various physiological changes to maintain efficient nutrient, gas, and waste exchange between the mother and fetus. These dynamic morphological changes are regulated by a plethora of factors, such as hormones, angiogenic regulators, and nutrient-related genes to adjust to the demands of the growing fetus [29]. Thus, it is not surprising that placental development precedes the period of rapid fetal growth, as shown in Figure 2.1 [43].

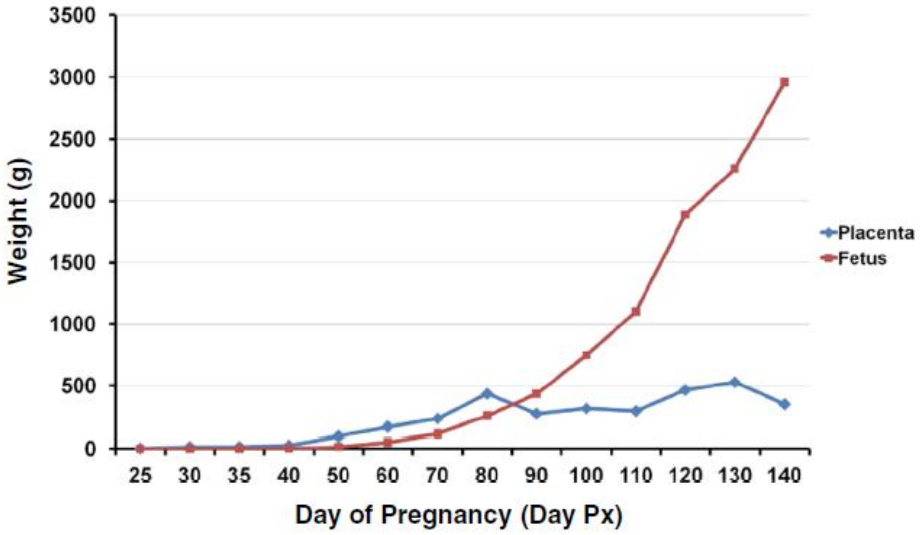


Figure 2.1 Placental and fetal weight throughout the sheep gestational period. Reprinted with permission from Bazer et al., 2012 [43].

Placentas are classified by their degree of invasiveness into the uterine wall and the distribution of their chorionic villi. The uterine endometrium of sheep, like other ruminant species, is characterized by aglandular caruncular and glandular intercaruncular regions [43, 44]. When placentation begins, fetal chorionic villi interdigitate with the maternal endometrium in the intercaruncular area to form an epitheliochorial interplacentomal region of the placenta. Interdigitation between fetal cotyledonary villi and maternal caruncular crypts results in the formation of placentomes, which become the primary sites of exchange between mother and fetus during gestation. Sheep are therefore classified as having a cotyledonary placenta. As early as gestational Day 14, chorionic binucleate cells migrate to the maternal uterine epithelium where they will later fuse with the maternal luminal epithelial cells to form a syncytial layer and ultimately a synepitheliochorial level of invasiveness [31, 43]. Thus, the placenta of ruminant livestock species is classified as both synepitheliochorial and cotyledonary.

The Peri-Implantation Period

Prior to placentation, the ovine conceptus goes through a period of rapid elongation followed by periods of apposition, adhesion, and implantation. Between Days 10 and 16 of gestation, the conceptus rapidly transforms from a spherical, to tubular, and then filamentous form [43, 45, 46]. This elongation process is essential for implantation, including apposition and adhesion involving the conceptus trophectoderm and the uterine luminal and superficial glandular epithelia (LE and sGE, respectively)

[43]. The fully elongated ovine conceptus begins implantation at approximately Day 15-16 of gestation.

As this filamentous conceptus becomes immobilized following apposition, cytoplasmic projections from conceptus trophectoderm cells interdigitate with uterine epithelial microvilli [43, 47]. Also during this time of apposition, the trophoblast extends finger-like villi, known as papillae, into the mouths of uterine glands for absorption of histotroph until Day 20 of gestation [43, 47-49]. These small areas of chorion located over the mouths of uterine glands develop into areolae and are common throughout the interplacentomal area of the ruminant placenta. Areolae function as a pouch-like structure that receive secretions, or histotroph, from the uterine glands to transport components of these secretions across the placenta and into the fetal circulation [43]. Preceding Day 25 of pregnancy, there is transitory loss of the uterine LE to allow apposition, adhesion, and attachment of the conceptus; the LE then begins restoration at approximately Day 25, and placentation progresses [43].

Placentome Formation

Following attachment of the trophoblast to uterine LE, fusion with the cotyledonary villi with the caruncular crypts occurs, giving rise to the placentomes, depicted in Figure 2.2 [26]. This interdigitation of cotyledonary villi into caruncular crypts begins around Day 24 of gestation with clusters of fetal binucleate cells initiating migration and syncytia formation [44]. In the beginning, cotyledonary villi are simplistic and fill the caruncular crypts to form an initial adherence. By approximately

Day 90 of gestation, cotyledonary villi reach their maximum length in the sheep placentome [44]. As gestation progresses to term, caruncular capillaries increase primarily in size, while cotyledonary capillaries proliferate by branching to form a complex vascular network of maximal surface area within the placentome, as shown in Figure 2.3 [26, 50].

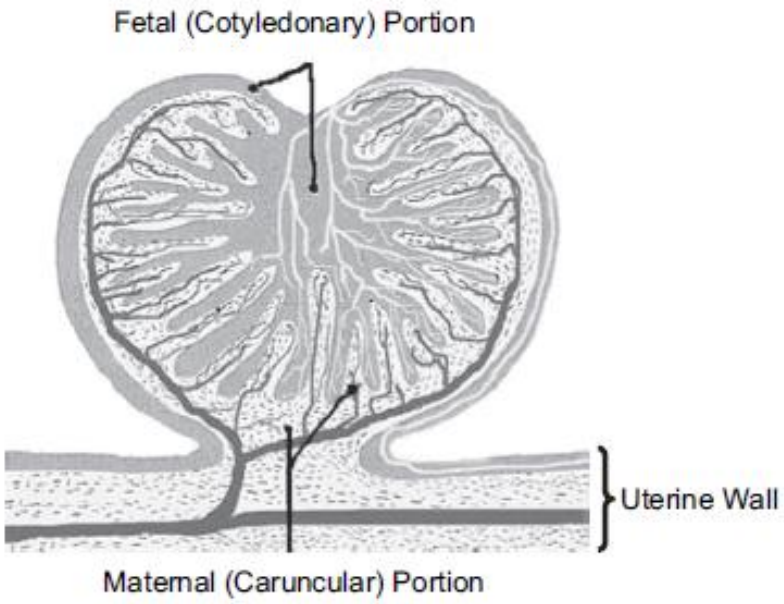


Figure 2.2. Schematic representation of the ovine placentome. Reprinted with permission from Reynolds et al., 2005 [26].

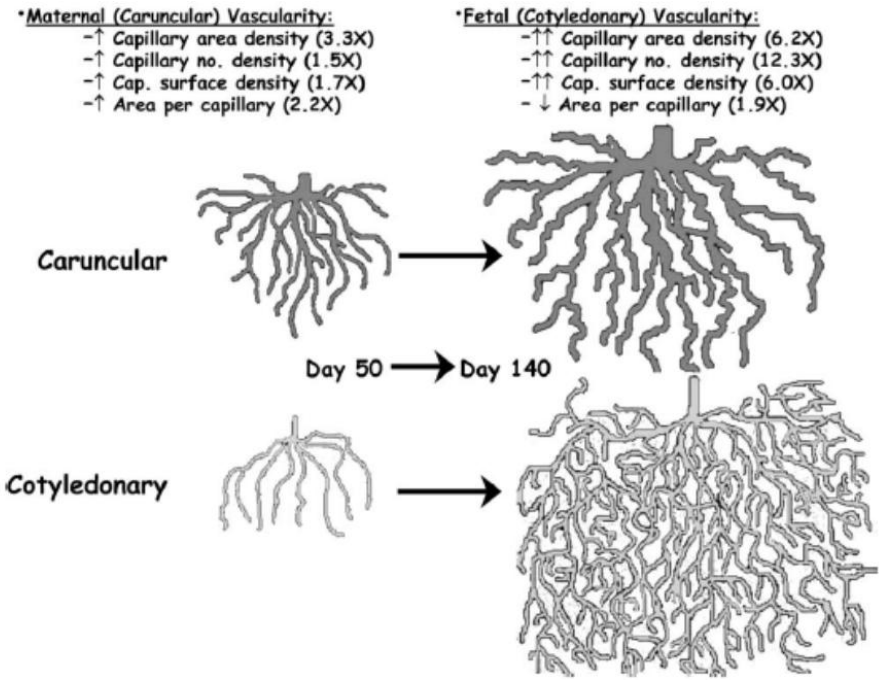


Figure 2.3. Model of angiogenesis in maternal caruncular and fetal cotyledonary portions of the sheep placentome during the final two-thirds of gestation. Reprinted with permission from Reynolds et al., 2005 [26].

Transfer Capacity of the Placenta

The capacity of the placenta to perform its principal functions of nutrient, waste, and gas exchange is achieved by a variety of mechanisms and structures, including fluid reservoirs within the extra-embryonic membranes, nutrient transporters, fetal-placental-uterine vasculature, and increased uteroplacental blood flow. This exchange process may be achieved by simple diffusion, passive diffusion, active transport, pinocytosis, or phagocytosis across the maternal and fetal-placental tissues and vasculature [44]. Proper placentation ensures adequate hematotrophic and histotrophic nutrition for fetal development via these mechanisms that involve the fetal fluids, placenta, uterus and fetal

vasculature [43]. Consequently, dynamic fluid changes within the various placental structures, particularly the allantois, allow for expansion of the placental membranes, ensuring maximal apposition and subsequently maximum placental surface area for transfer of nutrients and gases between the dam and conceptus.

The yolk sac is a transient organ that develops early in gestation, being highly vascularized by gestational Day 16 in the sheep, and serves as a nutrient reservoir and source of primordial germ cells and hematopoietic progenitor cells in the early conceptus [44]. This transient structure is soon replaced by the rapidly developing allantois [44]. Allantoic fluid is often thought of as simply a pool for fetal waste due to its anatomical connection to the fetal urachus; however, it functions largely as a reservoir for water, amino acids, proteins, and other nutrients [43, 51, 52]. Interestingly, previous work with ovine allantoic fluid has shown remarkable changes in the concentrations of amino acids between gestational Days 30 and 140, emphasizing the role of the allantoic sac as a nutrient reservoir [52]. The amniotic sac directly surrounds the fetus and functions to buoy the fetus for symmetrical development, prevent adherence of the fetal skin and amnion, and serves as a nutrient supply as the fetus consumes up to one liter of amniotic fluid daily [18, 43, 53]. Consequently, amniotic fluid, like allantoic fluid, is an available source of amino acids for the fetus [52] and ligation of the fetal esophagus to prevent amniotic fluid entry into the small intestine results in IUGR in sheep [54].

Uteroplacental blood flow plays an imperative role in facilitating adequate nutrient transport to the fetus. As gestation progresses the fetal demand for nutrients increases due to the exponential growth period of the fetus. This demand is achieved by

an increase in uteroplacental blood flow through both increased angiogenesis and vasodilation. Therefore, expression of angiogenic factors, such as the VEGFs, FGF2, and ANGPTs, and all of their respective receptors, along with the presence of vasodilatory molecules such as NO are imperative for proper development of the placental vasculature [26]. It should be noted that while the VEGFs, FGF2, and ANGPTs serve as major angiogenic factors, acid fibroblast growth factor, angiogenin, transforming growth factors, and many other factors can induce or stimulate angiogenesis [55] and may also play a role in placental angiogenesis.

Along with uteroplacental blood flow, a multitude of membrane transporters, are expressed by uterine epithelial and placental membranes to promote nutrient transport to the fetus [37, 56-59]. Transporter localization at both the maternal and fetal interfaces for transport across cell membranes is essential in the delivery of amino acids, polyamines, and other nutrients from the maternal circulation to the umbilical circulation [60]. Work in various species, such as sheep, humans, and rodents, has illustrated the necessity for placental nutrient transporters throughout gestation [40, 56-64].

Accordingly, it is widely accepted that proper placentation is crucial to growth and development of the fetus. Perturbations at any point during placental establishment may permanently alter fetal development. A common result of placental insufficiency is IUGR of the fetus [1, 4, 27, 65, 66]. Thus, numerous experimental models have been developed to elucidate the mechanisms regulating placentation and its impact on fetal development [1, 3, 4, 26, 38, 62, 65, 67-74]. The remainder of this review will discuss

blood flow, angiogenesis, and nutrient transport in the sheep placenta during a normal pregnancy, as well as in models of nutrient restriction from various species.

Angiogenesis and Vasodilation in the Ovine Placenta

As previously mentioned, uteroplacental blood flow plays an essential role in facilitating adequate nutrient transport to the fetus. The capacity for uteroplacental blood flow is influenced by two primary mechanisms, vascularity and vasodilation (increased volumetric capacity of the existing vasculature reduces resistance to blood flow). Placental vascularity is increased by angiogenesis, which is the development of new blood vessels from existing structures. Placental angiogenesis is largely regulated by the activity of the growth factor gene families, VEGFs, bFGFs the ANGs, and their respective receptors [26]. Increased vasculature and blood flow to the gravid uterine horn is evident as early as Day 24 of gestation [75]. This time point is correlated with the interdigitation of cotyledonary villi into maternal caruncular crypts [44]. Furthermore, as gestation progresses and growth of the fetus increases exponentially, the demand for uteroplacental blood flow increases and is fulfilled by both vasodilation and increased angiogenesis [43].

Uteroplacental blood flow

During early gestation, the rate of uterine blood flow increases about 4.0- to 6.0-fold between Days 11 and 30 of gestation [75]. A large majority of this increase occurs around Day 24 of gestation when the microvascular volume density increases in the

luminal caruncular tissue throughout the uterus (both the gravid and non-gravid uterine horns) [75]. From mid- to late gestation, an approximate 3.2-fold increase in uterine blood flow and a 19-fold increase in umbilical blood flow occurs [25]. This is concomitant with an approximate 0.5-fold increase in caruncular vascular density and 6.0-fold increase in cotyledonary vascular density [25]. Furthermore, as the placenta reaches its maximum size around Day 90, placental blood flow is approximately 63% of total uterine flow. In late gestation, close to term, 84% of the uterine blood flow passes through the caruncles and 94% of the total umbilical flow leaving the fetus travels through the cotyledons [76]. Collectively, placentomes serve as sites of high-throughput nutrient transfer [77] and provide the primary source of hematotrophic nutrient during gestation [78].

Thus, it is not surprising that throughout gestation the caruncular capillaries increase primarily in size, while cotyledonary capillaries proliferate by branching for maximal surface area within the placentome [26, 50, 79]. This is depicted in Figure 2.4 based on a study that determined vascularity of the caruncular and cotyledonary tissues of the sheep placentome [79]. In doing so, the following parameters were analyzed: tissue and shrinkage area, cross-sectional capillary area density or the total capillary area as a proportion of tissue area (abbreviated CAD), capillary number density or the total number of capillaries per unit of tissue area (CND), and capillary surface density or the total capillary circumference per unit of tissue area (CSD); the average cross-sectional area per capillary (APC) was also calculated by dividing the CAD by the CND [79]. There is a steady increase in CAD, CND, CSD, and APC in the caruncular tissue

throughout gestation. In cotyledonary tissue, however, there is a slight increase in CAD and CSD, an exponential increase in CND, but a decrease in APC. The decrease in APC within the cotyledon tissue is not surprising since these capillaries become highly branched and there is an increase in overall number of capillaries as gestation progresses [79].

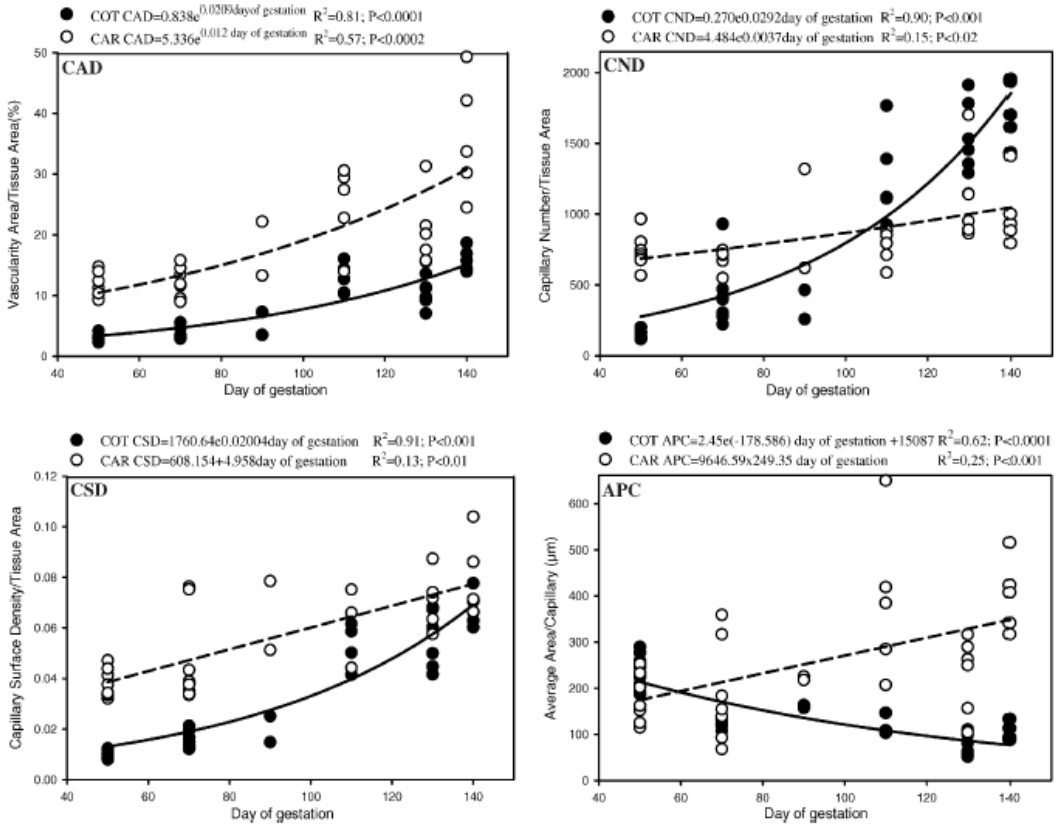


Figure 2.4. Regressions of measures of vascularity in caruncular and cotyledonary tissues of the sheep placentome. CAD, capillary area density (as a percentage); CND, capillary number density; CSD capillary surface density (in µm); APC, area per capillary (in μm^2). Reprinted with permission from the Society for the Study of Reproduction [79].

Vascular Endothelial Growth Factors

The VEGF family contributes to angiogenesis by stimulating permeability of the vasculature, as well as production and migration of vascular endothelial cell proteases [28, 80, 81]. Expression of *VEGF* and its receptor has been localized in placental and fetal tissues in various species, including the human, sheep, and mouse [28, 82-87]. At least three molecular forms of VEGFA ($VEGF_{120}$, $VEGF_{165}$, and $VEGF_{188}$) have been found in fetal placental tissues, with the most prominent form being $VEGF_{165}$ [88]. All forms possess vascular permeability and can stimulate angiogenesis [88].

During early gestation (Days 14 to 30) in sheep, there is no change in VEGF mRNA in caruncles [89]. Expression of mRNA for the VEGF receptor *FLT1* (also *VEGR-1*) in caruncles increases 2- to 2.5-fold between Days 20 and 28 and then 3.8-fold on Day 30 of gestation. In addition, mRNA levels for the receptor *KDR* (also *VEGFR-2*) increase 1.8- to 2.6-fold between Days 18 and 30 of gestation [89]. In the maternal caruncle, levels of *VEGF* mRNA increase approximately 2-fold between Days 50 and 130 of gestation, with peak mRNA expression at gestational Day 130 in the sheep [79]. Within the placental cotyledon, expression of *VEGF* increases from Days 60 to 140 of gestation [88] and expression levels remain elevated between Days 90 and 130 of gestation [79]. A linear increase in *FLT1* mRNA expression occurs in both the caruncles and cotyledons during the last two-thirds of gestation; however, mRNA levels and the rate of increase are greater in the caruncle. Furthermore, caruncular *KDR* mRNA levels increase from Days 50 to 110, and then declines throughout late gestation. However,

there is no change in *KDR* mRNA levels in cotyledons from Day 50 to Day 140 of gestation [79].

In mice, both homozygous and heterozygous VEGF-gene knockouts are embryonic lethal [90, 91]. Embryonic death is seen by Day 11 of gestation, along with abnormal heart, aorta, and vessel development in the fetal and extraembryonic vasculature, in the $VEGF^{-/-}$ mice [90]. Similar developmental defects are seen in the $VEGF^{+/-}$ mice, which die around Days 11-12 of gestation [91]. Individual homozygous knockouts of the VEGF receptors FLT1 and KDR both impair vascular development in the extraembryonic membranes and result in embryonic mortality by Day 8 of gestation [92, 93]. Conclusively, these studies exemplify the magnitude of VEGF and its receptors in fetal development.

Basic Fibroblast Growth Factor

Basic fibroblast growth factor (bFGF or FGF2) stimulates uterine and fetal arterial endothelial cells [26, 55]. In endothelial cells it stimulates production of collagenase and plasminogen activator and functions as a chemotactic and mitogenic factor [79, 94]. Similar to the expression of *VEGF*, no changes occur in levels of *FGF2* mRNA during early gestation (Days 14 to 30) in sheep, but levels of mRNA for its receptor *FGFR2* (also *FGFR2IIIc*) increase by 1.5-fold on Day 16 of gestation compared cyclic ewes [89]. No variation in expression of *FGF2* mRNA in the maternal caruncle occurs between Days 50 and 140 of gestation [79]. Cotyledonary expression of *FGF2* mRNA, however, increases exponentially from Day 50 to Day 140 of gestation [79].

Unlike VEGF-knockout mice, FGF2-gene knockouts are not embryonic lethal and only have mild defects in brain development, their ability to regulate blood pressure, and impaired wound healing [95-97]. In contrast, FGFR2-gene knockout mice die by approximately Day 4.5 of gestation and exhibit abnormal differentiation of the embryonic germ layers [97, 98]. Together, these studies demonstrate the importance of proper FGF signaling for normal development of the extraembryonic membranes and fetus.

Angiopoietins

The angiopoietins are another angiogenic factor family known to regulate development and growth of the placental vasculature [26, 99, 100]. Both *ANGPT1* and *ANGPT2* (also *ANG1* and *ANG2*, respectively) mRNAs are found throughout the reproductive tract and primarily function in vascular remodeling [26, 101]. ANGPT1 promotes organization of the microvasculature, as well as endothelial cell survival [100, 102]. Moreover, ANGPT1 induces both maturation and stabilization of vessels [103]. Expression of ANGPT2 is hypothesized to be stimulated by VEGF and FGF2 and to serve as a modulator of vascular growth when expressed coordinately with VEGF [101]. The angiopoietin receptor TIE2 (also TEK) is expressed primarily on endothelial cells and binds both ANGPT1 and ANGPT2 [104].

In the sheep, mRNA levels of *ANGPT1* increase by 2.1- to 2.5-fold on gestational Days 26 to 30 in caruncles [89]. Levels of *ANGPT2* mRNA do not differ until Day 30 of gestation, when there is a 2.4-fold increase in caruncular expression. A

1.8- to 2.3-fold increase in mRNA expression is also seen for the receptor *TIE2* between Days 18 to 30 of gestation [89]. Levels of *ANGPT1* mRNA continue to increase from Day 50 to 130 of gestation in caruncles and then decrease to Day 140 [79]. In the cotyledon, levels of *ANGPT1* mRNA increase drastically from Day 50 to Day 90, decrease to Day 110, and then remain steady from Day 110 to Day 140. Expression of *ANGPT2* mRNA steadily increase from mid- to late-gestation in both caruncular and cotyledonary tissues, with expression being significantly higher in the caruncle at Day 110. In contrast, expression of *TIE2* mRNA remains constant and similar in the caruncles and cotyledons from Day 50 to Day 140 [79].

Nitric Oxide Mediated Vasodilation

Endothelial cell-derived NO, synthesized from arginine by nitric oxide synthase (NOS), is essential in regulating vasodilation and angiogenesis in the placenta [105-108]. Both VEGF and FGF2 stimulate production of NO by endothelial cells, while NO is also capable of both stimulating and inhibiting expression of those angiogenic factors [26, 109]. NO can activate the soluble enzyme guanylate cyclase (GUCY1B3) in endothelial cells. Once activated, GUCY1B3 catalyzes the conversion of guanosine 5'-triphosphate (GTP) to guanosine 3',5'-monophosphate (cGMP) and thus regulates smooth muscle tone and blood flow [79]. In addition, endothelial nitric oxide synthase 3 (NOS3) is also hypothesized to interact with the VEGF family in the placenta, primarily to increase production and enhance function of the VEGF members during early pregnancy [89, 110]. During early gestation (Days 12-30), no change in mRNA levels for *GUCY1B3*

was observed in the ovine caruncle compared to cyclic ewes [89]. However, *NOS3* mRNA expression increases 2.5-fold on Day 18 and then 4.1-fold on Day 30 of gestation in caruncles, compared to cyclic ewes [89]. Furthermore, from Day 50 to Day 130 of gestation, levels of *GUCY1B3* mRNAs do not differ in either the caruncles or cotyledons based on day; however, there was a significance increase in *GUCY1B3* mRNAs on Day 140 in the cotyledonary tissue [79]. Levels of *NOS3* mRNAs remained similar in the cotyledonary tissue from Day 50 to 140, while levels in the caruncle remained steady from Day 50 to 70, increased significantly at Day 90, and then remained similar throughout Day 140 of gestation [79].

Results from studies of NOS3 knockout mice ($NOS3^{-/-}$) illustrated impaired placental nutrient transport and reduced fetal weight in response to loss of NOS3 compared to wild type controls [111]. This was concomitant with a significant increase in constriction of the uterine artery and a reduction in endothelium-dependent relaxation. Nutrient transport, as assessed by the unidirectional maternofetal ^{14}C-methylaminoisobutyric acid (MeAIB) clearance and sodium-dependent ^{14}C-MeAIB uptake into placental vesicles, was significantly lower in $NOS3^{-/-}$ mice [111]. A second study utilizing the $NOS3^{-/-}$ mouse model observed reduced fetal growth in conjunction with decreased umbilical blood flow and reduced umbilical venous diameter at Day 17.5 of gestation [112].

Coordination of angiogenic factors expression and that of their respective receptors throughout the entirety of gestation is imperative for proper development of placental vasculature [26]. During early gestation, growth and development of the

caruncular vasculature is essential in providing a framework for placental vascular growth. As gestation progresses, it is the proliferation of cotyledonary vessels that ensures adequate surface area for maximal nutrient and waste exchange between the dam and fetus. Disrupting the gestational environment, as in maternal nutrient restriction, can hinder the process of placental angiogenesis and ultimately lead to IUGR of the offspring [23, 113-116].

Select Nutrients and Growth Promoting Hormones in Ovine Placental Tissues and Fetal Fluids

Amino Acids and Their Transporters

Amino acids are fundamental for synthesis of peptides, proteins and other non-proteinaceous molecules of biological significance, such as NO and polyamines [52, 117]. With such a vast variety of physiological functions, it is not surprising that there are dynamic changes in concentrations of amino acids in maternal uterine arterial plasma, fetal plasma, and fetal fluids throughout pregnancy [52, 61]. These dynamic changes in concentrations of amino acids in fetal fluids, fetal plasma, and maternal plasma, as well as the variety of functions served by amino acids illustrate their necessity to the growing and developing fetus. Thus, proper transplacental exchange of amino acids is imperative throughout gestation.

The transport of amino acids across the placenta is largely regulated by members of the solute carrier (SLC) family of molecules [118-120]. The amino acid transport systems, listed Table 2.1, have been identified in mammals and are classified based on

whether they are sodium independent or sodium dependent, and if they show preference to cationic, anionic, or zwitterionic substrates [56, 121]. Amino acid transporters are often found on both the maternal and fetal membranes and many systems exhibit overlapping substrate affinities. Moreover, this is a rapidly changing field of study in which discovery of individual transporters frequently leads to identification of new members in a family of transporters [121].

Table 2.1. Amino acid transport systems in the placenta. Adapted from Grillo et al. (2008) [56].

	Transport System	**Protein**	**Substrates**
Sodium-Dependent Systems	A	SNAT(1,2,4)	Ala, Ser, Pro and Gly
	ASC	ASCT(1,2)	Ala, Ser and Cys
	N	SN1	His, Asn and Gln
	X_{GA}^-	EAAT(1-3)	Glu and Asp
	β	TAUT	Tau
	$B^{o,+}$	$ATB^{o,+}$	Cationic and neutral amino acids
	GLY	GLYT1	Gly and Sarcosine
Cationic Amino Acid Transport Systems	y^+	CAT 1-4	Cationic amino acids
Glycoprotein Associated Transport Systems	asc	Asc14F2hc	Small, neutral amino acids and D-serine
	$b^{o,+}$	$b^{o,+}$/rBAT	Cationic and neutral amino acids
	L	LAT1, LAT2/4F2hc	Neutral amino acids, branched-chain amino acids and Trp
	y^+L	y^+LAT1/4F2hc	Cationic amino acids
	x_c^-	xCT/4F2hc	Glutamate/cysteine exchange
	T	TAT1	Aromatic amino acids

Although identification and characterization of nutrient transporters in the placenta is growing, expression of the SLC transporter family in the sheep placenta throughout gestation has not been fully elucidated. However, studies have identified amino acid transporters in the sheep uterus and conceptus during the peri-implantation

period of pregnancy: (1) sodium-independent System y+ transports cationic amino acids (such as arginine) and is comprised of four transporters that are encoded by the genes *SLC7A1*, *SLC7A2*, *SLC7A3*, and *SLC7A4*; (2) Systems ASC and N transport neutral amino acids (such as glutamine) and are encoded by *SLC1A4* and *SLC1A5* (ASC) and *SLC38A3* and *SLC38A6* (N); (3) System L transports branched-chain and large neutral amino acids and is encoded by *SLC7A5*, *SLC7A8*, and *SLC43A2*; (4) System X_{AG}^- transports glutamate and aspartate and is encoded by *SLC1A1*, *SLC1A2*¸ and *SLC1A3*; (5) System $B^{o,+}$ transports neutral and basic amino acids and is encoded by *SLC6A14*; and (6) System B^o transports neutral amino acids and is encoded by *SLC6A19* [57, 58, 120]. Expression of transporters and the exchange of amino acids between the uterine tissues and conceptus trophectoderm during the peri-implantation of pregnancy are likely critical in establishing a proper environment for placentation and fetal development.

Changes in concentrations of amino acids in uterine luminal fluid are detected as early as Day 10 of gestation in the sheep [40]. When compared with uterine fluids from cyclic sheep, total recoverable glutamine, glutamate, arginine, asparagine, aspartate, leucine, histidine, beta-alanine, tyrosine, methionine, tryptophan, valine, phenylalanine, lysine, isoleucine, cysteine, and proline are greater in pregnant ewes between Days 10 and 16 of gestation. Moreover, a 3- to 23-fold increase in glutamine, glutamate, arginine, glycine, leucine, cysteine, and proline occurs between Days 10 and 14 of pregnancy and remain elevated at Day 16. Only modest changes (less than 2-fold) in

total recoverable citrulline, tyrosine, asparagine, tryptophan, methionine, cysteine, and valine are detectable between Days 3 and 16 of cyclic ewes [40].

Between Days 10 to 16 of the peri-implantation period of pregnancy, concentrations of glutamine, arginine, and leucine increase rapidly in uterine luminal fluids, coinciding with the period of rapid growth, elongation, and development of the conceptus [40]. These amino acids can induce cell proliferation by stimulating FKBP12-rapamycin complex-associated protein 1 (FRAP1, also mTOR) cell signaling and activating the protein kinase P70S6 [1, 40]. Additionally, through mTOR signaling, leucine and arginine stimulate outgrowth of the trophectoderm for implantation in mice [122, 123]. Metabolism of glutamine may provide energy for the early conceptus in the form of reducing equivalents [40-42]. Arginine's roles in NO signaling and as a precursor for polyamines for enhanced vasodilation and nutrient transport also make it imperative for development of the pre-implantation conceptus [4, 40].

In conjunction with the fluctuations in concentrations of amino acids in the uterine lumen during the peri-implantation period (Days 10 to 20) of gestation, expression of a multitude of amino acid transporters is evident throughout the endometrial LE/sGE, GE, and stroma, the caruncular LE and stroma, as well as the conceptus trophectoderm and endoderm (Table 2.2) [57, 58]. Expression of these transporters in the uterus during the peri-implantation period of pregnancy is most likely to increase concentrations of amino acids needed for conceptus development and survival [57]. In the peri-implantation conceptus, expression of these transporters is

Table 2.2. Localization of amino acid transporter in the sheep endometrium, caruncle, and conceptus during the peri-implantation stage.* Adapted from Gao et. al. 2009 [57, 58].

		Endometrial Expression			Caruncular Expression		Conceptus Expression	
Transporter	**Select Substrates**	**LE/sGE**	**GE**	**Stroma**	**LE**	**Stroma**	**Trophectoderm**	**Endoderm**
SLC1A1	Glu and Asp	+++	+++	-	+++	-	+	++
SLC1A2	Glu and Asp	+	+	+	+	+	+	+
SLC1A3	Glu and Asp	+	+	++	+	++	+	+
SLC1A4	Ala, Ser and Cys	++	++		++	+	+	++
SLC1A5	Ala, Ser and Cys	+	++	++	+	+	+	++
SLC38A3	His, Asn and Gln	+	+	+	+	+	+	+
SLC38A4	His, Asn and Gln	+	+	++	+	++	+	+
SLC38A6	His, Asn and Gln	-	-	++[†]	-	++[†]	+	+
SLC3A1	Cationic and neutral amino acids	+	+	+	+	+	+	+
SLC6A14	Cationic and neutral amino acids	+	+	+	+	+	+	+
SLC7A6	Cationic and neutral amino acids	+	+	+	+	+	++	++
SLC6A19	Neutral amino acids	+	+	+	+	+	++	++
SLC7A1	Cationic amino acids	++	++	+			+	+
SLC7A2	Cationic amino acids	+	+	+			+	+
SLC7A3	Cationic amino acids	+	+	+			+	+
SLC7A5	BCAA and large neutral amino acids	+	+++	+++	+	+	++	+++
SLC7A8	BCAA and large neutral amino acids	++	+	+	++	+	+	++
SLC43A2	BCAA and large neutral amino acids	++	++	+	++	+	++	++

*Expression denoted as: -, not expressed; +, weakly expressed; ++, moderately expressed; +++, abundantly expressed.
LE – luminal epithelium; sGE – superficial glandular epithelium; GE – glandular epithelium; BCCA – branch-chained.

[†]Authors noted that expression of SLC38A6 was only in cells distributed throughout the stroma that might represent an immune cell lineage [57].

thought to increase amino acids available to support growth and survival prior to implantation [57, 58].

Although limited work has been done in the late gestation placenta work utilizing 50% nutrient restriction and 100% dietary requirement control ewes identified temporal changes in expression of transporters patterns of expression for various amino acid transporters, including *SLC7A2, SLC7A5, SLC7A6* , and *SLC38A2* on gestational Days 50 to 125, with no differences seen based on treatment [124]. Expression of mRNA for the sodium-coupled neutral amino acid transporter *SLC38A2* is not different between Days 50 and 125 in nutrient restricted (NR) ewes; however, in placentomes of well-fed ewes *SLC38A2* expression increased from Days 50 to 75, decreased to Day 100 and then increased to Day 125. Conversely, expression of the amino acid transporters *SLC7A1*, *SLC7A7*, *SLC7A8,* and *SLC38A4* mRNAs did not differ as gestation progressed [124].

Fluctuations in concentrations of amino acids persist throughout gestation in fetal plasma, and fetal fluids. While there are fluctuations in the concentrations of select amino acids in the maternal circulation, the concentrations of total amino acids remain relatively constant throughout gestation [52]. This contrasts with the steady increase in concentrations of total amino acids throughout gestation. Total amino acids in amniotic fluid increase approximately 10-fold from Day 30 to Day 40, then another 10-fold from Day 40 to Day 60 of gestation, and will later double in amount between Day 100 and 120 of gestation just prior to the exponential phase of fetal growth. Similarly, total amino acids in the allantoic fluid increase approximately 9-fold between Day 80 and 100 of gestation when the placenta reaches its maximum weight and the period of rapid fetal

growth is about to begin [52]. Total content of amino acids in the amniotic and allantoic fluids are discussed here as they give a more accurate illustration of nutrient availability in these reservoirs at any given point during gestation since concentrations of amino acids do not reflect the significant changes in volumes of these fetal fluids throughout gestation.

As previously stated, the ovine fetus consumes up to one liter of amniotic fluid daily during late gestation. Thus, it is not surprising that some of the amino acids, such as glutamine, in the amniotic fluid may have an essential role in development of the fetal gut [52]. Although the fetus consumes large quantities of amniotic fluid and nutrient restriction causes IUGR, a comparison of amino acid concentrations and total quantities indicates that the allantois is by far the primary nutrient reservoir with amino acid concentrations being approximately 7 times greater than in amniotic fluid at Day 120 of gestation [52]. The allantois is comprised of maternal and fetal secretions and its composition is largely influenced by transplacental exchange of nutrients and wastes [52]. Citrulline, glutamine, serine, and alanine are the four most abundant amino acids in ovine allantoic fluid, predominantly during early gestation and the time of placental development. Alanine, serine, and glutamine have various functions which are required for DNA synthesis and, thus, ultimately cell proliferation [52]. These three amino acids are also gluconeogenic in sheep [125] and humans [52, 126]. Additionally, serine functions in one-carbon unit metabolism for the synthesis of 2'-deoxythymidylate for DNA synthesis and methylation [52, 127]. Glutamine serves as a major fuel source for the developing ovine fetus [52, 126] and is needed for synthesis of aminosugars,

nucleotides, and $NAD(P)^+$ [52, 128]. Citrulline is known to be an effective precursor for arginine. Moreover, the presence of argininosuccinate synthase and lyase in nearly all animal tissues, such as the placenta, allows the allantois to serve as a reservoir of citrulline for conversion to arginine for use by the fetal sheep [52, 117]. Citrulline may also function as an antioxidant, protecting against radical-induced oxidative damage and potentially aiding in the establishment of a protective fetal environment [52].

Additionally, the flux of essential amino acids (methionine, phenylalanine, leucine, isoleucine, valine, tryptophan, threonine, histidine, and lysine) across the placenta has been characterized by administering a bolus containing these nine amino acids into the maternal circulation [129, 130]. Not surprisingly, certain amino acids display a higher clearance rate than others. For example, methionine, phenylalanine, leucine, isoleucine, and valine have higher clearance rates than tryptophan, threonine, histidine, and lysine. Rapid clearance rates for methionine, phenylalanine, leucine, isoleucine, and valine are thought to be associated with the fact that these amino acids utilize the sodium-independent, glycoprotein associated L transport system [60, 130]. Further analysis of the five amino acids with more rapid clearance rates illustrates that the magnitude of flux across the placenta is impacted by the normal concentration of each amino acid in the maternal circulation [129, 130]. This also demonstrates a relatively low affinity for neutral amino-acid transporters and little transplacental clearance of basic amino acids [60, 129, 130].

Polyamines

Polyamines, such as putrescine, spermidine, and spermine, are synthesized from amino acids, mainly arginine, and function to stimulate DNA and protein synthesis, cell proliferation and differentiation, placental development, mammalian embryogenesis, signal transduction, and angiogenesis [38, 52, 131]. The regulation of gene expression, intracellular signal transduction, as well as the synthesis of DNA and proteins, occurs by the binding of polyamines to DNA, RNA, proteins, and other negatively charged intracellular molecules [131]. Additionally, polyamines function as endogenous scavengers of reactive oxygen species and have an essential role in the prevention of oxidative damage to DNA, proteins, and lipids. Ultimately all of these intracellular functions add to their importance in cellular proliferation and differentiation [131]. Importantly, inhibition of polyamine synthesis impairs placental growth and results in IUGR in both mice and rats [132-134].

Synthesis of polyamines from amino acids via the polyamine-synthetic pathway begins with the conversion of arginine to ornithine by arginase or with the conversion of proline to pyrroline-5-carboxylate, which is used to generate ornithine via ornithine aminotransferase [135]. This pathway is largely regulated by the rate-controlling enzyme ornithine decarboxylase (ODC1), which converts ornithine into putrescine. Putrescine may then be converted to spermidine by spermidine synthase or spermine by spermine synthase [131]. An alternative pathway for polyamine synthesis has also been recently discovered in mammalian cells, including ovine trophectoderm cells [45]. An in vivo ODC1 knockdown using morpholino antisense oligonucleotides during the pre-

implantation period illustrated that a deficiency in ODC1 function can be compensated for by the ADC/AGMAT pathway in the ovine conceptus. This ADC/AGMAT pathway involves arginine being metabolized to agmatine by arginine decarboxylase (ADC) and agmatine then being converted to putrescine by agmatinase [45].

During early pregnancy, levels of *ODC1* mRNA in the ovine endometrium decrease from Days 10 to 14 and then increase again at Day 20 [136]. The level of *ODC1* mRNA in the ovine conceptus increases between gestational Days 13 and 18, while ODC1 protein is more abundant in the trophectoderm than in the endoderm at this time [136]. Throughout gestation, concentrations of ornithine and polyamines are highest at Day 40, around the time of early placentome formation, in both the intercaruncular endometrium and placentomes [131]. This is concomitant with peak ODC1 and arginase activities in the intercaruncular endometrium, intercotyledonary placenta, and placentomes. Concentrations of polyamines in the allantoic fluid are greatest between Days 40 and 60, while peak concentrations in amniotic fluid occur between Days 100 and 140 of gestation [131].

Glucose and Its Transporters

Glucose serves as a primary energy source for the developing ovine conceptus during the peri-implantation period [40]. The concentration of glucose in uterine luminal fluid between Days 10 and 15 of gestation increases approximately 6-fold in order to meet the demands of the developing conceptus [40, 59]. During this time, glucose, as well as certain amino acids like arginine, glutamine, and leucine, can

stimulate the mTOR signaling pathway to enhance cell proliferation and function. As gestation progresses and the placenta is formed, the uteroplacental tissues consume 50% or more of the glucose received from the maternal circulation before it reaches the fetal circulation [36]. Indeed, from mid- to late pregnancy glucose requirements needed to meet the exponential growth in fetal mass increase greater than 6-fold [137].

In order to meet this increased demand for glucose, expression and availability of glucose transporters increase in the uteroplacental tissues. These consist largely of facilitative transporters of the SLC2A family (common name GLUT) and sodium-dependent transporters of the SLC5A family (common name SGLT) [59]. During the peri-implantation period, specifically Days 10 to 20 of gestation, the mRNAs and proteins of both SLC2A1 and SLC5A1 are greatest in the uterine LE and sGE [59]. The *SLC2A3* mRNA is not detectable in the uterine endometrium, while the *SLC2A4* mRNA and protein are localized to uterine stromal cells and GE. Additionally, expression of *SLC5A11* mRNA is abundant in uterine GE. Also at this time, all of these transporters (*SLC2A1*, *SLC2A3*, *SLC2A4*, *SLC5A1*, and *SLC5A11*) are expressed in the trophectoderm and endoderm of the conceptus [59].

Earlier work investigating the expression and characterization of glucose transporters in the ovine placenta focused on mid- to late-gestation. In a study utilizing placentas from ewes carrying twin pregnancies, the SLC2A1 and SLC2A3 (GLUT-1 and GLUT-3) proteins increase 2.3 and 2.9 times, respectively, between Days 75 and 140 of gestation [137]. This increase in protein is accompanied by increases in *SLC2A1* mRNA of 1.8 times, while *SLC2A3* mRNA increases 3.9 times. This study was unable to detect

SLC2A4 protein at Days 75, 110, and 140 of gestation in ewes [137]. Thus, as the demand for the energy substrate glucose increases throughout pregnancy, the expression and activity of its transporters in the placenta increase to ensure proper fetal-placental development.

Insulin

The pancreatic cells of the fetal lamb secrete the anabolic hormone, insulin, during the second half of gestation [138, 139]. As in adult mammals, insulin secretion in the fetal lamb is also dependent upon changes in nutrient availability and is thus involved in the relationship between fetal growth rates in response to fetal nutrient supply. Furthermore, glucose metabolism in the fetal pancreatic islet cells can have a profound impact on insulin release and insulin biosynthesis. However, glucose is not the only factor regulating insulin release, as norepinephrine and other catecholamines can impair insulin secretion in the fetus [139]. Due to the majority of fetal development occurring later in gestation, including development of the pancreas and insulin-sensitive tissues like the liver and skeletal muscle, it is not surprising that fetal plasma glucose levels and glucose uptake are higher at mid-gestation than near term [140]. Consequently, as the fetal pancreas and insulin-sensitive tissues become more developed and gestation progresses, concentrations of insulin in the fetal plasma increase [140]. As previously stated, insulin secretion is dependent upon nutrient availability. Thus, it is not surprising that pancreatic development and insulin secretion are altered in times of

maternal nutrient restriction. The implications of maternal nutrient restriction on fetal insulin secretion and pancreatic function will be discussed later in this review.

An earlier study illustrated that intravenous infusion of insulin into the fetal lamb in a normal, well-fed ewe for an 18 hour period during late gestation (sometime between Days 115 and 123) increased concentrations of oxygen, glucose, and lactate in fetal blood [141]. The increased uptake of glucose by the fetus following infusion also stimulated fetal uptake of amino acids. Increased uptake of those nutrients and amino acids could subsequently lead to increased oxidative metabolism by the fetus [141]. It is possible that infusion of insulin into the fetal system could increase fetal nutrient uptake in pregnancies of undernourished ewes. Means to ameliorate the effects of maternal under-nutrition during pregnancy will also be discussed later in this review.

Insulin-like Growth Factors

The insulin-like growth factors (IGF1 and IGF2), along with their associated receptors and binding proteins, are influential to both placental and fetal development. These growth factors serve to promote cellular proliferation and differentiation, as well as various metabolic functions. While the IGFs are known to regulate fetal development, gene deletion studies in rodent models initially illustrated their roles in placental development and function [142-145]. Studies were later conducted in the sheep to elucidate the mechanisms of action of IGFs in the ovine placenta and fetus [144].

During early gestation, levels of *IGF1* mRNA are low (compared to levels of *IGF1* expression during the estrous cycle) throughout the endometrial stroma, myometrium, and GE, with the highest expression being in the deep uterine glands [144]. Some expression of *IGF1* mRNA is detected in the caruncular tissue, but following the interdigitation of cotyledonary villi with the caruncular crypts this expression is lost. In contrast, levels of *IGF2* mRNA in the caruncular crypts increase between Days 25 and 35 of gestation and remain elevated through Day 55 (the last day studied by these authors) and high levels of expression are detectable in the interdigitating fetal cotyledonary villi. Expression of IGF type 1 receptor (*IGF1R*) mRNA is elevated in the deep uterine glands throughout the period of study (Days 13 to 55). Moderate expression of *IGF1R* occurs in the uterine sGE and caruncular crypts. Levels of *IGF1R* mRNA in the caruncle, like *IGF1*, decrease following interdigitation between caruncular and cotyledonary tissues. Additionally, expression of this receptor was not detected in the cotyledonary villi [144].

The IGFBPs can stimulate the effects of IGFs by helping transport IGFs to their receptors and by increasing half-life while remaining bound to the IGFs. They can also inhibit actions of IGF1 by preventing its binding to its receptor. *IGFBP2*, *IGFBP4*, *IGFBP5*, *IGFBP6*, and *IGFBP7* are all present in the stroma of the intercaruncular endometrial on Days 9 and 12 of gestation [146]. On Day 9 of gestation, *IGFBP5* mRNA is detected in the LE, while *IGFBP4*, *IGFBP5*, and *IGFBP7* mRNAs are localized in the smooth muscle of blood vessels within the endometrium [146]. *IGFBP1* and *IGFBP3* mRNAs are exclusively expressed in the LE and sGE of the intercaruncular

endometrium, as well as in the LE of the caruncles [146]. In a previously mentioned study, levels of *IGFBP2* are undetectable until Day 29 of gestation in sheep, at which time expression is restricted to the dense caruncular tissue close to the uterine LE and are co-localized with *IGFBP4* [144]. Expression of *IGFBP4* is also detectable within the capsule of the placentomes between Days 13 and 55 of gestation [144]. Moreover, levels of *IGFBP3* are high in uterine LE from Days 13 to 15 and then decrease dramatically [144]0. Elevated expression of *IGFBP3* is also detected in the caruncular crypts of the placentomes, but not in the cotyledonary villi [144]. Expression of *IGFBP1* is only seen during early pregnancy, at approximately Day 13, in the uterine LE and is undetectable by Day 21 [145]. Furthermore, *IGFBP5* mRNA is also localized to uterine LE and GE and expression increases throughout gestation [145].

IGF1 and IGF2 impact placental nutrient utilization and transfer of nutrients to the fetus [147]. Conversely, fetal nutrient levels can enhance IGF1 production [147]. IGF2, however, also enhances placental development in various species, such as the sheep [147]. The role of IGFs in fetal development, as well as the relationship between nutrient availability and IGF functions, will be discussed later in this review.

Malnourishment and Intrauterine Growth Restriction

Numerous genetic, physiological, and environmental factors impact uterine and placental development. Not surprisingly, disruption of placental physiology and the uterine milieu by any of these factors increases the likelihood of IUGR of the offspring, as depicted in Figure 2.5. IUGR may be defined as impaired growth and development of

the fetus or any of its organs during gestation. IUGR fetuses may be categorized under either symmetrical or asymmetrical with respect to growth patterns [4, 148]. The majority of IUGR fetuses fall into the asymmetrical category, which is often associated with insufficient placental structure and function [148]. Thus, an IUGR offspring may exhibit a seemingly normal growth trajectory, but have abnormal organ development that results in health implications later in life.

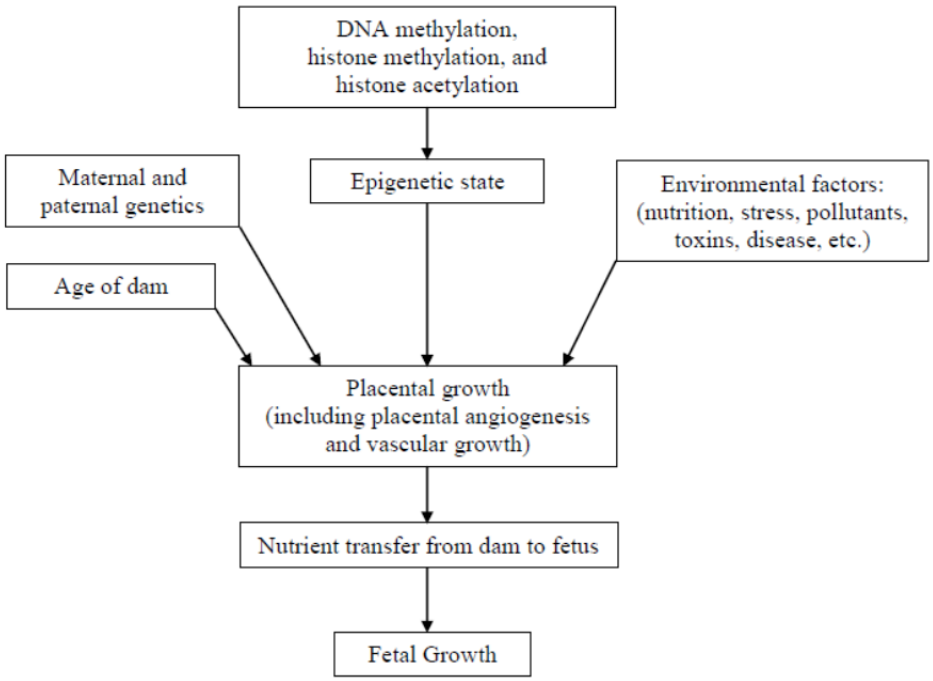


Fig 2.5. Factors affecting mammalian fetal growth. Adapted from Wu et al. (2006) [4].

Although the exponential growth of the fetus occurs primarily during the last third of gestation, insults to the gestational environment at any time point between

conception and parturition can dramatically affect development of the offspring and possibly result in termination of the pregnancy [4, 149]. In livestock, IUGR negatively influences neonatal adjustment, preweaning survival, postnatal growth, feed efficiency, overall health and body composition, meat quality, and reproductive performance [4].

In human pregnancies, small for gestational age (SGA) is often defined as a weight below the 10^{th} percentile for any given gestational age at birth [27, 150]. Similarly, IUGR is utilized to describe infants that are born smaller than normal. Despite advancements in prenatal care, the prevalence of IUGR infants born each year has remained relatively constant [151]. IUGR infants are at greater risk for perinatal morbidity and mortality, as well as increased incidences of diseases in postnatal life, such as coronary heart disease, hypertension, and adult-onset diabetes [5, 151].

The "developmental origins of disease" paradigm encompasses this concept that the interaction between the fetal genome and its intrauterine environment determines the offspring's risk of postnatal disease and its capability of thriving in its postnatal life [5, 6, 14, 15, 152, 153]. Accordingly, the fetus is considered to be in a state of developmental plasticity, indicating that one genotype can give rise to various phenotypes in response to its environmental condition [6]. Concomitantly, "the thrifty phenotype hypothesis" was developed in defense of the idea that the genetic component cannot fully explain the rapid increased risk for adult onset of disease which results from maternal nutrient restriction during gestation. This hypothesis proposes that in NR pregnancies a reduced fetal growth response may occur in anticipation for an inadequate or deprived postnatal environment [152, 154]. From this, the concept of fetal

programming during gestation is used to describe the phenomenon of unknown linkages between fetal and adult life [152, 155].

The prenatal growth trajectory is directly and indirectly influenced by maternal nutrition throughout gestation [4, 156-158]. Varying weather conditions, such as summer droughts and harsh winters, make maternal undernutrition during pregnancy a common occurrence for livestock producers. Indeed, maternal undernutrition leads to fetal undernutrition, thus impairing fetal growth. Lack of supplementation during times of undernutrition can further increase the risk of producing an IUGR offspring. Ewes grazing in the western United States often consume less than 50% of the National Research Council (NRC) dietary recommendations without receiving any supplements [4, 159, 160]. Since the primary breeding season for sheep occurs during the late-fall to early-winter months, ewes generally begin gestation when forage is low in quantity, as well as quality [4, 161]. While lack of adequate forage during the winter months may lead to gestational undernutrition, thermal stress in tropical and subtropical areas may also induce IUGR. Indeed, gestating animals have been shown to consume less feed and blood flow is diverted to the extremities for heat dissipation when environmental temperatures are elevated [3, 4, 162]. Furthermore, diverting blood flow to the extremities consequently means reducing nutrient transport to the uterus.

Malnourishment in humans is also a global crisis, prompting challenges in both developing and developed countries. The spectrum of malnutrition is immense, encompassing both under- and over-nutrition. The Food and Agriculture Organization (FAO) of the United Nations estimates that approximately 843 million people suffer

from chronic hunger, with even more of the global population suffering from diets that are deficient in total nutrient composition [163, 164]. Excessive consumption of nutrients results in an estimated 35% overweight and 12% obese adults in a population, as well as over 42 million overweight children worldwide [165, 166]. In the United States, more than one-third of the adult population is obese [167]. Collectively, malnourishment affects approximately 58% of the world population, of which a large portion are men and women of reproductive age. The cascade of consequences resulting from either maternal or paternal dietary alterations is summarized in Figure 2.6.

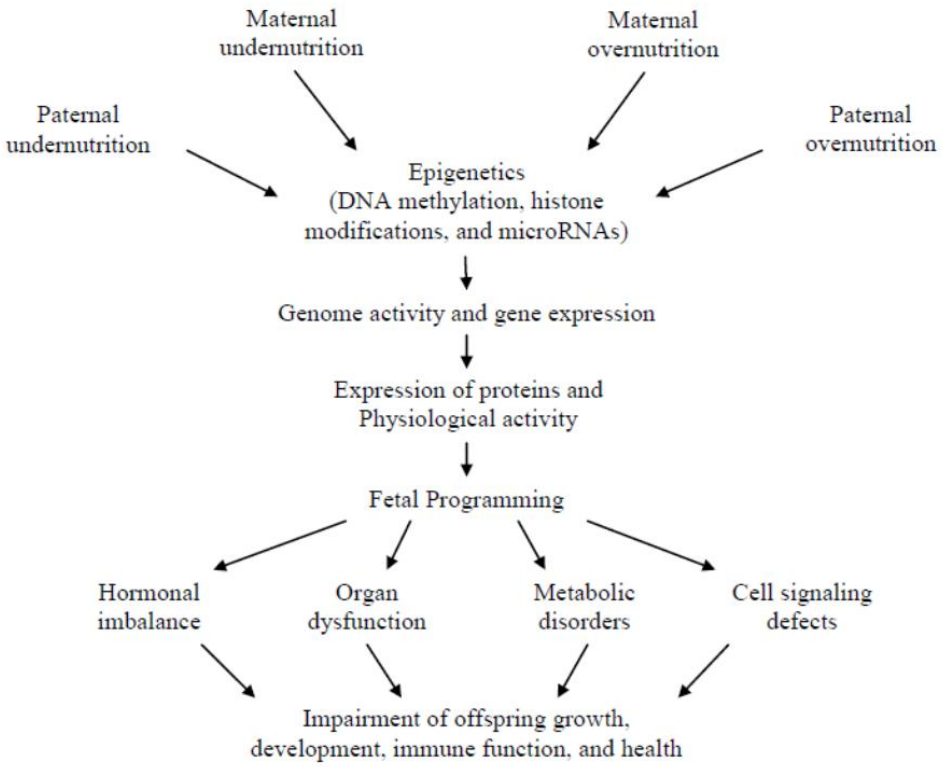


Fig 2.6. Consequences of maternal and paternal nutrition on fetal growth and development. Adapted from Wang et al. (2012) [13].

It is imperative to note that not all offspring of NR mothers are classified as IUGR. Indeed, at least one study in cattle has shown that gestational nutrient restriction induces IUGR in only a subset of offspring [168]. At mid-gestation fetuses of cows that had been nutrient restricted fell into two distinct groups: a non-IUGR group which possessed fetal weights similar to those of the control fed cows, and an IUGR group which possessed fetal weights significantly lower than for the control and NR non-IUGR groups. At this point during fetal development, the brain was significantly larger and the heart tended to be larger in the NR IUGR fetuses in comparison to the NR non-IUGR and control fetuses. However, in late gestation, realimentation of the diet at mid-gestation resulted in all NR pregnancies producing fetuses which were similar in weight to those that received the control diet. Therefore, this study suggests that a period of diminished fetal development during early gestation could go unnoticed if dams receive additional supplementation or a proper diet during late gestation (the period of exponential fetal growth) [168]. Similarly in sheep, this model of nutrient restriction during early gestation followed by proper nutrition until parturition results in an IUGR-like classification of fetuses at mid-gestation, but by late gestation fetuses are considered normal based upon weight [7, 38, 71].

The worldwide crisis of malnourishment in livestock and humans has led to development of models for research on nutrient restriction during pregnancy. Nutrient restriction models such as the one previously mentioned involving dietary realimentation during late gestation [7, 37, 38, 71, 168, 169], as well as models for global nutrient restriction throughout gestation [39, 62, 67, 68, 72, 170], have been developed in species

such as sheep, mice, and rats. Additionally, nutrient restriction models may also include diets specifically deficient in protein or even specific nutrients [63, 64, 171-177].

Impairment of Placental Angiogenesis by Nutrient Restriction

Uteroplacental blood flow is a critical mediator in the transfer of nutrients, wastes, and gases between the dam and fetus. In humans and livestock, uterine blood flow is correlated with placental function and fetal growth [178]. Thus, an exponential increase in uterine and umbilical blood flow must occur in order to facilitate nutrient availability to the developing fetus [116]. During the latter half of gestation, when the fetus is developing at a rapid rate, the absolute rate of uterine blood flow increases at least 3-fold in sheep, 4.5-fold in cattle, and 2.5-fold in humans [116, 179, 180].

Global Nutrient Restriction

Work in sheep demonstrated that while development of the vasculature increases in both the maternal and fetal portions of the placenta, increased vasularity occurs to a greater extent within the fetal component [79]. Additionally, work with severely undernourished adult ewes (diets were 30-40% that of control diets) showed a 17% reduction in uterine blood flow if the dietary insult occurred during mid-gestation, or an average 12% decrease in fetal weight was associated with a 20-33% reduction in uterine blood flow if the nutritional insult occurred late in gestation [116, 181-183]. Those results suggest that the timing of the nutritional insult exerts an effect upon uteroplacental vascularity.

Maternal nutrient restriction in the rat, cow, sheep, and pig results in differential expression of various angiogenic factors in the placenta throughout gestation, impacting placental function and ultimately blood flow to the fetus [23, 72, 169, 174, 175]. Interestingly, global nutrient restriction (60% of dietary requirements) in sheep from Day 50 to 130 of gestation induced IUGR, but surprisingly increased placental vascularity, although there was no difference in total placentome weight between treatment groups [23]. A decrease in expression of mRNAs for VEGF receptors at Day 130 occurred in the restricted group, but no difference in *VEGF* mRNA expression was detected. Therefore, the authors hypothesized that it is likely that a reduction in expression of VEGF receptors and possibly changes in the expression of other factors (such as the angiopoietins) and/or their receptors are responsible for modulating angiogenesis in NR pregnancies [23]. To date, however, the role of angiopoietins in the placentas of NR pregnancies has not been fully elucidated [29].

Nutrient Restriction Followed by Dietary Realimentation

As displayed in Figure 2.8, angiogenic factors such as the VEGF family can function through the MAPK/ERK1/2 and PI3K/Akt pathways, which stimulate angiogenesis in the mammalian placenta [169]. The role of these signaling pathways was evaluated in a sheep model implementing either a 50% NRC or a 100% NRC control diet beginning 60 days prior to conception and continuing until Day 30 of gestation [184]. Between Day 31 and 78 of gestation, all ewes were fed a diet providing 100% NRC requirements and necropsies were performed on Day 78. At this time,

increased vascularity was detected in the caruncular and cotyledonary tissues in response to nutrient restriction. Phosphorylated Akt and phosphorylated ERK1/2 were increased in the arteries of cotyledons, but not caruncles in response to nutrient restriction, indicating that members of the MAPK/ERK1/2, as well as the PI3K/Akt signaling pathways are increased in the arteries of cotyledonary, but not caruncular tissues in response to periconceptional nutrient restriction of ewes [184].

In a similar study, the researchers implemented either global 50% nutrient restriction or fed a control diet (100% NRC requirements) for beef cows from Day 30 to 125 of gestation, and then fed both treatment groups a 100% NRC diet until Day 250 of gestation [169]. Necropsies were performed on a subset of the cattle at Day 125, at which time the NR dams had increased levels of phosphorylated Akt and ERK1/2 in cotyledonary arteries, increased vascularity of the cotyledons, and enhanced placentome efficiency when compared to control fed dams. However, at Day 250 there was no difference in levels of phosphorylated Akt or ERK1/2 in the cotyledons or caruncles due to dietary treatments. Fetal weights only tended to be reduced in response to maternal undernutrition on Day 125, but fetal weights were similar for both dietary groups at Day 250. Thus, it is possible that an up-regulation of activities in the MAPK/ERK1/2 and PI3-K/Akt pathways enhance cotyledonary angiogenesis during early- to mid-gestation in response to nutrient restriction [169].

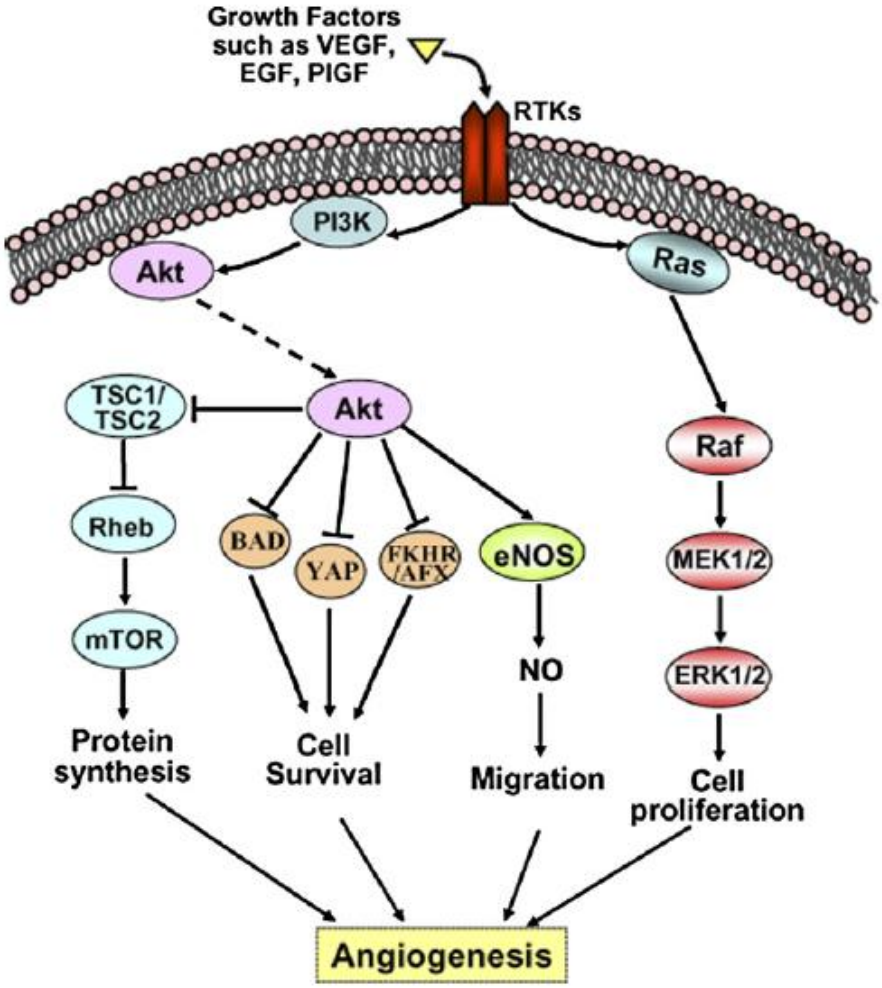


Figure 2.7. Roles of Akt and Erk1/2 signaling pathways in stimulating angiogenesis. This figure demonstrates that angiogenic and growth factors can bind to their receptors and stimulate the MAPK/ERK1/2 and PI3-K/Akt pathways which can ultimately enhance angiogenesis. Adapted from Zhu et al. (2006) [169].

Dietary Protein Restriction

Low protein diets reduce uteroplacental blood flow during pregnancy [175]. This is hypothesized to be partially due to a reduced response of the maternal uterine artery to VEGF. Indeed, rats fed a 9% casein diet (compared with an 18% casein control) from mating until Day 18 or 19 of gestation displayed a significant reduction in maximal uterine artery relaxation and overall response to VEGF. For comparison, the uterine arteries were also stimulated *in vitro* with phenylephrine to analyze vasoconstriction responses, and acetylcholine to analyze relaxation responses, and no difference was seen in response to those treatments between dietary groups. The uterine artery response to VEGF was reduced by inhibiting NOS in the control, but not the low protein diet. Thus, a low protein diet during gestation can result in an attenuated uterine artery vasodilatory response to VEGF. This may be partially due a reduction in the NO component of relaxation mechanisms induced by VEGF [175]. In a similar study during which rats were fed either 18% or 9% casein from mating until Day 19 of gestation, maximum relaxation of the mesenteric artery in response to acetylcholine was reduced in the pregnant rats fed the low protein diet [176]. This study demonstrated the sensitivity of the entire vascular network to dietary alterations during gestation [176].

In another study of a low protein diet, primiparous gilts were selected for either low or high concentrations of total cholesterol in plasma and then fed an isocaloric diet containing either 13% or 0.5% crude protein from mating until Day 40 or Day 60 of gestation [174]. Activity of total NOS, inducible nitric oxide synthase (iNOS), and constitutive nitric oxide synthase (cNOS) was 30-51% lower in the placentas of gilts fed

low protein diets to Day 40 or Day 60 of gestation. More specifically, gilts possessing high concentrations of total cholesterol in plasma and receiving the low protein diet had lower NOS, iNOS, and cNOS activities in their placentas at Day 40 and Day 60 of gestation in comparison to gilts selected for low concentrations of total cholesterol in plasma and also receiving the low protein diet. Low NOS activity in placentas of gilts receiving the low protein diet led to a 42% decrease in synthesis of citrulline from L-arginine. Furthermore, within the gilts fed the low protein diet, citrulline synthesis was decreased by 23% in gilts selected for high concentrations of total cholesterol in plasma compared to those selected for lower total cholesterol. This decrease in NOS, iNOS, and cNOS activities in response to low protein diets can impair NO synthesis and ultimately hinder placental angiogenesis [174].

Nutrient Restriction Alters Nutrient, Hormone and Growth Factor Availability

Amino Acids and Polyamines

Implementation of 50% global nutrient restriction in gestating ewes either throughout early to mid-gestation or from early to late gestation reduced concentrations of amino acids and polyamines in maternal and fetal blood, as well as allantoic and amniotic fluids at mid- and late-gestation [38]. Specifically, arginine-family member amino acids, branched-chain amino acids, and serine were reduced in these fluids. Dietary realimentation from mid- to late-gestation following 50% nutrient restriction during early gestation, increased concentrations of total amino acids and polyamines and prevented IUGR [38]. In studies comparing the genetically similar Baggs ewe with the

University of Wyoming (UW) ewe, nutrient restriction induced IUGR in UW ewes, but not Baggs ewes [67, 72, 170]. These populations are from two different physical environments as the Baggs ewes were raised in areas of limited nutrient availability while the UW ewes were raised in a more temperate and nutrient-rich environment. Amino acid analyses indicate that Baggs ewes had increased concentrations of amino acids in the fetal circulation compared to diet matched UW ewes, indicating that long-term selection for survival characteristics in a nutritionally limited environment can result in adaptive changes in placental function to increase amino acid transfer across the placenta and reduce the incidence of IUGR [67, 72, 170].

Functions of amino acids far surpass their most recognized role as foundational units for protein synthesis. Essential roles for amino acids during gestation also include regulation of hormone secretion, antioxidant function, and synthesis of nonprotein substances such as NO and polyamines [38, 126, 128, 131]. Importantly, arginine catabolism results in the production of NO, polyamines, agmatine, creatine, proline, and glutamate [185]. Catabolism of the amino acid arginine for the production of NO is illustrated in Figure 2.7 [108, 186, 187]. NO is synthesized from L-arginine by NOS and serves as a key regulator in placental angiogenesis, trophoblast growth, and development of the conceptus [188, 189]. Polyamines function to stimulate DNA and protein synthesis, cell proliferation and differentiation, signal transduction, and angiogenesis [38, 131]. Studies in both mice and rats have shown that inhibition of polyamine synthesis impairs placental development and results in IUGR offspring [132, 190].

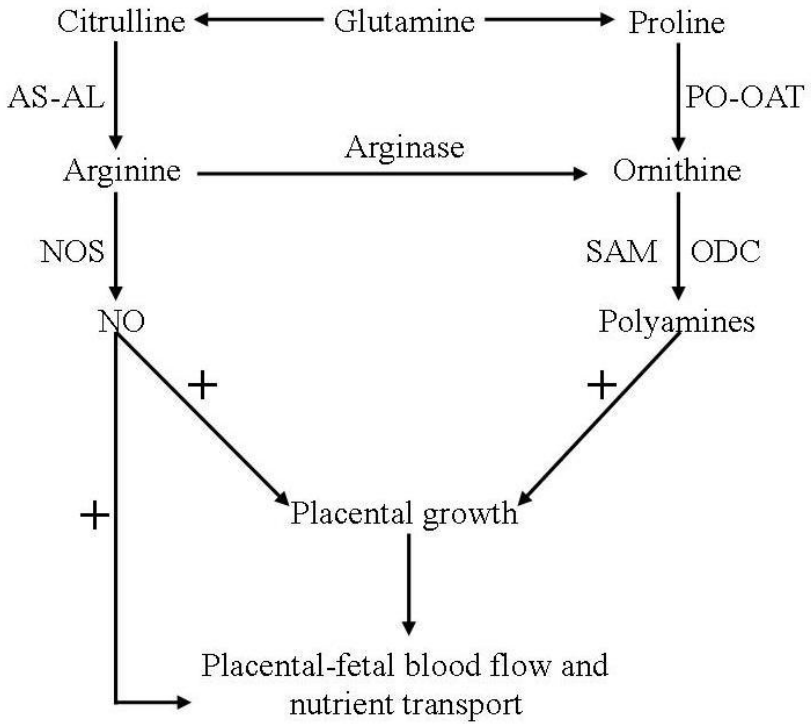


Figure 2.8. Roles of arginine, polyamines, and NO in placental development. Placental synthesis of polyamines and NO may be reduced in malnourished pregnancies, thereby reducing placental blood flow and transport of nutrients. Citrulline, synthesized from glutamine by the enzyme argininosuccinate synthase (AS-AL), may be used as a precursor for arginine synthesis. In turn, arginine may be catabolized into NO via NOS. Proline, also synthesized from glutamine, is used for the formation of ornithine via proline oxidase and ornithine aminotransferase (PO-OAT). Ornithine is then used for polyamine synthesis. Both NO and polyamines stimulate placental growth. Adapted from Wu et al. (2006) [4].

Glucose and Insulin

Fetal metabolism and use of glucose is highly dependent upon the availability of glucose and concentration of insulin in fetal plasma. As gestation progresses and the fetal pancreas develops (around the beginning of the second trimester [191]), use of glucose by insulin-sensitive tissues such as skeletal muscle and liver, increase the need for glucose. To meet these demands, the placenta must increase transport of glucose and gluconeogenic precursors (such as alanine, serine, and glutamine) to the fetus. IUGR pregnancies are associated with reduced glucose transport either by a smaller placenta or reduced transporter expression (to be discussed in a later section of this review) or both [191].

In a nutrient restriction model that utilized a treatment diet containing 70% of daily nutrient requirements between Days 26 and either Day 90 or Day 135, a significant decrease in fetal growth was associated with decreased concentrations of glucose and insulin in plasma of fetuses at Day 135, but not at Day 90 of gestation [192]. Levels of glucose in the amniotic and allantoic fluids were decreased at Day 90 and Day 135 of NR pregnancies. Additionally, maternal concentrations of glucose and insulin in plasma also decreased in response to nutrient restriction. Moreover, all of this was associated with decreased placental mass in NR ewes. Thus, a reduction in glucose and insulin, coupled with alterations in placental development and function, appeared to perturb fetal substrate delivery [192].

In sheep, a 50% maternal nutrient restriction from Days 28 to 115 of gestation does not alter glucose concentrations in the maternal serum, amniotic fluid, allantoic

fluid, although fetal weights were significantly reduced in NR ewes compared to control fed ewes at Day 115 [39]. Conversely, ewes being fed 50% NRC requirements beginning on Day 28 had lower serum glucose concentrations throughout gestation and produced lambs with significantly lower birth weights than control ewes [193]. Thus, nutrient restriction may alter glucose concentrations in the maternal circulation and subsequently lead to reduced birth weights in lambs. However, contrasting data suggests low fetal weights may not be a result of reduced maternal glucose concentrations and instead are a result of a decreased availability of amino acids and their metabolites [39].

Insulin-Like Growth Factors

A previously mentioned study in which ewes were fed only 70% of daily nutrient requirements between Days 26 and Day 90 or Day 135 of pregnancy decreased IGFBP2 protein in fetuses at Day 90 in response to dietary treatment [192]. However, levels of fetal IGFBP2 increased in NR pregnancies between Day 90 and 135 of gestation. A decrease in fetal plasma IGF1 was detected at Day 135 in response to nutrient restriction, even though maternal and fetal concentrations of IGF1 in plasma increased with advancing gestation in control fed pregnancies. Additionally, the declines in fetal insulin, glucose, IGF1, and IGFBP2 were associated with decreased fetal growth [192]. IGF1 is crucial to fetal growth, including development of pancreatic β cells which are responsible for insulin production so it is not surprising that decreased IGF1 is associated with decreased insulin and fetal growth.

An additional nutrient restriction study revealed alterations in placental expression of IGFBPs when pregnant ewes were fed 100% of their requirements and housed on straw bedding until Day 83 of gestation, at which time the ration was reduced and then completely withdrawn for a period of acute nutrient restriction on Days 85 to 90 of gestation [194]. During the withdrawal period, ewes were still housed on the straw bedding. Following the withdrawal period, a group of ewes was euthanized and another group of ewes were again given 100% of their dietary requirements until necropsies on Day 135 of gestation. At both Day 90 and Day 135 of pregnancy, expression of *IGFBP2* and *IGFB3* mRNAs were reduced in the placentas of NR ewes. However, no alterations in fetal IGF1 or insulin concentrations were detected [194].

As mentioned previously, IGF1 and IGF2, along with the IGFBPs, have critical roles in placental and fetal development. These growth factors function to enhance cellular proliferation and differentiation and various metabolic functions. Therefore, a decrease in IGFs and IGFBPs in response to nutrient restriction can perturb placental development and function, along with fetal development. Exogenous IGFs may improve some of these inhibitory effects in nutrient restriction models and will be discussed in a subsequent section of this review.

Nutrient Restriction Alters Expression of Placental Nutrient Transporters

Global Nutrient Restriction

Interestingly, although blood flow plays an essential role in nutrient transport to the fetus, the rate limiting step for delivery of amino acids and glucose is thought to be

expression and/or activity of their specific transporters [195]. In mice, a diet containing 80% of nutrient requirements reduced placental expression of glucose transporter *SLC2A1* mRNA at Day 16, but increased expression of *SLC2A1* and sodium-dependent amino acid transporter *SLC38A2* mRNAs at Day 19 of gestation [62]. Additionally, no change in fetal weight was observed at Day 16 although both placental and fetal weights were decreased in undernourished pregnancies at Day 19 of gestation [62].

In sheep, expression of *SLC2A1* (*GLUT1*) in the placenta increased while fetal blood glucose concentrations decreased at Day 78, but not Day 135 of pregnancy in response to 50% nutrient restriction during early- to mid-gestation [37]. Similarly, 50% nutrient restriction during pregnancy in rats decreased expression of glucose transporter *SLC2A3* without altering glucose concentrations in plasma of the fetus [68]. Therefore, expression of nutrient transporters may vary throughout gestation and may not always be reflected in variances in placental weight, fetal weight, or concentrations of nutrients in the fetal circulation.

Dietary Protein Restriction

Maternal dietary protein restriction in rats induces down-regulation of amino acid transport systems in the placenta early in gestation, before fetal growth restriction occurs [63, 64]. Implementation of a 5% casein diet (in comparison to a control diet of 20% casein) reduced amino acid ratios in fetal and maternal serum [63]. Transport of neutral amino acids by the sodium-dependent system A was reduced in pregnant rats on low protein diets; however, transport of neutral amino acids by sodium-dependent system

ASC was unaltered. Transport of anionic amino acids by sodium-dependent transporters was reduced on the basal membrane of placental trophoblast in pregnant rats on a low protein diet, while sodium-independent transporters of anionic amino acids were not altered. Additionally, the activity of transporters of cationic amino acids was reduced in placentas of rats fed a low protein diet [63]. In a second study, administration of a low protein diet to pregnant rats decreased activity of system A amino acid transporters in late gestation [64]. Specifically, levels of sodium-dependent amino acid transporter *SLC38A2* mRNA were reduced at gestational Day 21 in rats fed a low protein diet. The ubiquitous sodium-dependent system A transporter family has been hypothesized to have a fundamental role in fetal growth in rats [196]. Inhibition of the sodium-dependent system A transporter family by administration of 2-(methylamino)isobutyric acid between Days 7–20 of gestation resulted in a decrease in fetal weight via a mechanism that has not been fully elucidated [196]. It is possible that altered transporter activity is a causitive factor, instead of a compensatory mechanism in response to impaired fetal growth [64].

Dietary Supplements as a Means to Ameliorate IUGR

In addition to amino acids, certain pharmacological agents may be utilized to induce vasodilation during pregnancy. Sildenafil citrate has been shown to increase uterine blood flow in an ovariectomized sheep model [197]. Additionally, sildenafil citrate was shown to improve relaxation and decrease vasoconstriction in small arteries in myometrial biopsies taken from women with pregnancies complicated by fetal growth

restriction [198]. In order to increase uteroplacental blood flow and nutrient transport, three different doses of sildenafil citrate were given via subcutaneous injections from day 28 to 115 of gestation in both well-fed and undernourished ewes [39]. Sildenafil citrate (Viagra) inhibits phosphodiesterase-5A to increase intracellular cGMP, subsequently inducing vasodilation [135, 199]. Interestingly, administration of sildenafil citrate increased fetal weight at Day 115 in both undernourished and adequately fed ewes [39]. Concentrations of total amino acids and polyamines in amniotic and allantoic fluids and fetal serum, but not maternal serum, were increased in response to sildenafil citrate administration. Indeed, concentrations of over half of the amino acids in allantoic fluid, amniotic fluid, and fetal circulation in NR ewes were significantly increased following administration of sildenafil citrate. The increase in those nutrients in fetal fluids and circulation, but not maternal circulation further suggests increased availability of nutrients for fetal growth and not for maternal use or energy storage. It appears that the use of sildenafil citrate in challenged pregnancies may prevent perturbations in placental nutrient delivery and fetal development [39].

As previously discussed, amino acids and polyamines play numerous roles in placental and fetal development. Thus, using select nutrients to ameliorate the effects of undernutrition during gestation would be beneficial. Since arginine is a precursor for NO and polyamines (both of which have a demonstrated role in placental development and function), a number of studies have investigated the efficacy of arginine supplementation to ameliorate IUGR. In humans, L-arginine has also been shown to reduce placental apoptosis and improve fetal development in IUGR pregnancies [200].

In the undernourished sheep model, intravenous administration of L-arginine compared to administration of saline control between Day 60 of gestation and parturition enhanced birth weights of lambs from NR ewes by 21% [193]. Furthermore, the birth weights of lambs from NR ewes given L-arginine were similar to lambs from control-fed ewes [193]. In an additional study, L-arginine administration to NR ewes between Days 100 and 125 of gestation did not improve fetal weight on gestational Day 125. However, there was an increased mass of the fetal pancreas and peri-renal brown adipose tissue (BAT) in lambs from NR ewes administered L-arginine from Day 100 to 125 of gestation [201]. Results of those studies suggest that administration of L-arginine to NR ewes from gestational Day 60 until birth, but not from Day 100 to 125, can enhance the weight of lambs. It is not clear, however, whether it is the earlier administration (beginning on Day 60 instead of Day 100) or the continued administration until parturition (as opposed to performing necropsies on Day 125) that increased weights of lambs. Additionally, the postnatal implications of enhanced fetal pancreas and BAT mass as a result of administration of L-arginine later in gestation, Days 100 to 125, have yet to be elucidated.

L-citrulline, a neutral amino acid that serves as a precursor for L-arginine synthesis, is more easily transferred across the placenta. Due to its placental transport efficiency, L-citrulline may serve as a potential nutritional supplement to ameliorate impaired placental development and IUGR [52, 135, 202]. Indeed, intravenous administration of L-citrulline to ewes late in gestation was shown to be more effective at sustaining maternal and fetal arginine levels than the administration of L-arginine [202].

This study suggests the possible clinical use of arginine and citrulline as a means to prevent or repair insufficient placental development and function, as well as IUGR [202].

Taurine has also been supplemented to gestating rats consuming low protein diets to improve health of the offspring [203, 204]. Low protein diets consisting of 8% casein and control diets consisting of 20% casein were fed from gestational Day 1 until term when fetal organs were evaluated [203, 204]. Development of the endocrine pancreas, including β-cell mass and islet vascularization, appeared to be impaired as a result of low protein diets. Supplementation of low protein diets with taurine throughout gestation restored levels of angiogenic factors (VEGF and Flk-1) within pancreatic islets and endocrine cells of fetuses at birth and pups at 1 month of age [203]. Taurine supplementation to low protein diets from Day 12 of gestation until term increased fetal and cerebral weights, improved brain cortex structures (a greater number of synapses, increased glial cell proliferation, and a greater number of neurons) in IUGR offspring, and decreased amounts of cellular apoptosis detectable in the brain [204]. These results suggest that supplementation of low protein diets with taurine improves development and function of fetal organs such as the pancreas and brain.

Recent studies utilizing a model of IUGR induced by placental embolization between Days 113 and 120 of gestation followed by intra-amniotic infusion of IGF1 from Days 120 to 130 of gestation illustrated that IGF1 administration can increase fetal weight and enhance gut growth in those pregnancies [205, 206]. However, the weights of certain organs such as the spleen, liver, and thymus were reduced following IGF1

infusion, despite the weights of the fetuses being normal [205, 206]. Additionally, IGF1 treatment did not alter the concentrations of amino acids in blood or allantoic fluid, but decreased glutamine uptake by fetuses from blood [207]. A subsequent study using the same IUGR model revealed an 8% reduction in *IGF1* mRNA and a 30% reduction in IGF1 protein following IGF1 infusion into the lambs [208]. Levels of *IGFBP1, 2, and 3* mRNAs were reduced, but there was no change in levels of *IGF1* mRNA in the fetal gut. In contrast, IGF1 infusion increased hepatic *IGF1* expression [208]. Results of these studies illustrate tissue-specific regulation of expression of IGF1 and IGFBPs in response to IGF1 supplementation that enhances fetal weight.

Collectively, results from a variety of supplementation studies indicate that administration of sildenafil citrate, arginine, citrulline, taurine, and other amino acids or combinations of amino acids, as well as IGFs, may ameliorate some of the complications of IUGR and ultimately enhance fetal development in some species. Studies to further elucidate the mechanisms allowing for such enhanced transport capacity could lead to the practical use of these supplementations in malnourished gestating mothers.

The aim of the present studies was to establish an ovine model of adaptive placental nutrient transport and use this model to: (1) determine nutrient availability in maternal, fetal, and placental fluids and compare mRNA expression of nutrient transporters in the placentomes of IUGR and non-IUGR pregnancies; (2) examine morphology and histoarchitecture of placentomes from IUGR and non-IUGR pregnancies and contrast mRNA expression of angiogenic factors in the those placentomes; and (3) identify novel genes regulating placental growth and function.

CHAPTER III

ADAPTIVE PLACENTAL RESPONSE TO MATERNAL NUTRIENT RESTRICTION ALTERS FETAL GROWTH IN EWES

Introduction

Prolonged periods of maternal nutrient restriction, particularly during late gestation, result in IUGR [38, 39, 71, 209]. Delivery of nutrients to the fetus is dependent upon placental growth, uteroplacental blood flow, nutrient availability, and placental metabolism and transport capacity. In sheep, maternal nutrient restriction reduces uteroplacental blood flow [178] and amino acid availability for the fetus [38, 39]. In mice, maternal undernutrition alters the expression of placental transporters for glucose and system A amino acids [62], while feeding rats a low protein diet decreases system A transporter activity [63, 64], which precedes the development of IUGR. We have previously observed that the variation in birth weights of lambs is greater in ewes fed 50% of NRC requirements (mean = 4.84 kg; range = 2.5-7.1 kg; n=54) compared to those receiving 100% NRC requirements (mean = 6.28 kg; range = 5.2-7.2 kg; n=13) (Satterfield et al. unpublished observations). Similarly, in cattle, maternal nutrient restriction induces IUGR in only a subset of individuals [168]. In addition, studies comparing ewes adapted to harsh environmental conditions versus genetically similar ewes raised and selected under temperate conditions indicate that long-term selection for survival characteristics in a nutritionally limited environment can result in adaptive changes in placental function [67, 72, 170]. Collectivley, results suggest that adaptive

mechanisms of placental transport of nutrients exist to maintain normal fetal growth despite limited maternal resources.

Our objectives were to: 1) utilize natural population variance to establish an ovine model of adaptive placental nutrient transport for future investigations of critical mechanisms mediating fetal growth; and 2) determine nutrient availability in maternal and fetal fluids of NR ewes having IUGR and non-IUGR fetuses.

Materials and Methods

Experimental Design

All experimental and surgical procedures were approved by the Institutional Agricultural Animal Care and Use Committee of Texas A&M University. Prior to embryo transfer recipient Suffolk ewes of similar parity and frame size were fed 100% of their NRC requirements to maintain their body condition. Ewes were synchronized into estrus and a single embryo from superovulated Suffolk donor ewes of normal body condition was transferred into the uterus of each recipient ewe on Day 5.5 post-estrus. Pregnancy was diagnosed by ultrasound on Day 28 of gestation. On Day 35 of pregnancy, ewes were assigned randomly to a control-fed group (100% NRC) (n = 7) and a nutrient-restricted group (50% NRC requirements) (n = 24). Composition of the diet has been published previously [193]. All ewes were individually housed on concrete flooring from Days 28 to 125 of gestation and fed once daily at 0700. Beginning on Day 28 of gestation, body weight was determined every 7 days and feed intake was adjusted based on changes in body weight. Blood samples from the maternal

jugular vein were collected into vacuum tubes containing EDTA on Day 125 of gestation and plasma was harvested following centrifugation (2000 x *g* for 10 min at 4^{o}C) and stored at -20^{o}C until analyzed. On Day 125 of pregnancy (term = 147 days of gestation) ewes were necropsied and conceptus (fetal-placental unit) development assessed.

Tissue Collection and Handling Following Necropsy

At the time of necropsy ewes were euthanized using Beuthanasia. Immediately following euthanization, the uterus was removed and opened. A blood sample from the fetal umbilical vein was collected into an EDTA tube and plasma harvested. Following collection of blood samples, the fetus was removed, weighed, measured, and dissected. Samples from fetal organs were preserved in either 4% paraformaldehyde or snap frozen in liquid nitrogen and stored at -80^{o}C for analyses. A portion of the uteroplacental-unit was removed with placentomes being snap frozen in liquid nitrogen. The remaining placentomes were dissected, counted, and weighed.

Biochemical Analyses of Maternal, Fetal, and Placental Fluids

Concentrations of insulin in plasma were measured in duplicate by enzyme-linked immunoassay (EIA) (Catalog number 80-INSOV-E01, ALPCO Diagnostics) according to manufacturer's recommendations [201]. Non-esterified fatty acids (NEFA) were determined in duplicate using a commercial colorimetric assay (Wako Chemicals, Richmond, VA) [201]. Glucose, ammonia, and urea in plasma were analyzed in duplicate using enzymatic methods [210-212]. Amino acids and polyamines in plasma

were analyzed using HPLC as previously described [38]. To determine total quantities of nutrients in the fetal circulation, concentrations were multiplied by estimated blood volume. Blood volume exhibits a linear relationship with fetal weight [213] and was estimated at 110 ml/kg fetal weight [213, 214].

RNA Isolation and Real-Time PCR Analysis

Synthesis of cDNA from total cellular RNA (2 μg) using random primers (Invitrogen, Carlsbad, CA), oligo-dT primers, and SuperScript II Reverse Transcriptase (Invitrogen) was achieved as described previously [215]. Newly synthesized cDNA was acid-ethanol precipitated, resuspended in 20 μl water at a dilution of 100 ng, and stored at –20ºC for real-time PCR analysis. Primers under 200 bp were designed for each gene in order to maximize efficiency (Table 3.1). qPCR analysis of mRNAs was performed using an ABI PRISM 7700 (Applied Biosystems, Foster City, CA) with Power SYBR Green PCR Master Mix (Applied Biosystems) as the detector, according to manufacturer's recommendations and using methods described previously [146]. Cycle parameters for qPCR were 50ºC for 2 min, 95ºC for 10 min, and then 95ºC for 15 sec and 60ºC for 1 min for 40 cycles. Selected genes analyzed for microarray validation included: *solute carrier family 2, (facilitated glucose transporter), member 1 (SLC2A1), solute carrier family 2, (facilitated glucose transporter), member 3 (SLC2A3), solute carrier family 2, (facilitated glucose transporter), member 4 (SLC2A4), solute carrier family 5 (sodium/glucose cotransporter), member 5 (SLC5A1), solute carrier family 7 (cationic amino acid transporter, y+ system), member 1 (SLC7A1), solute carrier family*

7 (cationic amino acid transporter, y+ system), member 2 (SLC7A2), solute carrier family 7 (amino acid transporter light chain, L system), member 5 (SLC7A5), solute carrier family 7 (amino acid transporter light chain, y+L system), member 6 (SLC7A6), solute carrier family 7 (amino acid transporter light chain, y+L system), member 7 (SLC7A7), solute carrier family 7 (amino acid transporter light chain, L system), member 8 (SLC7A8), solute carrier family 38, member 1 (SLC38A1), solute carrier family 38, member 2 (SLC38A2), and *solute carrier family 38, member 4 (SLC38A4).*

Template input was optimized from serial dilutions of placentomal cDNA for each gene to ensure that the amplification reaction achieved 95-105% efficiency, and that the amount of input chosen was based on being within the linear range of efficiency. Final reactions for *SLC2A1* and *SLC2A3* used 2.5 ng, *SLC5A1, SLC7A2, SLC7A6, SLC7A7, SLC7A8, SLC38A1,* and *SLC38A2* used 5 ng, and *SLC2A4, SLC7A1, SLC7A5,* and *SLC384* used 10 ng of input. Data were analyzed using 7200HT SDS software (version 2.3, Applied Biosystems). The relative quantification of gene expression across treatments was evaluated using the comparative CT method as previously described [146].

Table 3.1. Primers utilized for quantitative real-time PCR analyses of solute carrier family members.

Target[a]	Forward/reverse primers (5'3')[b]	Length of amplicon (bp)	GenBank accession no.[c]
SLC2A1	AACTGTGCGGACCCTATGTC	149	XM_004001865
	GTCACTTTGGCTTGCTCCTC		
SLC2A3	TGTGGTGTCTGTGTTCTTGG	160	NM_001009770
	TTTCAAAGAAGGCCACAAAG		
SLC2A4	GGCATGGGTTTCCAGTATGTG	62	XM_004012643
	ACCGCGAATAGAAGAAAGACGTA		
SLC5A1	GCTGGAGCCTGCGTAACAG	64	NM_001009404
	TGAATGTCCTCGTCTTCTGCAT		
SLC7A1	CCTAGCGCTCCTGGTCATCA	56	AF212146
	GGGCGTCCTTGCCAAGTA		
SLC7A2	GCAGAGCAGCGCTGTCTTT	62	XM_002698665
	ACTGTCCAGAGTGACGATTTTCC		
SLC7A5	GGTGAACCCTGGTACGAATTTAGT	64	NM_174613
	TCCACGCTCGAGAGGTATCTG		
SLC7A6	CATTTGTGAACTGCGCCTATGT	72	NM_001075937
	CCAGGACCTTGGCATAAGTGA		
SLC7A7	TCAGGCTTGCCCTTCTACTTCT	64	NM_001075151
	GGAGCCAAAGAGGTCGTTTG		
SLC7A8	GGCCATGATCCACGTGAAG	65	NM_001192889
	GGGTGGAGATGCATGTGAAGA		
SLC38A1	CAAATTTGGGCTGCCCTTT	60	XM_002687321
	GGGAATGCTTACCAGGGAAAA		
SLC38A2	CAGCTATAGTTCCAACAGCGACTTC	77	NM_001082424
	CATCGGCATAATGGCTTTTCA		
SLC38A4	TGCTTCATGCTTACAGCAAAGTG	63	NM_001205943
	CAGCCAGGCGTACCATGAG		
TUBA	GGTCTTCAAGGCTTCTTGGT	54	AF251146
	CATAATCGACAGAGAGGCGT		

[a]The amplification target.

[b]The forward and reverse DNA oligos used for amplification of the target. Forward and reverse primers do not necessarily indicate the in vivo direction of transcription.

[c]The accession numbers to the ovine or bovine sequences used for primer design.

Statistical Analyses

Regression analyses were conducted using the PROC-MIXED procedures of the Statistical Analysis System (SAS Institute, Cary, NC). All other measures were subjected to least-squares analysis of variance using the General Linear Models procedures of the Statistical Analysis System. Data are presented as least-squares means with overall standard error of the mean (SE). There was no effect of fetal sex in the statistical model; therefore it was removed from the statistical model. Differences in means were considered to be statistically significant when a P value was ≤ 0.05 while a P value of ≤ 0.1 was considered to indicate a tendency toward significance. Data from quantitative real-time PCR analysis were subjected to least-squares analysis of variance using the general linear models procedures of the Statistical Analysis System (SAS Institute, Cary, NC).

Results

Assessment of Fetal Weight Correlations

Based on results of a prior study, the present study was completed to exploit natural population variance in NR ewes to identify subpopulations of IUGR and non-IUGR fetuses as a first step to elucidate adaptive mechanisms for placental transport of nutrients. The complete distribution of fetal weights from control and NR ewes is shown in Fig. 3.1. To determine if maternal weight prior to the onset of nutrient restriction was correlatedwith fetal weight, regression analyses were conducted for NR ewes. Fetal weight was not correlated with maternal weight at Day 35 (P = 0.26) (Fig. 3.2A) or at

Day 125 of gestation (P = 0.11) (data not shown). Further, fetal weight did not correlate with maternal weight changes between Days 35 and 125 of gestation (P = 0.45) (data not shown). However, as expected, total placentome weight was correlated ($R^2 = 0.419$; $P < 0.0005$) with fetal weight within NR ewes (Fig. 3.2B). Given that the correlation between maternal and fetal weights were not significant, the six fetuses with the highest weights (NR non-IUGR) and the six fetuses with the lowest weights (NR IUGR) from NR ewes and all fetuses from control ewes were utilized in all subsequent comparisons. The inclusion of all control animals allowed evaluation of a sentinel cohort of ewes representing average parameters of growth and development of fetuses from ewes with adequate nutrition. Mean fetal weights for NR IUGR lambs (2.8 ± 0.1 kg) were lower ($P < 0.05$) than weights for control (4.0 ± 0.1 kg) and NR non-IUGR (4.1 ± 0.1 kg) fetuses (Fig. 3.3). Mean placentome weights for NR IUGR pregnancies (401 ± 40 g) were lower ($P < 0.005$) than weights of placentomes from control (587 ± 40 g) and NR non-IUGR (630 ± 40 g) pregnancies (data not shown).

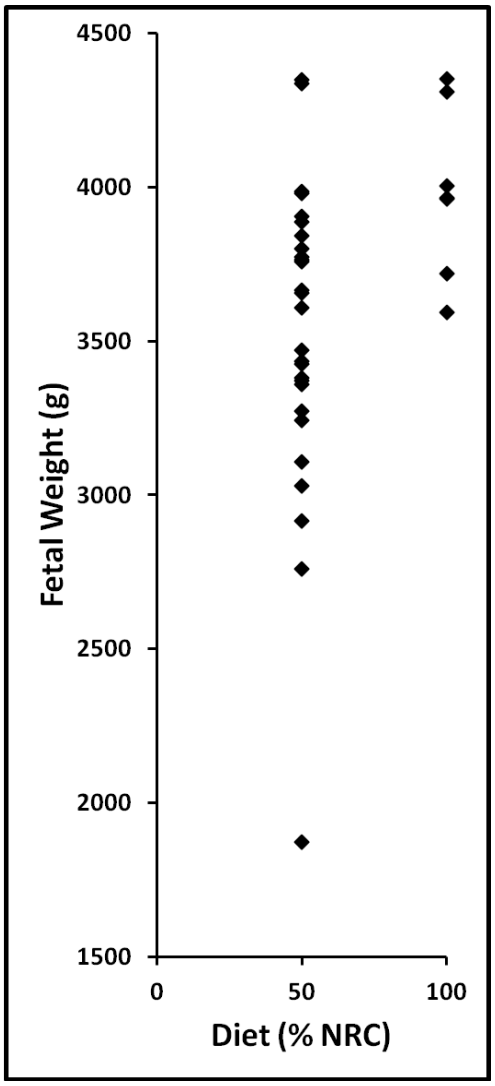
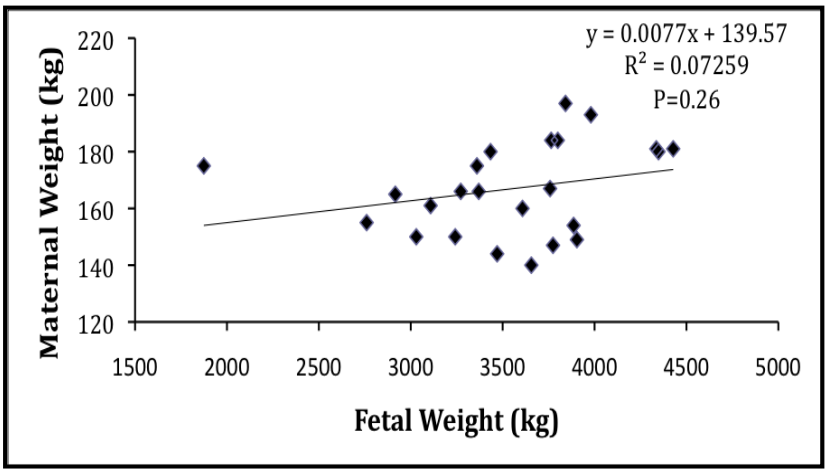


Figure 3.1. The distribution of fetal weights of lambs from nutrient-restricted (50% NRC) and control (100% NRC) ewes collected on Day 125 of gestation.

[A]



[B]

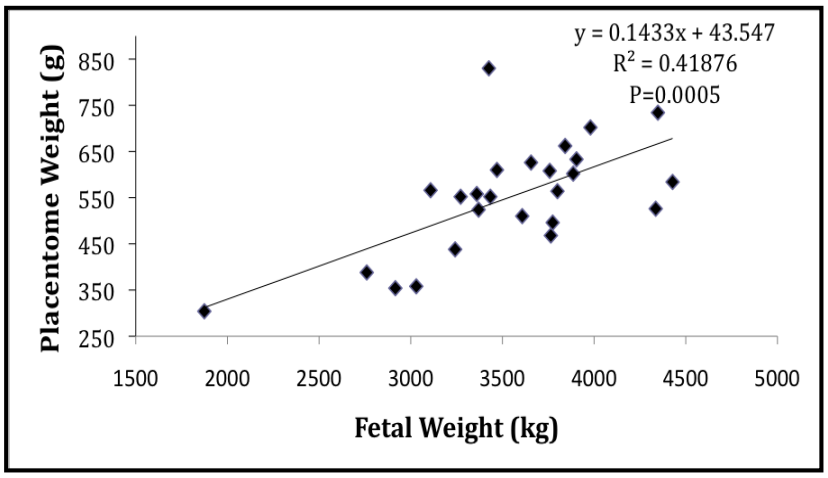


Figure 3.2. Regression analyses of fetal weight against maternal weight and weights of placentomes. [A] Fetal weight was not correlated ($P = 0.26$) with maternal weight. [B] Fetal weight was positively correlated ($P < 0.0005$) with placentome weight on Day 125 of gestation.

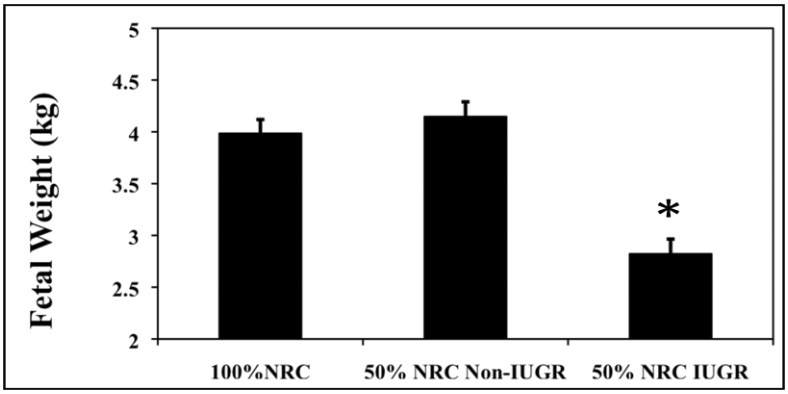


Figure 3.3. Mean fetal weights for control, NR IUGR and NR non-IUGR fetuses on Day 125 of gestation. Mean weight of the six heaviest (non-IUGR) fetuses from NR ewes was similar ($P > 0.10$) to the mean weight of fetuses from control-fed ewes. In contrast, mean weight of the six lightest (IUGR) fetuses from NR ewes was lower ($P < 0.05$) than weights of both NR non-IUGR and control fetuses.

Concentrations of Select Nutrients in Maternal Plasma

Concentrations of select nutrients in maternal plasma are presented in Table 3.2. Concentrations of ammonia, urea, putrescine, spermidine, and insulin were not affected ($P > 0.1$) by maternal nutrient restriction and were not different ($P > 0.1$) between NR ewes having IUGR and non-IUGR fetuses. Concentrations of glucose in maternal plasma were less ($P < 0.01$) for NR non-IUGR compared to NR IUGR ewes and control ewes. Concentrations of NEFAs in maternal plasma were greater ($P < 0.01$) for NR non-IUGR compared to NR IUGR and control ewes. The total of all amino acids, as well as concentrations of glycine, were less ($P < 0.05$) in plasma from control ewes compared to NR ewes. In contrast, concentrations of tryptophan in maternal plasma were greater ($P < 0.05$) for control than NR ewes. Concentrations of serine in plasma were greater ($P < 0.05$) for NR IUGR versus NR non-IUGR and control ewes. Concentrations of glutamate, isoleucine, and cysteine in maternal plasma were greater ($P < 0.05$) for NR non-IUGR compared to NR IUGR and control ewes. Concentrations of methionine tended to be greater ($P = 0.1$) in plasma of NR non-IUGR compared to NR IUGR and control ewes. Concentrations of histidine and leucine in maternal plasma were greater ($P < 0.05$) for NR non-IUGR compared to control ewes, with concentrations of those amino acids being intermediate in NR IUGR ewes.

Table 3.2. Concentrations of total and select nutrients in maternal plasma.

	100% NRC	50% NRC non-IUGR	50% NRC IUGR	SE	P-value
Ammonia (µmol/L)	148	129	181	52	
Urea (µmol/L)	4691	4515	4374	780	
Glucose (µmol/L)	4840[a]	2862[b]	4583[a]	382	P<0.01
Insulin (ng/ml)	0.54	0.35	0.85	0.17	
NEFA (mmol/L)	0.61[a]	1.17[b]	0.72[a]	0.1	P<0.01
Putrescine (nmol/L)	2802	2863	3580	332	
Spermidine (nmol/L)	1802	1455	2355	569	
Amino Acids (µmol/L)					
Asp	5.3	5.8	7.1	0.9	
Glu	62[a]	83[b]	58[a]	6	P<0.05
Asn	28	34	31	3	
Ser	73[a]	74[a]	98[b]	7	P<0.05
Gln	348	403	359	31	
His	56[a]	79[b]	65[a,b]	7	P=0.07
Gly	603[a]	902[b]	1040[b]	101	P<0.05
Thr	49	56	47	6	
Cit	192	211	226	21	
Arg	218	228	226	19	
b-Ala	30	30	31	5	
Tau	72	76	76	13	
Ala	165	170	137	27	
Tyr	57	61	56	3	
Trp	41[a]	29[b]	32[b]	3	P<0.05
Met	28[a]	32[b]	28[a]	1	P=0.1
Val	108	124	125	8	
Phe	43	49	47	2	
Ile	64[a]	91[b]	70[a]	5	P<0.01
Leu	85[a]	111[b]	101[a,b]	5	P<0.05
Orn	72	64	62	15	
Lys	91	120	116	12	
Cys	121[a]	162[b]	114[a]	12	P<0.05
Pro	153	174	135	18	
Total AA	2764[a]	3368[b]	3287[b]	161	P<0.05

Means in a row without a common superscript letter are significantly different.

Select Nutrients in the Fetal Circulation

Concentrations of select nutrients in fetal plasma are presented in Table 3.3. Concentrations of spermidine in the fetal circulation were less ($P < 0.05$) in NR IUGR fetuses compared to NR non-IUGR and control fetuses. Concentrations of the amino acid serine tended to be greater ($P = 0.06$) in the circulation of NR non-IUGR fetuses versus control and NR IUGR fetuses.

Total amounts of select nutrients in the fetal circulation are presented in Table 3.4. Concentrations of urea were lower ($P = 0.05$) in plasma of NR IUGR compared to NR non-IUGR and control fetuses. Total amounts of insulin, putrescine, and spermidine were lower ($P < 0.05$) in the circulation of NR IUGR fetuses compared to NR non-IUGR and control fetuses. Total amino acids in the fetal circulation were less ($P < 0.01$) for NR IUGR versus NR non-IUGR and control fetuses. Specifically, serine, glutamine, histidine, threonine, arginine, alanine, tyrosine, tryptophan, methionine, phenylalanine, isoleucine, leucine, ornithine, lysine, and proline were lower ($P < 0.05$) in plasma of NR IUGR fetuses than NR non-IUGR and control fetuses. In addition, concentrations of valine and cysteine were lower ($P < 0.05$) in plasma from NR IUGR versus NR non-IUGR fetuses. Concentrations of isoleucine and cysteine were greater ($P < 0.05$) in plasma from NR non-IUGR than control fetuses. Concentrations of asparagine, glutamate, glycine, citrulline, beta-alanine, and taurine were not different ($P > 0.10$) among groups of fetuses, nor were concentrations of ammonia, glucose, and NEFAs ($P > 0.10$) in plasma.

Table 3.3. Concentrations of select nutrients in plasma from fetuses.

	100% NRC	50% NRC non-IUGR	50% NRC IUGR	SE	P-value
Ammonia (µmol/L)	86.0	140.1	292.6	75.7	
Urea (µmol/L)	5558	5001	4918	643.0	
Glucose (µmol/L)	23.9	36.0	27.7	8.9	
Insulin (ng/ml)	0.28	0.23	0.14	0.06	
NEFA (mmol/L)	0.09	0.10	0.23	0.06	
Putrescine (µmol/L)	2.45	2.37	1.93	0.21	
Spermidine (µmol/L)	3.03[a]	2.01[a]	0.27[b]	1.29	P<0.05
Amino Acids (µmol/L)					
Asp	26.7	26.4	35.3	3.7	
Glu	47.3	51.3	74.8	13.3	
Asn	92.8	101.7	93.3	11.1	
Ser	638.3[a]	798.6[b]	604.4[a]	56.2	P=0.06
Gln	977	1016	912.5	97.6	
His	103.4	103.8	95.4	8.8	
Gly	683.7	850.2	730.1	141.4	
Thr	250.8	304.8	211	36.6	
Cit	218.6	217.8	227.6	21.9	
Arg	290.3	298.6	224.1	25.7	
b-Ala	355.3	274.3	290.0	48.6	
Tau	152.9	155.8	164.5	38.2	
Ala	378.2	361.8	331.1	23.7	
Tyr	108	117.6	104.3	9.5	
Trp	101	107.8	87	8.0	
Met	56.3	54.2	48.7	5.0	
Val	235	251.1	268.8	14.4	
Phe	116.2	127	130.2	6.3	
Ile	93.5[a]	113.2[b]	98.4[a,b]	6.1	P=0.09
Leu	173.6	178.9	176.1	13.3	
Orn	151.4	147	153.2	9.3	
Lys	171.4	193.2	168.7	15.7	
Cys	90.8	113.1	99.3	8.4	
Pro	318.6[a]	317[a]	260.5[b]	14.2	P<0.05
Total AA	5422	5851	5230	397.0	

Means in a row without a common superscript are significantly different.

Table 3.4. Total amounts of select nutrients in plasma from fetuses.

	100% NRC	50% NRC non-IUGR	50% NRC IUGR	SE	P-value
Ammonia (µmol)	36.8	64.6	87.3	26.4	
Urea (µmol)	2,443[a]	2,284[a]	1,536[b]	286.0	P=0.05
Glucose (µmol)	10.9	15.9	9.0	3.4	
Insulin (ng)	122[a]	106[a]	46[b]	26.3	P<0.05
NEFA (mmol)	39.4	42.7	64.6	17.6	
Putrescine (µmol)	1.07[a]	1.08[a]	0.62[b]	0.08	P<0.01
Spermidine (µmol)	1.23[a]	0.96[a]	0.09[b]	0.56	P<0.05
Amino Acids (µmol)					
Asp	11.7	12.1	10.5	1.0	
Glu	20.7	23.5	21.1	2.6	
Asn	40.7[a]	46.2[a]	27.9[b]	2.2	P<0.01
Ser	280.6[a]	364.2[a]	189.7[b]	28.1	P<0.01
Gln	429.6[a]	461.5[a]	284.1[b]	41.7	P<0.05
His	45.3[a]	47.2[a]	29.1[b]	3.2	P<0.01
Gly	304.8	383.3	223.3	63.0	
Thr	110.0[a]	136.3[a]	64.6[b]	14.6	P<0.05
Cit	96.0	98.9	71.3	9.8	
Arg	127.1[a]	135.4[a]	69.7[b]	10.8	P<0.01
b-Ala	156.7	122.9	93.9	19.6	
Tau	67.2	69.5	46.1	12.7	
Ala	166.7[a]	164.1[a]	101.8[b]	10.5	P<0.01
Tyr	47.7[a]	53.2[a]	31.8[b]	4.1	P<0.01
Trp	44.6[a]	48.7[a]	26.9[b]	3.6	P<0.01
Met	24.7[a]	24.4[a]	14.9[b]	2.1	P<0.01
Val	103.2[a,b]	113.9[a]	83.0[b]	4.2	P<0.05
Phe	51.1[a]	57.7[a]	40.2[b]	3.2	P<0.01
Ile	41.0[a]	51.5[b]	30.4[c]	1.7	P<0.01
Leu	76.6[a]	81.1[a]	54.1[b]	6.2	P<0.05
Orn	66.7[a]	66.7[a]	47.2[b]	4.2	P<0.01
Lys	75.1[a]	87.4[a]	51.4[b]	5.9	P<0.01
Cys	39.8[a]	51.6[b]	33.1[a]	3.9	P<0.05
Pro	139.8[a]	144.0[a]	86.8[b]	6.9	P<0.0001
Total AA	2,387.3[a]	2,649.5[a]	1,613.1[b]	181.7	P<0.01

Total amount of each of the select nutrients was calculated by multiplying concentration by blood volume, which was estimated at 110 ml/kg fetal weight. Means in a row without a common superscript differ significantly.

Expression of Amino Acid and Glucose Transporters in the Placenta

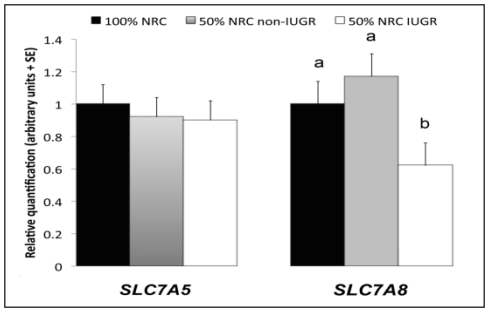
As a first step in determining adaptive mechanisms potentially responsible for enhanced nutrient availability to fetuses, we determined expression of mRNAs for select amino acid transporters in the placentomes of ewes. Steady-state levels of mRNA for large neutral amino acid transporters are shown in Fig. 3.4A. *SLC7A8* mRNA expression was greater ($P < 0.05$) in placentomes from NR non-IUGR compared to NR IUGR pregnancies, but intermediate ($P > 0.10$) between NR ewes having an IUGR or non-IUGR fetuses. In contrast, expression of *SLC7A5* mRNA in placentomes was not different ($P > 0.10$) among fetal types. Steady-state levels of mRNAs for cationic amino acid transporters are shown in Fig. 3.4B. *SLC7A2* mRNA levels were greater ($P < 0.05$) in placentomes from control ewes as compared to NR ewes having either an IUGR or a non-IUGR fetus. *SLC7A6* mRNA expression was greater ($P < 0.05$) for both control and NR non-IUGR placentomes as compared to placentomes from NR ewes having an IUGR fetus. Expression of *SLC7A7* mRNA was higher ($P < 0.05$) for placentomes from NR non-IUGR pregnancies compared to NR IUGR pregnancies, but were intermediate for control ewes and did not differ ($P > 0.10$) between from NR ewes having either IUGR or non-IUGR fetuses. *SLC7A1* mRNA expression was not affected ($P > 0.10$) by type of fetus. Steady-state levels of mRNAs for sodium coupled neutral amino acid transporters (SNATs) are shown in Fig. 3.4C. *SLC38A2* mRNA was more abundant ($P < 0.05$) in both control and NR non-IUGR placentomes compared to NR-IUGR placentomes. In contrast, *SLC38A1* and *SLC38A4* mRNAs in placentomes were not different in abundance ($P > 0.10$) among fetal types. Steady-state levels of mRNAs for glucose

transporters *SLC2A1*, *SLC2A3*, *SLC2A4*, and *SLC5A1* were not different ($P > 0.10$) among fetal types (data not shown).

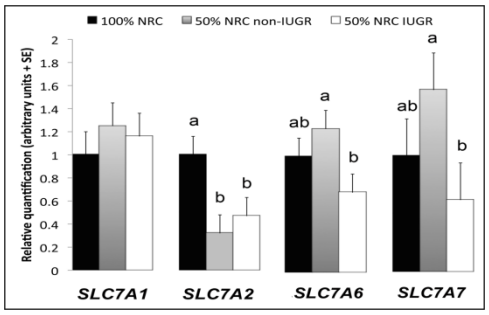
Discussion

The placenta mediates delivery of nutrients for fetal growth. Perturbations resulting in impaired placental development or function result in IUGR [216]. In a series of previous studies using the undernourished sheep model, we detected a range of fetal weights that were greater for NR ewes than ewes fed to meet 100% of NRC requirements, when confounding variables such as maternal size, genotype, and fecundity are controlled. Therefore, we aimed to develop a model to investigate mechanisms by which the placenta adapts to maternal nutrient restriction to support normal versus restricted fetal growth using a population variance approach. As a first step, we determined quantities of select nutrients in maternal and fetal fluids in well-fed and NR ewes having either IUGR or non-IUGR fetuses and the expression of a number of amino acid transporter family members hypothesized to be involved in placental and/or fetal development. Results of the present study indicated that quantities of a number of amino acids and their metabolites are reduced in plasma of NR IUGR versus NR non-IUGR and control fetuses in NR ewes. Interestingly, a number of these nutrients can be metabolized to form products involved in regulating angiogenesis and blood flow. Further, enhanced fetal growth in NR ewes having non-IUGR fetuses was associated with increased expression of mRNAs in placentomes for a number of amino acid transporters, including the neutral amino acid transporters SLC7A8 and SLC38A2,

[A]



[B]



[C]

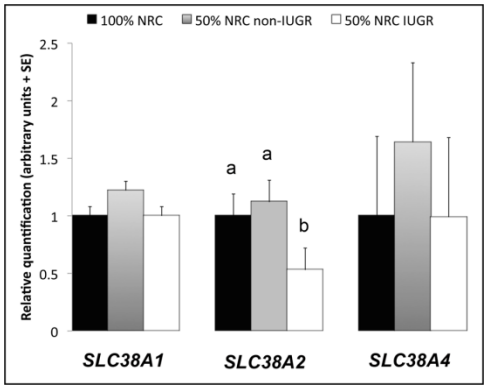


Figure 3.4. Steady-state levels of mRNAs encoding select amino acid transporters in the ovine placentome. [A] Analyses of steady-state levels of mRNAs for large neutral amino acid transporters *SLC7A5* and *SLC7A6* indicated that expression of *SLC7A6* mRNA is greater ($P<0.05$) for placentomes from control and NR non-IUGR than NR-IUGR pregnancies. [B] Steady-state mRNA levels for the cationic amino acid transporters *SLC7A1*, *SLC7A2*, *SLC7A7* and *SLC7A8* were assessed and expression of *SLC7A7* and *SLC7A8* was greater ($P<0.05$) in placentomes from NR non-IUGR than NR IUGR pregnancies. Results also indicated that *SLC7A2* mRNA is less abundant in placentomes of NR IUGR and NR non-IUGR pregnancies compared to placentomes from control ewes. [C] Analyses of steady-state levels of mRNA for sodium coupled neutral amino acid transporters (SNATs) *SLC38A1*, *SL38A2*, and *SLC38A4 revealed* that expression of *SLC38A2* is greater ($P<0.05$) in placentomes from control and NR non-IUGR pregnancies than NR IUGR pregnancies. For each gene, columns lacking a similar letter differ statistically ($P<0.05$).

as well as the cationic amino acid transporters SLC7A6 and SLC7A7, above those for IUGR fetuses.

In agreement with our prior observations, results from the present study indicated that despite an identical nutrient restriction there was a wide range in fetal weights at Day 125 of gestation for NR ewes. Indeed, the upper quartile of NR ewes had fetuses that were similar in weight to well-fed control ewes. These results are similar to those observed in beef cattle, whereby nutrient restriction from early- to mid-gestation resulted in both IUGR and non-IUGR fetuses at mid-gestation [168]. Further, the IUGR pregnancies were characterized by smaller cotyledonary weights and reduced placentomal surface area [168] which is similar to results from the present study, further supporting a large body of literature indicating that placental weight is positively correlated with fetal weight. Placental adaptation to meet the increasing demands of the growing fetus has been observed under a variety of conditions in humans and mice [62, 217-219]. Importantly, these adaptive changes are not simply characterized by an increase in placental size or blood flow, but also by changes in specific nutrient transport mechanisms [62, 64, 217, 220]. Although placental blood flow is imperative for optimal nutrient delivery, expression and/or activity of specific transporters is the rate limiting step for delivery of many nutrients, including glucose and amino acids [195]. In both humans and rats, compromised pregnancies are associated with specific alterations in transporter availability and function [64, 221]. In rats, maternal dietary protein deprivation results in down-regulation of placental amino acid transport systems prior to the detection of fetal growth restriction [63, 64]. These observations are in line with

results from the present study indicating increased expression of transporters for both neutral and cationic amino acids in the placentomes of NR ewes having a non-IUGR fetus versus an IUGR fetus.

Placental growth precedes fetal growth, thus the ability to respond to the increased demand for nutrients during late gestation can be undermined by poor placental development earlier in gestation [116, 222]. Interestingly, a number of amino acids and their metabolites may play a central role in regulating placental angiogenesis and vasodilation. NO, a product of arginine catabolism, plays a crucial role in regulating placental angiogenesis and fetal-placental blood flow during gestation [108, 186, 187]. In addition, arginine, proline, and glutamate can be metabolized to form ornithine, a precursor of polyamine synthesis [135]. Polyamines stimulate gene expression, cell proliferation and differentiation, DNA and protein synthesis, and angiogenesis [131]. Inhibition of polyamine synthesis in mice and rats results in impaired placental growth and IUGR [132-134]. In sheep, maternal supplementation with arginine throughout mid-gestation increases fetal growth in undernourished ewes and ewes carrying multiple fetuses [105, 193]. Results of the present study further support the hypothesis that select nutrients are essential for optimal fetal growth directly and/or via enhanced growth of the placenta. Indeed, arginine, proline, glutamate, ornithine, methionine, putrescine, and spermidine were all increased in the fetal circulation of NR non-IUGR compared to IUGR lambs. Further, increased expression of mRNAs for amino acid transporters in placentomes known to transport arginine, proline, glutamate, and ornithine was detected in non-IUGR pregnancies of NR ewes. Insulin is a critical metabolic regulator that

stimulates cells to take up nutrients such as glucose and amino acids. In well-fed sheep, infusion of insulin to fetuses in late-gestation increased amino acid uptake, but failed to increase weight of lambs [141]. Failure to increase fetal weight following treatment with insulin has also been observed in the pig [223]. However, in the rhesus monkey, long-term insulin infusion to the fetus resulted in a 33% increase in body mass [224]. Similarly, in the rat, infusion of long-acting insulin into the fetus during late gestation resulted in a 10% increase in pup weight at birth [225]. In sheep, restriction of placental growth by carunclectomy impairs insulin secretion from the late-gestation sheep fetus [226]. In the present study, there were increased concentrations of insulin in the fetal circulation of lambs from NR non-IUGR and control fed pregnancies. As the sheep placenta does not transport appreciable quantities of insulin [227], the increases in insulin likely result from either increased secretion by the fetus or reduced utilization. Increased quantities of insulin in the fetal circulation may contribute to increases in amino acids in the fetal circulation as insulin promotes amino acid transport across the placenta [228-231]. The observation that concentrations of glucose were lower in maternal plasma of NR non-IUGR ewes than control or NR IUGR ewes is interesting, especially given that concentrations of glucose in the fetal circulation were not different among fetal types. This suggests that ewes with NR non-IUGR fetuses may have an altered metabolic state resulting in enhanced glucose utilization or transport to the conceptus to meet metabolic demands of the fetus or for conversion to fructose by the placenta. How this might impact nutrient delivery to the fetus is currently unknown.

Consequently, it may be advantageous to measure fructose in the fetal circulation of control fed and NR pregnancies.

In conclusion, results of the present study establish a model for investigation of mechanisms for placental adaptation in an effort to increase nutrient delivery to the conceptus despite limited nutrient availability for the ewe. Results support previous findings from our laboratory and others highlighting critical roles for amino acids and their metabolites in supporting normal fetal growth and development and the critical role for amino acid transporters in nutrient delivery to the fetus. Future studies to determine differences in placental vascularization, placental blood flow, nutrient transporter activity, and the long-term consequences of maternal nutrient restriction in both normal and IUGR offspring are needed.

CHAPTER IV

ALTERED PLACENTAL MORPHOLOGY AND EXPRESSION OF ANGIOGENIC FACTORS ARE ASSOCIATED WITH COMPENSATORY GROWTH IN AN OVINE MODEL OF INTRAUTERINE GROWTH RESTRICTION

Introduction

The mammalian placenta serves a multitude of tasks to ensure proper fetal development during gestation. Due to its high degree of plasticity, the placenta undergoes various physiological changes to maintain efficient nutrient, gas, and waste exchange between the mother and fetus. Such changes can include alterations in uteroplacental blood flow, maternal nutrient partitioning, activity and availability of nutrient transporters, and/or metabolism by both the placenta and fetus. Accordingly, it is widely acknowledged that proper placentation is fundamental to the growth and development of the fetus.

Uteroplacental blood flow plays an essential role in facilitating sufficient nutrient transport from the maternal circulation to the fetus. As gestation progresses the demand for uteroplacental blood flow increases due to the exponential growth of the fetus, particularly in late gestation. This demand for increased vasculature and blood flow is attained by both enhanced vasodilation and increased angiogenesis [43]. In the cotyledonary placentas of ruminants, structures known as placentomes support hematotrophic nutrient flow between maternal and fetal vasculatures [77].

Expression of vascular endothelial growth factors, basic fibroblast growth factors, angiopoietins, and their respective receptors are known to be imperative to the proper development of placental vasculature [26]. The VEGF family stimulates permeability of the vasculature, as well as production and migration of vascular endothelial cell proteases which are involved in degradation of the extracellular matrix and are essential of the angiogenic process [28, 80, 81]. Expression of *VEGF* and its receptors have been localized in placental and fetal tissues in multiple species, including the sheep, mouse and human [28, 82-87]. Within endothelial cells, basic fibroblast growth factor (bFGF or FGF2) stimulates both collagenase and plasminogen activator protease production for the conversion of plasminogen to plasmin for degradation of blood clots, and FGF2 functions as a chemotactic and a mitogenic factor [79, 94]. The angiopoietins, *ANGPT1* and *ANGPT2* (also *ANG1* and *ANG2*, respectively), also function in vascular remodeling, promoting endothelial cell survival, organization of the microvasculature, and inducing both maturation and stabilization of blood vessels to enhance and sustain adequate blood flow [26, 99-103]. Additionally, endothelium-derived NO is essential for the regulation of both vasodilation and angiogenesis in the placenta [105-108]. The growth factors VEGF and FGF2 stimulate production of NO by endothelial cells, while NO is also capable of regulating expression of these angiogenic factors [26].

Disruptions to placental establishment, development, and function at any point during gestation may permanently alter fetal development. A common consequence of placental insufficiency is IUGR of the fetus [1, 4, 27, 65, 66]. Interestingly, work in

cattle [168] and sheep (Satterfield et al. unpublished observations) have shown that maternal nutrient restriction only induces IUGR in a subset of individuals. Furthermore, studies utilizing two populations of genetically similar ewes raised in either harsh or temperate environmental conditions illustrate that adaptive placental changes can occur in response to long-term exposure to a nutritionally limited environment for population preservation and survival [67, 72, 170]. The purpose of this study was to investigate differences in placentomal morphology and expression of angiogenic factors in placentas from NR ewes having either IUGR or non-IUGR pregnancies.

Materials and Methods

Animals

Mature Suffolk ewes (Ovis aries) were observed for estrus (designated as Day 0) in the presence of a vasectomized ram and used in experiments only after exhibiting at least two estrous cycles of normal duration (16–18 Days) as described previously [232]. Ewes were maintained and cared for at the Texas A&M Nutrition and Physiology Center. All experimental procedures were in compliance with the Guide for the Care and Use of Agriculture Animals in Research and Teaching and approved by the Institutional Animal Care and Use Committee of Texas A&M University.

Experimental Design and Tissue Collection

Prior to embryo transfer recipient Suffolk ewes of similar parity and frame size were fed 100% of their NRC requirements to maintain their body condition. Ewes were

synchronized into estrus and a single embryo from superovulated Suffolk donor ewes of normal body condition was transferred into the uterus of each recipient ewe on Day 5.5 post-estrus. Pregnancy was diagnosed by ultrasound on Day 28 of gestation. On Day 35 of pregnancy, ewes were assigned randomly to a control-fed group (100% NRC) (n = 7) and a nutrient-restricted group (50% NRC requirements) (n = 24). Composition of the diet has been published previously [193]. All ewes were individually housed on concrete flooring from Days 28 to 125 of gestation and fed once daily at 0700. Beginning on Day 28 of gestation, body weight was determined every 7 days and feed intake was adjusted based on changes in body weight. Blood samples from the maternal jugular vein were collected into vacuum tubes containing EDTA on Day 125 of gestation and plasma was harvested following centrifugation (2000 x *g* for 10 min at 4^{o}C) and stored at -20^{o}C until analyzed. On Day 125 of pregnancy (term = 147 days of gestation) ewes were necropsied and conceptus (fetal-placental unit) development assessed. At the time of necropsy ewes were euthanized using Beuthanasia.

Following euthanization, the fetus was removed, weighed, measurements made, and dissected to obtain organs. A portion of the uteroplacental-unit was removed with placentomes being snap frozen in liquid nitrogen. The remainder of the uteroplacental unit was filled with warmed PBS with lidocaine and maternal and fetal arteries were catheterized to allow for perfusion of placentomes with Carnoy's solution as previously described by Borowicz and colleagues [79]. Placentomes were dissected, counted, and weighed following perfusion. Lastly, ewes were divided into 3 groups, control, NR non-IUGR and NR IUGR, with the NR ewes divided into upper and lower quartiles (NR non-

IUGR and NR IUGR) based on fetal weights, as described in Chapter III.

RNA Isolation and Real-Time PCR Analysis

Total cellular RNA was isolated from frozen placentomes using Trizol reagent (Gibco-BRL, Bethesda, MD) according to manufacturer's instructions. The quantity and quality of total RNA will be determined by spectrometry and by denaturing agarose gel electrophoresis, respectively, in accordance with the manufacturer's instructions. Total RNA samples were digested with RNase-free DNase I and cleaned up using the RNeasy MinElute Cleanup Kit (Qiagen, Valencia, CA). Synthesis of cDNA from total cellular RNA (2 µg) using random primers (Invitrogen, Carlsbad, CA), oligo-dT primers, and SuperScript II Reverse Transcriptase (Invitrogen) was achieved as described previously [215]. Newly synthesized cDNA was acid-ethanol precipitated, resuspended in 20 µl water at a dilution of 100 ng, and stored at –20ºC for real-time PCR analysis. Primers under 100 bp were designed for each gene in order to maximize efficiency (Table 4.1). qPCR analysis of mRNAs was performed using an ABI PRISM 7700 (Applied Biosystems, Foster City, CA) with Power SYBR Green PCR Master Mix (Applied Biosystems) as the detector, according to manufacturer's recommendations and using methods described previously [146]. Cycle parameters for qPCR were 50ºC for 2 min, 95ºC for 10 min, and then 95ºC for 15 sec and 60ºC for 1 min for 40 cycles. Selected genes analyzed for microarray validation included: *vascular endothelial growth factors (VEGFA, VEGFB, and VEGFC),kinase insert domain receptor (a type III receptor kinase) (KDR), fms-related tyrosine kinases (FLT1, and FLT4), fibroblast*

growth factor 2 (basic) (FGF2), fibroblast growth factor receptors (FGFR1, FGFR2, FGFR3, and FGFR4), angiopoietins (ANGPT1, and ANGPT2), tyrosine kinase, endothelial (TEK), tyrosine kinase with immunoglobulin-like and EGF-like domains 1 (TIE1), guanylate cyclase 1, soluble, beta 3 (GUCY1B3), nitric oxide synthase 3 (endothelial cell) (NOS3), placental growth factor (PGF), sphingosine-1-phosphate receptor 1 (S1PR1), and sphingosine kinase 1 (SPHK1.)

Template input was optimized from serial dilutions of placentomal cDNA for each gene to ensure that the amplification reaction achieved 95-105% efficiency, and that the amount of input chosen was based on being within the linear range of efficiency. Final reactions for *VEGFA, VEGFB, VEGFC, KDR, FLT1, FLT4, FGF2, FGFR1, FGFR4, ANGPT2,* and *GUCY1B3* used 2.5 ng, *FGFR2, FGFR3, ANGPT1, TIE1, NOS3, PGF, S1PR1,* and *SPHK1* used 5 ng, and *TEK* used 10 ng of input. Data were analyzed using 7200HT SDS software (version 2.3, Applied Biosystems). The relative quantification of gene expression across treatments was evaluated using the comparative CT method as previously described [146].

Table 4.1. Primers utilized for quantitative real-time PCR analysis of angiogenic factors.

Target[a]	Forward/reverse primers (5'3')[b]	Length of amplicon (bp)	GenBank accession no.[c]
VEGFA	GCCCACTGAGGAGTTCAACATC	62	NM_174216
	GCTGGCTTTGGTGAGGTTTG		
VEGFB	TCCAAGGCTGCCATCCA	57	NM_174487
	GACCCCTCTTGGTCTCCTCAT		
VEGFC	CCAGGCTGCAAACAAGACTTG	60	NM_174488
	CATCTGCAGACGTGGTTATTCC		
KDR	GCTTGGCCCGGGATATTTA	57	AF534634
	GGCGAGCATCTCCTTTTCTG		
FLT1	TGGATTTCAGGTGAGCTTGGA	68	XM_012184655
	TCACCGTGCAAGACAGCTTC		
FLT4	GAGCATCGCGCCCTGAT	63	XM_002688493
	CACGTTGAGGTGGTTACCAATG		
FGF2	CCAGTTGGTATGTGGCACTGA	61	NM_001009769
	GGTCCTGTTTTGGGTCCAAGT		
FGFR1	GAGGTGCTGCACTTAAGGAATGT	65	NM_001110207
	TTACCCGCCAAGCATGTATACTC		
FGFR2	TGAAGCAGTGGGAATTGACAAG	64	NM_001205310
	CAACATCTTCACGGCCACAGT		
FGFR3	GACGGCGGGCGCTAA	61	NM_174318
	GGTGACATTGCGCAAGGAT		
FGFR4	TCCTTGCTTCTGCACAACGT	60	NM_001192584
	GCCATTGCTGGAGGTCATG		
ANGPT1	AAATGAAAAGCAGAACTACAGGTTGTAT	77	NM_001076797
	GCAAGATCAGGCTGCTCTGTT		
ANGPT2	TCCGTCCAGCAGATTTCTAAACT	62	NM_001098855
	GGAAACAGGGCAAGACATTGTC		
TEK	CCTCGGAGGCAGGAAGAT	62	NM_173964
	TCAGGCAGGTCATTCCCG		
TIE1	CATCCGGGCCATGATCA	71	NM_173965
	AGGCATACTCTTTCAGCATCTTGA		
GUCY1B3	CACCACGCACGGTCCAT	55	NM_174641
	GGCCAGCAATCTCCATCATATC		
NOS3	CGGAACAGCACAAGAGTTACAAGAT	100	NM_001129901
	GTGTTGCTGGACTCCTTTCTCTTC		

Table 4.1. Cont'd.

Target[a]	Forward/reverse primers (5'3')[b]	Length of amplicon (bp)	GenBank accession no.[c]
PGF	CCATGTGGCTTCAGCTTGAG	57	NM_173950
	AGGCCTGCCCATCACAAA		
S1PR1	CGCCATTGAGCGCTACATC	62	NM_001013585
	GGAACCTGTTGCTCCCATTG		
SPHK1	TGGCCGCTTCTTTGAACTATTAC	69	XM_002696204
	TGCAGTTGGTCAGGAGGTCTT		
TUBA	GGTCTTCAAGGCTTCTTGGT	54	AF251146
	CATAATCGACAGAGAGGCGT		

[a]The amplification target.

[b]The forward and reverse DNA oligonucleotides used for amplification of the target. Forward and reverse primers do not necessarily indicate the in vivo direction of transcription.

[c]The accession number to the ovine or bovine sequence used for primer design.

Histological Analyses

Placentomes were sectioned (5 microns) and stained with Masson's trichrome stain as previously described [233]. This procedure stains nuclei black, cytoplasm and muscle fibres red, and extracellular matrix (ECM) components blue. For this stain, placentome sections were deparaffinized in CitraSolv (Fisher Scientific; Fairlawn, NJ) and rehydrated through a graded alcohol series to distilled water. Tissues were then incubated for 1 h at 55^{o}C in Bouin's solution (71% (v/v) picric acid, 24% formaldehyde (40%), and 5% (v/v) glacial acetic acid) and rinsed in water. Slides were incubated sequentially at room temperature for 5 min each in Weigert's iron haematoxylin (50% (v/v) ethanol (95%), 4% (v/v) ferric chloride (29% aqueous), 1% (v/v) hydrochloric acid, and 1% (w/v) haematoxylin), biebrich scarlet-acid fuchsin solution (90% (v/v) biebrich scarlet (1% aqueous), 9% (v/v) acid fuchsin (1% aqueous), and 1% (v/v) glacial acetic

acid), phosphomolybdic–phosphotungstic acid solution (2.5% (v/v) phosphomolybdic acid, 2.5% (w/v) phosphotungstic acid), aniline blue solution (2.5% (w/v) aniline blue, 2% (v/v) glacial acetic acid), and then in 1% glacial acetic acid (v/v) for 5 min. Slides were then dehydrated through alcohol to xylene, and coverslips fixed with Permount (Fisher Scientific, Fair Lawn, NJ). Photomicrographs of stained tissues were captured using a Nikon Ni-E motorized research microscope with Apochromat Lamda 10X and 20X objectives. Images were assembled using Adobe Photoshop.

Immunohistochemistry

Immunohistochemical localization of cytokeratin, desmin, and von Willebrand factor proteins in ovine placentomes was performed as described previously [120]. Localization of cytokeratin and desmin was performed using mouse monoclonal anti-Cytokeratin (ab49779; AbCam) and mouse monoclonal anti-Desmin at 1:500 (ab6322; AbCam) with substitution of the primary antibody with nonimmune mouse IgG (Sigma). Localization of von Willebrand factor was performed using rabbit polyclonal anti-von Willebrand Factor (ab6994; AbCam) at 1:500, with substitution of the primary antibody with nonimmune rabbit IgG (Sigma). A Vectastain ABC anti-mouse or anti-rabbit kit (Vector Laboratories) was used for detection of all proteins following antigen retrieval with boiling citrate buffer as described previously [234]. Immunoreactive protein was visualized using diaminobenzidine tetrahydrochloride (Sigma) as the chromagen. Sections were dehydrated and coverslips affixed with Permount (Fisher).

Statistical Analysis

Data were subjected to least-squares analysis of variance using the General Linear Models procedures of the Statistical Analysis System (SAS Institute, Cary, NC) and are presented as least-squares means with overall standard error of the mean (SE). There was no effect of fetal sex in the statistical model; therefore it was removed from the statistical model. Differences in means were considered to be statistically significant when a P value was ≤ 0.05 while a P value of ≤ 0.1 was considered to indicate a tendency toward significance. Data from quantitative real-time PCR analysis were subjected to least-squares analysis of variance using the general linear models procedures of the Statistical Analysis System (SAS Institute, Cary, NC).

Results

Expression of mRNAs for Angiogenic Factors in the Placentome

To begin investigating differences in vasculature in the ovine placentomes in NR non-IUGR, IUGR, and control pregnancies, we determined mRNA expression of select angiogenic factors and associated genes. Steady-state mRNA levels of genes in the VEGF family are shown in Fig. 4.1A. Steady-state mRNA levels of *VEGFA* were higher ($P<0.05$) in placentomes from ewes having NR non-IUGR fetuses compared to either control ewes or NR ewes having IUGR fetuses. *VEGFB* mRNA levels in placentomes of NR ewes with non-IUGR fetuses were intermediate and did not differ ($P>0.10$) from control or NR IUGR fetuses, but expression between control and NR IUGR placentomes was different ($P<0.05$). *VEGFC* mRNA levels in placentomes were

not different (P>0.10) among fetal phenotypes. Expression of the receptor, *FLT1*, in placentomes of control ewes tended to be lower (P<0.10) than that for placentomes of IUGR or non-IUGR ewes. Steady state levels of *KDR* mRNA tended (P<0.10) to be less for placentomes from control versus NR non-IUGR pregnancies, but did not differ (P>0.10) from NR non-IUGR placentomes. No differences (P>0.10) were detected for *FLT4* mRNA in placentomes from the three groups. Steady-state levels of mRNAs for the fibroblast growth factor family are shown in Fig. 4.1B. No differences (P>0.10) were detected among placentomes from the three fetal phenotypes for mRNAs for *FGF2* or its receptors *FGFR1*, *FGFR2*, *FGFR3*, and *FGFR4*. Similarly, steady-state levels of mRNAs for the angiopoietin family are shown in Fig. 4.1C. Steady-state levels of mRNAs for *ANGPT1*, *ANGPT2*, and the receptor *TEK* were not different (P>0.10) for placentomes among groups. Steady state levels of *TIE mRNA* did, however, tend (P<0.10) to be less in placentomes from control ewes versus NR non-IUGR pregnancies, but did not differ from those for NR non-IUGR pregnancies. Furthermore, expression was not different (P>0.10) among treatments for *GUCY1B3*, *NOS3*, *PGF*, *S1PR1*, or *SPHK1* mRNA levels (data not shown).

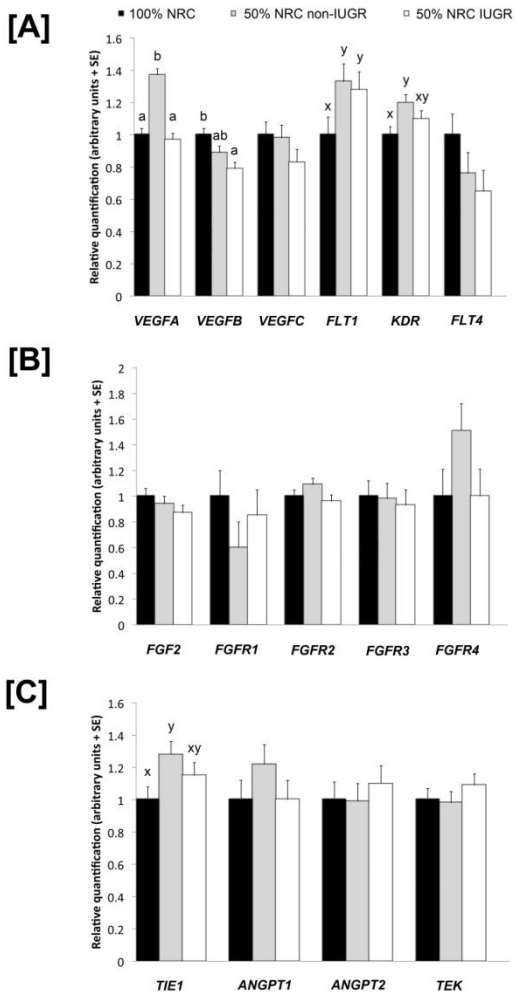


Figure 4.1. Steady state levels of mRNAs for angiogenic factors assessed via real time RT-PCR. [A] Expression of the growth factor *VEGFA* mRNA was greater (P<0.05) in placentomes from NR non-IUGR fetuses than either control ewes or NR ewes having IUGR fetuses. *VEGFB* mRNA levels in placentomes of NR ewes with non-IUGR fetuses were intermediate and did not differ (P>0.10) from control or NR IUGR fetuses, which did differ (P<0.05) significantly from each other. *VEGFC* mRNA levels in placentomes were not different (P>0.10) among groups. Expression of *FLT1* mRNA in placentomes of control ewes was lower (P<0.05) than for NR ewes. Steady state levels of *KDR* and *TIE1* mRNAs tended (P<0.10) to be less in placentomes from control ewes versus those from NR non-IUGR pregnancies, but did not differ (P>0.10) from those of NR non-IUGR pregnancies. [B] No statistically significant differences (P>0.10) were detected in levels of *FGF2* nor *FGFR1*, *FGFR2*, *FGFR3*, and *FGFR4 mRNAs*. [C] Steady state levels of *TIE1* mRNA tended (P<0.10) to be less in placentomes from control ewes versus NR non-IUGR pregnancies, but did not differ (P>0.10) from those of NR non-IUGR pregnancies. Expression of *ANGPT1*, *ANGPT2*, and *TEK* did not differ (P>0.10) significantly between groups.

Alterations in Histoarchitecture of the Placentome

Placentomes of the NR IUGR pregnancies were strikingly less dense than those from either control or NR non-IUGR ewes, as evidenced by a lack of caruncular crypt development. In both the NR non-IUGR and control placentomes there were numerous thick and highly branched crypts with closer juxtapostition to the fetal cotyledonary tissues than for the NR IUGR placentomes. Interestingly, concomitant with a decrease in caruncular crypt development there was an increased thickness of the caruncular capsule in the NR IUGR placentomes.

Localization of Immunoreactive Cytokeratin, Desmin, and von Willebrand Factor in the Placentome

Immunohistochemical staining detected the presence of cytokeratin protein, a marker of intermediate filaments located within the intracytoplasmic cytoskeleton of epithelial cells, at the fetal maternal interface of the placentome (Figure 4.3). Specifically, immunoreactive cytokeratin was more abundant in the syncytia of the control and non-IUGR placentomes and less abundant in the IUGR placentomes.

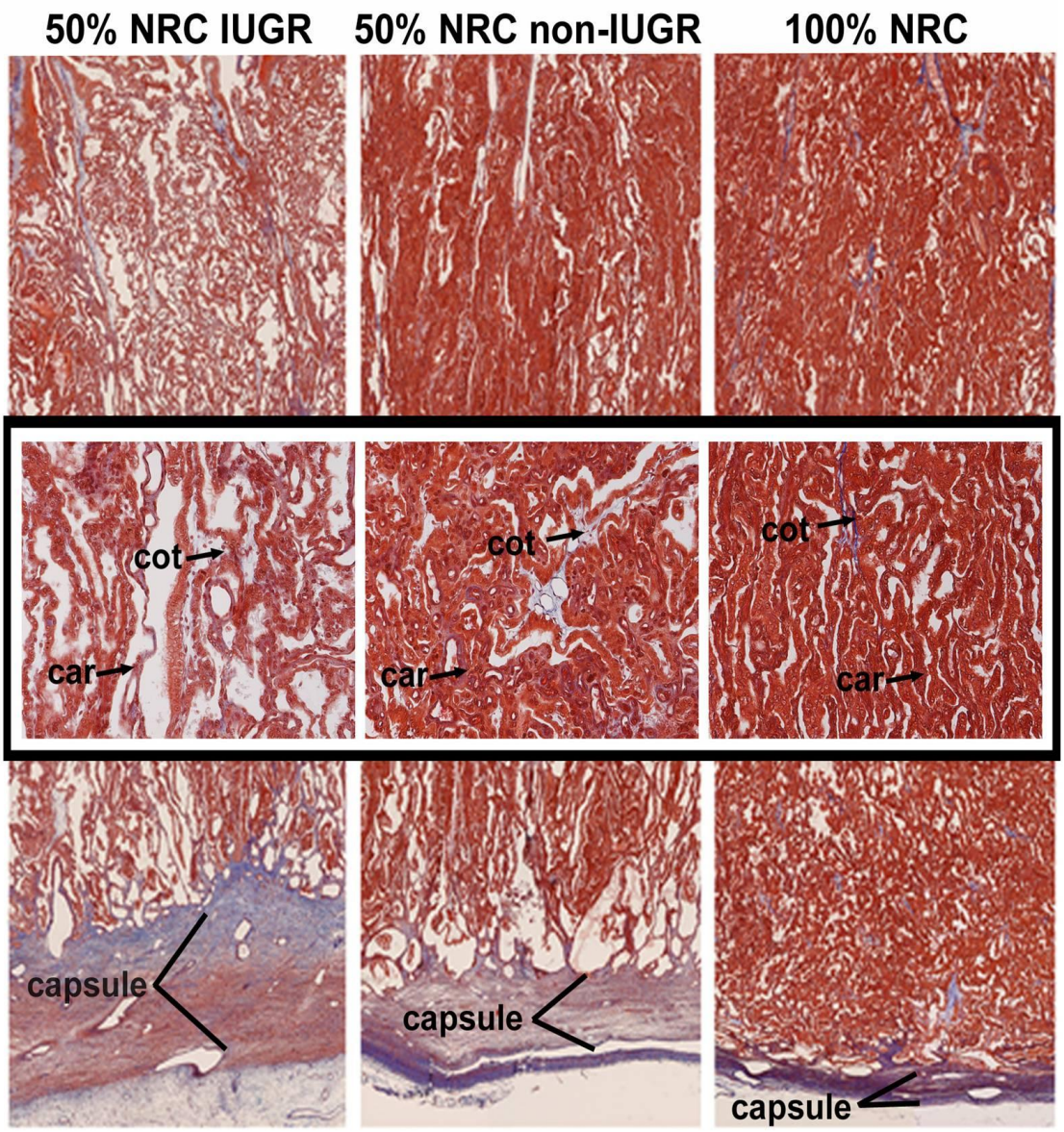


Figure 4.2. Histoarchitecture of the placentomes from 50% NR IUGR, 50% NR non-IUGR and 100% NRC control ewes. CAR denotes caruncle and COT denotes cotyledon. Width of each field of view is 940 µm for low magnification and 640 µm for high magnification.

Desmin, a common marker of vascular development and intermediate filaments of the sarcomere, was detectable in samples from all ewes in this study. However, amounts of immunoreactive desmin protein localized to blood vessels throughout the caruncular and cotyledonary portions of placentomes were markedly more abundant in control and non-IUGR pregnancies. This suggests that IUGR pregnancies are associated with a lack of vascular development. Immunoreactive vWF is used to evaluate the presence of endothelial cells. In the present study, immunoreactive vWF was most abundant in placentomes of the control ewes compared to that for ewes receiving 50%NRC. The presence of vWF protein was detected throughout the placentome, but was most abundant within the cotyledons. While still detectable in placentomes from NR ewes having both non-IUGR and IUGR pregnancies, levels were markedly lower than controls. Collectively, suggesting that NR may compromise vascular development in cotyledonary tissue.

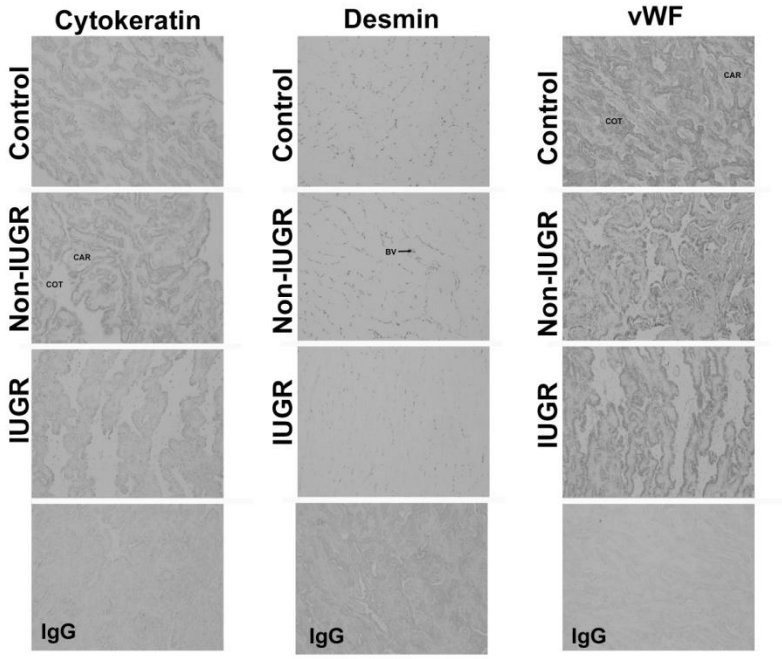


Figure 4.3. Immunohistochemical staining for cytokeratin, desmin, and von Willebrand Factor in placentomes of 100%NRC, 50% NR non-IUGR, and 50% NR IUGR pregnancies. Cytokeratin was localized to the fetal-maternal interface of the placentome and the abundance of immunoreactive cytokeratin was greater in the syncytia of control and non-IUGR placentomes and less in the IUGR placentomes. Desmin was detectable in samples from all ewes and it was localized to blood vessels throughout the caruncular and cotyledonary portions of placentomes and was markedly more abundant in control and non-IUGR pregnancies. Immunoreactive vWF were most abundant in placentomes of control ewes compared to those receiving 50%NRC, and vWF protein was detected throughout the placentome, but most abundant within the cotyledons. CAR denotes caruncle; COT denotes cotyledon; and BV denotes blood vessel. Images are taken at 10X with the width of each field of view being 940 µm.

Discussion

The exponential increase in fetal weight which occurs late in gestation must be supported by an extensive vascular network in the placenta. Thus, angiogenesis is essential to placental formation and function throughout pregnancy in both the maternal caruncular and fetal cotyledonary tissues. Alterations in placental angiogenesis and blood flow in response to inadequate maternal nutrition has been shown to induce IUGR of the fetus in a variety of species, including the sheep, cow, rat, and pig [23, 72, 116, 169, 174, 175]. Results from the present study suggest that placentome morphology and expression of angiogenic growth factors vary in response to maternal nutritional challenge during pregnancy. More specifically, placentomes in NR non-IUGR pregnancies are similar in morphology to placentomes of control fed ewes, with more intimate contact between maternal and fetal tissues than in the placentomes of NR IUGR pregnancies. Those morphological differences likely support enhanced exchange of nutrients, gases, and wastes across the placenta either through increased hematotrophic support and/or via enhanced apposition of maternal and fetal interfaces to improve transport efficiency.

VEGFs play an essential role in angiogenesis by stimulating the production and migration of vascular endothelial cell protease and increasing permeability of the vasculature [28, 80, 81]. More specifically, VEGFA is known to stimulate strong angiogenesis when bound to its receptors FLT1 (also VEGFR1) and KDR (also VEGFR2), with KDR displaying a stronger affinity to VEGFA than FLT1 [235]. Conversely, VEGFB binds to FLT1, but not to KDR or FLT4 (also VEGFR3). The *in*

vivo functions of VEGFB are less clear, but it has been reported to stimulate anigogenesis in fetal tissues such as the heart, lungs, and brown fat [235, 236]. It is also hypothesized that VEGFB can dimerize with VEGFA to increase angiogenic activity [235, 237, 238]. VEGFC stimulates endothelial cell proliferation and migration when bound to KDR or FLT4, and increases vascular permeability when bound to KDR [235].

In the present study, an up-regulation of *VEGFA* mRNA was observed in placentomes from NR non-IUGR ewes compared to both the NR IUGR and control fed ewes at Day 125 of gestation. In addition, an up-regulation of *VEGFB* mRNA expression was seen in the placentomes of control fed ewes compared to the NR IUGR pregnancies, but levels of *VEGFB* in the placentomes from NR non-IUGR were intermediate between these two groups. Collectively, these results suggest that VEGF function was elevated in placentomes from NR non-IUGR ewes compared to NR IUGR ewes. A previous study found no difference in VEGF mRNA expression in NR ewes compared to controls using a slightly milder and shorter period of nutrient restriction [22]. The differences observed between these two studies highlights the potential benefits of using this model to investigate placental factors regulating fetal growth. Indeed, it is likely that the previous study failed to identify differences in VEGF gene expression within NR ewes that correlated with differential rates of fetal growth. It is further likely that the up-regulation of VEGFA and maintenance of VEGFB mRNA expression serve as compensatory factors to enhance placental development and function in NR ewes having normal weight fetuses.

Angiogenic factors, such as the VEGF family, can function through MAPK/ERK1/2 and PI3-K/Akt pathways to stimulate angiogenesis within the placenta. Cell proliferation and growth are also regulated by the MAPK/ERK1/2 and PI3-K/Akt pathways. In cattle, nutrient restriction from Days 30 to 125, followed by dietary realimentation to Day 250 of gestation demonstrated an up-regulation of the MAPK/ERK1/2 and PI3-K/Akt pathways in the cotyledons of NR pregnancies at Day 125 but not Day 250 of gestation [169]. Moreover, fetal weights only tended to be reduced in response to maternal undernutrition on Day 125, but were similar in both dietary groups at Day 250. Thus, this up-regulation of proliferative factors was associated with a tendency for reduced fetal weights at Day 125 of gestation. However, this was mitigated once the diet was restored and did not persist to term. The authors suggest that the up-regulation of MAPK/ERK1/2 and PI3-K/Akt pathways enhanced cotyledonary angiogenesis during early to mid-gestation to ameliorate the effects of nutrient restriction to prevent growth restriction [169]. Yet, it is also possible that the nutrient restriction stimulus induced an inappropriate up-regulation which led to a tendency for smaller fetal weights that was then mitigated once the diet was restored. These results differ from the ones of the present study in which an up-regulation of *VEGFA* expression in the placenta is associated with fetal weights in the NR non-IUGR and control ewes, but greater than weights of NR IUGR fetuses.

FGF2 enhances proliferation of uterine and fetal arterial endothelial cells [26, 55]. Within the endothelial cells, FGF2 stimulates collagenase and plasminogen activator protease [79, 94]. Additionally, FGF2 is a chemotactic and mitogenic factor

for endothelial cells [79, 94]. In humans, there is an increase in VEGFA, FGF2, and endothelial NOS3 proteins, localized to cytotrophoblasts, syncytiotrophoblasts, extravillous trophoblasts, vascular smooth muscle cells, chorionic villous stromal cells, and villous vascular endothelial cells of the placenta of IUGR pregnancies [239]. In the present study, however, no significant changes in levels of mRNAs for *FGF2* or its receptors, *FGFR1-4* were detected, suggesting that at gestational Day 125 this angiogenic factor is not impacted by maternal nutrient restriction and does not play a role in the increased nutrient delivery for enhanced fetal growth in the NR non-IUGR compared to the NR IUGR pregnancies.

Furthermore, there were no significant changes in expression of *ANGPT1* and *ANGPT2*, or their receptors, *TIE1* and *TEK*. ANGPT1 functions to maintain adequate blood flow through maturation and stabilization of vessels [103], while ANGPT2 modulates vascular growth when expressed with VEGF [101]. Similarly, no change in mRNA levels of *placental growth factor (PGF)* was detected in the placentomes. An *in vitro* study has shown that vasodilation is increased when PGF is bound to the FLT1 receptor [240]. The lysosphingolipid sphingosine 1-phosphate (S1P) biochemical pathway is another key pathway in mediating angiogenesis and previous work has illustrated coordinated temporal regulation of several members of this pathway at key sites of angiogenesis within the pregnant ovine uterus [241]. The present study assessed steady-state mRNA levels for the kinase *SPHK1*, which phosphorylates sphingosine to generate sphingosine 1-phosphate, and the receptor *S1PR1*, in placentomes and saw no changes between groups for either of these genes. Consequently, like FGF2 and its

receptors, the ANGPT family and S1P pathway does not appear to be impacted by the nutrient restriction stimulus, again suggesting that these genes are not involved in the mechanisms which increase nutrient delivery and fetal size in the NR non-IUGR compared to the NR IUGR pregnancies at Day 125.

The relationship between the vasodilatory molecule NO and the VEGF and FGF2 angiogenic factors is essential in regulating both vasodilation and angiogenesis in the placenta [105-108]. Vasodilation may be induced when NO activates GUCY1B3, an enzyme present in endothelial cells that catalyzes the conversion of GTP to cGMP [79]. The functions of the VEGFs can be further stimulated by increased activity of NOS3 [89, 110]. However, no changes in expression of *GUCY1B3* or *NOS3* mRNAs were detected among the three groups of ewes in this study, suggesting that these genes are not associated with the placental mechanisms regulating fetal growth in control or NR pregnancies at Day 125 of gestation.

Formation of placentomes involves interdigitation of the cotyledonary villi into the caruncular crypts during early gestation. As interdigitation progresses, clusters of fetal binucleate cells migrate to the maternal uterine epithelium where they fuse with the maternal epithelial cells to form a syncytial layer. Throughout gestation, capillaries within the cotyledons increase primarily in size, while capillaries in the cotyledon proliferate and branch to maximize surface area [26, 50, 79]. Once these cotyledonary capillaries become highly branched, there is a decrease in area per capillary, but an increase in overall capillary number and overall surface area as gestation progresses [79]. Intimate contact between the maternal caruncle and fetal cotyledon is imperative

to the exchange of nutrients, wastes, and gases between the dam and fetus since hematotrophic transfer of nutrients and gasses is primarily conveyed through placentomes. Moreover, failure of placentome development results in loss of the fetus [242].

In the present study, placentomes of NR IUGR pregnancies were markedly less dense than those of either controls or NR non-IUGR pregnancies. In sheep, maximal juxtaposition between the endometrial and placental microvasculatures is achieved by Day 40 of gestation [243, 244]. As gestation progresses, the placentomes continues to develop and interdigitation of the caruncular and cotyledonary tissues increases [244]. The strikingly reduced density of caruncular crypts, coupled with noticeably less contact and juxtapostition between the maternal caruncular and the fetal cotyledonary tissues in the NR IUGR placentomes further illustrate an impediment in the transfer of nutrients to the fetus.

Cytokeratin protein was detected by immunohistochemical staining at the placental-maternal interface of the placentome. Cytokeratins are typically localized to the intracytoplasmic skeleton of epithelial tissues and localized to uninucleate and binucleate trophoblast cells, as well as the syncytial plaques within the ovine placentome [245]. In this study, cytokeratin protein was specifically increased in the syncytia of placentomes from control and NR non-IUGR ewes and decreased in placentomes from NR IUGR ewes, indicating reduced syncytial plaque formation in these placentomes. Desmin serves as a common marker of vascular development as it stains intermediate filaments of the sarcomere. This localization of desmin suggests a lack of vascular

development in NR IUGR pregnancies. Immunoreactive vWF was used to evaluate presence of endothelial cells in placentomes [246]. Similar to desmin, reduced levels of immunoreactive vWF indicate a decreased abundance of endothelial cells, and thus a lack of vascular development, within the cotyledons in response to nutrient restriction.

In conclusion, the results of this study suggest that illustrates that enhanced fetal growth rates in NR pregnancies is associated with a compensatory elevation of VEGFA mRNA expression. Further, the placental histoarchitecture of placentomes from NR non-IUGR pregnancies more closely mirrors that of well-fed control animals compared to NR IUGR pregnancies. The mechanisms and timing by which the NR non-IUGR placentomes develop in a manner similar to well-fed controls warrants further investigation.

CHAPTER V

MICROARRAY ANALYSIS PORTRAYS AN ADAPTIVE PLACENTAL RESPONSE TO NUTRIENT RESTRICTION IN EWES

Introduction

Maternal nutrient restriction during pregnancy impairs placental and fetal growth in humans and livestock species, often resulting in intrauterine growth restriction (IUGR) [1-4]. Indeed, undernutrition in ruminant livestock species is a global challenge, with the nutrient intake of ewes frequently being less than 50% of the National Research Council (NRC) recommendations [4, 159]. IUGR is a leading cause of neonatal morbidity and mortality in livestock species, as well as humans, with the clinical definition of IUGR being below the 10^{th} percentile for birth [1, 3, 4, 65]. The intrauterine environment is not only a major determinant of fetal growth in utero, but also of great importance in the etiology of chronic disease during adult life [1, 6]. In response to reduced nutrient delivery from the dam, the fetus undergoes a number of epigenetic adaptations to reset critical metabolic and physiologic functions that will allow for enhanced survival in postnatal life [6, 247]. The mechanisms regulating this adaptation in fetal growth, development, and programming are not fully understood.

Placental growth and development occur primarily during the first half of gestation and is significantly affected by maternal nutrition and other environmental stressors [248, 249]. During the latter half of gestation, vascularization of the placenta increases markedly, especially within the cotyledonary portion of placentomes, to

develop sufficient absorptive area for nutrient exchange [26, 28, 50, 249]. The functional capacity obtained during placental development is necessary to support the substantial fetal growth which occurs late in gestation. Throughout pregnancy, the placenta facilitates transportation of maternal nutrients to the fetus; however, nutrient delivery is dynamic and also dependent upon nutrient availablity, uteroplacental blood flow, placental metabolism and transport capacity of the uterus and placenta. Not surprisingly, a significant positive correlation exists between placental and fetal weights and between uteroplacental blood flow and fetal weight in various species [65, 70, 168, 250]. Interestingly, the highly adaptable placenta is hypothesized to undergo developmental and functional compensation during times of suboptimal nutrition [251].

Although maternal nutrient restriction results in smaller offspring at birth than those from adequately fed ewes, a wider variation in lamb weights has been observed within ewes that received 50% NRC than within those that received 100% NRC requirements. Similarly, placental weights vary greatly between uniformly treated ewes [69]. Previous work from our lab has shown that lamb birth weights in ewes fed at 50% of NRC requirements (mean = 4.84 kg; range = 2.5-7.1 kg; n=54) vary more in comparison to those receiving 100% NRC (mean = 6.28 kg; range = 5.2-7.2 kg; n=13) (Satterfield et al. unpublished observations). A subsequent study from our lab also resulted in a large variation in fetal weights within the lambs from nutrient restricted ewes (CHAPTER III). Within this study, lambs from ewes receiving 50% NRC requirements were further divided into the top and bottom quartiles based on fetal weights; the six largest (NR non-IUGR) and six smallest (NR IUGR) fetuses. Available

evidence suggests that adaptive mechanisms exist in a subset of nutrient restricted ewes that allows them to support normal fetal growth despite limited nutrient availability. Therefore, the objective of the present study was to utilize a discovery based approach to identify novel placental genes associated with differential rates of fetal growth within nutrient restricted ewes.

Materials and Methods

Animals

Mature Suffolk ewes (Ovis aries) were observed for estrus (designated as Day 0) in the presence of a vasectomized ram and used in experiments only after exhibiting at least two estrous cycles of normal duration (16–18 days). Ewes were maintained and cared for at the Texas A&M Nutrition and Physiology Center. All experimental procedures were in compliance with the Guide for the Care and Use of Agriculture Animals in Research and Teaching and approved by the Institutional Animal Care and Use Committee of Texas A&M University.

Experimental Design and Tissue Collection

Prior to embryo transfer recipient Suffolk ewes of similar parity and frame size were fed 100% of their NRC requirements to maintain their body condition. Ewes were synchronized into estrus and a single embryo from superovulated Suffolk donor ewes of normal body condition was transferred into the uterus of each recipient ewe on Day 5.5 post-estrus. Pregnancy was diagnosed by ultrasound on Day 28 of gestation. On Day 35

of pregnancy, ewes were assigned randomly to a control-fed group (100% NRC) (n = 7) and a nutrient-restricted group (50% NRC requirements) (n = 24). Composition of the diet has been published previously [193]. All ewes were individually housed on concrete flooring from Days 28 to 125 of gestation and fed once daily at 0700. Beginning on Day 28 of gestation, body weight was determined every 7 days and feed intake was adjusted based on changes in body weight. Blood samples from the maternal jugular vein were collected into vacuum tubes containing EDTA on Day 125 of gestation and plasma was harvested following centrifugation (2000 x *g* for 10 min at 4^{o}C) and stored at -20^{o}C until analyzed. On Day 125 of pregnancy (term = 147 days of gestation) ewes were necropsied and conceptus (fetal-placental unit) development assessed. At the time of necropsy ewes were euthanized using Beuthanasia.

Following euthanization, the fetus was removed, weighed, measurements made, and dissected to obtain organs. A portion of the uteroplacental-unit was removed with placentomes being snap frozen in liquid nitrogen. The remainder of the uteroplacental unit was filled with warmed PBS with lidocaine and maternal and fetal arteries were catheterized to allow for perfusion of placentomes with Carnoy's solution as previously described by Borowicz and colleagues [79]. Placentomes were dissected, counted, and weighed following perfusion. Lastly, ewes were divided into 3 groups, control, NR non-IUGR and NR IUGR, with the NR ewes divided into upper and lower quartiles (NR non-IUGR and NR IUGR) based on fetal weights, as described in Chapter III.

RNA Extraction and Affymetrix GeneChip Array Analysis

Total cellular RNA was isolated from frozen placentomes using Trizol reagent (Gibco-BRL, Bethesda, MD) according to manufacturer's instructions. The quantity and quality of total RNA was determined by spectrometry and by Bioanalyzer, respectively, in accordance with the manufacturer's instructions. Total RNA samples were digested with RNase-free DNase I and cleaned up using the RNeasy MinElute Cleanup Kit (Qiagen, Valencia, CA). Both quality and quantity of RNA were determined using the Agilent Bioanalyzer (Agilent Technologies, Santa Clara, CA, USA) and the NanoDrop 1000 (Thermo Fisher Scientific, Inc., Wilmington, DE, USA) respectively. Only samples with an RNA integrity number > 8.0 were used for microarray analysis.

Microarray analysis were perform on placentomes from NR non-IUGR and NR IUGR pregnancies using methods previously described [252]. A Gene Chip One-cycle Target Labeling Kit (Affymetrix, Santa Clara, CA, USA) was used to label total RNA, which was then hybridized to the Affymetrix GeneChip Bovine and Ovine Genome 1.0 ST Arrays. Hybridization quality was assessed using GCOS 1.4 (Affymetrix). Hybridization probes for the Affymetrix GeneChip Bovine and Ovine Genome 1.0 ST Arrays (Affymetrix) were prepared using 10 mg of total RNA and the One-Cycle Target Labeling and Control Reagent package (Affymetrix). The GeneChip Hybridization, Wash, and Stain Kit (Affymetrix) and a Fluidic Station 450 (Affymetrix) were used for the hybridization, wash, and staining process. All steps were carried out according to the manufacturer's protocol. The processed arrays were scanned with a GeneChip Scanner 3000 (Affymetrix).

Array output was normalized via the robust multiarray method, and probe sets were filtered based on expression calls, as previously described [252, 253]. Data analysis was conducted using the GeneSpring GX Software (Agilent Technologies) using ANOVA (PZ0.05) with a Benjamini and Hochberg false discovery rate multiple test correction to determine differentially expressed genes in placentomes from NR non-IUGR and NR IUGR pregnancies.

Database for Annotation, Visualization, and Integrated Discovery

DAVID version 6.7 (http://david.abcc.ncifcrf.gov/home.jsp) facilitates the use of microarray gene lists to generate specific functional annotations of biological processes affected by treatment in microarray experiments [232, 254, 255]. DAVID was utilized, as previously described, to annotate biological themes in response to dietary treatment [232]. All differentially expressed genes identified were both significantly ($P \leq 0.05$) and numerically (1.5-fold change or greater) different and homologous to a known and annotated human gene for use in the DAVID analysis. The background list utilized in the program included all genes assigned a human accession number that were present on the bovine or ovine oligo array. With Gene Ontology (GO) terms identified as biological mechanism, cellular component, and molecular function, along with protein domain and biochemical pathway membership, DAVID generated biological themes by grouping similar terms, ultimately creating functional annotation clusters associated with effects of dietary treatment [232].

cDNA Synthesis and Real-Time PCR Analysis

Synthesis of cDNA from total cellular RNA (2 µg) using random primers (Invitrogen, Carlsbad, CA), oligo-dT primers, and SuperScript II Reverse Transcriptase (Invitrogen) was achieved as described previously [215]. Newly synthesized cDNA was acid-ethanol precipitated, resuspended in 20 µl water at a dilution of 100 ng, and stored at –20ºC for real-time PCR analysis. Primers under 100 bp were designed for each gene in order to maximize efficiency (Table 5.1). qPCR analysis of mRNAs was performed using an ABI PRISM 7700 (Applied Biosystems, Foster City, CA) with Power SYBR Green PCR Master Mix (Applied Biosystems) as the detector, according to manufacturer's recommendations and using methods described previously [146]. Cycle parameters for qPCR were 50ºC for 2 min, 95ºC for 10 min, and then 95ºC for 15 sec and 60ºC for 1 min for 40 cycles. Selected genes analyzed for microarray validation included: anterior gradient protein 2 homolog (*AGR2)*, UDP-Gal:betaGlcNAc beta 1,3-galactosyltransferase, polypeptide 2 (*B3GALT2*), cell adhesion molecule 1 (*CADM1*), leukocyte antigen CD37 (*CD37)*, T-lymphocyte activation antigen CD86 (*CD86*), C-X-C motif chemokine 10 (*CXCL10*), chemokine (C-X-C motif) ligand 14 (*CXCL14*), cathepsin S (*CTSS*), dihydropyrimidine dehydrogenase (*DPYD*), glycine amidinotransferase (L-arginine:glycine amidinotransferase) (*GATM*), histone deacetylase 11 (*HDAC11*), interleukin 12 receptor, beta 2 (*IL12RB2*), lipase, endothelial (*LIPG*), nucleoporin 210kDa (*NUP210*), solute carrier family 44, member 4 (*SLC44A4*), solute carrier organic anion transporter family, member 1C1 (*SLCO1C1*), secreted phosphoprotein 1 (*SPP1*), stanniocalcin 1 (*STC1*), and sulfatase 2 (*SULF2*).

Template input was optimized from serial dilutions of placentomal cDNA for each gene to ensure that the amplification reaction achieved 95-105% efficiency, and that the amount of input chosen was based on being within the linear range of efficiency. Final reactions for *CADM1, GATM, HDAC11, LIPG, SLC44A4* used 2 ng, *B3GALT2, CD37, CD86, CTSS,CXCL10, CXCL14, DPYD, IL12RB2, NUP210, SLCO1C1*and *STC1* used 2.5 ng, *SPP1* used 5 ng, *SULF2* used 10 ng, and *AGR2* used 12.5 ng of input. Data were analyzed using 7200HT SDS software (version 2.3, Applied Biosystems). The relative quantification of gene expression across treatments was evaluated using the comparative CT method as previously described [146]. Statistical analysis of each qPCR gene analyzed the NR non-IUGR and NR IUGR placentomes for validation of the Affymetrix GeneChip Bovine and Ovine Genome 1.0 ST Arrays. Mean gene expression values from placentomes from control fed ewes have been included for informative comparisons only and were not included in the statistical analysis.

Table 5.1. Sequences of primers used for quantitative real-time PCR analyses for microarray validation.

Amplification target	Forward primer (5'3')[a]	Reverse primer (5'3')[a]	Length of amplicon (bp)[b]	GenBank accession no.
AGR2	CCTCTCTCCTGATGGCCAGTAT	CAGTCAGGGATGGGTCAACAA	55	NM_001040500
B3GALT2	ACTACTAATCGCTGCGGAACCT	GCCCCAAGTTTGCCGAAT	64	NM_001076188
CADM1	AAGCCCCAGCCTGTGATG	ACGGCATGTTGAGGCATTTC	62	NM_001038558
CD37	TTCGTGGGCTTGTCCTTCAT	CTGAGACGGCCAGGACCTT	58	NM_001046011
CD86	GGCCGCACAAGCTTTGA	TTTGATTTGAACGTTGTGGAGTCT	60	NM_001038017
CTSS	TGGGAGCCCTGGAAGCA	TGCACTCAGAGACACCAGCTTT	59	NM_001033615
CXCL10	CCGTGGACTTCGGTTTTCCTA	GCAGGAGTAGTAGCAGCTGAT ATG G	66	NM_001046551
CXCL14	CCGCTACAGCGACGTGAA	CCTCGCAGTGCGGGTACTT	56	NM_001034410
DPYD	TGCTCCAGGTATGCAGTGCTAT	TTTGAGGCCAGTGCAGTAGTCTT	71	NM_174041
GATM	CCGAAGCGCTGCACTACA	CACCCATCCCGTTACAGTTCTT	56	NM_001045878
GRP	CCAGTGGGAAGAAGCGACAA	CGGGCCCCCTTTGCT	57	NM_001101239
HDAC11	CACGGCCCGCATCATC	ATGAGCCCCAGGCTGTACAG	54	NM_001102056
IL12RB2	ATGGTGGGCGTTCTCTCAAT	GAGGGCCAAAAGGAGAACAAA	63	NM_174645
LIPG	GAGGGACGGCTGCAAGGT	TGGCAGCAGTCGGTACCA	55	XM_002697766
NUP210	CAGTGATGTTTTTTATTTGTGTCAGTTC	ATTTGCTCCAATCATTTCCCAGTA	92	NM_001191461
SLC44A4	GACCCCATGGAGCAAGTGA	GCCCTGGAAGACGCACAT	57	NM_001083442
SLCO1C1	GTGCCTGGTGGCTTGGTTA	GGCACAGCTGCCAAAAGAGT	58	NM_001191509
SPP1	TTCTGCCTCTTGGGCATTG	CTGCCAGAACTGGTCGGTTT	56	NM_001009224
STC1	TGTGATCCGGCCTGCTATG	ACTGATGAACGGTGACAAGTCAA	61	NM_176669
SULF2	CCCACCACCGCCTGAA	GGATGATGTTGGGACGAATGT	63	NM_001192938
TUBA	GGTCTTCAAGGCTTCTTGGT	CATAACGACAGAGAGGCGT	54	AF251146

[a] Forward and reverse primers do not necessarily indicate the in vivo direction of transcription.

[b] The length of the amplicon created during PCR.

Cloning of Partial cDNAs

Partial cDNAs were amplified by RT-PCR using placentomal or endometrial total RNA isolated from Day 125 pregnant ewes and specific primers (Table 5.2) using methods described previously [232, 256]. PCR amplification was conducted as follows for *CTSS*, *IL12RB2,* and *STC*: 1) 95°C for 5 min; 2) 95°C for 30 s; 58°C for 30 s (for *CTSS*, and *IL12RB2*), and 60°C (for *STC1*); and 72°C for 30 s for 35 cycles; and 3) 72°C for 7 min. The partial cDNAs of the correct predicted size were cloned into pCRII using a T/A Cloning Kit (Invitrogen) and the sequence of each verified using an ABI PRISM Dye Terminator Cycle Sequencing Kit and ABI PRISM automated DNA sequencer (Perkin-Elmer Applied Biosystems).

Table 5.2. Sequences of primers used for RT-PCR and cloning.

Amplification target	**Forward/reverse primers (5'3')[a]**	**Length of amplicon (bp)[b]**	**GenBank accession no.**
CTSS	CCTGGAAGCACAAGTGAAGC	330	X62001
	GAATGGCTCGCGTCTATACC		
IL12RB2	TGTGCAGGAATACGTGGTGG	585	NM_174645
	CAACGCATTGAGAGAACGCC		
STC1	TGATCAGTGCTTCTGCAACC	478	NM_176669
	TCACAGTCCAGTAGGCTTCG		

[a] Forward and reverse primers do not necessarily indicate the *in vivo* direction of transcription.

[b] The length of the amplicon created during PCR.

In Situ Hybridization

Localization of mRNAs in the ovine placentome was determined by radioactive in situ hybridization analysis as described previously [232, 256]. Exposure times were as follows: three weeks for *CTSS,* and *STC1*; and six weeks for *IL12RB2*. Images of representative fields were recorded under bright-field or dark-field illumination using a Nikon Ni-E motorized research microscope with Apochromat Lamda 4X, 10X, 20X and 40X objectives.

Statistical Analysis

Data were subjected to least-squares analysis of variance using the General Linear Models procedures of the Statistical Analysis System (SAS Institute, Cary, NC) and are presented as least-squares means with overall standard error of the mean (SE). There was no effect of fetal sex in the statistical model; therefore it was removed from the statistical model. Differences in means were considered to be statistically significant when a P value was ≤ 0.05 while a P value of ≤ 0.1 was considered to indicate a tendency toward significance. Data from quantitative real-time PCR analysis for placentomes from NR non-IUGR and NR IUGR pregnancies were subjected to least-squares analysis of variance using the general linear models procedures of the Statistical Analysis System (SAS Institute, Cary, NC).

Results

Microarray Analysis

To capitalize on our observed natural population variance in response to maternal nutrient restriction we conducted a gene expression array to identify novel genes in the placentomal transcriptome that regulate placental growth and/or function. A summary of this approach identified 103 differentially expressed genes in placentomes from ewes having NR non-IUGR versus NR IUGR fetuses (Table 5.3). Within this set of differentially expressed genes, 15 genes were upregulated and 88 genes were down-regulated in placentomes having NR non-IUGR fetuses compared to those having IUGR fetuses.

Validation of Selected Genes

A summary comparison of differentially expressed genes selected for validation of the microarray can be found in Table 5.4. Steady-state mRNA levels of *IL12RB2*, *NUP210*, and *SLCO1C1* were higher ($P<0.05$) and *B3GALT2* tended ($P<0.10$) to be higher in NR non-IUGR compared to NR IUGR placentomes (Fig. 5.1). In contrast, *CADM1*, *CD86*, *CTSS*, *CXCL10*, *DPYD*, *GATM*, *SLC44A4*, *STC1* and *SULF2* mRNA levels were increased ($P<0.05$), and *CD37* and *SPP1* tended to be increased ($P<0.10$) in placentomes from ewes having NR IUGR fetuses compared to NR non-IUGR fetuses (Fig. 5.2).

Table 5.3. Placentomal mRNA levels for selected genes identified using the microarray analysis.

Gene Symbol	Gene Name	Fold Change
20ALPHA-HSD	Placental and ovarian 20alpha hydroxysteroid dehydrogenase protein	-2.13
A4IFS4	Pregnancy-associated glycoprotein 16	2.11
ADH6	Alcohol dehydrogenase 6 (class V)	-1.99
AGR2	Anterior gradient protein 2 homolog	-2.32
AGTR2	Angiotensin II receptor, type 2	-1.89
ALDH1A1	Aldehyde dehydrogenase 1 family, member A1	-1.54
B3GALT2	Beta-1,3-galactosyltransferase 2	1.57
B3GNT3	UDP-GlcNAc:betaGal beta-1,3-N-acetylglucosaminyltransferase 3	1.74
BCL2L15	Bcl-2-like protein 15	-2.17
BOLA-DRA	Major histocompatibility complex, class II, DR alpha	-1.69
BOLA-DRB3	Major histocompatibility complex, class II, DRB3	-1.65
C1QB	Complement C1q subcomponent subunit B	-1.67
C4ORF19	Uncharacterized protein C4orf19	-1.60
CADM1	Cell adhesion molecule 1	-1.58
CCKN	Cholecystokinin Precursor	1.77
CD200R1	Cell surface glycoprotein CD200 receptor 1	-1.51
CD37	Leukocyte antigen CD37	-1.59
CD86	T-lymphocyte activation antigen CD86	-1.51
CFD	Complement factor D	-1.55
CH3L1	Chitinase 3-like protein 1 Precursor	-2.13
CHRM2	Muscarinic acetylcholine receptor M2	-1.53
CP	Ceruloplasmin (ferroxidase)	-2.25
CPE	Carboxypeptidase E	-1.60
CR2	Complement receptor type 2	-2.93
CST6	Cystatin-M	-1.59
CTSS	Cathepsin S	-1.54
CXCL10	C-X-C motif chemokine 10	-1.85
CXCL14	C-X-C motif chemokine 14	-2.17
CYP26A1	Cytochrome P450, family 26, subfamily A, polypeptide 1	-1.57
CYP4F22	Cytochrome P450 4F22	1.51
DLK1	Protein delta homolog 1	-1.62
DPYD	Dihydropyrimidine dehydrogenase	-1.51
EHF	Ets homologous factor	-1.60
EMP1	Epithelial membrane protein 1	-1.53

Table 5.3. Cont'd

Gene Symbol	Gene Name	Fold Change
EVI2B	Protein EVI2B	-1.59
FAM134B	Protein FAM134B	-1.71
FBN2	Fibrillin-2	-1.54
FCGR3	Low affinity immunoglobulin gamma Fc region receptor III	-1.61
FGFR1	Fibroblast growth factor receptor 1	-1.60
FOLR2	Folate receptor beta	-1.54
GATA6	Transcription factor GATA-6	-1.66
GATM	Glycine amidinotransferase, mitochondrial	-2.01
GHR	Growth hormone receptor	-1.55
GPR115	Probable G-protein coupled receptor 115	-1.66
GPR151	Probable G-protein coupled receptor 151	2.20
GRM7	Metabotropic glutamate receptor 7	-2.66
HDAC11	Histone deacetylase 11	1.58
HTR4	Serotonin 5-HTA receptor	-2.78
IL12RB2	Interleukin-12 receptor subunit beta-2	1.88
INHBA	Inhibin, beta A	-1.85
KLF5	Krueppel-like factor 5	-1.54
KNG2	Kininogen-2	-1.68
LBP	Lipopolysaccharide binding protein	-1.68
LIPG	Endothelial lipase	2.00
Mamu-DRA	Mamu class II histocompatibility antigen, DR alpha chain	-1.78
MCEMP1	Mast cell-expressed membrane protein 1	1.70
MICB	MHC class I polypeptide-related sequence B	-1.75
MILR1	Allergin-1	-1.57
MIR186	MicroRNA mir-186	-2.16
MIR29A	MicroRNA mir-29a	1.53
MIR329B	MicroRNA mir-329b	-1.55
MS4A8A	Membrane-spanning 4-domains subfamily A member 8A	-1.72
MSLN	Mesothelin	-1.56
MSR1	Macrophage scavenger receptor types I and II	-1.65
MUC16	Mucin-16	-1.64
NUP210	Nuclear pore membrane glycoprotein 210	1.77
OCIAD2	OCIA domain-containing protein 2	-1.55
OGN	Mimecan	-1.88
OR52E1	Olfactory receptor 52E1	1.53
OSTP	Osteopontin Precursor	-1.52

Table 5.3. Cont'd

Gene Symbol	Gene Name	Fold Change
P2RY12	P2Y purinoceptor 12	-1.61
PAM	Peptidyl-glycine alpha-amidating monooxygenase	-1.58
PDCD2L	Programmed cell death protein 2-like	1.83
PDZK1IP1	PDZK1-interacting protein 1	-1.61
PEBP4	Phosphatidylethanolamine-binding protein 4	-2.46
PPAP2A	Lipid phosphate phosphohydrolase 1	-1.58
PRR15	Proline-rich protein 15	-1.70
QSOX1	Quiescin Q6 sulfhydryl oxidase 1	-1.63
RBP4	Retinol-binding protein 4	-1.59
RNASE6	Ribonuclease K6	-1.67
S100A7	S100 calcium binding protein A7	-1.51
SAA3	Serum amyloid A 3	-2.08
SDS	L-serine dehydratase/L-threonine deaminase	-1.66
SERPINE2	Glia-derived nexin	-1.62
SESN3	Sestrin-3	-1.61
SIGLEC1	Sialic acid binding Ig-like lectin 1, sialoadhesin	-1.54
SLC1A1	Excitatory amino acid transporter 3	-1.72
SLC26A3	Chloride anion exchanger	-1.82
SLC37A2	Sugar phosphate exchanger 2	-1.68
SLC44A4	Choline transporter-like protein 4	-2.19
SLC7A2	Low affinity cationic amino acid transporter 2	-2.22
SLC7A9	B(0,+)-type amino acid transporter 1	-1.82
SLCO1C1	Solute carrier organic anion transporter family member 1C1	1.78
SPP1	Secreted phosphoprotein 1	-1.56
STC1	Stanniocalcin-1	-1.91
SULF2	Extracellular sulfatase Sulf-2	-1.64
TC2N	Tandem C2 domains nuclear protein	-2.26
TFEC	Transcription factor EC	-1.51
TFPI2	Tissue factor pathway inhibitor 2	-1.64
THBS4	Thrombospondin-4	-1.62
TIMD4	T-cell immunoglobulin and mucin domain containing 4	-1.59
VNN1	Pantetheinase	-1.53
WNT11	Protein Wnt-11	-1.81

Steady-state mRNA levels for *CXCL14*, *HDAC11*, and *LIPG* were not statistically different (P>0.10). Further, we previously reported that levels of *AGR2* mRNA in placentomes were greater (P<0.05) for ewes producing NR IUGR fetuses than for ewes producing NR non-IUGR fetuses (data not shown) [257]. These results validate gene expression based on transcriptional profiling analyses and indicate that genes are differentially expressed in NR non-IUGR compared with NR IUGR placentomes.

Expression of *IL12RB2* mRNA was weak in the placentomes of control or NR IUGR associated pregnancies. However, expression of *IL12RB2* mRNA was detected in scattered cells throughout the caruncular stroma of the placentomes of NR non-IUGR pregnancies. The relative abundance of *STC1* and *CTSS* mRNAs was greater in the cotyledonary tissue of NR IUGR associated placentomes as compared to that of NR non-IUGR or controls. Expression of *STC1* was detected in a diffuse pattern throughout the cotyledonary tissue, while *CTSS* appeared to be more abundant at the fetal maternal interface of the cotyledon and caruncle.

Table 5.4. Comparison of expression of placentomal mRNAs for selected genes identified using microarray or quantitative real-time PCR analyses.

Gene Symbol[a]	Gene Name	Microarray Fold Change[b]	qPCR Fold Change	qPCR P-Value
AGR2	Anterior gradient protein 2 homolog	-2.32	-2.69	0.05
B3GALT2	Beta-1,3-galactosyltransferase 2	1.57	1.67	0.09
CADM1	Cell adhesion molecule 1	-1.58	-2.14	0.03
CD37	Leukocyte antigen CD37	-1.59	-1.73	0.07
CD86	T-lymphocyte activation antigen CD86	-1.51	-1.84	0.01
CTSS	Cathepsin S	-1.54	-1.88	0.03
CXCL10	C-X-C motif chemokine 10	-1.85	-3.01	0.03
CXCL14	C-X-C motif chemokine 14	-2.17	-2.79	0.12
DPYD	Dihydropyrimidine dehydrogenase	-1.51	-2.12	0.03
GATM	Glycine amidinotransferase, mitochondrial	-2.01	-2.84	0.02
HDAC11	Histone deacetylase 11	1.58	1.85	0.17
IL12RB2	Interleukin-12 receptor subunit beta-2	1.88	6.40	0.00
LIPG	Endothelial lipase	2.00	2.02	0.28
NUP210	Nuclear pore membrane glycoprotein 210	1.77	2.50	0.03
SLC44A4	Choline transporter-like protein 4	-2.19	-3.07	0.03
SLCO1C1	Solute carrier organic anion transporter family member 1C1	1.78	2.04	0.05
SPP1	Secreted phosphoprotein 1	-1.56	-3.41	0.09
STC1	Stanniocalcin 1	-1.91	-1.54	0.03
SULF2	Extracellular sulfatase Sulf-2	-1.64	-2.74	0.03

[a]Official gene symbols were used as abbreviations.
[b]Microarray fold-changes are significant ($P<0.05$).

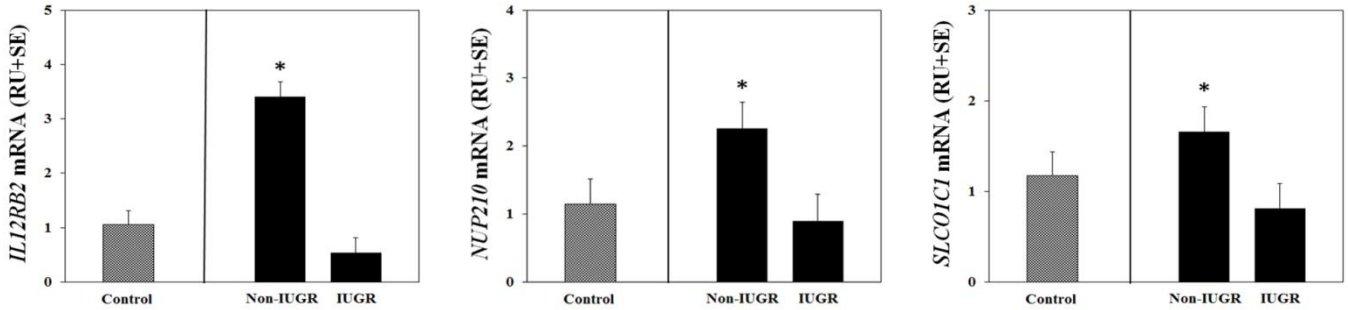


Figure 5.1. Steady state levels of *IL2RB2*, *NUP210*, and *SLCO1C1* mRNAs from an affymetrix microarray were assessed via real time RT-PCR. Expression of the genes *IL2RB2*, *NUP210*, and *SLCO1C1* was greater ($P<0.05$) in placentomes from NR non-IUGR fetuses compared to NR ewes having IUGR fetuses. Mean gene expression values from placentomes from control fed ewes have been included for informative comparisons only and were not included in the statistical analysis.

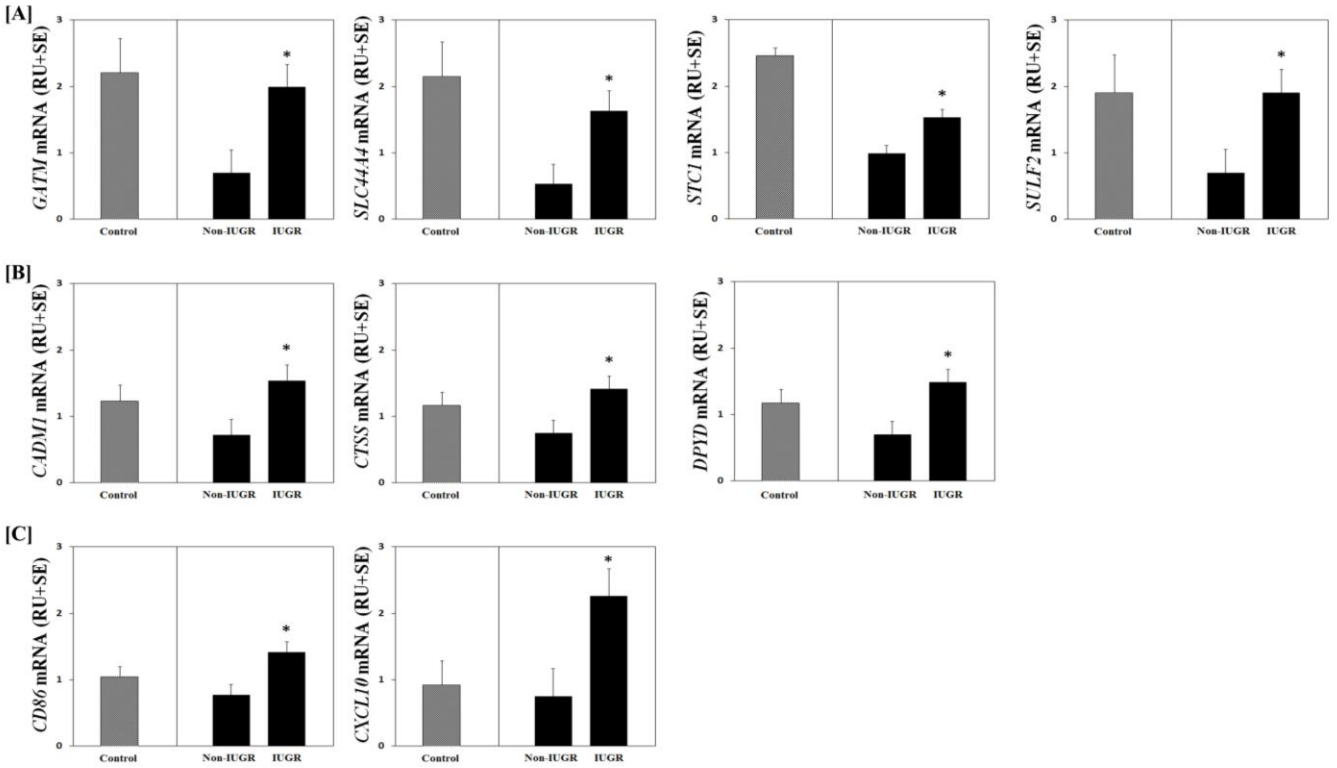


Figure 5.2. Steady state levels of *CADM1, CTSS, DPYD, GATM, SLC44A4, STC1,* and *SULF2* mRNAs from an affymetrix microarray were assessed via real time RT-PCR. [A] *GATM, SLC44A4, STC1* and *SULF2* mRNAs in placentomes were greater (P<0.05) in placentomes from ewes producing NR IUGR fetuses compared to placentomes from ewes producing NR non-IUGR fetuses. [B] *CADM1, CTSS,* and *DPYD* mRNAs in placentomes were greater (P<0.05) for ewes producing NR IUGR fetuses than for ewes producing NR non-IUGR fetuses. [C] *CD86* and *CXCL10* mRNAs in placentomes were greater (P<0.05) for ewes producing NR IUGR fetuses than for ewes producing NR non-IUGR fetuses. Mean gene expression values from placentomes from control fed ewes have been included for informative comparisons only and were not included in the statistical analysis.

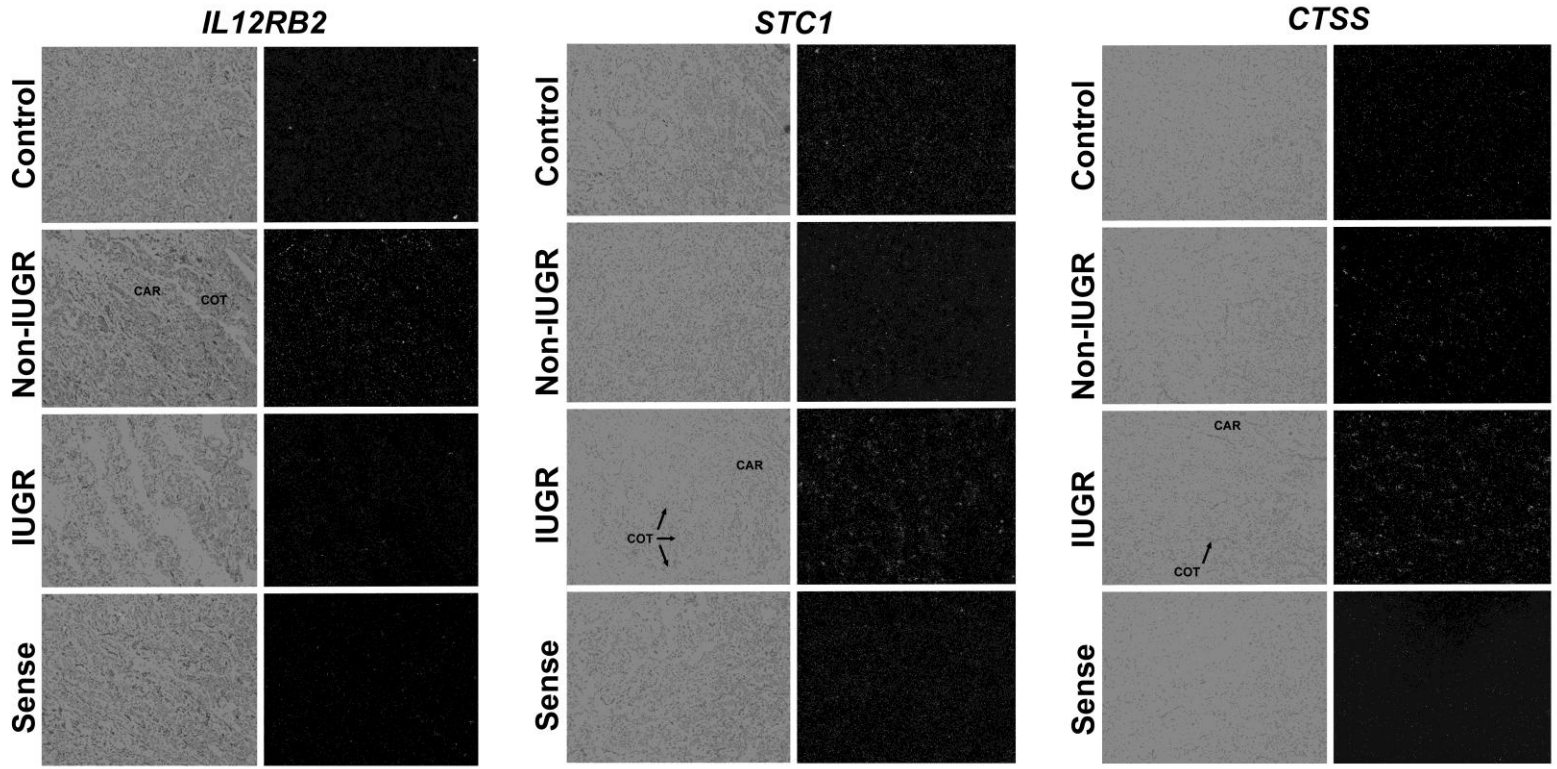


Figure 5.3. Localization of *IL12RB2*, *STC1*, and *CTSS* mRNA in placentomes of 100%NRC, 50% NR non-IUGR, and 50% NR IUGR pregnancies. Expression of *IL12RB2* mRNA was weak in the placentomes of control or NR IUGR associated pregnancies. However, expression of *IL12RB2* mRNA was detected in scattered cells throughout the caruncular stroma of the placentomes of NR non-IUGR pregnancies. The relative abundance of *STC1* and *CTSS* mRNAs was greater in the cotyledonary tissue of NR IUGR associated placentomes as compared to that of NR non-IUGR or controls. Expression of *STC1* was detected in a diffuse pattern throughout the cotyledonary tissue, while *CTSS* appeared to be more abundant at the fetal-maternal interface of the cotyledon and caruncle. Corresponding bright and dark field images of representative cross sections are shown. CAR denotes caruncle and COT denotes cotyledon. Corresponding bright and dark field images of representative cross sections are shown. All photomicrographs are shown at the same width of field (940 µm).

Bioinformatics

DAVID bioinformatic analyses were performed to identify biological processes potentially regulating the differential rates of placental growth and/or function between NR non-IUGR and NR IUGR ewes. DAVID analysis of the 15 genes upregulated in NR non-IUGR pregnancies identified three weakly enriched functional annotation clusters, which were associated with biological terms such as transmembrane region, integral to membrane, intrinsic to membrane, receptor, cell surface receptor linked signal transduction, signal peptide, alternative splicing, and splice variant (Table 5.5).

Conversely, thirty-three enriched clusters were identified by DAVID analysis of the 88 down-regulated genes from NR non-IUGR placentomes. The 10 most highly enriched clusters are presented in Table 5.6. Interestingly, of the ten most enriched clusters, two were associated with response to nutrients, while five were associated with immune response. Clusters associated with a response to nutrient levels featured biological terms such as response to nutrient, response to extracellular stimulus, amino acid transport, amine transport, amino acid transmembrane transporter activity, carboxylic acid transport, organic acid transport, and amine transmembrane transporter activity. Those clusters related to immune responses featured biological terms such as positive regulation of immune response, immune effector process, immunoglobulin-like, immunoglobulin domain, activation of immune response, complement activation, activation of plasma proteins involved in acute inflammatory response, humoral immune response, lymphocyte mediated immunity, adaptive immune response, leukocyte

mediated immunity and adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains.

Table 5.5. Functional annotation clusters of biological terms representing NR non-IUGR placentomes.

Annotation Cluster[a]	Enrichment Score[b]	Biological Terms[c]
1	1.27	transmembrane region (7)
		Transmembrane (7)
		GO:0016021 ~ integral to membrane (7)
		GO:0031224 ~ intrinsic to membrane (7)
2	0.51	receptor (3)
		GO:0007166 ~ cell surface receptor linked signal transduction (3)
		topological domain:Extracellular (3)
3	0.15	signal (3)
		signal peptide (3)
		alternative splicing (3)
		splice variant (3)

[a]The three most significant annotation clusters identified from the gene list submitted for analysis through DAVID. [b]The enrichment score is determined through DAVID and ranks the significance of each annotation cluster based on the relatedness of the terms and the genes associated with them. [c]This column summarizes the biological terms in the annotation clusters. The gene ontology (GO) terms were gathered based on the known annotation of the submitted genes with respect to biological process, cellular component, and molecular function; as well as biological pathway membership and protein domains. The number in parentheses indicates the number of differentially expressed genes contributing to the clustered term.

Table 5.6. Functional annotation clusters of biological terms representing NR IUGR placentomes.

Annotation Cluster[a]	Enrichment Score[b]	Biological Terms[c]
1	3.4	GO:0031667 ~ response to nutrient levels (7)
		GO:0007584 ~ response to nutrient (6)
		GO:0009991 ~ response to extracellular stimulus (7)
2	2.01	GO:0050778 ~ positive regulation of immune response (6)
		GO:0002252 ~ immune effector process (5)
		GO:0048584 ~ positive regulation of response to stimulus (6)
		immune response (5)
3	2.01	transmembrane region (32)
		transmembrane (32)
		membrane (37)
		GO:0031224 ~ intrinsic to membrane (36)
		GO:0016021 ~ integral to membrane (33)
4	1.91	GO:0005624 ~ membrane fraction (11)
		GO:0005626 ~ insoluble fraction (11)
		GO:0000267 ~ cell fraction (12)
5	1.67	Ig-like V-type (5)
		CD80-like, immunoglobulin C2-set (3)
		IG (4)
		Immunoglobulin subtype (4)
6	1.66	GO:0006865 ~ amino acid transport (4)
		GO:0015837 ~ amine transport (4)
		GO:0015171 ~ amino acid transmembrane transporter activity (3)
		GO:0046942 ~ carboxylic acid transport (4)
		GO:0015849 ~ organic acid transport (4)
		GO:0005275 ~ amine transmembrane transporter activity (3)
7	1.62	Immunoglobulin-like (7)
		Immunoglobulin-like fold (7)
		Immunoglobulin domain (6)
8	1.61	GO:0042803 ~ protein homodimerization activity (6)
		GO:0046983 ~ protein dimerization activity (7)
		GO:0042802 ~ identical protein binding (7)
9	1.54	GO:0051605 ~ protein maturation by peptide bond cleavage (4)
		GO:0002253 ~ activation of immune response (4)
		GO:0016485 ~ protein processing (4)
		GO:0006956 ~ complement activation (3)
		GO:0002541 ~ activation of plasma proteins involved in acute inflammatory response (3)
		GO:0051604 ~ protein maturation (4)

Table 5.6. Cont'd

Annotation Cluster[a]	Enrichment Score[b]	Biological Terms[c]
9	1.54	innate immunity (3)
		GO:0006959 ~ humoral immune response (3)
		Complement and coagulation cascades (3)
		GO:0006508 ~ proteolysis (5)
10	1.31	GO:0002449 ~ lymphocyte mediated immunity (3)
		GO:0002250 ~ adaptive immune response (3)
		GO:0002460 ~ adaptive immune response based on somatic recombination of immune receptors built from immunoglobulin superfamily domains (3)
		GO:0002443 ~ leukocyte mediated immunity (3)

[a]The 10 most significant annotation clusters identified from the gene list submitted for analysis through DAVID. [b]The enrichment score is determined through DAVID and ranks the significance of each annotation cluster based on the relatedness of the terms and the genes associated with them. [c]This column summarizes the biological terms in the annotation clusters. The gene ontology (GO) terms were gathered based on the known annotation of the submitted genes with respect to biological process, cellular component, and molecular function; as well as biological pathway membership and protein domains. The number in parentheses indicates the number of differentially expressed genes contributing to the clustered term.

Discussion

Microarray analysis of placentomes from NR ewes identified novel candidate genes that may regulate development and/or function of the placentome which give rise to differing rates of fetal growth. Placentomal genes expressed later in gestation, in this case gestational Day 125, are likely indicative of either prior changes in placental development which set a pathway(s) in motion or factors regulating the substantial rate of fetal growth that occurs during the final trimester. Indeed, previous studies using models of nutrient restriction in pregnant ewes have shown that throughout gestation, genes such as nutrient transporters [37, 124] and angiogenic factors [26, 28, 115] are essential in regulating proper fetal development. Likewise, data from various pregnancy

models in livestock, humans, and mice also illustrate the importance of placental development and gene function on fetal development [116, 178, 248].

Previous work from our laboratory using the same nutrient restriction model has shown that the expression of various nutrient transporters (CHAPTER III) and angiogenic factors (CHAPTER IV) are up-regulated in NR non-IUGR placentomes in comparison to their NR IUGR counterparts. This, along with the increased placental and fetal weights in NR non-IUGR pregnancies led to the hypothesis that adaptive mechanisms exist in a subset of NR ewes to maintain normal fetal growth despite limited maternal nutrient availability. However, results from the present study suggest that enhanced fetal growth in NR ewes is associated with an altered immune response, rather than solely a compensatory up-regulation of genes involved in placental development and function. Furthermore, the nutrient transporters and angiogenic factors (CHAPTER III and IV, respectively) that were up-regulated in the NR non-IUGR compared to NR IUGR placentomes were not detected in this microarray. This is likely due to the selection criteria of a 1.5-fold change or greater, as many of the previously discussed genes exhibited smaller fold changes. While these select nutrient transporters and angiogenic factors significantly impact placental function and fetal development, we increased the stringency of our selection criteria of this microarray in order to elucidate novel genes influencing placental development and function.

DAVID bioinformatic analysis of the 15 genes up-regulated in NR non-IUGR pregnancies identified only three functional annotation clusters. Those clusters featured GO terms such as integral to membrane, intrinsic to membrane, and cell surface receptor

linked signal transduction. Select genes found in those clusters included interleukin-12 receptor subunit beta-2 (IL12Rβ2), nuclear pore membrane glycoprotein 210 (NUP210), solute carrier organic anion transporter family member 1C1 (SLCO1C1), beta-1,3-galactosyltransferase 2 (β3GALT2), and endothelial lipase (LIPG).

The interleukin-12 receptor is known to be expressed primarily on natural killer (NK) and activated T cells, with the β2 subunit being restricted to Th1 lymphocytes [258-260]. Thus, when acting with its ligand Il-12, IL12Rβ2 may mediate differentiation of Th1 lymphocytes [258, 261]. During implantation and pregnancy, there appears to be shift towards a greater population of Th2 lymphocytes at the maternal-fetal interface [262]. Th1 lymphocytes produce cytokines that can compromise pregnancy. The increase in cytokines produced by Th2 lymphocytes are thought to inhibit inflammatory Th1 responses at the maternal-fetal interface to allow implantation and pregnancy to occur [262]. While there is an up-regulation of *IL12Rβ2* mRNA in the placentomes of NR non-IUGR pregnancies, the present study did not investigate the amount of IL-12 in placentomes or at the maternal-fetal interface throughout the placenta, the presence of other IL-12 receptors has also not been assessed and further work is needed to fully elucidate the implications of the increased *IL12Rβ2* mRNA in the placentomes of NR non-IUGR pregnancies.

Approximately 30 proteins known as nucleoporins serve as building blocks for nuclear pore complexes (NPCs) at fusion sites between the inner and outer nuclear membranes. NUP210 is one of only three integral membrane proteins in the various components of the NPC [263]. The complete function of NUP210 is not clear; however,

in mice it has been shown to be involved in epithelial cell development in various organs, and is required for myogenic and neuronal differentiation, serving a role in cell fate determination and regulation of gene expression [263, 264]. Therefore, it is possible that NUP210 in the placentomes during late gestation regulate cell fate determination and expression of genes that allow this subpopulation of ewes to produce NR non-IUGR fetuses. To our knowledge this is the first study showing *NUP210* mRNA expression in the placenta and further work to assess localization is still needed.

The thyroid hormones (TH), triiodothyronine (T_3) and thyroxine (T_4), are imperative to normal *in utero* growth and development, largely by being essential for increasing fetal mass and terminal tissue differentiation [265]. Expression of the organic anion transporter SLCO1C1 (also known as OATP1C1 and OATP14) is primarily at the blood-brain barrier and blood-cerebrospinal fluid barrier for transport of TH to the developing brain and to be expressed in human Leydig cells [266, 267]. In the rat brain, SLCO1C1 possess the highest affinity for T_4 [266, 268]. Interestingly, SLCO1C1 was recently found to be strongly expressed in the villous stroma of the rat placenta [266]. In that same study, SLCO1C1 was found to work with the thyroid hormone transporter MCT8 in a compensatory manner during times of TH deficiency in either the mother or fetus throughout gestation. Permeability of the placenta to TH is partly dependent on species and placental type. Humans and rodents, having a hemochorial placenta, are relatively permeable to T_3 and T_4, while livestock, possessing epitheliochorial and synepitheliochorial placentas, are thought to be seemingly impermeable to maternal THs

[265]. Moreover, concentrations of TH are low in human IUGR infants and in IUGR offspring from placental insufficiency and NR animal models [265, 269-273].

In the present study 88 genes were down-regulated in placentomes from NR non-IUGR versus NR IUGR conceptuses. Interestingly, a number of these genes appeared to display similar patterns of expression between the NR IUGR and control placentomes, with these genes being down-regulated in the NR non-IUGR pregnancies. DAVID analysis of these genes revealed 33 functional annotation clusters. Not surprisingly, the most significant cluster featured the GO terms response to nutrient levels, response to nutrient, and response to extracellular stimulus. However, of the 10 most significant annotation clusters identified, half were related to immune response, with GO terms such as positive regulation of immune response, immune effecter process, activation of immune response, humoral immune response, lymphocyte mediated immunity, leukocyte mediated immunity, and adaptive immune response.

Establishment and maintenance of pregnancy in all mammalian species involves an intricate balance of immune cells, particularly a balance of pro- and anti-inflammatory cytokines regulated by the maternal immune system, at the maternal-placental interface [274]. This balance is largely regulated by the presence of progesterone which allows for local inhibition of immune responses at the maternal-placental interface without resulting in systemic immunosuppression [275]. In a clinical setting, an increase in inflammatory cytokines, such as TNF-α, and the chemokine IL-8 is seen in placentas of IUGR pregnancies [276, 277]. Umbilical artery ligation in sheep induces a fetoplacental inflammatory response, characterized by increased pro-

inflammatory cytokines, and ultimately results in IUGR at Day 116 of gestation [278]. Overall, work illustrating immune responses in the placentas of IUGR pregnancies are limited. Furthermore, to our knowledge, data on the immunological profile of the placenta in response to maternal nutrient restriction is lacking and the present study presents novel genes regulating immune responses within the placental of NR pregnancies which result in IUGR.

The rate-limiting enzyme for creatine synthesis, glycine amidinotransferase (GATM), decreases production of NO by competing with the inducible form of NO synthase for the amino acid arginine [279]. Additionally, *GATM* is an imprinted gene in human and mouse placentas [280, 281], with expression being exclusively from the maternal allele in extraembryonic tissues of mice [281]. Importantly, a genome-wide survey discovered increased expression of *GATM* of placentas from women that gave birth to an IUGR fetus [282]. Expression of *GATM* is also seen on bovine endometrial $CD14^+$ cells, potentially serving roles characteristic of M2 activated macrophages such as tissue remodeling and immune regulation for promoting pregnancy [283]. It is hypothesized that expression of *GATM* in the placenta for production of phosphocreatine might reduce the impact of sudden high-energy demands from the fetus on the gestating dam [281].

Stanniocalcin 1 is a glycoprotein responsible for regulating calcium and phosphate homeostasis in a paracrine manner, with a role in regulating calcium and phosphate transport in the kidney and intestine [284, 285]. During pregnancy in sheep, *STC1* is involved in regulation of placental and fetal growth and differentiation with

expression appearing in the endometrial glands on Day 18 of gestation and increases until Day 80 [286]. Levels of *STC1* remain elevated in the uterine glands through gestational Day 120. Localization of *STC1* in the glands is associated with the secretion of STC1 protein into the glands and uterine lumen and transport via the placental areolae into fetal circulation and allantoic fluid [286]. In the study by Song et al. [286], expression of *STC1* mRNA was not detected in the placentomes of ewes from Days 30, 40, 60, 80, 100, 120, or 140 of gestation. However, in the present study we detected low levels of *STC1* mRNA in the placentome at Day 125 of gestation. It is probable that the stress of under-nutrition stimulates up-regulation of STC1 in the NR IUGR compared the NR non-IUGR pregnancies but its overall function in the placentome is still unclear.

Uterine remodeling is initiated during the very early stages of gestation and continues until parturition to ensure proper implantation and placentation needed for normal fetal development. This tissue remodeling is partially supported by the degradation of the extracellular matrix and catabolism of intracellular hormones stimulated by a group of peptidases known as cathepsins [287, 288]. Expression of various cathepsins has been detected in ovine uteroplacental tissues throughout gestation. Cathepsin S, in particular, was found in both the intercaruncular endometrium, as well as the placentome through Day 120 of gestation [287]. More specifically, expression of *CTSS* increased in the stratum compactum stroma but declined in the caruncular stroma during gestation [287]. Our data indicate that mRNA levels of *CTSS* are increased in NR IUGR compared to NR non-IUGR placentomes, with *CTSS* being localized in the cotyledonary villi and being more abundant at the fetal-

maternal interface of the cotyledon and caruncle of the placentome at gestational Day 125.

In addition to its role as a cysteine protease, CTSS is also essential to major histocompatibility complex (MHC) class II antigen presentation and proteolysis [289, 290]. CTSS-deficient mice (*CTSS*$^{-/-}$) have normal populations of B and T cells but have an impaired ability to degrade the invariant chain (Ii), which is necessary for MHC class II molecules to acquire antigenic peptides and undergo peptide binding [291, 292]. While the expression of MHC class II molecules in the placentome is not fully understood, there is expression of MHC class I during late gestation, around the time of parturition [293, 294]. Additionally, parturition in cattle is associated with increased apoptosis, degradation of the extracellular matrix, and an innate immune response [295]. These physiological processes and complexes at late gestation align with the genes and functional annotation clusters discovered in the present study. CTSS's role as a peptidase suggests that an increase in CTSS in the placentomes of NR IUGR pregnancies during late gestation may represent a failed attempt to enhance vascular or tissue remodeling to improve placental function. However, as it also functions in MHC class II antigen presentation and proteolysis, its exact role in the placentomes of NR ewes at this point in gestation is not clear and warrants further investigation.

Epigenetic alterations, such as DNA methylation, during fetal development can profoundly influence the susceptibility of offspring to postnatal diseases through a phenomenon known as fetal programming. Therefore, it is a common clinical practice for gestating mothers to be supplemented with methyl donors such as folate and choline

[1]. Previous work in our laboratory with ewes revealed that the sodium-dependent choline transporter SLC44A4 is up-regulated in the endometrium of the uterus during early pregnancy and in response to exogenous progesterone [232]. Studies in rodents and humans have shown that a deficiency in choline during early pregnancy can lead to neural tube defects and other brain defects during postnatal life [296]. Thus, an increase in expression of *SLC44A4* mRNA in NR IUGR compared to NR non-IUGR placentomes could be an attempt to prevent choline deficiency in IUGR lambs in response to nutrient restriction by attempting to increase mRNA levels similar to that seen in control pregnancies. However, levels of choline were not measured in these studies.

In conclusion, results of the present study indicate that in a subpopulation of NR ewes, placentomal genes expressed late in gestation are associated with an altered immune response that is associated with enhanced fetal growth. This altered immune response may work in conjunction with increased expression of certain nutrient transporters and angiogenic factors, along with increased fetal nutrient availability to enhance fetal growth in NR non-IUGR pregnancies (Fig. 3.4 and 4.1; Table 3.3 and 3.4). Future studies are necessary to investigate the immune cell profile and immunological forces at play within the placentas of compromised and adaptive pregnancies.

CHAPTER VI

SUMMARY

Maternal nutrient restriction during pregnancy impairs placental and fetal growth in humans and livestock species, often resulting in IUGR [1-4]. Indeed, undernutrition in ruminant livestock species is a global challenge, with the nutrient intake of ewes frequently being less than 50% of the National Research Council (NRC) recommendations [4, 159]. IUGR is a leading cause of neonatal morbidity and mortality in livestock species, as well as humans, with approximately 5% of human infants in the U.S. suffering complications of IUGR each year [1, 3, 4, 65]. The intrauterine environment is not only a major determinant of fetal growth in utero, but also of great importance in the etiology of chronic disease during adult life [1, 6]. As a result of maternal malnutrition, the fetus is hypothesized to adapt to a thrifty phenotype, through which it attempts to alter the function of its organs to maximize the chances of its survival in a postnatal life that is nutrient deficient [6, 247]. However, the mechanisms regulating this adaptation in fetal growth, development, and programming are not fully understood.

Available evidence suggests that, in the face of maternal nutrient restriction, a subset of the population is capable of adapting to this insult in order to increase delivery of nutrients to the fetus. In previous studies, our laboratory determined that variation in lamb birth weights is greater in ewes fed at 50% of the NRC requirements (mean = 4.84 kg; range = 2.5-7.1 kg; n=54) compared to those receiving 100% NRC (mean = 6.28 kg;

range = 5.2-7.2 kg; n=13) (Satterfield et al. unpublished observations). Likewise, in a bovine model, maternal nutrient restriction induced IUGR in only a subset of individuals [168]. Furthermore, research comparing ewes raised and selected under temperate conditions to genetically similar ewes that have adapted to harsh environmental conditions indicated that long-term selection for survival characteristics in a nutritionally limited environment can result in adaptive changes in placental function [67, 72, 170]. Collectively, results suggest that adaptive mechanisms of placental nutrient transport exist to maintain normal fetal growth despite limited maternal resources in certain individuals.

As a first step to elucidate the mechanisms involved in an adaptive placental response to maternal nutrient restricion, we established an ovine model of maternal nutrient restriction followed by subpopulation delineation. Briefly, ewes were synchronized into estrus and a single blastocyst from superovulated Suffolk donor ewes of normal body condition was transferred into the uterus of a recipient ewe. Pregnant ewes received either 50% NRC or 100% NRC from Day 35 of gestation to necropsy on Day 125. Maternal weight did not correlate with fetal weight; therefore, the six heaviest (NR non-IUGR) and six lightest (NR IUGR) fetuses from NR (representing the upper and lower quartiles for fetal growth, respectively), as well as the seven control fetuses, were compared with respect to various indices of placental development and gene expression. Mean weights of NR IUGR fetal lambs were lower than weights for control and NR non-IUGR fetal lambs.

In the first study we aimed to determine quantities of select nutrients in maternal and fetal fluids in well-fed and NR ewes having either IUGR or non-IUGR fetuses. We then quantified the expression of a number of amino acid transporter family members which transport nutrients hypothesized to be involved in placental and/or fetal development. Results of this study (CHAPTER III) indicate that quantities of 18 out of 24 amino acids and related hormones and metabolites are reduced in the fetal circulation in NR IUGR versus NR non-IUGR and control ewes. Moreover, enhanced fetal growth in NR ewes having non-IUGR fetuses was associated with increased placentomal mRNA expression of a number of amino acid transporters above levels observed in those having an IUGR fetus. Interestingly, many of the amino acids that were decreased by maternal nutrient restriction, as well their metabolites, are involved in placental angiogenesis and vasodilation [4, 40]. As example, arginine can be catabolized into NO which has a critical role in promoting placental angiogenesis and increasing fetal-placental blood flow during gestation [108, 186, 187]. Additionally, arginine, proline, and glutamate can be metabolized to form ornithine, a precursor of polyamine synthesis [135]. Polyamines serve a multitude of functions such as stimulating gene expression, cell proliferation and differentiation, DNA and protein synthesis, and angiogenesis [131]. In rodent models, inhibition of polyamine synthesis results in impaired placental growth and IUGR [132-134]. An increase in arginine, proline, glutamate, ornithine, methionine, putrescine, and spermidine in the fetal circulation of NR non-IUGR lambs compared to IUGR lambs was concomitant with increased expression of amino acid transporters known to transport arginine, proline, glutamate, and ornithine in the placentomes of NR non-IUGR

pregnancies. These observations are similar to previous studies in humans and rats which demonstrated that growth restricted pregnancies are associated with specific alterations in nutrient transporter availability and function [64, 221].

Insulin also serves as a critical metabolic regulator that stimulates the uptake of nutrients such as glucose and amino acids into cells. Our results identified increased concentrations of insulin in the circulation of NR non-IUGR and control fetuses compared to IUGR fetuses. Since the sheep placenta does not transport appreciable quantities of insulin [227], this likely resulted from either increased secretion or reduced utilization of insulin by the fetus. Furthermore, insulin promotes amino acid transport across the placenta [228-231] and thus may contribute to the detected increases in amino acids in the circulation of NR non-IUGR lambs. Reduced concentrations of glucose in maternal plasma of NR non-IUGR ewes compared to both control and NR IUGR ewes was interesting as there was no difference in concentrations of glucose in plasma due to phenotype of fetus. Additionally, since excess glucose not utilized by the fetus can be converted to fructose [43], it would be advantageous to measure fructose in the fetal circulation, as well as concentrations of both fructose and glucose in the allantoic fluid.

Angiogenesis in both the maternal and fetal portions of the placenta is a vital part of placental development [28]. The primary role of the placenta is physiological exchange of nutrients, wastes, and gases between the dam and conceptus. Indeed, all nutrients, wastes, and gases exchanged between the dam and conceptus must be transported through the placenta, primarily through the placental vasculature [25, 28].

Fetal weight is typically correlated with both placental weight and uteroplacental blood flow and uteroplacental blood flow may be reduced by maternal nutrient restriction [70].

We hypothesized that, in the face of maternal nutrient restriction, the remarkable adaptations by the placenta to increase delivery of nutrients to the fetus involve alterations in morphology of placentomes and expression of major angiogenic factors in placentomes. The major factors regulating angiogenesis are the VEGFs, FGF2, the ANGPT family, and their respective receptors [26]. Indeed, histological analysis of placentomes in our second study (CHAPTER IV) illustrated that placentomes of the NR IUGR pregnancies were strikingly less dense than those for control and NR non-IUGR pregnancies, as evidenced by a lack of caruncular crypt development. There were numerous, thick and highly branched crypts with closer juxtaposition to the fetal cotyledonary tissues in both the NR non-IUGR and control placentomes compared to the NR IUGR placentomes. Along with the decrease in caruncular crypt development, there was also an increased thickness of the caruncular capsule in the NR IUGR placentomes.

The exponential increase in fetal growth which occurs late in gestation must be supported by an extensive vascular network in the placenta. Thus, angiogenesis in both the maternal caruncular and fetal cotyledonary tissues is fundamental to placental formation and function throughout pregnancy. Importantly, perturbations in placental angiogenesis and blood flow in response to inadequate maternal nutrition induce IUGR of the fetus in a variety of species, including sheep, cow, rat, and pig [23, 72, 116, 169, 174, 175]. Similarly, in our second study (CHAPTER IV), up-regulation of *VEGFA* mRNA was detected in placentomes from NR non-IUGR ewes compared to both NR

IUGR and control ewes. In addition, up-regulation of *VEGFB* mRNA expression was detected in placentomes of control ewes compared to NR IUGR pregnancies, but expression of *VEGFB* in placentomes from NR non-IUGR was intermediate. However, these results contrast with those from a study that implemented a nutrient restricted diet containing 60% of dietary requirements in ewes from Day 50 to Day 130 of gestation and found no change in expression of *VEGF* mRNA in placentomes [23]. A decrease in expression of mRNAs for VEGF receptors in placentomes at Day 130 in response to nutrient restriction was also reported for that study [23]. The increased expression of *VEGFA* in the NR non-IUGR placentomes found in the present study may be part of a compensatory mechanism in response to the fetal nutrient deprivation that promote compensatory vascular development to increase transport of nutrients to the fetus. Additionally, the division of the nutrient restricted pregnancies into NR non-IUGR and NR IUGR groups offers a more focused assessment of expression of VEGF as it relates to placental growth and function, while the previous study [23] utilized a non-segregated population of lambs from nutrient restricted mothers, which may account for the observed differences between the two studies.

Results of immunohistochemical staining for cytokeratin, desmin, and vWF suggested that nutrient restriction may compromise the histoarchitecture of cotyledonary tissue, particularly in IUGR pregnancies. This is first illustrated by an increase in immunoreactive cytokeratin in the syncytia of placentomes from control and non-IUGR ewes compared to IUGR placentomes. Additionally, immunoreactive desmin was detectable in placentomes from all ewes and was distinctively localized to blood vessels

throughout the caruncular and cotyledonary components of placentomes. The abundance of desmin was markedly less in placentomes from NR IUGR ewes. This localization of desmin suggests that IUGR pregnancies are compromised, in part, due to a lack of vascular development. Furthermore, vWF protein was detected throughout the placentomes, but most abundant within the cotyledons. Additionally, vWF was more abundant in placentomes from control ewes compared to NR ewes.

Lastly, we utilized an Affymetrix Bovine/Ovine Gene 1.0 ST array to capitalize on natural population variance in fetal weights in response to nutrient restriction, in an effort to identify novel factors regulating placental growth and function (CHAPTER V). Interestingly, based on statistical significance and a fold change of 1.5 or greater, 15 genes were upregulated and 88 genes were down-regulated in placentomes having NR non-IUGR fetuses compared to those having IUGR fetuses. Bioinformatic analyses, performed using DAVID, of genes upregulated in NR non-IUGR pregnancies identified three functional annotation clusters, which featured GO terms such as integral to membrane, intrinsic to membrane, and cell surface receptor linked signal transduction. Thirty-three clusters were identified by DAVID analysis of the down-regulated genes from NR non-IUGR placentomes. These clusters were associated with response to nutrient levels, immune response, and positive regulation of response to stimulus. Indeed, of the ten most enriched clusters, two were associated with response to nutrients, while five were associated with immune response.

An intricate balance of immune cells, particularly a balance of pro- and anti-inflammatory cytokines regulated by the maternal immune system, at the maternal-

placental interface is imperative to the establishment and maintenance of pregnancy [274]. Placentas from pregnancies of IUGR infants are associated with an increase in inflammatory cytokines, such as TNF-α, and the chemokine IL-8 [276, 277]. In an ovine model of IUGR by umbilical artery ligation, a fetoplacental inflammatory response is characterized by increased pro-inflammatory cytokines [278]. Work illustrating immune responses at the maternal-placental interface in response to nutrient restriction is limited and the present study presents novel genes regulating immune responses within the placenta of nutrient restricted pregnancies which result in IUGR. Future studies are necessary to investigate the immune cell profile and immunological forces at play within the placentas of compromised and adaptive pregnancies.

In conclusion, these studies suggest that in the face of maternal nutrient restriction, a subset of the population of ewes is capable of adapting to this insult in order to increase delivery of nutrients to the fetus. Accordingly, such adaptive mechanisms in the placentomes of NR non-IUGR pregnancies include increased expression of select nutrient transporters and angiogenic factors, thick and highly branched caruncular crypts with close juxtaposition to the fetal cotyledonary tissues, and an altered immune response. Results also support previous findings from our laboratory and others highlighting critical roles for amino acids and their metabolites in supporting normal fetal growth and development and the critical role for amino acid transporters in nutrient delivery to the fetus.

Future studies to determine differences in placental vascularization, blood flow, nutrient transporter activity, as well as the immune cell profile and immunological forces

at play within the placentas of compromised and adaptive pregnancies are needed. Furthermore, it would be exceedingly advantageous to analyze placentome morphology, blood flow, and gene expression earlier in gestation when placentome development is occurring. This would grant the opportunity to elucidate adaptive mechanisms during early placental development that potentially lead to subsequent IUGR late in gestation.

REFERENCES

1. Wu G, Bazer FW, Cudd TA, Meininger CJ, Spencer TE. Maternal nutrition and fetal development. J Nutr 2004; 134:2169-2172.
2. Bell AW, Ehrhardt RA. Regulation of placental nutrient transport and implications for fetal growth. Nutr Res Rev 2002; 15:211-230.
3. Wallace JM, Regnault TR, Limesand SW, Hay WW, Jr., Anthony RV. Investigating the causes of low birth weight in contrasting ovine paradigms. J Physiol 2005; 565:19-26.
4. Wu G, Bazer FW, Wallace JM, Spencer TE. Board-invited review: intrauterine growth retardation: implications for the animal sciences. J Anim Sci 2006; 84:2316-2337.
5. Barker DJ. The developmental origins of well-being. Philos Trans R Soc Lond B Biol Sci 2004; 359:1359-1366.
6. Barker DJP. The origins of the developmental origins theory. J Intern Med 2007; 261:412-417.
7. Ford SP, Hess BW, Schwope MM, Nijland MJ, Gilbert JS, Vonnahme KA, Means WJ, Han H, Nathanielsz PW. Maternal undernutrition during early to mid-gestation in the ewe results in altered growth, adiposity, and glucose tolerance in male offspring. J Anim Sci 2007; 85:1285-1294.
8. Gilbert JS, Cox LA, Mitchell G, Nijland MJ. Nutrient-restricted fetus and the cardio-renal connection in hypertensive offspring. Expert Rev Cardiovasc Ther 2006; 4:227-237.
9. Gilbert JS, Lang AL, Grant AR, Nijland MJ. Maternal nutrient restriction in sheep: hypertension and decreased nephron number in offspring at 9 months of age. J Physiol 2005; 565:137-147.
10. Gilbert JS, Lang AL, Nijland MJ. Maternal nutrient restriction and the fetal left ventricle: decreased angiotensin receptor expression. Reprod Biol Endocrinol 2005; 3:27.
11. Hyatt MA, Gardner DS, Sebert S, Wilson V, Davidson N, Nigmatullina Y, Chan LL, Budge H, Symonds ME. Suboptimal maternal nutrition, during early fetal liver development, promotes lipid accumulation in the liver of obese offspring. Reproduction 2011; 141:119-126.
12. Wallace JM, Milne JS, Green LR, Aitken RP. Postnatal hypothalamic-pituitary-adrenal function in sheep is influenced by age and sex, but not by prenatal growth restriction. Reprod Fertil Dev 2011; 23:275-284.
13. Wang J, Wu Z, Li D, Li N, Dindot SV, Satterfield MC, Bazer FW, Wu G. Nutrition, epigenetics, and metabolic syndrome. Antioxid Redox Signal 2012; 17:282-301.
14. Armitage JA, Khan IY, Taylor PD, Nathanielsz PW, Poston L. Developmental programming of the metabolic syndrome by maternal nutritional imbalance: how strong is the evidence from experimental models in mammals? J Physiol 2004; 561:355-377.

15. Gluckman PD, Hanson MA. The developmental origins of the metabolic syndrome. Trends Endocrinol Metab 2004; 15:183-187.
16. Barker DJ, Clark PM. Fetal undernutrition and disease in later life. Rev Reprod 1997; 2:105-112.
17. Ashworth S. Global sheep industry: developing trends. published 2012; PMID: PMID.
18. Bazer FW, Spencer TE, Thatcher WW. Growth and development of the ovine conceptus. J Anim Sci 2012; 90:159-170.
19. Eleftheriades M, Creatsas G, Nicolaides K. Fetal growth restriction and postnatal development. Ann N Y Acad Sci 2006; 1092:319-330.
20. Gicquel C, Le Bouc Y. Hormonal regulation of fetal growth. Horm Res 2006; 65 Suppl 3:28-33.
21. Sizonenko SV, Borradori-Tolsa C, Bauthay DM, Lodygensky G, Lazeyras F, Huppi P. Impact of intrauterine growth restriction and glucocorticoids on brain development: insights using advanced magnetic resonance imaging. Mol Cell Endocrinol 2006; 254-255:163-171.
22. Mari G, Hanif F. Intrauterine growth restriction: how to manage and when to deliver. Clin Obstet Gynecol 2007; 50:497-509.
23. Redmer DA, Wallace JM, Reynolds LP. Effect of nutrient intake during pregnancy on fetal and placental growth and vascular development. Domest Anim Endocrinol 2004; 27:199-217.
24. Meschia G. Circulation to female reproductive organs. Handbook of Physiology 1992; 3:241-269.
25. Reynolds LP, Redmer DA. Utero-placental vascular development and placental function. J Anim Sci 1995; 73:1839-1851.
26. Reynolds LP, Borowicz PP, Vonnahme KA, Johnson ML, Grazul-Bilska AT, Wallace JM, Caton JS, Redmer DA. Animal models of placental angiogenesis. Placenta 2005; 26:689-708.
27. Resnik R. Intrauterine growth restriction. Obstet Gynecol 2002; 99:490-496.
28. Reynolds LP, Redmer DA. Angiogenesis in the placenta. Biol Reprod 2001; 64:1033-1040.
29. Belkacemi L, Nelson DM, Desai M, Ross MG. Maternal undernutrition influences placental-fetal development. Biol Reprod 2010; 83:325-331.
30. Fowden AL, Ward JW, Wooding FP, Forhead AJ, Constancia M. Programming placental nutrient transport capacity. J Physiol 2006; 572:5-15.
31. Carver TD, Hay WW, Jr. Uteroplacental carbon substrate metabolism and O2 consumption after long-term hypoglycemia in pregnant sheep. Am J Physiol 1995; 269:E299-308.
32. Ross JC, Fennessey PV, Wilkening RB, Battaglia FC, Meschia G. Placental transport and fetal utilization of leucine in a model of fetal growth retardation. Am J Physiol 1996; 270:E491-503.
33. Harding JE, Evans PC, Gluckman PD. Maternal growth hormone treatment increases placental diffusion capacity but not fetal or placental growth in sheep. Endocrinology 1997; 138:5352-5358.

34. Wallace JM, Bourke DA, Aitken RP, Milne JS, Hay WW, Jr. Placental glucose transport in growth-restricted pregnancies induced by overnourishing adolescent sheep. J Physiol 2003; 547:85-94.
35. Jansson T, Ylven K, Wennergren M, Powell TL. Glucose transport and system A activity in syncytiotrophoblast microvillous and basal plasma membranes in intrauterine growth restriction. Placenta 2002; 23:392-399.
36. Ward JW, Wooding FB, Fowden AL. Ovine feto-placental metabolism. J Physiol 2004; 554:529-541.
37. Ma Y, Zhu MJ, Uthlaut AB, Nijland MJ, Nathanielsz PW, Hess BW, Ford SP. Upregulation of growth signaling and nutrient transporters in cotyledons of early to mid-gestational nutrient restricted ewes. Placenta 2011; 32:255-263.
38. Kwon H, Ford SP, Bazer FW, Spencer TE, Nathanielsz PW, Nijland MJ, Hess BW, Wu G. Maternal nutrient restriction reduces concentrations of amino acids and polyamines in ovine maternal and fetal plasma and fetal fluids. Biol Reprod 2004; 71:901-908.
39. Satterfield MC, Bazer FW, Spencer TE, Wu G. Sildenafil citrate treatment enhances amino acid availability in the conceptus and fetal growth in an ovine model of intrauterine growth restriction. J Nutr 2010; 140:251-258.
40. Gao H, Wu G, Spencer TE, Johnson GA, Li X, Bazer FW. Select nutrients in the ovine uterine lumen. I. Amino acids, glucose, and ions in uterine lumenal flushings of cyclic and pregnant ewes. Biol Reprod 2009; 80:86-93.
41. Wales RG, Du ZF. The metabolism of glutamine by the preimplantation sheep conceptus and its interaction with glucose. Reprod Fertil Dev 1994; 6:659-667.
42. Rieger D, Loskutoff NM, Betteridge KJ. Developmentally related changes in the metabolism of glucose and glutamine by cattle embryos produced and co-cultured in vitro. J Reprod Fertil 1992; 95:585-595.
43. Bazer FW, Song G, Kim J, Dunlap KA, Satterfield MC, Johnson GA, Burghardt RC, Wu G. Uterine biology in pigs and sheep. J Anim Sci Biotechnol 2012; 3:23.
44. Lamming GE (ed.). Marshall's Physiology of Reproduction. Chapman & Hall; 1994.
45. Wang X, Ying W, Dunlap KA, Lin G, Satterfield MC, Burghardt RC, Wu G, Bazer FW. Arginine decarboxylase and agmatinase: an alternative pathway for de novo biosynthesis of polyamines for development of mammalian conceptuses. Biol Reprod 2014; 90:84.
46. Wang X, Frank JW, Little DR, Dunlap KA, Carey Satterfield M, Burghardt RC, Hansen TR, Wu G, Bazer FW. Functional role of arginine during the peri-implantation period of pregnancy. I. Consequences of loss of function of arginine transporter SLC7A1 mRNA in ovine conceptus trophectoderm. FASEB J 2014.
47. Guillomot M, Flechon JE, Wintenberger-Torres S. Conceptus attachment in the ewe: an ultrastructural study. Placenta 1981; 2:169-182.
48. Guillomot M, Guay P. Ultrastructural features of the cell surfaces of uterine and trophoblastic epithelia during embryo attachment in the cow. Anat Rec 1982; 204:315-322.

49. Wooding FB. The role of the binucleate cell in ruminant placental structure. J Reprod Fertil Suppl 1982; 31:31-39.
50. Wilson ME. Role of placental function in mediating conceptus growth and survival. J. Anim. Sci. 2002; 80:E195-E201.
51. Wu G, Bazer FW, Datta S, Johnson GA, Li P, Satterfield MC, Spencer TE. Proline metabolism in the conceptus: Implications for fetal growth and development. Amino Acids 2009; 35:153-168.
52. Kwon H, Spencer TE, Bazer FW, Wu G. Developmental changes of amino acids in ovine fetal fluids. Biol Reprod 2003; 68:1813-1820.
53. Bazer FW, Johnson GA. Pig blastocyst-uterine interactions. Differentiation 2014.
54. Trahair JF, Harding R. Restitution of swallowing in the fetal sheep restores intestinal growth after midgestation esophageal obstruction. J Pediatr Gastroenterol Nutr 1995; 20:156-161.
55. Folkman J, Klagsbrun M. Angiogenic factors. Science 1987; 235:442-447.
56. Grillo MA, Lanza A, Colombatto S. Transport of amino acids through the placenta and their role. Amino Acids 2008; 34:517-523.
57. Gao H, Wu G, Spencer TE, Johnson GA, Bazer FW. Select nutrients in the ovine uterine lumen. IV. Expression of neutral and acidic amino acid transporters in ovine uteri and peri-implantation conceptuses. Biol Reprod 2009; 80:1196-1208.
58. Gao H, Wu G, Spencer TE, Johnson GA, Bazer FW. Select nutrients in the ovine uterine lumen. III. Cationic amino acid transporters in the ovine uterus and peri-implantation conceptuses. Biol Reprod 2009; 80:602-609.
59. Gao H, Wu G, Spencer TE, Johnson GA, Bazer FW. Select nutrients in the ovine uterine lumen. ii. glucose transporters in the uterus and peri-implantation conceptuses. Biol Reprod 2009; 80:94-104.
60. Battaglia FC, Regnault TR. Placental transport and metabolism of amino acids. Placenta 2001; 22:145-161.
61. Battaglia FC. In vivo characteristics of placental amino acid transport and metabolism in ovine pregnancy--a review. Placenta 2002; 23 Suppl A:S3-8.
62. Coan PM, Vaughan OR, Sekita Y, Finn SL, Burton GJ, Constancia M, Fowden AL. Adaptations in placental phenotype support fetal growth during undernutrition of pregnant mice. J Physiol 2010; 588:527-538.
63. Malandro MS, Beveridge MJ, Kilberg MS, Novak DA. Effect of low-protein diet-induced intrauterine growth retardation on rat placental amino acid transport. Am J Physiol 1996; 271:C295-303.
64. Jansson N, Pettersson J, Haafiz A, Ericsson A, Palmberg I, Tranberg M, Ganapathy V, Powell TL, Jansson T. Down-regulation of placental transport of amino acids precedes the development of intrauterine growth restriction in rats fed a low protein diet. J Physiol 2006; 576:935-946.
65. Marsal K. Intrauterine growth restriction. Curr Opin Obstet Gynecol 2002; 14:127-135.
66. Pardi G, Marconi AM, Cetin I. Placental-fetal interrelationship in IUGR fetuses--a review. Placenta 2002; 23 Suppl A:S136-141.

67. Jobgen WS, Ford SP, Jobgen SC, Feng CP, Hess BW, Nathanielsz PW, Li P, Wu G. Baggs ewes adapt to maternal undernutrition and maintain conceptus growth by maintaining fetal plasma concentrations of amino acids. J Anim Sci 2008; 86:820-826.
68. Lesage J, Hahn D, Leonhardt M, Blondeau B, Breant B, Dupouy JP. Maternal undernutrition during late gestation-induced intrauterine growth restriction in the rat is associated with impaired placental GLUT3 expression, but does not correlate with endogenous corticosterone levels. J Endocrinol 2002; 174:37-43.
69. Mellor DJ. Nutritional and placental determinants of foetal growth rate in sheep and consequences for the newborn lamb. Br Vet J 1983; 139:307-324.
70. Owens JA, Falconer J, Robinson JS. Effect of restriction of placental growth on umbilical and uterine blood flows. Am J Physiol 1986; 250:R427-434.
71. Vonnahme KA, Hess BW, Hansen TR, McCormick RJ, Rule DC, Moss GE, Murdoch WJ, Nijland MJ, Skinner DC, Nathanielsz PW, Ford SP. Maternal undernutrition from early- to mid-gestation leads to growth retardation, cardiac ventricular hypertrophy, and increased liver weight in the fetal sheep. Biol Reprod 2003; 69:133-140.
72. Vonnahme KA, Hess BW, Nijland MJ, Nathanielsz PW, Ford SP. Placentomal differentiation may compensate for maternal nutrient restriction in ewes adapted to harsh range conditions. J Anim Sci 2006; 84:3451-3459.
73. Meyer KM, Koch JM, Ramadoss J, Kling PJ, Magness RR. Ovine surgical model of uterine space restriction: interactive effects of uterine anomalies and multifetal gestations on fetal and placental growth. Biol Reprod 2010; 83:799-806.
74. Reynolds LP, Borowicz PP, Vonnahme KA, Johnson ML, Grazul-Bilska AT, Redmer DA, Caton JS. Placental angiogenesis in sheep models of compromised pregnancy. J Physiol 2005; 565:43-58.
75. Reynolds LP, Redmer DA. Growth and microvascular development of the uterus during early pregnancy in ewes. Biol Reprod 1992; 47:698-708.
76. Makowski EL, Meschia G, Droegemueller W, Battaglia FC. Measurement of umbilical arterial blood flow to the sheep placenta and fetus in utero. Distribution to cotyledons and the intercotyledonary chorion. Circ Res 1968; 23:623-631.
77. Mott JC. Control of the foetal circulation. J Exp Biol 1982; 100:129-146.
78. Reynolds LP, Biondini ME, Borowicz PP, Vonnahme KA, Caton JS, Grazul-Bilska AT, Redmer DA. Functional significance of developmental changes in placental microvascular architecture. Endothelium 2005; 12:11-19.
79. Borowicz PP, Arnold DR, Johnson ML, Grazul-Bilska AT, Redmer DA, Reynolds LP. Placental growth throughout the last two thirds of pregnancy in sheep: vascular development and angiogenic factor expression. Biol Reprod 2007; 76:259-267.
80. Reynolds LP, Killilea SD, Redmer DA. Angiogenesis in the female reproductive system. FASEB J 1992; 6:886-892.
81. Ferrara N, Davis-Smyth T. The biology of vascular endothelial growth factor. Endocr Rev 1997; 18:4-25.

82. Dumont DJ, Fong GH, Puri MC, Gradwohl G, Alitalo K, Breitman ML. Vascularization of the mouse embryo: a study of flk-1, tek, tie, and vascular endothelial growth factor expression during development. Dev Dyn 1995; 203:80-92.
83. Sharkey AM, Charnock-Jones DS, Boocock CA, Brown KD, Smith SK. Expression of mRNA for vascular endothelial growth factor in human placenta. J Reprod Fertil 1993; 99:609-615.
84. Barleon B, Hauser S, Schollmann C, Weindel K, Marme D, Yayon A, Weich HA. Differential expression of the two VEGF receptors flt and KDR in placenta and vascular endothelial cells. J Cell Biochem 1994; 54:56-66.
85. Cooper JC, Sharkey AM, McLaren J, Charnock-Jones DS, Smith SK. Localization of vascular endothelial growth factor and its receptor, flt, in human placenta and decidua by immunohistochemistry. J Reprod Fertil 1995; 105:205-213.
86. Cheung CY, Brace RA. Developmental expression of vascular endothelial growth factor and its receptors in ovine placenta and fetal membranes. J Soc Gynecol Investig 1999; 6:179-185.
87. Reynolds LP, Redmer DA. Expression of the angiogenic factors, basic fibroblast growth factor and vascular endothelial growth factor, in the ovary. J Anim Sci 1998; 76:1671-1681.
88. Cheung CY, Brace RA. Ovine vascular endothelial growth factor: nucleotide sequence and expression in fetal tissues. Growth Factors 1998; 16:11-22.
89. Grazul Bilska A, Borowicz P, Johnson M, Minten M, Bilski J, Wroblewski R, Redmer D, Reynolds L. Placental development during early pregnancy in sheep: vascular growth and expression of angiogenic factors in maternal placenta. Reproduction 2010; 140:165-174.
90. Carmeliet P, Ferreira V, Breier G, Pollefeyt S, Kieckens L, Gertsenstein M, Fahrig M, Vandenhoeck A, Harpal K, Eberhardt C, Declercq C, Pawling J, et al. Abnormal blood vessel development and lethality in embryos lacking a single VEGF allele. Nature 1996; 380:435-439.
91. Ferrara N, Carver-Moore K, Chen H, Dowd M, Lu L, O'Shea KS, Powell-Braxton L, Hillan KJ, Moore MW. Heterozygous embryonic lethality induced by targeted inactivation of the VEGF gene. Nature 1996; 380:439-442.
92. Fong GH, Rossant J, Gertsenstein M, Breitman ML. Role of the Flt-1 receptor tyrosine kinase in regulating the assembly of vascular endothelium. Nature 1995; 376:66-70.
93. Shalaby F, Rossant J, Yamaguchi TP, Gertsenstein M, Wu XF, Breitman ML, Schuh AC. Failure of blood-island formation and vasculogenesis in Flk-1-deficient mice. Nature 1995; 376:62-66.
94. Klagsbrun M, D'Amore PA. Regulators of angiogenesis. Annu Rev Physiol 1991; 53:217-239.
95. Ortega S, Ittmann M, Tsang SH, Ehrlich M, Basilico C. Neuronal defects and delayed wound healing in mice lacking fibroblast growth factor 2. Proc Natl Acad Sci U S A 1998; 95:5672-5677.

96. Miller DL, Ortega S, Bashayan O, Basch R, Basilico C. Compensation by fibroblast growth factor 1 (FGF1) does not account for the mild phenotypic defects observed in FGF2 null mice. Mol Cell Biol 2000; 20:2260-2268.
97. Reynolds LP, Grazul-Bilska AT, Redmer DA. Angiogenesis in the female reproductive organs: pathological implications. Int J Exp Pathol 2002; 83:151-163.
98. Arman E, Haffner-Krausz R, Chen Y, Heath JK, Lonai P. Targeted disruption of fibroblast growth factor (FGF) receptor 2 suggests a role for FGF signaling in pregastrulation mammalian development. Proc Natl Acad Sci U S A 1998; 95:5082-5087.
99. Suri C, Jones PF, Patan S, Bartunkova S, Maisonpierre PC, Davis S, Sato TN, Yancopoulos GD. Requisite role of angiopoietin-1, a ligand for the TIE2 receptor, during embryonic angiogenesis. Cell 1996; 87:1171-1180.
100. Patan S. TIE1 and TIE2 receptor tyrosine kinases inversely regulate embryonic angiogenesis by the mechanism of intussusceptive microvascular growth. Microvasc Res 1998; 56:1-21.
101. Koh GY, Kim I, Kwak HJ, Yun MJ, Leem JC. Biomedical significance of endothelial cell specific growth factor, angiopoietin. Exp Mol Med 2002; 34:1-11.
102. Hayes AJ, Huang WQ, Mallah J, Yang D, Lippman ME, Li LY. Angiopoietin-1 and its receptor Tie-2 participate in the regulation of capillary-like tubule formation and survival of endothelial cells. Microvasc Res 1999; 58:224-237.
103. Carmeliet P. Mechanisms of angiogenesis and arteriogenesis. Nat Med 2000; 6:389-395.
104. Maisonpierre PC, Suri C, Jones PF, Bartunkova S, Wiegand SJ, Radziejewski C, Compton D, McClain J, Aldrich TH, Papadopoulos N, Daly TJ, Davis S, et al. Angiopoietin-2, a natural antagonist for Tie2 that disrupts in vivo angiogenesis. Science 1997; 277:55-60.
105. Lassala A, Bazer FW, Cudd TA, Datta S, Keisler DH, Satterfield MC, Spencer TE, Wu G. Parenteral administration of L-arginine enhances fetal survival and growth in sheep carrying multiple fetuses. J Nutr 2011; 141:849-855.
106. Wu G, Bazer FW, Burghardt RC, Johnson GA, Kim SW, Knabe DA, Li P, Li X, McKnight JR, Satterfield MC, Spencer TE. Proline and hydroxyproline metabolism: implications for animal and human nutrition. Amino Acids 2011; 40:1053-1063.
107. Wu G. Amino acids: metabolism, functions, and nutrition. Amino Acids 2009; 37:1-17.
108. Sladek SM, Magness RR, Conrad KP. Nitric oxide and pregnancy. Am J Physiol 1997; 272:R441-463.
109. Kimura H, Esumi H. Reciprocal regulation between nitric oxide and vascular endothelial growth factor in angiogenesis. Acta Biochim Pol 2003; 50:49-59.
110. Parenti A, Morbidelli L, Cui XL, Douglas JG, Hood JD, Granger HJ, Ledda F, Ziche M. Nitric oxide is an upstream signal of vascular endothelial growth

factor-induced extracellular signal-regulated kinase1/2 activation in postcapillary endothelium. J Biol Chem 1998; 273:4220-4226.
111. Kusinski LC, Stanley JL, Dilworth MR, Hirt CJ, Andersson IJ, Renshall LJ, Baker BC, Baker PN, Sibley CP, Wareing M, Glazier JD. eNOS knockout mouse as a model of fetal growth restriction with an impaired uterine artery function and placental transport phenotype. Am J Physiol Regul Integr Comp Physiol 2012; 303:R86-93.
112. Kulandavelu S, Whiteley KJ, Bainbridge SA, Qu D, Adamson SL. Endothelial NO synthase augments fetoplacental blood flow, placental vascularization, and fetal growth in mice. Hypertension 2013; 61:259-266.
113. Hagen AS, Orbus RJ, Wilkening RB, Regnault TR, Anthony RV. Placental expression of angiopoietin-1, angiopoietin-2 and tie-2 during placental development in an ovine model of placental insufficiency-fetal growth restriction. Pediatr Res 2005; 58:1228-1232.
114. Luther J, Milne J, Aitken R, Matsuzaki M, Reynolds L, Redmer D, Wallace J. Placental growth, angiogenic gene expression, and vascular development in undernourished adolescent sheep. Biol Reprod 2007; 77:351-357.
115. Redmer DA, Aitken RP, Milne JS, Reynolds LP, Wallace JM. Influence of maternal nutrition on messenger RNA expression of placental angiogenic factors and their receptors at midgestation in adolescent sheep. Biol Reprod 2005; 72:1004-1009.
116. Reynolds LP, Caton JS, Redmer DA, Grazul-Bilska AT, Vonnahme KA, Borowicz PP, Luther JS, Wallace JM, Wu G, Spencer TE. Evidence for altered placental blood flow and vascularity in compromised pregnancies. J Physiol 2006; 572:51-58.
117. Wu G, Morris SM, Jr. Arginine metabolism: nitric oxide and beyond. Biochem J 1998; 336 (Pt 1):1-17.
118. Broer S. Apical transporters for neutral amino acids: physiology and pathophysiology. Physiology (Bethesda) 2008; 23:95-103.
119. Broer S. Amino acid transport across mammalian intestinal and renal epithelia. Physiol Rev 2008; 88:249-286.
120. Satterfield MC, Gao H, Li X, Wu G, Johnson GA, Spencer TE, Bazer FW. Select nutrients and their associated transporters are increased in the ovine uterus following early progesterone administration. Biol Reprod 2010; 82:224-231.
121. Regnault TR, de Vrijer B, Battaglia FC. Transport and metabolism of amino acids in placenta. Endocrine 2002; 19:23-41.
122. Martin PM, Sutherland AE. Exogenous amino acids regulate trophectoderm differentiation in the mouse blastocyst through an mTOR-dependent pathway. Dev Biol 2001; 240:182-193.
123. Martin PM, Sutherland AE, Van Winkle LJ. Amino acid transport regulates blastocyst implantation. Biol Reprod 2003; 69:1101-1108.
124. Dunlap K, Brown J, Keith A, Satterfield M. Factors controlling nutrient availability to the developing fetus in ruminants. Journal of Animal Science and Biotechnology 2015; 6:1-10.

125. Clark MG, Filsell OH, Jarrett IG. Gluconeogenesis in isolated intact lamb liver cells. Effects of glucagon and butyrate. Biochem J 1976; 156:671-680.
126. Wu G. Amino Acids Biochemistry and Nutrition. CRC Press; 2013.
127. Snell K, Fell DA. Metabolic control analysis of mammalian serine metabolism. Adv Enzyme Regul 1990; 30:13-32.
128. Flynn NE, Meininger CJ, Haynes TE, Wu G. The metabolic basis of arginine nutrition and pharmacotherapy. Biomed Pharmacother 2002; 56:427-438.
129. Battaglia FC. Placental transport: a function of permeability and perfusion. Am J Clin Nutr 2007; 85:591S-597S.
130. Paolini CL, Meschia G, Fennessey PV, Pike AW, Teng C, Battaglia FC, Wilkening RB. An in vivo study of ovine placental transport of essential amino acids. Am J Physiol Endocrinol Metab 2001; 280:E31-39.
131. Kwon H, Wu G, Bazer FW, Spencer TE. Developmental changes in polyamine levels and synthesis in the ovine conceptus. Biol Reprod 2003; 69:1626-1634.
132. Lopez-Garcia C, Lopez-Contreras AJ, Cremades A, Castells MT, Marin F, Schreiber F, Penafiel R. Molecular and morphological changes in placenta and embryo development associated with the inhibition of polyamine synthesis during midpregnancy in mice. Endocrinology 2008; 149:5012-5023.
133. Pendeville H, Carpino N, Marine JC, Takahashi Y, Muller M, Martial JA, Cleveland JL. The ornithine decarboxylase gene is essential for cell survival during early murine development. Mol Cell Biol 2001; 21:6549-6558.
134. Ishida M, Hiramatsu Y, Masuyama H, Mizutani Y, Kudo T. Inhibition of placental ornithine decarboxylase by DL-alpha-difluoro- methyl ornithine causes fetal growth restriction in rat. Life Sci 2002; 70:1395-1405.
135. Wu G, Bazer FW, Datta S, Johnson GA, Li P, Satterfield MC, Spencer TE. Proline metabolism in the conceptus: implications for fetal growth and development. Amino Acids 2008; 35:691-702.
136. Gao H, Wu G, Spencer TE, Johnson GA, Bazer FW. Select nutrients in the ovine uterine lumen. V. Nitric oxide synthase, GTP cyclohydrolase, and ornithine decarboxylase in ovine uteri and peri-implantation conceptuses. Biol Reprod 2009; 81:67-76.
137. Ehrhardt RA, Bell AW. Developmental increases in glucose transporter concentration in the sheep placenta. Am J Physiol 1997; 273:R1132-1141.
138. Aldoretta PW, Carver TD, Hay WW, Jr. Maturation of glucose-stimulated insulin secretion in fetal sheep. Biol Neonate 1998; 73:375-386.
139. Limesand SW, Rozance PJ, Zerbe GO, Hutton JC, Hay WW, Jr. Attenuated insulin release and storage in fetal sheep pancreatic islets with intrauterine growth restriction. Endocrinology 2006; 147:1488-1497.
140. Molina RD, Carver TD, Hay WW, Jr. Ontogeny of insulin effect in fetal sheep. Pediatr Res 1993; 34:654-660.
141. Milley JR, Papacostas JS, Tabata BK. Effect of insulin on uptake of metabolic substrates by the sheep fetus. Am J Physiol 1986; 251:E349-356.
142. Baker J, Liu JP, Robertson EJ, Efstratiadis A. Role of insulin-like growth factors in embryonic and postnatal growth. Cell 1993; 75:73-82.

143. DeChiara TM, Robertson EJ, Efstratiadis A. Parental imprinting of the mouse insulin-like growth factor II gene. Cell 1991; 64:849-859.
144. Reynolds TS, Stevenson KR, Wathes DC. Pregnancy-specific alterations in the expression of the insulin-like growth factor system during early placental development in the ewe. Endocrinology 1997; 138:886-897.
145. Wathes DC, Reynolds TS, Robinson RS, Stevenson KR. Role of the insulin-like growth factor system in uterine function and placental development in ruminants. J Dairy Sci 1998; 81:1778-1789.
146. Satterfield MC, Hayashi K, Song G, Black SG, Bazer FW, Spencer TE. Progesterone regulates FGF10, MET, IGFBP1, and IGFBP3 in the endometrium of the ovine uterus. Biol Reprod 2008; 79:1226-1236.
147. Fowden AL. The insulin-like growth factors and feto-placental growth. Placenta 2003; 24:803-812.
148. Regnault TR, Galan HL, Parker TA, Anthony RV. Placental development in normal and compromised pregnancies-- a review. Placenta 2002; 23 Suppl A:S119-129.
149. Jonker FH. Fetal death: comparative aspects in large domestic animals. Anim Reprod Sci 2004; 82-83:415-430.
150. Lubchenco LO, Hansman C, Dressler M, Boyd E. Intrauterine Growth as Estimated from Liveborn Birth-Weight Data at 24 to 42 Weeks of Gestation. Pediatrics 1963; 32:793-800.
151. Ergaz Z, Avgil M, Ornoy A. Intrauterine growth restriction-etiology and consequences: what do we know about the human situation and experimental animal models? Reprod Toxicol 2005; 20:301-322.
152. Gluckman PD, Hanson MA. Developmental origins of disease paradigm: a mechanistic and evolutionary perspective. Pediatr Res 2004; 56:311-317.
153. Gluckman PD, Hanson MA. Living with the past: evolution, development, and patterns of disease. Science 2004; 305:1733-1736.
154. Hales CN, Barker DJ. The thrifty phenotype hypothesis. Br Med Bull 2001; 60:5-20.
155. Lucas A. Programming by early nutrition in man. Ciba Found Symp 1991; 156:38-50; discussion 50-35.
156. Ferguson JD. Nutrition and reproduction in dairy herds. Vet Clin North Am Food Anim Pract 2005; 21:325-347.
157. Robinson JJ, Sinclair KD, McEvoy TG. Nutritional effects on foetal growth. Anim. Sci. 1999; 68:315-331.
158. Rehfeldt C, Nissen PM, Kuhn G, Vestergaard M, Ender K, Oksbjerg N. Effects of maternal nutrition and porcine growth hormone (pGH) treatment during gestation on endocrine and metabolic factors on sows, fetuses, and pigs, skeletal muscle development, and postnatal growth. Domest Anim Endocrinol 2004; 27:267-285.
159. NRC NRC. Nutrient Requirements for Sheep. In. Washington, DC: Natl Acad Press; 1985.

160. Thomas VM, Kott RW. A review of Montana winter range ewe nutrition research. Sheep Goat Res J 1995; 11:17-24.
161. Hoaglund CM, Thomas VM, Petersen MK, Kott RW. Effects of supplemental protein source and metabolizable energy intake on nutritional status in pregnant ewes. J Anim Sci 1992; 70:273-280.
162. Reynolds LP, Ferrell CL, Nienaber JA, Ford SP. Effects of chronic environmental heat stress on blood flow and nutrient uptake of the gravid bovine uterus and foetus. J Agric Sci Camb 1985; 104:289-297.
163. (FAO) UNFaAO. The State of Food Insecurity in the World 2013. In, vol. 2014; 2013.
164. Wu G, Fanzo J, Miller DD, Pingali P, Post M, Steiner JL, Thalacker-Mercer AE. Production and supply of high-quality food protein for human consumption: sustainability, challenges, and innovations. Ann N Y Acad Sci 2014; 1321:1-19.
165. Organization WH. Overweight and Obesity. In, vol. 2014; 2008.
166. Organization WH. Childhood overweight and obesity on the rise. In, vol. 2014; 2010.
167. Ogden CL, Carroll MD, Kit BK, Flegal KM. Prevalence of childhood and adult obesity in the United States, 2011-2012. JAMA 2014; 311:806-814.
168. Long NM, Vonnahme KA, Hess BW, Nathanielsz PW, Ford SP. Effects of early gestational undernutrition on fetal growth, organ development, and placentomal composition in the bovine. J Anim Sci 2009; 87:1950-1959.
169. Zhu MJ, Du M, Hess BW, Means WJ, Nathanielsz PW, Ford SP. Maternal nutrient restriction upregulates growth signaling pathways in the cotyledonary artery of cow placentomes. Placenta 2007; 28:361-368.
170. Burt BE, Hess BW, Nathanielsz PW, Ford SP. Flock differences in the impact of maternal dietary restriction on offspring growth and glucose tolerance in female offspring. Soc Reprod Fertil Suppl 2007; 64:411-424.
171. Schoknecht PA, Newton GR, Weise DE, Pond WG. Protein restriction in early pregnancy alters fetal and placental growth and allantoic fluid proteins in swine. Theriogenology 1994; 42:217-226.
172. Wu G, Pond WG, Ott T, Bazer FW. Maternal dietary protein deficiency decreases amino acid concentrations in fetal plasma and allantoic fluid of pigs. J Nutr 1998; 128:894-902.
173. Rees WD, Hay SM, Buchan V, Antipatis C, Palmer RM. The effects of maternal protein restriction on the growth of the rat fetus and its amino acid supply. Br J Nutr 1999; 81:243-250.
174. Wu G, Pond WG, Flynn SP, Ott TL, Bazer FW. Maternal dietary protein deficiency decreases nitric oxide synthase and ornithine decarboxylase activities in placenta and endometrium of pigs during early gestation. J Nutr 1998; 128:2395-2402.
175. Itoh S, Brawley L, Wheeler T, Anthony FW, Poston L, Hanson MA. Vasodilation to vascular endothelial growth factor in the uterine artery of the pregnant rat is blunted by low dietary protein intake. Pediatr Res 2002; 51:485-491.

176. Koumentaki A, Anthony F, Poston L, Wheeler T. Low-protein diet impairs vascular relaxation in virgin and pregnant rats. Clin Sci (Lond) 2002; 102:553-560.
177. Sinclair KD, Allegrucci C, Singh R, Gardner DS, Sebastian S, Bispham J, Thurston A, Huntley JF, Rees WD, Maloney CA, Lea RG, Craigon J, et al. DNA methylation, insulin resistance, and blood pressure in offspring determined by maternal periconceptional B vitamin and methionine status. Proc Natl Acad Sci U S A 2007; 104:19351-19356.
178. Lang U, Baker RS, Braems G, Zygmunt M, Kunzel W, Clark KE. Uterine blood flow--a determinant of fetal growth. Eur J Obstet Gynecol Reprod Biol 2003; 110 Suppl 1:S55-61.
179. Reynolds LP, Ferrell CL, Robertson DA, Ford SP. Metabolism of the gravid uterus, foetus and uteroplacenta at several stages of gestation in cows. J Agric Sci Camb 1986; 106:437-444.
180. Konje JC, Howarth ES, Kaufmann P, Taylor DJ. Longitudinal quantification of uterine artery blood volume flow changes during gestation in pregnancies complicated by intrauterine growth restriction. BJOG 2003; 110:301-305.
181. Chandler KD, Leury BJ, Bird AR, Bell AW. Effects of undernutrition and exercise during late pregnancy on uterine, fetal and uteroplacental metabolism in the ewe. Br J Nutr 1985; 53:625-635.
182. Leury BJ, Bird AR, Chandler KD, Bell AW. Glucose partitioning in the pregnant ewe: effects of undernutrition and exercise. Br J Nutr 1990; 64:449-462.
183. Newnham JP, Kelly RW, Patterson L, James I. The influence of maternal undernutrition in ovine twin pregnancy on fetal growth and Doppler flow-velocity waveforms. J Dev Physiol 1991; 16:277-282.
184. Zhu MJ, Du M, Hess BW, Nathanielsz PW, Ford SP. Periconceptional nutrient restriction in the ewe alters MAPK/ERK1/2 and PI3K/Akt growth signaling pathways and vascularity in the placentome. Placenta 2007; 28:1192-1199.
185. Wu G, Bazer FW, Davis TA, Kim SW, Li P, Marc Rhoads J, Carey Satterfield M, Smith SB, Spencer TE, Yin Y. Arginine metabolism and nutrition in growth, health and disease. Amino Acids 2009; 37:153-168.
186. Reynolds LP, Redmer DA. Angiogenesis in the placenta. Biol Reprod 2001; 64:1033-1040.
187. Bird IM, Zhang L, Magness RR. Possible mechanisms underlying pregnancy-induced changes in uterine artery endothelial function. Am J Physiol Regul Integr Comp Physiol 2003; 284:R245-258.
188. Kwon H, Wu G, Meininger CJ, Bazer FW, Spencer TE. Developmental changes in nitric oxide synthesis in the ovine placenta. Biol Reprod 2004; 70:679-686.
189. Maul H, Longo M, Saade GR, Garfield RE. Nitric oxide and its role during pregnancy: from ovulation to delivery. Curr Pharm Des 2003; 9:359-380.
190. Ishida M, Hiramatsu Y, Masuyama H, Mizutani Y, Kudo T. Inhibition of placental ornithine decarboxylase by DL-alpha-difluoro-methyl ornithine causes fetal growth restriction in rat. Life Sci 2002; 70:1395-1405.

191. Hay WW, Jr. Placental-fetal glucose exchange and fetal glucose metabolism. Trans Am Clin Climatol Assoc 2006; 117:321-339; discussion 339-340.
192. Osgerby JC, Wathes DC, Howard D, Gadd TS. The effect of maternal undernutrition on ovine fetal growth. J Endocrinol 2002; 173:131-141.
193. Lassala A, Bazer FW, Cudd TA, Datta S, Keisler DH, Satterfield MC, Spencer TE, Wu G. Parenteral administration of L-arginine prevents fetal growth restriction in undernourished ewes. J Nutr 2010; 140:1242-1248.
194. McMullen S, Osgerby JC, Milne JS, Wallace JM, Wathes DC. The effects of acute nutrient restriction in the mid-gestational ewe on maternal and fetal nutrient status, the expression of placental growth factors and fetal growth. Placenta 2005; 26:25-33.
195. Jones HN, Powell TL, Jansson T. Regulation of placental nutrient transport--a review. Placenta 2007; 28:763-774.
196. Cramer S, Beveridge M, Kilberg M, Novak D. Physiological importance of system A-mediated amino acid transport to rat fetal development. Am J Physiol Cell Physiol 2002; 282:C153-160.
197. Zoma WD, Baker RS, Clark KE. Effects of combined use of sildenafil citrate (Viagra) and 17beta-estradiol on ovine coronary and uterine hemodynamics. Am J Obstet Gynecol 2004; 190:1291-1297.
198. Wareing M, Myers JE, O'Hara M, Baker PN. Sildenafil citrate (Viagra) enhances vasodilatation in fetal growth restriction. J Clin Endocrinol Metab 2005; 90:2550-2555.
199. Jobgen WS, Fried SK, Fu WJ, Meininger CJ, Wu G. Regulatory role for the arginine-nitric oxide pathway in metabolism of energy substrates. J Nutr Biochem 2006; 17:571-588.
200. Shen SF, Hua CH. Effect of L-arginine on the expression of Bcl-2 and Bax in the placenta of fetal growth restriction. J Matern Fetal Neonatal Med 2011; 24:822-826.
201. Satterfield MC, Dunlap KA, Keisler DH, Bazer FW, Wu G. Arginine nutrition and fetal brown adipose tissue development in nutrient-restricted sheep. Amino Acids 2011.
202. Lassala A, Bazer FW, Cudd TA, Li P, Li X, Satterfield MC, Spencer TE, Wu G. Intravenous administration of L-citrulline to pregnant ewes is more effective than L-arginine for increasing arginine availability in the fetus. J Nutr 2009; 139:660-665.
203. Boujendar S, Arany E, Hill D, Remacle C, Reusens B. Taurine supplementation of a low protein diet fed to rat dams normalizes the vascularization of the fetal endocrine pancreas. J Nutr 2003; 133:2820-2825.
204. Liu J, Liu L, Chen H. Antenatal taurine supplementation for improving brain ultrastructure in fetal rats with intrauterine growth restriction. Neuroscience 2011; 181:265-270.
205. Bloomfield FH, Bauer MK, van Zijl PL, Gluckman PD, Harding JE. Amniotic IGF-I supplements improve gut growth but reduce circulating IGF-I in growth-restricted fetal sheep. Am J Physiol Endocrinol Metab 2002; 282:E259-269.

206. Bloomfield FH, Breier BH, Harding JE. Fate of (125)I-IGF-I administered into the amniotic fluid of late-gestation fetal sheep. Pediatr Res 2002; 51:361-369.
207. Bloomfield FH, van Zijl PL, Bauer MK, Harding JE. Effects of intrauterine growth restriction and intraamniotic insulin-like growth factor-I treatment on blood and amniotic fluid concentrations and on fetal gut uptake of amino acids in late-gestation ovine fetuses. J Pediatr Gastroenterol Nutr 2002; 35:287-297.
208. Shaikh S, Bloomfield FH, Bauer MK, Phua HH, Gilmour RS, Harding JE. Amniotic IGF-I supplementation of growth-restricted fetal sheep alters IGF-I and IGF receptor type 1 mRNA and protein levels in placental and fetal tissues. J Endocrinol 2005; 186:145-155.
209. Scheaffer AN, Caton JS, Redmer DA, Reynolds LP. The effect of dietary restriction, pregnancy, and fetal type in different ewe types on fetal weight, maternal body weight, and visceral organ mass in ewes. J Anim Sci 2004; 82:1826-1838.
210. Fu WJ, Haynes TE, Kohli R, Hu J, Shi W, Spencer TE, Carroll RJ, Meininger CJ, Wu G. Dietary L-arginine supplementation reduces fat mass in Zucker diabetic fatty rats. J Nutr 2005; 135:714-721.
211. Wu G. Urea synthesis in enterocytes of developing pigs. Biochem J 1995; 312 (Pt 3):717-723.
212. Wu G, Bazer FW, Tou W. Developmental changes of free amino acid concentrations in fetal fluids of pigs. J Nutr 1995; 125:2859-2868.
213. Brace RA. Fetal blood volume responses to acute fetal hemorrhage. Circ Res 1983; 52:730-734.
214. Brace RA. Blood volume and its measurement in the chronically catheterized sheep fetus. Am J Physiol 1983; 244:H487-494.
215. Stewart MD, Johnson GA, Gray CA, Burghardt RC, Schuler LA, Joyce MM, Bazer FW, Spencer TE. Prolactin receptor and uterine milk protein expression in the ovine endometrium during the estrous cycle and pregnancy. Biol Reprod 2000; 62:1779-1789.
216. Sibley CP, Turner MA, Cetin I, Ayuk P, Boyd CA, D'Souza SW, Glazier JD, Greenwood SL, Jansson T, Powell T. Placental phenotypes of intrauterine growth. Pediatr Res 2005; 58:827-832.
217. Sibley CP, Brownbill P, Dilworth M, Glazier JD. Review: Adaptation in placental nutrient supply to meet fetal growth demand: implications for programming. Placenta 2010; 31 Suppl:S70-74.
218. Coan PM, Vaughan OR, McCarthy J, Mactier C, Burton GJ, Constancia M, Fowden AL. Dietary composition programmes placental phenotype in mice. J Physiol 2011.
219. Myatt L. Placental adaptive responses and fetal programming. J Physiol 2006; 572:25-30.
220. Rosario FJ, Jansson N, Kanai Y, Prasad PD, Powell TL, Jansson T. Maternal protein restriction in the rat inhibits placental insulin, mTOR, and STAT3 signaling and down-regulates placental amino acid transporters. Endocrinology 2011; 152:1119-1129.

221. Jansson T, Powell TL. IFPA 2005 Award in Placentology Lecture. Human placental transport in altered fetal growth: does the placenta function as a nutrient sensor? -- a review. Placenta 2006; 27 Suppl A:S91-97.
222. Steyn C, Hawkins P, Saito T, Noakes DE, Kingdom JC, Hanson MA. Undernutrition during the first half of gestation increases the predominance of fetal tissue in late-gestation ovine placentomes. Eur J Obstet Gynecol Reprod Biol 2001; 98:165-170.
223. Spencer GS, Hill DJ, Garssen GJ, Macdonald AA, Colenbrander B. Somatomedin activity and growth hormone levels in body fluids of the fetal pig: effect of chronic hyperinsulinaemia. J Endocrinol 1983; 96:107-114.
224. Susa JB, McCormick KL, Widness JA, Singer DB, Oh W, Adamsons K, Schwartz R. Chronic hyperinsulinemia in the fetal rhesus monkey: effects on fetal growth and composition. Diabetes 1979; 28:1058-1063.
225. Angervall L, Karlsson K, Martinson A. Effects on rat fetuses of intrauterine injections of insulin. Diabetologia 1981; 20:558-562.
226. Owens JA, Gatford KL, De Blasio MJ, Edwards LJ, McMillen IC, Fowden AL. Restriction of placental growth in sheep impairs insulin secretion but not sensitivity before birth. J Physiol 2007; 584:935-949.
227. Rankin JH, Jodarski G, Shanahan MR. Maternal insulin and placental 3-O-methyl glucose transport. J Dev Physiol 1986; 8:247-253.
228. Jansson N, Greenwood SL, Johansson BR, Powell TL, Jansson T. Leptin stimulates the activity of the system A amino acid transporter in human placental villous fragments. J Clin Endocrinol Metab 2003; 88:1205-1211.
229. Jones HN, Jansson T, Powell TL. Full-length adiponectin attenuates insulin signaling and inhibits insulin-stimulated amino Acid transport in human primary trophoblast cells. Diabetes 2010; 59:1161-1170.
230. Karl PI, Alpy KL, Fisher SE. Amino acid transport by the cultured human placental trophoblast: effect of insulin on AIB transport. Am J Physiol 1992; 262:C834-839.
231. Hiden U, Lang I, Ghaffari-Tabrizi N, Gauster M, Lang U, Desoye G. Insulin action on the human placental endothelium in normal and diabetic pregnancy. Curr Vasc Pharmacol 2009; 7:460-466.
232. Satterfield MC, Song G, Kochan KJ, Riggs PK, Simmons RM, Elsik CG, Adelson DL, Bazer FW, Zhou H, Spencer TE. Discovery of candidate genes and pathways in the endometrium regulating ovine blastocyst growth and conceptus elongation. Physiol Genomics 2009; 39:85-99.
233. Gray CA, Burghardt RC, Johnson GA, Bazer FW, Spencer TE. Evidence that absence of endometrial gland secretions in uterine gland knockout ewes compromises conceptus survival and elongation. Reproduction 2002; 124:289-300.
234. Taylor KM, Gray CA, Joyce MM, Stewart MD, Bazer FW, Spencer TE. Neonatal ovine uterine development involves alterations in expression of receptors for estrogen, progesterone, and prolactin. Biol Reprod 2000; 63:1192-1204.

235. Yamazaki Y, Morita T. Molecular and functional diversity of vascular endothelial growth factors. Mol Divers 2006; 10:515-527.
236. Tammela T, Enholm B, Alitalo K, Paavonen K. The biology of vascular endothelial growth factors. Cardiovasc Res 2005; 65:550-563.
237. Miquerol L, Gertsenstein M, Harpal K, Rossant J, Nagy A. Multiple developmental roles of VEGF suggested by a LacZ-tagged allele. Dev Biol 1999; 212:307-322.
238. Eriksson U, Alitalo K. Structure, expression and receptor-binding properties of novel vascular endothelial growth factors. Curr Top Microbiol Immunol 1999; 237:41-57.
239. Barut F, Barut A, Gun BD, Kandemir NO, Harma MI, Harma M, Aktunc E, Ozdamar SO. Intrauterine growth restriction and placental angiogenesis. Diagn Pathol 2010; 5:24.
240. Osol G, Celia G, Gokina N, Barron C, Chien E, Mandala M, Luksha L, Kublickiene K. Placental growth factor is a potent vasodilator of rat and human resistance arteries. Am J Physiol Heart Circ Physiol 2008; 294:H1381-1387.
241. Dunlap KA, Kwak HI, Burghardt RC, Bazer FW, Magness RR, Johnson GA, Bayless KJ. The sphingosine 1-phosphate (S1P) signaling pathway is regulated during pregnancy in sheep. Biol Reprod 2010; 82:876-887.
242. Mellor DJ, Mitchell B, Matheson IC. Reductions in lamb weight caused by pre-mating carunclectomy and mid-pregnancy placental ablation. J Comp Pathol 1977; 87:629-633.
243. Cross JC, Baczyk D, Dobric N, Hemberger M, Hughes M, Simmons DG, Yamamoto H, Kingdom JC. Genes, development and evolution of the placenta. Placenta 2003; 24:123-130.
244. Lawn AM, Chiquoine AD, Amoroso EC. The development of the placenta in the sheep and goat: an electron microscope study. J Anat 1969; 105:557-578.
245. Kadyrov M, Garnier Y, Gantert M, Kramer BW, Kaufmann P, Huppertz B. Cytokeratin antibodies as differential markers of trophoblast and fetomaternal syncytial plaques in the sheep placentome. Placenta 2007; 28:1107-1109.
246. Blann AD, Taberner DA. A reliable marker of endothelial cell dysfunction: does it exist? Br J Haematol 1995; 90:244-248.
247. Ford SP, Long NM. Evidence for similar changes in offspring phenotype following either maternal undernutrition or overnutrition: potential impact on fetal epigenetic mechanisms. Reprod Fertil Dev 2011; 24:105-111.
248. Bell AW, Ehrhardt RA. Placental Regulation of Nutrient Partitioning During Pregnancy. Nutrition and Reproduction 1998; 8:229-254.
249. Heasman L, Clarke L, Stephenson TJ, Symonds ME. The influence of maternal nutrient restriction in early to mid-pregnancy on placental and fetal development in sheep. Proceedings of the Nutrition Society 1999; 58:283-288.
250. Morrison JL. Sheep models of intrauterine growth restriction: fetal adaptations and consequences. Clin Exp Pharmacol Physiol 2008; 35:730-743.
251. Cross JC, Mickelson L. Nutritional influences on implantation and placental development. Nutr Rev 2006; 64:S12-18; discussion S72-91.

252. Spencer TE, Forde N, Dorniak P, Hansen TR, Romero JJ, Lonergan P. Conceptus-derived prostaglandins regulate gene expression in the endometrium prior to pregnancy recognition in ruminants. Reproduction 2013; 146:377-387.
253. Irizarry RA, Hobbs B, Collin F, Beazer-Barclay YD, Antonellis KJ, Scherf U, Speed TP. Exploration, normalization, and summaries of high density oligonucleotide array probe level data. Biostatistics 2003; 4:249-264.
254. Dennis G, Jr., Sherman BT, Hosack DA, Yang J, Gao W, Lane HC, Lempicki RA. DAVID: Database for Annotation, Visualization, and Integrated Discovery. Genome Biol 2003; 4:P3.
255. Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nature Protocols 2009; 4:44-57.
256. Spencer TE, Stagg AG, Joyce MM, Jenster G, Wood CG, Bazer FW, Wiley AA, Bartol FF. Discovery and characterization of endometrial epithelial messenger ribonucleic acids using the ovine uterine gland knockout model. Endocrinology 1999; 140:4070-4080.
257. Del Curto HM, Satterfield MC, Johnson GA, Dunlap KA. Anterior Gradient Homolog 2 and Its Potential Roles in Ovine Pregnancy. Texas A&M University; 2014.
258. Meng X, Guo A, Gong W, Jia W, Luo X, Zhai J, Dou Y, Cai X. Molecular characterization, tissue distribution and expression analysis of interleukin-12 receptor beta2 chain in sheep. Gene 2012; 499:124-129.
259. Rogge L, Barberis-Maino L, Biffi M, Passini N, Presky DH, Gubler U, Sinigaglia F. Selective expression of an interleukin-12 receptor component by human T helper 1 cells. J Exp Med 1997; 185:825-831.
260. Rogge L, Papi A, Presky DH, Biffi M, Minetti LJ, Miotto D, Agostini C, Semenzato G, Fabbri LM, Sinigaglia F. Antibodies to the IL-12 receptor beta 2 chain mark human Th1 but not Th2 cells in vitro and in vivo. J Immunol 1999; 162:3926-3932.
261. Barbulescu K, Becker C, Schlaak JF, Schmitt E, Meyer zum Buschenfelde KH, Neurath MF. IL-12 and IL-18 differentially regulate the transcriptional activity of the human IFN-gamma promoter in primary CD4+ T lymphocytes. J Immunol 1998; 160:3642-3647.
262. Wegmann TG, Lin H, Guilbert L, Mosmann TR. Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon? Immunol Today 1993; 14:353-356.
263. D'Angelo MA, Gomez-Cavazos JS, Mei A, Lackner DH, Hetzer MW. A change in nuclear pore complex composition regulates cell differentiation. Dev Cell 2012; 22:446-458.
264. Olsson M, Ekblom M, Fecker L, Kurkinen M, Ekblom P. cDNA cloning and embryonic expression of mouse nuclear pore membrane glycoprotein 210 mRNA. Kidney Int 1999; 56:827-838.
265. Forhead AJ, Fowden AL. Thyroid hormones in fetal growth and prepartum maturation. J Endocrinol 2014; 221:R87-R103.

266. Sun YN, Liu YJ, Zhang L, Ye Y, Lin LX, Li YM, Yan YQ, Chen ZP. Expression of organic anion transporting polypeptide 1c1 and monocarboxylate transporter 8 in the rat placental barrier and the compensatory response to thyroid dysfunction. PLoS One 2014; 9:e96047.
267. Hagenbuch B. Cellular entry of thyroid hormones by organic anion transporting polypeptides. Best Pract Res Clin Endocrinol Metab 2007; 21:209-221.
268. Sugiyama D, Kusuhara H, Taniguchi H, Ishikawa S, Nozaki Y, Aburatani H, Sugiyama Y. Functional characterization of rat brain-specific organic anion transporter (Oatp14) at the blood-brain barrier: high affinity transporter for thyroxine. J Biol Chem 2003; 278:43489-43495.
269. Wrutniak C, Cabello G. Changes in the concentration of thyroxine in the plasma of rat fetuses during late gestation: influence of ligation of the maternal uterine vein and artery. J Endocrinol 1983; 99:233-238.
270. Thorpe-Beeston JG, Nicolaides KH, Felton CV, Butler J, McGregor AM. Maturation of the secretion of thyroid hormone and thyroid-stimulating hormone in the fetus. N Engl J Med 1991; 324:532-536.
271. Kilby MD, Verhaeg J, Gittoes N, Somerset DA, Clark PM, Franklyn JA. Circulating thyroid hormone concentrations and placental thyroid hormone receptor expression in normal human pregnancy and pregnancy complicated by intrauterine growth restriction (IUGR). J Clin Endocrinol Metab 1998; 83:2964-2971.
272. Rae MT, Rhind SM, Kyle CE, Miller DW, Brooks AN. Maternal undernutrition alters triiodothyronine concentrations and pituitary response to GnRH in fetal sheep. J Endocrinol 2002; 173:449-455.
273. Pereira DN, Procianoy RS. Effect of perinatal asphyxia on thyroid-stimulating hormone and thyroid hormone levels. Acta Paediatr 2003; 92:339-345.
274. Clark DA, Arck PC, Chaouat G. Why did your mother reject you? Immunogenetic determinants of the response to environmental selective pressure expressed at the uterine level. Am J Reprod Immunol 1999; 41:5-22.
275. Hansen PJ. Regulation of uterine immune function by progesterone--lessons from the sheep. J Reprod Immunol 1998; 40:63-79.
276. Hahn-Zoric M, Hagberg H, Kjellmer I, Ellis J, Wennergren M, Hanson LA. Aberrations in placental cytokine mRNA related to intrauterine growth retardation. Pediatr Res 2002; 51:201-206.
277. Bartha JL, Romero-Carmona R, Comino-Delgado R. Inflammatory cytokines in intrauterine growth retardation. Acta Obstet Gynecol Scand 2003; 82:1099-1102.
278. Bertucci MC, Loose JM, Wallace EM, Jenkin G, Miller SL. Anti-inflammatory therapy in an ovine model of fetal hypoxia induced by single umbilical artery ligation. Reprod Fertil Dev 2011; 23:346-352.
279. Bronte V, Zanovello P. Regulation of immune responses by L-arginine metabolism. Nat Rev Immunol 2005; 5:641-654.
280. Yu Y, Singh U, Shi W, Konno T, Soares MJ, Geyer R, Fundele R. Influence of murine maternal diabetes on placental morphology, gene expression, and function. Arch Physiol Biochem 2008; 114:99-110.

281. Sandell LL, Guan XJ, Ingram R, Tilghman SM. Gatm, a creatine synthesis enzyme, is imprinted in mouse placenta. Proc Natl Acad Sci U S A 2003; 100:4622-4627.
282. McMinn J, Wei M, Schupf N, Cusmai J, Johnson EB, Smith AC, Weksberg R, Thaker HM, Tycko B. Unbalanced placental expression of imprinted genes in human intrauterine growth restriction. Placenta 2006; 27:540-549.
283. Oliveira LJ, McClellan S, Hansen PJ. Differentiation of the endometrial macrophage during pregnancy in the cow. PLoS One 2010; 5:e13213.
284. Yeung BH, Law AY, Wong CK. Evolution and roles of stanniocalcin. Mol Cell Endocrinol 2012; 349:272-280.
285. Uuskula L, Mannik J, Rull K, Minajeva A, Koks S, Vaas P, Teesalu P, Reimand J, Laan M. Mid-gestational gene expression profile in placenta and link to pregnancy complications. PLoS One 2012; 7:e49248.
286. Song G, Bazer FW, Wagner GF, Spencer TE. Stanniocalcin (STC) in the endometrial glands of the ovine uterus: regulation by progesterone and placental hormones. Biol Reprod 2006; 74:913-922.
287. Song G, Bazer FW, Spencer TE. Differential expression of cathepsins and cystatin C in ovine uteroplacental tissues. Placenta 2007; 28:1091-1098.
288. Song G, Spencer TE, Bazer FW. Cathepsins in the ovine uterus: regulation by pregnancy, progesterone, and interferon tau. Endocrinology 2005; 146:4825-4833.
289. Riese RJ, Wolf PR, Bromme D, Natkin LR, Villadangos JA, Ploegh HL, Chapman HA. Essential role for cathepsin S in MHC class II-associated invariant chain processing and peptide loading. Immunity 1996; 4:357-366.
290. Riese RJ, Mitchell RN, Villadangos JA, Shi GP, Palmer JT, Karp ER, De Sanctis GT, Ploegh HL, Chapman HA. Cathepsin S activity regulates antigen presentation and immunity. J Clin Invest 1998; 101:2351-2363.
291. Shi GP, Villadangos JA, Dranoff G, Small C, Gu L, Haley KJ, Riese R, Ploegh HL, Chapman HA. Cathepsin S required for normal MHC class II peptide loading and germinal center development. Immunity 1999; 10:197-206.
292. Hsieh CS, deRoos P, Honey K, Beers C, Rudensky AY. A role for cathepsin L and cathepsin S in peptide generation for MHC class II presentation. J Immunol 2002; 168:2618-2625.
293. Davies CJ, Eldridge JA, Fisher PJ, Schlafer DH. Evidence for expression of both classical and non-classical major histocompatibility complex class I genes in bovine trophoblast cells. Am J Reprod Immunol 2006; 55:188-200.
294. Davies CJ, Fisher PJ, Schlafer DH. Temporal and regional regulation of major histocompatibility complex class I expression at the bovine uterine/placental interface. Placenta 2000; 21:194-202.
295. Streyl D, Kenngott R, Herbach N, Wanke R, Blum H, Sinowatz F, Wolf E, Zerbe H, Bauersachs S. Gene expression profiling of bovine peripartal placentomes: detection of molecular pathways potentially involved in the release of foetal membranes. Reproduction 2012; 143:85-105.

296. Zeisel SH. Choline: critical role during fetal development and dietary requirements in adults. Annu Rev Nutr 2006; 26:229-250.