ANTERIOR GRADIENT HOMOLOG 2 AND ITS POTENTIAL ROLES IN OVINE PREGNANCY

A Thesis

by

HANNAH M. DEL CURTO

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

MASTER OF SCIENCE

Chair of Committee,	Kathrin A. Dunlap
Committee Members,	Michael C. Satterfield
	Gregory A. Johnson
Head of Department,	H. Russell Cross

August 2014

Major Subject: Physiology of Reproduction

Copyright 2014 Hannah M. Del Curto

ABSTRACT

Anterior gradient homolog 2 (AGR2) is a proto-oncogene that encodes a secreted protein with biological roles in cell migration, differentiation, and growth, and is also implicated in epithelial barrier function and integrity. AGR2 is up-regulated in prostate cancer, breast cancer, lung cancer, renal carcinomas and endometrial carcinomas, and it contributes to the survival of cells undergoing physiological stress. We previously reported that *AGR2* expression in the ovine endometrium increased between days 9 and 12 post-mating, was stimulated by progesterone, and associated with conceptus elongation. *In situ* hybridization studies localized *AGR2* mRNA to the luminal epithelium (LE) of the ovine endometrium. Further, *AGR2* mRNA expression is greater from Days 14 to 16 of pregnancy than in the cycle, suggesting a role for conceptus-derived products in regulation of *AGR2* expression in the endometrium. Using osmotic pumps to infuse pregnancy levels of hormones into the uterus of cyclic ewes, we found that interferon tau (IFNT) along with cortisol and prostaglandins (PGE2, PGF2α, and PGI2) or both cortisol and prostaglandins increased ($P<0.05$) expression of *AGR2* mRNA in the endometrium compared to controls receiving vehicle and ewes receiving IFNT, cortisol, or prostaglandins alone. A role for AGR2 in placental function during later pregnancy has not been described for any species. Using a model of maternal nutrient restriction, we utilized natural population variance in fetal growth to identify novel genes associated with the development of either intrauterine growth restricted (IUGR) or non-IUGR pregnancies in sheep. Interestingly, microarray analysis of

placentomes from IUGR and non-IUGR pregnancies at Day 125 of pregnancy found *AGR2* was up-regulated ($P<0.05$) in IUGR pregnancies. Quantitative real-time PCR analysis confirmed that *AGR2* mRNA was 2.7-fold higher in placentomes from IUGR pregnancies. Collectively, results suggest that *AGR2* is involved in growth and development of the early conceptus. Additionally, elevated AGR2 expression may be associated with increased levels of physiological stress in the IUGR pregnancy. During late pregnancy, a reduction in the proliferative actions of *AGR2* may be necessary for enhanced placental function in the nutritionally restricted ewe.

ACKNOWLEDGEMENTS

The pursuit of a Master's of Science degree has been one in which I have not only learned about academic and research topics in reproductive sciences, but perhaps just as importantly, I have learned about life, myself, and it has been a process that has helped me identify my goals, objectives, and passions for the future. For those whom have helped me along this journey I am forever grateful.

First and foremost I would like to thank my major professor, Dr. Kathrin Dunlap. Your training and interests in my future have gone far beyond instruction in an academic form. I am thankful for you sharing your knowledge and expertise in the animal science field, and I am also thankful for your friendship and for having a listening ear and a patient understanding especially when I needed it most.

Thank you to for the extra time taken by my committee members and professors: Dr. Michael Satterfield, Dr. Greg Johnson, and Dr. Fuller Bazer. I appreciate your teaching, questioning, and support throughout my education. Each of your strong work ethics, and passion for the field is something to aspire to.

This work would not be possible without the tremendous support of fellow graduate students. A special thanks to Ashley Keith for her numerous hours spent helping me in the lab or studying for exams. I am extremely thankful for your guidance and willingness to help no matter what the question. Churchill Wang for helping with lab techniques and technical advice, Jacob Brown for assistance with lab and sheep work, and Michelle Bedenbaugh for your friendship and for helping to keep me organized. To

all lab members of the Bazer, Satterfield, Dunlap, Johnson, and Wu labs your knowledge, humor, and kindness have not only made this project possible but also enjoyable. I wish you the best in all your future endeavors!

Finally, to my family and friends for your love and understanding in helping me reach my goals. Sam, your kindness, patience, and all the little things you do help me every day. Your love and support has been a great source of stability for me throughout this process. To my brother and sister for supporting and pushing me when I need it; and to my parents for being my sounding board and for your pride and belief in me. Thank you for your quite support and for always reminding me to keep things in perspective and realize what is truly important in life.

TABLE OF CONTENTS

LIST OF FIGURES

FIGURE Page

LIST OF TABLES

CHAPTER I

INTRODUCTION

Previous reports from our laboratory have demonstrated that exogenous progesterone treatment advanced blastocyst morphology. The study included ewes being bred at Day 0 of estrus and then assigned randomly to receive daily intramuscular injections of either corn oil (CO) vehicle as control or 25 mg progesterone per day for 36 hours post mating to hysterectomy at Day 9 (Study 1). A second study was also conducted in which ewes were bred at Day 0 of estrus and assigned randomly to receive CO treatment or progesterone treatment through day 12 of gestation. Additionally, a third treatment group received daily progesterone injections until day 8 in which they then received injections of RU486 (progesterone receptor antagonist) until hysterectomy performed on Day 12 (Study 2). It was concluded that injections of P4 beginning on Day 1.5 after mating increased blastocyst diameter 2.2-fold on Day 9 (Study 1) and accelerated the morphological transformation of spherical blastocysts into filamentous conceptuses on Day 12 (Study 2)[1]. Microarray analysis was conducted to identify differences in the endometrial transcriptome between days 9 and 12 of pregnancy and between P4 and CO-treated ewes. Anterior Gradient Homolog 2 (AGR2) was among the novel genes present in this microarray. For validation of AGR2, steady-state levels of endometrial mRNA was determined by slot blot hybridization analysis. Steady-state mRNA levels of *AGR2* increased on day 12 versus day 9 ($P<0.05$), and *AGR2* was found to a greater degree in ewes treated with progesterone versus those with the corn oil

treatment (Figure 1.1). The finding of AGR2 in this study led us to further question the role of AGR2 and, in turn, its relevance in ovine pregnancy.

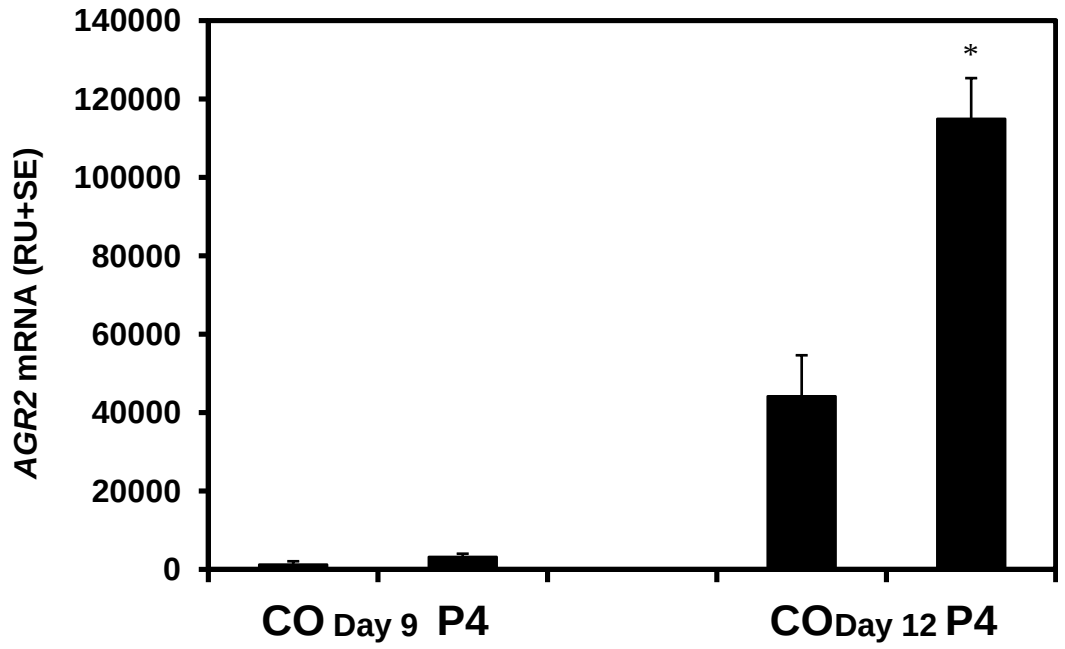


Figure 1.1. Effects of treatment with CO or P4 on endometrial mRNA levels. Steady-state levels of endometrial mRNAs were determined by slot blot hybridization analysis. Data are presented as LSM with SE *Significant effect of treatment ($P<0.05$).

Further work identified AGR2 as a relevant factor in later pregnancy. It is well established that fetal environment correlates to metabolic syndrome. However, therapeutic means for treatment of harsh gestational conditions and thus the facilitation of nutrient transfer are not established. Understanding nutritional support of embryonic and fetal development are critical for both clinicians and animal agriculturalists to ensure

enhanced fertility, pregnancy survival, and fetal growth [2]. Inadequate delivery of nutrients results in intrauterine growth restriction (IUGR), a leading cause of neonatal morbidity and mortality[3, 4]. Because our laboratory has a profound interest in understanding gestational stress and subsequently fetal growth retardation, studies were performed to evaluate mechanisms by which placental fetal nutrient transfer could be supplemented. For example, previous reports from our laboratory have established both supplemental arginine and treatment with Viagra to be viable options to enhance placental function and nutrient delivery[5]. Adequate placental blood flow is critical to ensure proper nutrient delivery between mother and fetus. Additionally, utero-placental blood flow is known to increase markedly during the second and third trimesters of pregnancy. Thus enhanced placental blood flow could combat the negative consequences observed in IUGR pregnancies[6]. Satterfield et al., observed that treatment with sildenafil citrate (Viagra) induces vasodilation through inhibition of type 5 phosphodiesterase (PDE5)[7]. This study included ewes receiving daily subcutaneous injections of Viagra from day 28 to 115 gestation in both a nutrient restricted population (ewes fed 50% NRC) and adequately fed group (ewes fed 100% NRC). An important finding of this study was that ewes receiving Viagra treatment displayed enhanced fetal weights at day 115 of gestation by approximately 14% compared to ewes who did not receive Viagra treatment regardless of diet. Additionally, in conjunction with enhanced fetal weights increased amino acids and polyamines were also present in the fetal blood and placental fluid indicating Viagra treatment has the potential to influence the trafficking of nutrients between mother and fetus. Thus these novel findings shed light

on potential therapeutic means to enhance placental blood flow and thus further define and combat the negative consequences of IUGR[5]. Other findings by the laboratory have demonstrated that arginine plays a critical role in conceptus development. Arginine is metabolized to ornithine, proline, and nitric oxide and can influence pregnancy success. Nitric oxide can act as a vasodilator and positively impact angiogenesis while ornithine and proline are substrates for synthesis of polyamines [2]. Using our nutrient restricted model in which ewes were fed either 100% NRC requirements or were nutrient restricted and fed 50% NRC a study was performed to analyze the impact of parenteral administration of L-arginine. In nutrient restricted ewes treatment with arginine increased concentrations of arginine, ornithine, proline, methionine and numerous other critical amino acids. Additionally, ewes treated with arginine exhibited enhanced birth weights 21% greater than that of the control. Just as importantly, arginine treated nutrient restricted ewes produced lambs with birth weights comparable to the adequately fed ewes[8]. Thus these novel findings again lay the foundation for potential theraputic means for treatment of IUGR.

Interestingly, we repeatedly observed that fetal weights are more variable within nutrient restricted ewes than well-fed controls. This observation elucidates the existence for a dynamic range of responses to a suboptimal nutritional environment allowing for placental adaptations to support normal fetal growth in a subpopulation of nutrient restricted ewes. In order to further analyze placental adaptations to maternal nutrient restriction and continue to evaluate the consequences of an inadequate gestational environment, an additional study was performed to elucidate potential mechanisms

regulating fetal nutrient availability and growth. Singleton pregnancies were generated by embryo transfer. Pregnant ewes received either 50% NRC (n=24) or 100% NRC (n=7) as a control from Day 28 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 nutrient-restricted ewes, as well as the 7 control fetuses, were compared to investigate differences in nutrient availability[9]. AGR2 was among the novel genes differentially expressed in this studies microarray.

The finding of AGR2 in multiple unique studies led us to further question AGR2's relevance in ovine pregnancy. Thus the goal of this research is to establish localization of AGR2 throughout gestation and lay the foundation for which future mechanistic studies to examine function can be designed. As a result the following studies are designed to elucidate potential roles of AGR2 throughout the course of ovine pregnancy.

CHAPTER II*

LITERATURE REVIEW

Conceptus Development and Implantation

Early Pregnancy Events

Establishment and maintenance of a healthy pregnancy that will result in a live offspring is the ultimate goal of the reproductive system. Early pregnancy is a critical time for embryonic growth and survival. In humans, it is estimated that the likelihood of a woman conceiving during one menstrual cycle is 30% and of these pregnancies only 50% to 60% are likely to survive to 20 weeks gestation [10, 11]. Among these described early pregnancy losses, 75% can be attributed to implantation failure [11]. Early implantation is not only a critical time in human fertility and reproduction but it is also an important window of time in domestic livestock species. In cattle, greater than 20% of reproductive losses occur during the period of maternal recognition of pregnancy (approximately Day 15) [12]. In sheep, percentages of embryonic wastage are difficult to estimate and likely higher due to multiple ovulations [13]. From a production standpoint it is important to understand the mechanisms underlying conceptus development and implantation to effectively minimize pregnancy loss and thus positively impact the bottom line. In summary, the peri-implantation period is the critical time in which most

* Part of the data reported in this chapter is reprinted with permission from "Nutrition and reproduction: links to epigenetics and metabolic syndrome in offspring." by DelCurto, H., Wu, G., and Satterfield, M.C. 2013. *Curr Opin Clin Nutr Metab Care*, 16(4):385-91. Copyright (2013) by Lippincott Williams and Wilkins

embryonic deaths occur due to deficiencies in uterine function and failure of the conceptus to develop appropriately and initiate pregnancy recognition. Further, deficiencies in uterine function and conceptus development that do not directly result in early embryonic death may still result in placentation deficiencies resulting in loss of advanced pregnancies[14]. Although physiological mechanisms behind placentation and implantation are diverse among species, the early steps that occur between the trophoblast and maternal uterine endometrial luminal epithelium are similar [15]. Early implantation is a highly coordinated process that involves blastocyst hatching, shedding of the zona pellucida, apposition, adhesion, and either attachment or endometrial invasion depending on the species [16]. Prior to implantation the uterine wall has a unique histology. The uterine wall is made up of two components: endometrium and myometrium. The myometrium serves as the smooth muscle component of the uterus while the endometrium is the sight of the inner mucosal lining of the uterus which will eventually make up the maternal component of the placenta [17]. The ovine endometrium consists of luminal epithelium (LE), glandular epithelium (GE), the stratum compactum and stratum spongiosum of the stroma, blood vessels, and immune cells. Endometrial anatomy and function is conserved among most species. The endometrial mucosa is composed of a mono or pseudostratified epithelium which on the surface consists of secretory cells containing microvilli and ciliated cells. The mucosa and stroma are separated from one another by the basal lamina. The stroma is a highly vascularized region that contains coiled and/or branched glands whose ducts will enter into the uterine lumen. In regards to implantation, it is during the blastocyst stage that

the trophectoderm (Tr) is developmentally competent to attach to the luminal epithelium (LE). The LE is of critical importance to establish juxtacrine communication necessary to initiate the implantation cascade [15]. Attachment and implantation is a unique process, which can be characterized as either non-invasive or invasive depending on whether the conceptus invades the uterine LE to imbed into the stroma [18]. In rodents and primates the conceptus enters the receptive uterus and almost immediately attaches, invades, and expands rapidly; extraembryonic membranes are formed post implantation (invasive implantation) [19]. Domestic animals exhibit a noninvasive implantation. Thus the conceptus does not invade the stroma. Unique differences can also be witnessed in interactions between the maternal and fetal tissues at the maternal-fetal interface. For example, in swine, intimate contact between the LE and chorion derived from trophectoderm is maintained throughout pregnancy (epitheliochorial placenta). Conversely, implantation in ruminants is characterized by the formation of binucleate trophectoderm cells that migrate and fuse with the uterine LE to form plaques of multinucleated syncytia (synepitheliochorial placenta) [20]. The pregnant sheep is not only an ideal model for studying early pregnancy in our economically important ruminant livestock species, but is also a model that is used extensively in understanding human pregnancy. Researchers have the ability to analyze maternal-fetal interaction in the ewe, in part, because of the ability to surgically analyze and maintain catheters in the maternal and fetal vasculature [10]. The following is a brief review of the general events leading to implantation in the ewe.

Pregnancy Recognition

The events of implantation can be divided into four basic phases: shedding of the zona pelluicida (phase 1), precontact and blastocyst orientation (phase 2) apposition (phase 3), and adhesion (phase 4). The morula enters the uterus from the oviduct on day 4 post mating. The blastocyst is formed on day 6, and the zona pellucida, which is thought to prevent the trophoblast from connecting and attaching to the LE is shed on day 8 or 9[16]. During this time the blastocyst evolves from a spherical form (200 um) on day 9 to an elongated and filamentous "trophoblastic vesicle" form, elongating to 25 cm or more by day 17 [15, 16]. Following the shedding of the zona pellucida on day 11 the blastocyst begins the process of elongation. Elongation of the ovine conceptus is a prerequisite for implantation. At day 12, the blastocyst is approximately 10-22 mm in length and at day 14, it has reached 10 cm. Ultimately, the blastocyst will reach length of 25cm by day 17, and will have taken on a long filamentous form[15]. This elongation process is critical for the initiation, synthesis and secretion of interferon tau (IFNT), the pregnancy recognition signal of ruminants [21]. Maternal recognition of pregnancy is critical for establishment and maintenance of early pregnancy. This communication between the signaling conceptus and the maternal reproduction system may be established by luteotrophic mechanisms (hormone acts directly on the corpus luteum to maintain luteal function) or antiluteolytic mechanisms (the hormone prevents uterine release of luteolytic prostaglandin F2α) [18, 22]. Maternal recognition of pregnancy results in the maintenance of a functional corpus luteum (CL) that produces progesterone and thus is permissive to actions of IFNT, growth factors, and cytokines [18]. Interferon

tau ultimately silences expression of estrogen receptor α. This effect triggers a cascade which prevents estrogen from upregulating oxytocin receptor (OXTR) expression in the uterine LE and sGE and thus hinders oxytocin release, ultimately preventing the release of pulsatile PGF2α which prevents CL regression [20].

Apposition

The next phase in the implantation process involves apposition in which the conceptus becomes closely associated with the endometrial LE. Initial site of apposition is at the location of the developing embryo and then is spread across the elongating conceptus [16]. At approximately day 14, the embryo becomes immobilized and there is close contact between the apical membranes of both maternal and fetal cell types; thus in this situation, the trophectoderm is closely pressed against the apical membranes of endometrial cells, and these apical membranes actually physically imprint into the apical surface of the trophectoderm cell. However, the embryo may still be recovered from the uterus intact by lavage at this time [15]. Interestingly, between days 15 and 18, finger like projections known as papillae are formed in sheep and cattle (not goat) trophectoderm. These structures extend into the superficial ducts of the uterine glands and are hypothesized to be necessary for anchoring the conceptus and, by close attachment, facilitating the absorption of a rich nutrient supply from histotrophic secretion of glands. Histotroph is produced in the superficial GE and mid to deep-gland epithelium and includes: nutrient transport proteins, ions, mitogens, cytokines, lymphokines, enzymes, hormones, growth factors, proteases and protease inhibitors, amino acids, glucose, fructose, vitamins and other substances[23]. Therefore, the uterine

glands not only produce histotroph which is essential for conceptus development both pre and postimplantation, but also may be critical in anchoring the conceptus against the uterine epithelia which allows the initiation of cellular changes during implantation [24, 25].

Adhesion

If the preceding phases of pregnancy, described above, are all established and the condition of synchrony is met implantation adhesion may begin. At this time, uterine microvilli penetrate cytoplasmic projections of trophoblast cells [16]. Adhesion progresses along the uterine horn and is generally complete at approximately day 22 of pregnancy. This interlocking of microvilli will maintain close attachment of the two tissues throughout pregnancy [24]. The engagement of adhesion molecules at the LE and Tr surfaces is necessary to transduce cytoplasmic signals essential to maintain both maternal and fetal interactions and stimulate the formation of the placenta [26]. One of the cellular components necessary for establishment of cell contact is the involvement of glycosylated membrane proteins. During the apposition stage the glycocalyx, located on the apical membrane surface of trophectoderm cells, increases in thickness. A glycoprotein coat is always present at the utero-trophoblast interface throughout pregnancy, although it becomes abbreviated during the apposition and adhesion phases of implantation[24]. Progesterone is known to play an important role during this time in order to establish and maintain pregnancy. Progesterone receptor protein expression is not detectable in the endometrial LE and GE in sheep after days 11 and 13[27]. During the implantation period it is common to see a loss in progesterone receptors in

mammalian species [28]. It is reasonable to conclude that adhesion and implantation may be regulated by the loss of epithelial cell progesterone receptors and specific factors implicated in this process[15]. For example, MUC1 is a component of the glycocalyx that is abundant on the microvilli extending from the apical cell surface of the endometrial LE. MUC1 contains a large amount of glycans and removal of this antiadhesive barrier is believed to be necessary for exposure of other glycoproteins involved in adhesion. Thus, down regulation of MUC1 is responsible for exposing integrin receptors for adhesion molecules and initiating the implantation cascade between day 9 and 17 of ovine pregnancy [29]. Additionally, GlyCAM-1, a sulfated glycoprotein that mediates leukocyte-endothelial cell adhesion [30] has also been identified to show a temporal and spatial pattern in the uterus of cyclic and pregnant ewes, thus linking the glycoprotein to be a potential regulator of implantation[15]. Osteopontin [also known as secreted phosphoprotein 1 (SPP1)], ia a small integrin-binding ligand, which has numerous physiological roles is also linked to pregnancy[31]. In sheep, SPP1 is part of the histotroph secreted from endometrial glands during pregnancy [32]. Ultimately, binding of SPP1 to the conceptus trophoblast and uterine LE facilitates migration and adhesion of the conceptus as it elongates [31, 32]. Additional molecules necessary for implantation include: LGALS15 (lectin, galactoside binding, soluble 15) [33]; and IGFBP1 (insulin like growth factor binding protein 1) [34], which aid in attachment between the trophectoderm and LE. Early pregnancy events depicted in (Figure 2.1).

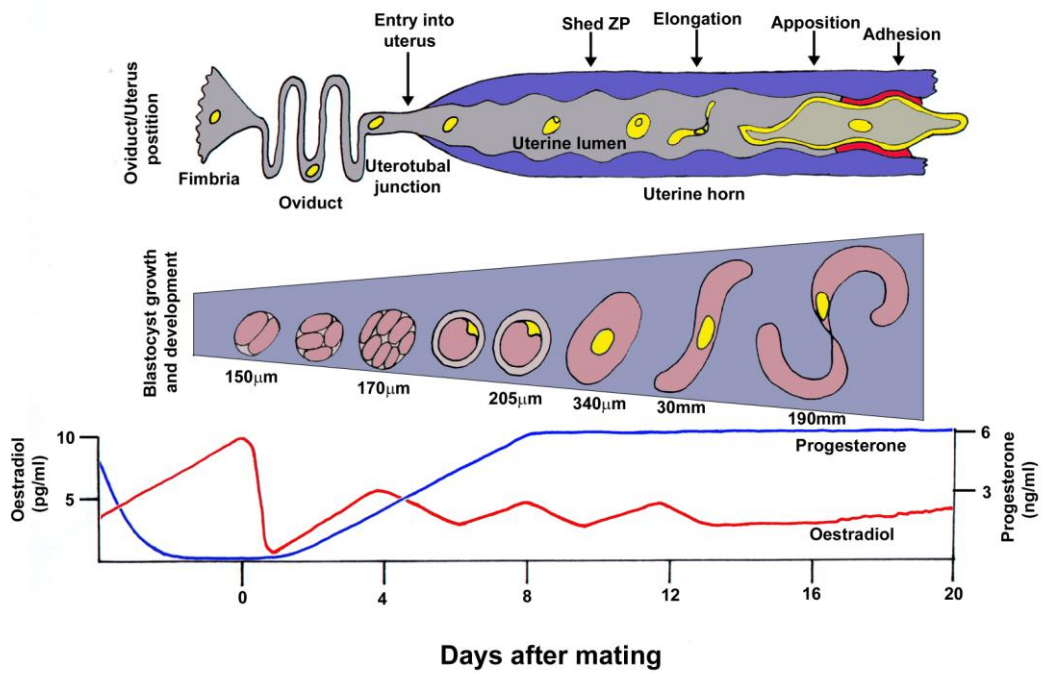


Figure 2.1. Early pregnancy events in sheep. This schematic describes relationships between development of the embryo and conceptus with respect to hormonal status and position within the maternal uterine environment. Embryos enter the uterus on Days 4-5 post-fertilization (Day 0), reach the blastocyst stage on Day 6 and hatch from the zona pellucida by Day 9. The blastocyst transitions from a spherical to a tubular form by Day 11. Elongation to a filamentous conceptus occurs between Days 12 and 16 during which time the conceptus is apposed to the uterine LE and begins adhesion around Day 16. Ultimately, the conceptus will occupy the entire ipsilateral uterine horn and elongate into the contralateral uterine horn. The hormone profile of the maternal environment during early pregnancy is dominated by progesterone. Copied from (Spencer *et al.* 2007).

Binucleate Cells

Binucleate cells play a major role in placental function in ruminants. Formation of binucleate cells begins in the trophectoderm as soon as elongation of the conceptus is complete [25]. Upon differentiation these binucleate cells lose contact with the basement membrane and apical tight junctions allowing them to migrate within the epithelial

sheet[35]. Binucleate cells migrate from the trophectoderm into the uterine LE where they fuse with individual LE cells to form trinucleate cells which are developed in the uterine epithelium on day 16 of pregnancy. In sheep, as opposed to cattle, these trinucleate cells are capable of fusions with multiple binucleate cells to produce syncytial plaques which ultimately results in a tissue with both fetal and maternal components [36]. Continued migration and fusion of the binucleate cells enlarge the synctium. Wooding et al., has shown that all binucleate cells migrate and migration is at a constant 4-5% of the trophectoderm to form the synctium [37]. Binucleate cells are important for the formation of placentomes and also for production of steroid hormones and proteins necessary for pregnancy. For the sheep, migration and fusion places the syncytial layer opposed to the maternal tissue and thus the binucleate cell granules are moved to a position where they can be released by rapid exocytosis into the maternal circulation[37].

The Placenta

Function and Evolution of the Placenta

The placenta is the vital organ in which transport of nutrients, gases, and waste products between fetal and maternal circulation occurs [4, 38]. The placenta has two evolutionary roles conserved among all species. The first is it generates a large surface area for nutrient exchange by the epithelial barrier and fetal blood vessels, all of which make up the chorioallantoic placenta. The second basic function is that the trophoblast cells interact closely with the uterus which produces histotroph. This interaction allows for the production of growth factors, cytokines, and hormones necessary to facilitate

blood flow and nutrient delivery to the fetus [39]. Additionally, the placenta also serves as a barrier to protect the fetus from the maternal immune system [40]. The first recorded scientific description of the placenta was documented by Ionian physician, Diogenes of Apollonia, in 480 BC. He was the first person to describe the placenta as an organ of fetal nutrition. Others believed that the fetus obtained its nutrients by sucking on the uterine lining known as "uterine paps." This theory continued well into the Middle Ages. Later, Aristotle dismissed this theory due to the fact that the fetus was surrounded by membranes and thus had no direct contact with the uterine lining. Instead he drew a correlation to the chick embryo and hypothesized the placenta was like a "yolk sac" and provided the nutritional support of the embryo. Galen, a Roman physician, and brilliant anatomist, agreed with the ideas of Aristotle, and theorized the embryo was nourished by direct connection with the maternal blood via the umbilical cord. Advances in science, philosophy, and art, happened rapidly during the Renaissance, and eventually gave rise to more detailed understanding and classifications of placental types [41].

Placental Classifications

The earliest most primitive type of chorioallantoic placenta is found in egg-laying animals in which the chorioallantoic membrane, making up the underlining of the eggshell, is necessary for gas exchange and transport of calcium from the shell to the fetus [39]. The mammalian placenta is certainly much more complex and takes on a variety of other physiological roles such as production of hormones and growth factors, vascularization, directing methylation patterns, and regulating expression of specific genes, all of which alter maternal physiology and uterine environment [42]. Although

the steps of early implantation are conserved among most species, placental structure is quite diverse among mammalian species, specifically among the typical research models, including: humans, rodents, and ruminants. Mechanisms of placentation are characterized by the degree of interaction and number of interposed layers between the maternal and fetal circulation as well as according to their distribution of chorionic villi [43]. Placental conformation has a wide range of variations. The simplest superficial epitheliochorial placenta is displayed in domestic animals in which the uterine LE remains intact throughout pregnancy and the embryo is restricted to the uterine lumen. Conversely, rodents and humans demonstrate endotheliochorial and hemochorial placentas in which the conceptus invades the LE and embeds in the stroma [26]. There are several distinct placental types. The diffuse placentas, found in the pigs, have a uniform distribution of chorionic villi that cover the surface of the chorion. Rodents and primates utilize a discoid placenta which is characterized by having two distinct discs that contain chorionic villi that interface with the endometrium for exchange of gas, nutrients, and metabolic waste [43]. The sheep (ruminant) has a distinct fetal component of the placenta formed from the fusion of the avascular chorion to the vascular allantois. Consequently, this placenta type is classified as being primarily cotyledonary [44].

The Ovine Placenta

Early pregnancy is a critical time for pregnancy survival. Upwards of 30% of pregnancy loss can occur in most species at this time, and this total could reach 50% in humans [45]. Ruminants have a cotyledonary placenta in which hematotrophic nutrition is primarily conveyed from the uterus to the fetus through placentomes, which are

structures formed through the interdigitation of fetal cotyledons and maternal caruncles. Cotyledons are highly branched villous tree-like folds of fetal chorioallantois which are lined by syncytial plaques and protrude into maternal endometrial caruncular crypts (aglandular endometrial areas consisting of stroma covered by a single lay of LE. At approximately day 17 of gestation attachment is initiated. Chorionic epithelium which is overlying the maternal caruncles associates and interdigitates with caruncular tissue. Resultantly, by gestational day 40 in sheep there is maximal juxtaposition of endometrial and placental microvasculatures [39]. Failure of placentome development results in loss of the fetus [46] because these structures provide a source of hematotrophic nutrition where maternal and fetal blood vessels are in very close proximity for exchanging oxygen and nutrients (Figure 2.2) [47].

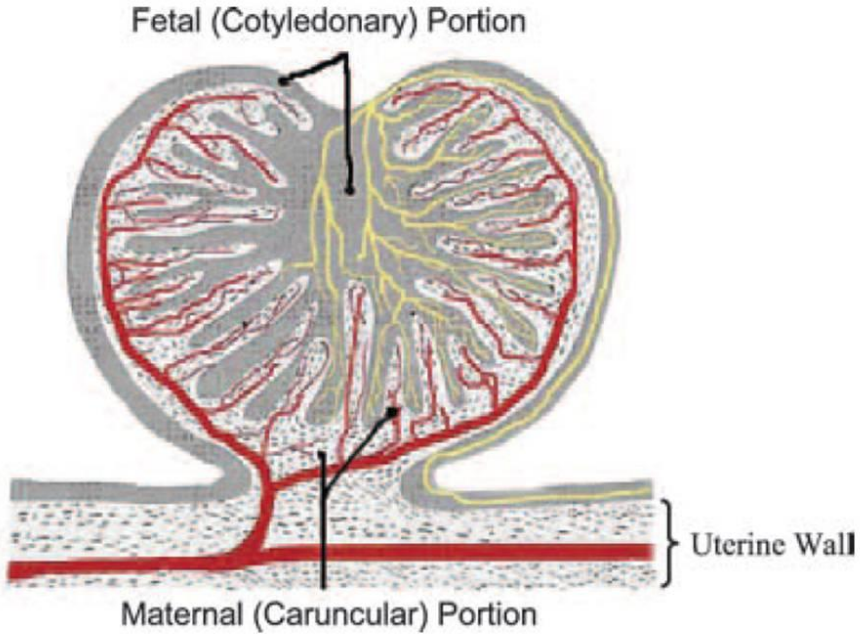


Figure 2.2. Schematic Representation of the Sheep Placentome. The maternal (caruncular) portion is represented by the strippled areas, and the fetal (cotyledonary) area, is represented by the greyish areas. The vascular supply for each portion of the placentome is represented by the red (maternal, caruncular) or yellow (fetal, cotyledonary) vessels. Figure adapted from Reynolds et al., Journal of Physiology, 2005.

Angiogenesis in the Placenta

The importance of the placenta has been observed and studied since ancient time. Multiple factors are necessary for proper placental growth and utilization. Lawrence Lingo (1972) described the placenta saying:

> 'The fetal "lifeline" thus includes an adequate maternal placental circulation and supply of blood nutrients, a placenta that transports and metabolizes various substances properly and a functional fetal placental circulation'

Angiogenesis, the formation of new blood vessels from existing vasculature, is a critical factor in placentation [48] which ensures adequate blood flow and thus enhanced fetal

growth. Multiple factors can effect angiogenesis and in turn blood flow and fetal nutrient delivery such as genotype, number of fetuses, maternal nutrient intake, and environmental stress[49]. Vascularization and angiogenesis in the placenta bring about morphological changes that alter both number and surface density of capillaries to ultimately influence blood flow in the umbilical and uterine circulations [47]. Increased placental blood flow is critical to increased transplacental exchange, which is necessary for the exponential fetal growth that occurs during the last trimester of pregnancy. Ultimately, fetal growth is dependent on the growth of placental and endometrial vascular beds, and thus a large increase in uterine and umbilical blood flows [50]. This is confirmed in analyzing situations of intrauterine growth restriction (IUGR). It has been observed that in human IUGR pregnancies the third trimester is characterized by impaired uterine and umbilical blood flows [4]. Reduced blood flow ultimately leads to impaired fetal nutrient delivery which has both short term and long term consequences[49]. In a normal pregnancy fetal de-oxygenated blood is carried to the placenta by the arterial system. This blood will eventually become oxygenated in the villous capillary network and then be transported back by the venous system to the fetus. Increased uterine blood flow is a primary mechanism for transport of nutrients from mother to fetus [44].

Utilization of the Sheep Model to Study Placental Angiogenesis

The pregnant sheep has been utilized extensively as a model of placental angiogenesis and to understand placental blood flow occurring in human pregnancy. While a great deal of knowledge has been generated from numerous animal models there

have been limitations in the ability to obtain repetitive human samples from both the maternal and fetal components of the placenta. This inability limits the usefulness of studying placenta and fetal interactions under steady-state conditions [10]. The ruminant model rectifies this limitation. The pregnant sheep has specifically been used as a model to investigate maternal-fetal interactions, because the sheep possesses the ability to surgically place and maintain catheters in both maternal and fetal vasculature[51].

With the advantage characterized by the ovine model a great deal of knowledge has been generated in regards to placental angiogenesis. Reduced placenta vascular development and increased vascular resistance has been linked to early embryonic mortality [45]. In modeling of the ovine placenta vascular density has been shown to increase continuously throughout gestation. This is different compared to the development of the fetal placental cotyledons whose growth remains relatively constant until midgestation at which time they then show accelerated growth correlated with rapid fetal growth [45].

Angiogenic Factors

Factors shown to be necessary in regulating placental angiogenesis include: vascular endothelial growth factor (VEGF), fibroblast growth factor (FGF), and the angiopoietin (ANG) protein families [45, 52]. The VEGFs are critical components of placental angiogenesis due to their ability to stimulate necessary angiogenic processes such as stimulating vascular permeability, as well as vascular endothelial cell protease production and migration [53, 54]. VEGFs have been shown to induce angiogenesis in both normal conditions as well as pathological processes such as wound healing,

coronary ischemia, and tumor growth. To no surprise, VEGF is a critical factor during pregnancy. VEGF has been shown to correlate with and play a role in development of brain ventricles, kidney glomeruli, and placental tissues during late pregnancy in mice. In the sheep, VEGF can be found in placental tissues throughout pregnancy[45]. FGFs are unique in that they not only influence placental angiogenesis but also various other developmental functions. Specifically FGF have been shown to stimulate differentiation of embryonic germ layers [45, 55]. Throughout gestation, FGF is produced in both fetal and maternal placental tissue. Additionally, both VEGF and FGF may play a role in regulating placental blood flow [45]. For instance, VEGF and FGF have been found as contributors in inducing nitric oxide (NO) production. Nitric oxide is a potent vasodilator that is necessary in stimulating estrogen-induced increases in uterine blood flow[56]. Furthermore, other angiogenic factors are necessary for placental development. The ANG family is a necessary regulator of vascular development and can have both a stimulatory and inhibitory effect on vascular growth and differentiation[45]. The intricate interaction of placental development, blood flow, and environmental conditions which influence this interaction are the target of numerous research endeavors aimed at improving pregnancy development. Understanding factors that regulate placental angiogenesis and in turn overall health of offspring is the first step in finding solutions to associated problems.

Nutritional Regulation of Reproduction

Impact of Nutritional Inadequacies

Suboptimal nutrition is a far-reaching societal challenge. Whether it is the 925 million people who are undernourished or the billion adults who are overweight, nutritional inadequacies have a significant negative global impact[57]. Importantly, many of these nutritionally compromised individuals are reproductively mature adults, whereas others are developing children still capable of reproduction in the future. Pregnancy is a particularly sensitive period to suboptimal nutrition. From a production livestock standpoint nutrition plays a major role in productivity. And in cases of suboptimal nutrition the fetus may never be able to reach its genetic potential [58]. Epidemiological studies have demonstrated links between maternal undernutrition/overnutrition and the cause of chronic metabolic disease in adult offspring[59]. Metabolic syndrome has been defined as a cluster of disorders, including obesity, hyperglycemia [fasting serum glucose (>6.1 mM)], hyperinsulinemia, hyperlipidemia, hypertension, and insulin resistance (impaired response of cells or tissues to physiological concentrations of insulin)[60]. These epidemiological observations have been recapitulated in controlled experimental studies using a variety of model systems, and have collectively given rise to the concept of fetal programming[4, 61]. Fetal programming proposes that alterations in fetal nutrition and endocrine status impacts development; permanently changes structure and metabolism; and as a result influences an individual's susceptibility to disease[62]. These changes are mediated at the level of the epigenome, and are observed as stable and sometimes

inheritable alterations of genes through covalent modifications of DNA and core histones without changes in DNA sequences [63].

Intrauterine Growth Retardation

Maternal nutrient intake and subsequent fetal growth are intricately correlated and associated with pregnancy success and overall lifelong health of the offspring [57]. A potential hindrance to this interaction occurs in a situation of intrauterine growth retardation (IUGR) defined as impaired growth and development of the mammalian embryo/fetus or its organs during pregnancy. Multiple factors contribute to an IUGR pregnancy. These factors could be naturally occurring restrictions, due to insufficient uterine capacity, which pose limitations on conceptus growth and development, as well as, dysfunction or insufficiency of the endometrium and/or placenta. Alternatively, environmental factors such as nutritional hardships, heat stress, disease, and toxins, are potentially controllable factors that can also contribute to development of an IUGR offspring [64-66]. The occurrence of IUGR poses financial strains on both livestock enterprises and the human population alike. In humans, IUGR affects 5 to 10% of all births. It is estimated that the costs associated with IUGR equate to approximately $2 billion incurred due to immediate care expenses. Total costs are likely to be much higher when the price of lifelong care is taken into account [67]. In livestock enterprises IUGR is a significant factor that impacts productivity. One half of all pre weaning lamb deaths occur on the day of birth and the largest contributor to perinatal mortality is low birth weight. It has been estimated that lambs weighing less than 4 pounds experience a 65% mortality rate compared to lambs weighing 9 to 12 pounds at birth that only exhibit a 8%

mortality rate[68]. Thus further understanding of the causes of IUGR and subsequently methods of prevention is of critical importance in both the human and livestock populations.

IUGR in Livestock

From a production livestock context IUGR remains a critical problem due to the limited understanding of nutritional regulation of fetal growth[4]. Simplistically, it is easy to predict that more offspring lead to more total livestock product, and ultimately greater profit for the producer. Certainly, recent advances in the field of reproductive technologies, such as embryo transfer, have tested this theory. One would be quick to hypothesize that producing twins in cattle would be an easy way to increase profit; as the overhead costs for maintaining a single-calving cow accounts for more than 50% of the total costs of production (Guerra-Martinez 1990). Thus, one would assume that by doubling the amount of offspring produced by a single cow this would positively impact the bottom line. However, this hypothesis does not take into account maternal reproductive health and adequate uterine space as well as management of a cow with two calves after birth. In cattle, cows that carry twins lose on average 12% body weight during the last trimester of gestation and twinning also reduces fetal growth and consequent birth weight of offspring. This could lead to decrease weight at sale [4]. Thus, careful consideration must be demonstrated in analyzing enhanced production technologies.

The sheep industry displays a similar situational paradigm. Under ideal environmental and management conditions multiple offspring pregnancies increase the

prolificacy of the species. However, it is important to note, that increased number of fetuses can contribute to placental insufficiencies and thus lower birth weights [69]. In fact, in sheep, IUGR can be induced by a multitude of factors. As mentioned, prolificacy is an important factor in intensive sheep production however the economic benefits of high prolificacy ewes are usually not fully exploited because multi-fetal pregnancies are associated with IUGR. Other natural factors can affect birth weight in sheep such as maternal age. Lambs born to relatively young ewes and relatively old ewes generally possess lower birth weights [70]. Seasonality can also be an indicator of birth weight. Lambs born in the fall and summer are generally lighter than those born in the spring and winter; which can possibly be explained by alterations in melatonin and seasonal effects on gestation. Additionally, gestation occurring in the warmer months where ewes shuttle blood flow to the periphery of the body to dissipate heat rather than to the core could ultimately decrease placental blood flow and placental growth[71]. Finally, altitude is another factor that affects lamb birth weights. Lambs that are born at high altitude may experience hypobaric hypoxia and be lighter than lambs born at a lower altitude. All of these situations can ultimately lead to IUGR offspring who possess reduced survival ability (Figure 2.3) [70].

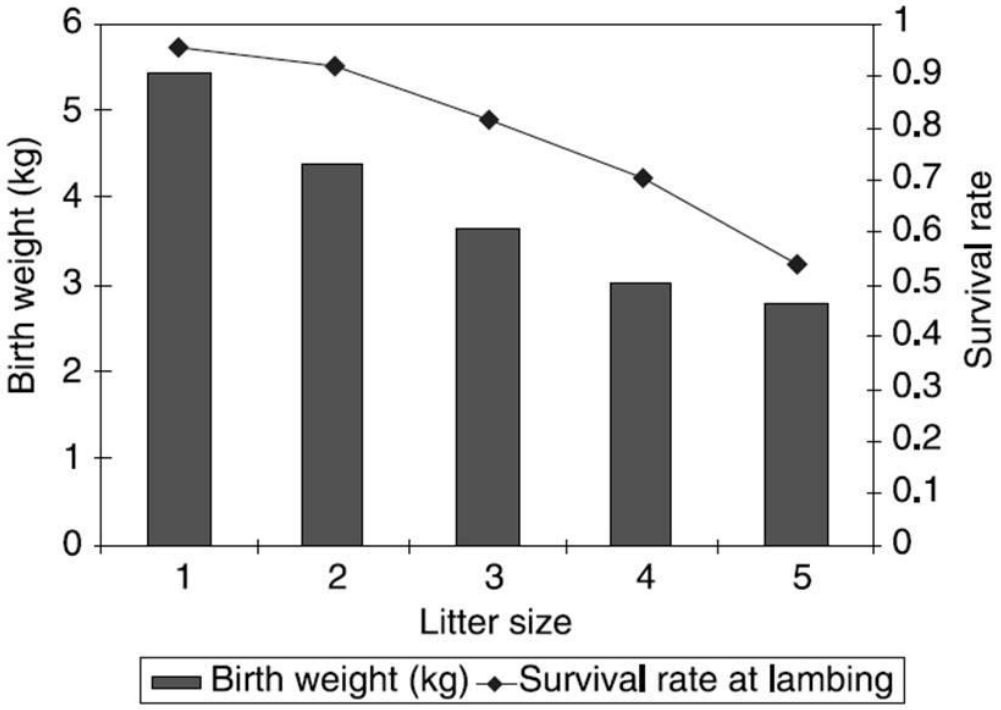


Figure 2.3. Birth weights and perinatal survival rates for lambs. Lambs (n=4 781) born to Afec-Assaf ewes (Volcanic Center, Israel) according to litter size (after Gootwine and Rozov (2006)). Copied from (Gootwine et al., 2007)

Maternal nutrition is arguably the most important and potentially manageable factor that contributes to pregnancy and maternal health [72]. Various livestock species are produced in different environmental conditions. Swine are commonly produced in a confinement setting and are fed specifically formulated plant based diets (intensive systems). On the other hand ruminants, such as cattle and sheep, are more commonly managed under grazing systems. However, the quality of the grazing system is directly dependent on availability of nutrients, which is usually limited during the winter months

and times of dry conditions. Thus nutrient availability may be limited for adequate growth and development as well as proper gestation and lactation [73] [4]. The sheep, in particular, is a seasonal breeder which enters estrus in fall and early winter. Therefore, most of gestation occurs in the winter months, a time of low forage quality and limited nutrient availability [74]. In fact, Thomas and Knott reported, that without supplementation, the nutrient consumption of grazing ewes in the Western United States is often less than 50% of the National Research Council (NRC) recommendations [75].

Researchers and care providers alike try to find efficient and non invasive means for preventing the continuous occurrence of IUGR and premature delivery. Thus using nutritional means are an attractive option for non-invasive prevention. In order to fully utilize nutritional intervention one must understand the mechanisms by which nutrition mediates fetal growth. Using humans as research models is an unethical option, thereby the sheep is an attractive alternative. The sheep has a relatively long gestational period compared to other laboratory models (approximately 147 days), a single pregnancy can be sustained in a sheep, and there are many similarities to humans with respect to fetal size, organ development, and physiology and maturity at birth [76]. To begin, nutritional status and maternal weight/ adiposity have been shown to be an indicator of successful pregnancy in the sheep model. Ewes that were lighter and thinner at mating, and thus exhibited low nutrient intake, had lambs with an associated 13% decreased birth weight [77]. Ultimately, the hypothesis is that these alterations in fetal growth may be mediated by a hindrance of the placental growth trajectory [77]. While limited studies have been conducted to correlate these findings in women; a study conducted in Chinese women

found that being severely underweight at conception equated to a significant decrease in birth weight and an 80% higher risk of IUGR [78]. A great deal of research has also been conducted in the ewe with respect to the interaction with nutritional and placental development and morphology. Recognizing that placental development is a major indicator of birth weight, researchers have found an ever occurring commonality among studies. The morphology of the placenta is altered under nutrient scarce conditions, with the most notable change being increased development of the fetal cotyledon. This change is thought to reflect an adaptive compensatory response to maximize transplacental exchange and thus fetal growth[76, 79, 80].

Maternal Undernutrition and Metabolic Syndrome

The impact of intrauterine growth retardation is not only substantial in domestic livestock but is also a global problem in the human population. There are approximately 350,000 low birth weight infants born each year in the United States. On average each one of these infants required immediate medical care at a cost of $57,000 per infant[67], which equates to a total of nearly $2 billion per year. This factor does not take into account the long-term costs of continuous health care and special education that may be required as the low birth weight infants that survive the neonatal period have an increased risk for lifelong complications including neurological and developmental disabilities. Ultimately, the long-term costs of premature and growth restricted infants is estimated to be $363,000 per surviving baby[81]. The economic impact also does not take into account the psychological stress more likely to be experienced by mothers of low birth weight offspring [82]. The impact of maternal nutrient deprivation and the

resultant long-term consequences to health of the human offspring have been extensively studied since the seminal findings by Barker et al. related to the Dutch Famine [83]. Intensive study of The Dutch Famine (1944-1945) found that not only does gestational undernutrition have critical effects on adult health but also the timing of exposure in relation to stage of pregnancy determines severity of effects in addition to the organs that are compromised. Organs affected are based on tissue development taking place during that specific time of gestation [84]. In regards to growth, women exposed to famine during the second or third trimester had offspring with reduced weight, length, and head circumference [85]. Currently, much of the work has now shifted to understanding the epigenetic mechanisms by which the fetus programs critical metabolic set points in response to maternal dietary cues. It should be noted that inadequate nutrition during pregnancy not only occurs due to a lack of food, but may also result from severe nausea and vomiting, known as hyperemesis gravidarium early or closely spaced pregnancies, and multifetal pregnancies, commonly associated with assisted reproductive technologies [57]. Luther et al., specifically addressed fetal programming concerns associated with adolescent pregnancies. Adolescents who become pregnant have a greater risk of premature delivery, low birth weight, neonatal mortality, and maternal death, due in part, to poor nutrient stores [86]. Furthermore, propensity to adult disease is not always predicted by growth rate in utero, assessed using standard gross measures. Birth weight is a valuable tool for clinicians to assess the level of nutrition available to the fetus during pregnancy. Primary growth of the fetus occurs in the third trimester, thus growth effects due to nutrient restriction are more visible at this time [4]. However,

this does not mean that maternal undernutrition only has a major impact during the third trimester of pregnancy. In fact, the effect of gestational undernutrition critically depends on the stage of pregnancy, with early gestation being a critical time for developing metabolism and overall offspring health [83]. Thus, long-term health consequences can occur in the absence of changes in birth weight [83].

But maternal nutrient restriction does not only have negative consequences. In this discussion, it is important to take into account that restricted fetal growth could be an adaptation to a problematic uterine environment that allows the fetus a greater chance of survival [87]. This adaptation, however, could involve the partitioning of nutrients to organs critical for survival such as the brain and heart, while other organs less critical to survival (pancreas and kidney) are nutritionally compromised [88]. This situation gives rise to what is termed the "thrifty phenotype," which proposes that there is an association between poor nutrition in early life and permanent changes in glucose and insulin metabolism [89] Early postnatal nutrition also may affect the function and development of the gut microbiota, and having deficient digestive microbiota in early life could contribute to problems with immunity and overall health later in life [90].

A growing body of experimental evidence from animal models has given rise to relevant and interesting findings in regard to the effect of global or specific maternal nutrient restriction on the development of adult metabolic disease. For example, global nutrient restriction has been shown to increase the risk for obesity, cardiovascular disease, and type 2 diabetes in a variety of animal models [4, 91]. Similarly, maternal protein restriction results in hypertension and vascular dysfunction in offspring in a sex-

specific manner [92, 93]. Interestingly, high cholesterol in offspring from rats exposed to a protein-restricted diet coincided with repressive histone modifications at the cholesterol 7 alpha hydroxylase promoter[94]. In sheep, feeding a diet deficient in specific B vitamins and methionine during the periconceptional period of pregnancy may resulted in hypertension and adiposity, predominantly observed in male offspring[95]. This was associated with global changes in methylation status in the liver of these offspring.

Importance of Gestational and Early Life Nutrition

The challenges associated with pregnancy undernutrition are evident. Not only can this nutritional status pose health problems in mature adults, but just as concerning, these problems may manifest themselves in the offspring of obese or undernourished individuals. Multiple factors can affect epigenetics and gene expression, which can ultimately program development and long-term health of offspring[4, 57]. Therefore, gestational and early life nutrition are critical to determine long-term developmental fate. Using a model of maternal nutrient restriction in the ewe we have observed numerous developmental and physiological differences between adequately fed and nutrient restricted mothers. Among our unique observations, AGR2 arose as a novel gene that was identified in this research model. Throughout pregnancy AGR2 is expressed in the uterus and is present in the placentome. The goal of this research is to determine AGR2's potential roles throughout pregnancy in conditions of differential nutrient intake and ultimately determine effective means of addressing the concerns associated with unbalanced early life nutrition.

Anterior Gradient Homolog 2

Introduction to AGR2

Anterior gradient homolog 2 (AGR2) has a wide range of evolutionarily conserved roles. AGR2 protein expression is known to induce metastasis, act as a p53 tumor suppressor inhibitor and survival factor, and participate in neoplastic transformation [96]. Currently, there is intensive research being conducted on AGR2 and its role in cancer and tumor development. When AGR2 is downregulated in humans it serves as a candidate for inflammatory bowel disease; and decreased *AGR2* mRNA equates to increased risk of Chrohn's disease. Conversely, when AGR2 is upregulated it contributes to neoplasm development and elevated AGR2 levels have been described in various tumor tissues. This includes non hormonal type tumors such as those found in the esophagus, gastrointestinal tract, and lungs; as well as hormonal tumors such as breast, prostate, and ovarian tumors [96-98]. These hormonal tumors provide a direct correlation to hormonal changes during pregnancy. Multiple highly conserved genes important in early embryonic development, such as those belonging to the Wnt and Hedgehog pathways, have been found to significantly influence tumor development [99]. AGR2 may also be involved in such developmental gene pathways and thus could play a significant role in pregnancy. However, the roles of AGR2 in pregnancy have not been evaluated. The purpose of this review is to shed light on the known function and characteristics of AGR2 and thus possibly elute its potential physiological roles during pregnancy.

Characterization of the Anterior Gradient Gene Family

Anterior gradient (AGR) genes were first described in *Xenopus laevis* (XAG) where their expression is responsible for the development of the cement gland [96, 100]. *Agrs* encode for proteins belonging to the protein disulphide isomerase family (PDI), which accelerate the folding of other proteins by means of disulphide bond formation. The Agr family of genes are intriguing due to their ability to be secreted from the cell and their subsequent role in cell growth and differentiation, specifically in embryonic and tumor development [101]. By dissection of X. laevis embryos, XAG-1, XAG-2, and XAG-3 each were identified to be first expressed in the dorsal ectoderm of late gastrula embryos. Specifically, studies show that XAG-2 works to determine the fate of the dorso-anterior ectoderm and forms the cement gland. The cement gland forms columnar epithelium which serves as the area of attachment of the frog embryo to solid support[102]. Interestingly, the newt AG homolog (nAG) was the first protein discovered that could promote limb regeneration [96]. AGR1, also named ERP18/19, could be considered the founding member of the AGR family. Its function involves its ability to form mixed disulphides with proteins in the endoplasmic reticulum however there is still little known about this specific AGR.[103]. AGR1 is found within complex invertebrates[97]. AGR2 and AGR3 seem to have emerged in chordates and thus far their expression is confined to vertebrates. Most research has been done on validating the AGR2 protein, the homologus AGR1 and AGR3 have also been identified [97]. AGR3 shares 71% sequence similarity with AGR2. AGR3 was initially identified in estrogen receptor positive breast tumors and is upregulated in some ovarian cancers

[104]. Still, many tumors that express AGR3 can be AGR2 negative suggesting their expression is not necessarily coupled [97]. Additional studies are needed to further identify the role of the AGR family in biology.

AGR2 Interaction with Amphiregulin

Although mainly localized in the ER, AGR2 has the ability to play other intracellular roles, such as to induce epidermal growth factor (EGF) receptor ligand amphiregulin (AREG)[105] and interact with the AAA+ protein Reptin [100]. Thus, several studies have revealed that AGR2 supports many of the transformed properties of adenocarcinoma cell lines through potential activation of the Hippo signaling pathway and induced expression of AREG[105-107]. Work by Dong et al., found that AGR2 and AREG are co-expressed in human adenocarcinoma cells. AREG is an EGFR ligand that is expressed in higher vertebrates [105]. Specifically, AREG has been identified in the sheep mammary gland suggesting a role for amphiregulin in normal mammary development [108]. Dong et al., discovered that AGR2 expression stimulates AREG and the EGFR signaling pathway which is responsible for increased cell proliferation and anchorage-independent growth. Additionally, the Hippo pathway, which serves to regulate cell proliferation, apoptosis and functions to regulate organ size, is another potential AGR2 induced pathway. Yes-associated protein (YAP1) is a coactivator of transcription in the Hippo pathway and is responsible for AREG expression in breast epithelial cell lines[109]. Dong et al., showed that AGR2 induces AREG expression through YAP1 dephosphorylation [105].

AGR2 Interaction with Reptin

Recently, a protein named Reptin was identified as an AGR2 binding protein in a yeast two-hybrid screen and validated as an interacting protein with AGR2 in human cells [110]. Reptin belongs to a family of proteins known as AAA+ ATPases (ATPases associated with various cellular activity) [111]. Discovered by several laboratories in the late 1990's, Reptin is also known as Ruvbl12 or Tip48 [112]. Reptin is a necessary protein in various cellular functions. The protein Reptin is critical in assembly of protein complexes that play a role in regulation of cellular energetic metabolism, transcription, chromatin remodeling, DNA damage response, and nonsense mediated RNA decay [113]. Of noteworthiness, Reptin expression and regulation appears to follow a sexual dimorphism, specifically in the liver, as Reptin mRNA levels have been revealed to be four fold higher in male mouse livers versus expression in the female liver [114]. Reptin has been shown to be overexpressed in liver tumors and a high Reptin level is correlated with a poor prognosis [115]. Although findings of Reptin in other cancerous tumors are limited, Reptin, like AGR2, has been specifically identified in breast cancer. As previously mentioned, Reptin interacts with AGR2 by specific binding and the ATP binding domains and thus conformation of Reptin play a role in driving the stability of the AGR-Reptin complex [110]. Specifically, AGR2 uses a divergent-binding loop to bind to Reptin, and then Reptin uses two allosterically interacting ATP binding motifs to control its binding activity toward AGR2. Because both proteins are identified in cancer, AGR2 and Reptin are both thought of as potential anticancer therapeutic targets. By using biochemically based screening assays and by analyzing the substrate binding loops

of AGR2 or ATP binding motifs of Reptin there is a potential for scientific advancements that could allow for the development of small molecules that regulate the AGR2-Reptin binding complex [110]. Further research is sure to be conducted to determine greater detail and specificity of the Reptin- AGR2 interaction.

AGR2 Interaction with Caudal Type Homeobox 2

Additional studies have also linked AGR2 with the expression of the transcription factor caudal related homeobox 2 (CDX2). Dong et al., screened for markers of intestinal development and found that expression of CDX2 was induced upon expression of wild-type AGR2 in IEC-6 (a rat small intestinal jejunum cell line) cells [105]. The induction of CDX2 expression by AGR2 stimulation is interesting in a reproductive context because CDX2 regulates trophectoderm differentiation as well as the trophoblast specific gene, interferon tau (IFNT), in ruminants [116]. Interferon Tau (IFNT) is the pregnancy recognition signal in all ruminants [20]. Although a great deal is known about IFNT, its transcriptional mechanisms have not been fully characterized. In recent years great progress has been made in detecting uterine-derived factors and key placental specific transcriptional regulators which makes IFNT an interesting molecule in determining maternal-fetal interactions during early pregnancy[117, 118]. In regards to CDX2, a great deal is known about its role in embryonic development. CDX2 is isolated to the trophectoderm and is essential for ensuring correct segregation of the inner cell mass and trophectoderm cell lineages in blastocysts, as CDX2 ensures the repression of Oct4 and Nanog in the trophectoderm [119]. Interestingly, Tead4 (a transcription factor necessary for trophectoderm formation) along with its coactivator

protein Yap, can induce and lead to changes in expression of CDX2[120]. This finding correlates to the interaction of AGR2 with its downstream target AREG. Both AREG and CDX2 are downstream targets of YAP activation and both are involved in the Hippo signaling pathway (Figure 2.4).

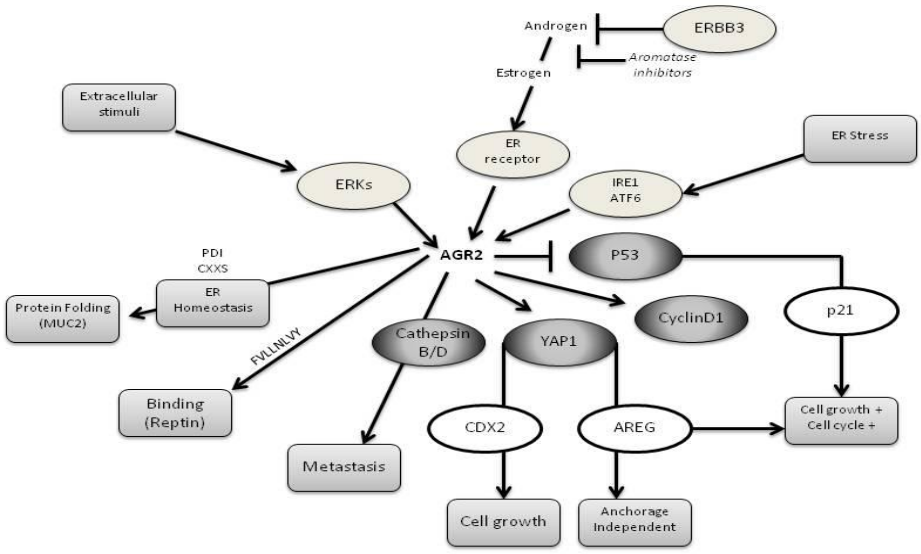


Figure 2.4. AGR2 biological pathways. AGR2 is presented as the central point of this picture. Pathway intermediates known to regulate AGR2 expression are indicated in light grey ovals. AGR2-dependent functions are indicated in dark grey ovals. Potential (not experimentally demonstrated) intermediates are indicated as empty ovals. Physiological/pathophysiological inputs or outputs are represented by grey boxes. Adapted from Chevet et al., 2013.

AGR2's Role in Cancer

AGR2 has garnered major scientific attention recently due to its potential role in tumor development and cancers. Numerous reports have analyzed hormone dependent cancers such as breast, prostate, and ovarian cancer, as well as non-hormonal types such as esophagus, gastrointestinal tract and lung cancer [96]. Recent cell culture experiments have demonstrated that when cultured under serum depleted conditions and/or hypoxia, AGR2 was activated to promote angiogenesis, motility and invasion. Therefore, these findings demonstrate evidence that AGR2 contributes to the survival of cells undergoing physiological stress [121]. In a reproductive context, AGR2 has been associated with breast cancer. In breast cancer cell lines AGR2 has been linked with estrogen receptor alpha (ERα). In fact, estrogen has been shown to cause AGR2 to become transcriptionally activated by direct binding of the ER [122]. AGR2 knockout mouse models indicate that in the mammary gland the gene promotes lobuloalveolar development by stimulating cell proliferation[98, 123]. When AGR2 is secreted it has been found to play a role in cell adhesion and facilitating communication pathways elicited by a metastatic tumor[123]. Tamoxifen is an antagonist to the estrogen receptor in breast tissues; however in other tissues, such as the endometrium, it can be viewed as an agonist. Tamoxifen is used as a primary therapy in treating estrogen hormone receptor-positive breast cancers serving as an anti-estrogen therapy[124]. While Tamoxifen is a proven cancer therapy intrinsic and acquired resistance can be a problem. AGR2 is an identified gene that might mediate the resistance of breast cancer to Tamoxifen [96, 97]. Although AGR2 has been strongly correlated with estrogen in

breast cancer cells other regulatory factors are likely to exist. This is demonstrated by Innes et al., finding inconsistency of AGR2 expression in breast cancer scenarios [125]. Poor prognosis is indicated by AGR2 expression in estrogen-positive breast cancers. However, AGR2 was present in one third of ER-negative cases and was not consistently expressed in ER-positive breast cancers; suggesting the mechanism(s) by which AGR2 is regulated in these cancer cell lines remain to be fully understood [125]. In regards to prostate cancer, both estrogens and androgens stimulate AGR2 expression [104]. AGR2 mRNA is present in normal prostate tissue. However, AGR2 expression markedly increases in cancerous conditions of the prostate. Although the fact that AGR2 is present in not only normal prostate tissue, but also normal colon, pancreatic, and lung tissue, limit its use in cancer treatment, progress has been made in identifying AGR2's role in prostate cancer [96]. Two forkhead transcriptional factors, Foxa1 and Foxa2 positively regulate the AGR2 promoter in prostate cancer. Additionally, ErbB3 binding protein 1 (EBP1) has been found to suppress the invasive ability of cells by inhibiting AGR2 expression. This binding protein has been implicated as a negative regulator of androgen receptor signaling[126]. Immunohistochemical analysis has also demonstrated that AGR2 expression is restricted to secretory epithelial cells of the prostate gland [127]. Recent findings using immunohistochemical staining of ovarian tissues have demonstrated that AGR2 protein was expressed at a basal level in normal ovary surface epithelium[128]. Conversely, cancerous tissues present moderate to strong staining of AGR2. Park et al., demonstrated that AGR2 enhanced cell growth and migration of ovarian cancerous cells. AGR2 can also be detected in the serum of mucinous ovarian

tumor patients, which suggests AGR2 could be a potential biomarker for patients with ovarian cancer [128]. The primary research focus revolves around AGR2's role in cancer, and thus certainly, comparisons can be drawn between cancer and embryonic development. It is currently hypothesized that in order for a new tumor to develop, the cancer cell is likely to resurrect early embryonic behavioral programs. Movement and development of the embryo can be likened to metastasis [129]. In new tumor development cells acquire both genetic and epigenetic changes that allow them to proliferate and to stimulate angiogenesis[130]. Cancer cells can thus take on a phenotype similar to that of early embryonic cells and express unique embryonic transcription factors. These transcription factors have the ability to allow cancer cells to move, become invasive and resist programmed cell death comparable to what is witnessed in the early stages of pregnancy[129]. Thus this correlation provides an intriguing idea to base future research pursuits.

CHAPTER III

ANTERIOR GRADIENT HOMOLOG 2 AND ITS POTENTIAL ROLES IN OVINE PREGNANCY

Introduction

Anterior gradient homolog 2 (AGR2) has a wide range of evolutionarily conserved roles. AGR2 protein expression is known to induce metastasis, acts as a p53 tumor suppressor inhibitor and survival factor, and participates in neoplastic transformation[96]. Currently, there is intensive research being conducted in regards to AGR2's role in cancer and tumor development. When AGR2 is downregulated it is an autoimmune regulator of inflammatory disease. In humans it serves as a candidate for inflammatory bowel disease; and decreased *AGR2* mRNA correlates to increased risk of Chrohn's disease. Conversely, when AGR2 is upregulated it contributes to neoplasm development, and elevated AGR2 levels have been described in various tumor tissues. This includes non hormonal dependent tumors such as those found in the esophagus, gastrointestinal tract, and lungs; as well as hormonal dependent tumors such as breast, prostate, and ovarian tumors [96-98]. These hormonal tumors provide a correlative link to hormonal changes during pregnancy. Multiple highly conserved genes important in early embryonic development, such as those belonging to the Wnt and Hedgehog pathways, have been found to significantly influence tumor development [99]. AGR2 may be involved in such developmental gene pathways and thus could play a significant role in pregnancy. However, the roles of AGR2 in pregnancy have not been evaluated.

Our laboratory has demonstrated that *AGR2* expression in the ovine endometrium increased between days 9 and 12 post mating, and was stimulated by progesterone. Additionally, using a model of maternal nutrient restriction AGR2 was found to be a gene present in the placentome during late gestation. These findings led to further evaluation of AGR2 and its characterization throughout ovine pregnancy.

Materials and Methods

Animals

Mature Suffolk ewes (*Ovis aries*) were observed daily for estrus in the presence of vasectomized rams and used in experiments only after they exhibited at least two estrous cycles of normal duration (16–18 days). All experimental and surgical procedures were in compliance with the Guide for the Care and Use of Agriculture Animals and approved by the Institutional Animal Care and Use Committee of Texas A&M University and Washington State University (Study 2).

Experimental Designs

Study 1: At estrus (day 0), ewes were mated to either an intact or vasectomized ram as described previously [131] and then hysterectomized (n = 5 ewes/d) on day 10, 12, 14, or 16 of the estrous cycle or day 10, 12, 14, 16, 18, or 20 of pregnancy. Pregnancy was confirmed on day 10–16 after mating by the presence of a morphologically normal conceptus(es) in the uterus. At hysterectomy, several sections (~0.5 cm) from the midportion of each uterine horn ipsilateral to the corpus luteum were fixed in fresh 4% paraformaldehyde in PBS (pH 7.2). After 24 h, fixed tissues were changed to 70% ethanol for 24 h and then dehydrated and embedded in Paraplast-Plus

(Oxford Labware, St. Louis, MO). The remaining endometrium was physically dissected from myometrium, frozen in liquid nitrogen, and stored at −80 C for subsequent RNA or protein extraction. In monovulatory pregnant (PX) ewes, uterine tissue samples were marked as either contralateral or ipsilateral to the ovary bearing the corpus luteum. No tissues from the contralateral uterine horn were used for study. Uterine flushes were clarified by centrifugation (3000 × *g* for 30 min at 4 C) and frozen at −80 C for Western blot analysis.

Study 2: Ewes (n= 40) were detected for estrus using a vasectomized ram. Using a surgical approach described previously [132, 133], ewes on day 10 after estrus were subjected to a midventral laparotomy, and the lumen of each uterine horn received a vinyl catheter (0007760; Durect Corp, Cupertino, California) connected to an Alzet 2ML1 osmotic pump (Durect Corp) secured within the infundibulum of the oviduct and mesovarium. Ewes (n=5 per treatment) received pumps containing the following: 1) 2 mL of vehicle as a control [CX; 2% ethanol (vol/vol) in saline]; 2) 156 ng cortisol (CORT) in 2 mL of vehicle; 3) The amount of cortisol pumped into the uterine lumen on a daily basis (23 ng) mimics cortisol production by an elongating day 14 ovine conceptus [133]. Recombinant ovine IFNT was prepared as described previously [134], and the amount of recombinant IFNT pumped into the uterine lumen on a daily basis (14.4 µg) mimics IFNT production by an elongating day 14 ovine conceptus, which is 600 ng/h [135]. Our previous studies found that infusion of that amount of IFNT in the uterine lumen each day mimics effects of the conceptus on endometrial expression of hormone receptors and IFNT-stimulated genes during early pregnancy in ewes [132,

136]. At necropsy on day 14 after estrus, the female reproductive tract was excised and endometrium was physically dissected from myometrium, frozen in liquid nitrogen, and stored at -80°C for subsequent RNA extraction. 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 at Washington State University.

Study 3: At estrus (day 0), ewes were mated to an intact ram and then hysterectomized (n = 5 ewes/day) on either Day 40, 60, 80, 100, or 120 of pregnancy (gestation period is 147 days). At hysterectomy, the uterus was trimmed free of cervix and oviduct and opened along the mesometrial border. Several sections (~0.5 cm) of interplacentomal and placentomal areas of uteroplacental tissues were fixed in fresh 4% paraformaldehyde in PBS (pH 7.2). Placentomes were then removed by physical dissection, and intercaruncular endometrium was dissected from the myometrium. Samples of intercaruncular endometrium and placentomes were frozen in liquid nitrogen and stored at −80 °C for RNA extraction.

Study 4: Mature Suffolk ewes of similar parity and frame size were fed 100% of NRC requirements to maintain their body condition prior to embryo transfers. Ewes were synchronized into estrus and a single embryo from a superovulated donor ewe of normal body condition was transferred into the recipient uterus on post-estrus Day 6. Pregnancy was determined by ultrasonography on Day 28. On Day 35 of gestation, ewes were randomly assigned to receive either 50% of their daily nutrient requirements (n=24) or 100% of their daily nutrient requirements as control (n=7), based on the

National Research Council guidelines. The ewes were kept in separate pens and individually fed once daily from Day 35 to 125. Weights were collected weekly beginning on Day 28 to adjust feed intake with weight gain or loss. On Day 125 of gestation (term = 147) ewes were necropsied and conceptus (fetal-placental unit) development was measured. Following euthanization, the fetus was removed, weighed, measured, and dissected. A portion of the uteroplacental-unit was removed and placentomes were dissected, counted, and weighed. Placentomes were then either snap frozen in liquid nitrogen and stored at -80°C or preserved in 4% paraformaldehyde. (Figure 3.1)

Study 5: Mature Suffolk ewes of similar parity and frame size were fed 100% of their NRC requirements to maintain their body condition prior to embryo transfers. Ewes were synchronized into estrus and a single embryo from a superovulated donor ewe of normal body condition was transferred into the recipient uterus on post-estrus Day 6. Pregnancy was determined by ultrasonography on Day 28. On Day 35 of gestation, ewes were randomly assigned to receive either 50% of their daily nutrient requirements (n=32) or 100% of their daily nutrients requirements as control (n=32), based on the National Research Council requirements. Ewes were randomly assigned to necropsy on Day 50, 75, 100, 125, or term (Day 147), (n=4-7 per day and diet) and conceptus (fetal-placental unit) development was measured. Ewes were maintained and cared for at the Texas A&M Nutrition and Physiology Center.

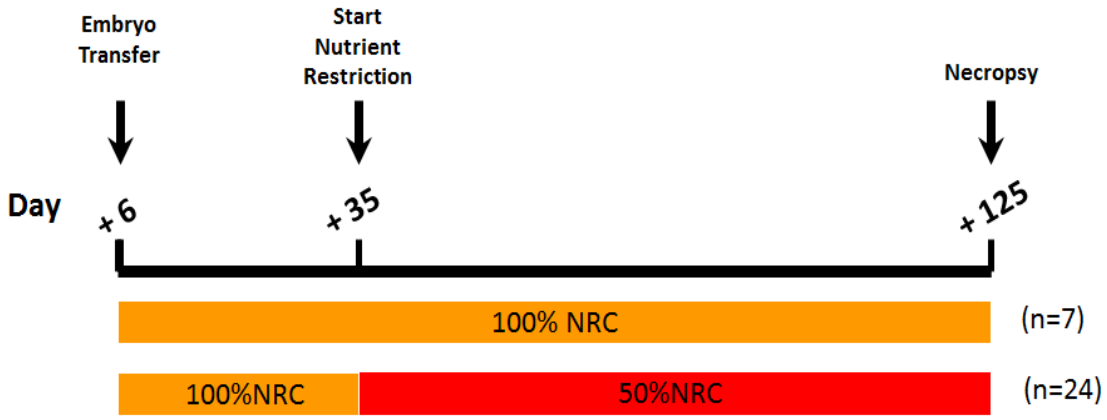


Figure 3.1. Experimental model. Singleton pregnancies were generated in mature Suffolk ewes of similar weight and body composition (n=31) via embryo transfer. Embryos were collected from superovulated Suffolk ewes mated to genetically similar sires. Pregnant ewes were individually housed from Day 28 to 125 of gestation. Beginning on Day 35 and continuing to necropsy, ewes were fed 50% (n=24) or 100% (n=7) of NRC requirements. Ewes fed 100% NRC are identified as control.

RNA Extraction and Affymetrix GeneChip Array Analysis

As previously conducted by Spencer et al., total RNA was extracted from approximately 100 mg of placentome using TRIzol reagent as per manufacturer's instructions (Invitrogen) and by column DNAse treatment, and clean-up was performed (Qiagen)[137]. 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. 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.

Hybridization probes for the Affymetrix GeneChip Bovine and Ovine Genome 1.0 ST Arrays were prepared using 10 mg of total RNA and the One-Cycle Target Labeling and Control Reagent package. The GeneChip Hybridization, Wash, and Stain Kit and a Fluidic Station 450 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.

Array output was normalized via the robust multiarray method, and probe sets were filtered based on expression calls, as previously described [137, 138]. 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.

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 [139-141]. DAVID was utilized, as previously described, to annotate biological themes in response to dietary treatment [141]. All differentially expressed genes identified to be both significantly ($P \leq 0.05$) and numerically (1.5-fold change or greater) different and homologous to a known and annotated human gene were used 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 as identified through 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 [141].

RNA Isolation and Real-Time PCR

Total cellular RNA was isolated from frozen placentome using Trizol reagent (Gibco-BRL) according to the manufacturer's instructions. The quantity and quality of total RNA was determined by spectrometry and denaturing agarose gel electrophoresis, respectively. Total RNA from each sample was reverse transcribed in a total reaction volume of 20 μl. Briefly, total RNA (4 μg) was combined with primer mix containing oligo (dT) primer (0.2 μg/ml), random hexamer primer (300 μg/ml) (Invitrogen), and dNTP mix (10 mM each), and incubated at 65°C for 5 min. A reverse transcriptase mix containing 5× first-strand buffer, 100 mM DTT (dithiothreitol) and SuperScript II reverse transcriptase (Invitrogen) was added to the reaction, and reverse transcription was performed under the following conditions: 25°C for 10 min, 42°C for 60 min, and 70°C for 5 min. Control reactions in the absence of reverse transcriptase were prepared for each sample to test for contamination with genomic DNA. The resulting cDNA was stored at −20°C for further analysis.

Real-time PCR was performed using ABI prism 7900HT system (Applied Biosystems) with Power SYBR Green PCR Master Mix (Applied Biosystems). Specific oligonucleotide primers were designed by Oligo 5 program (Molecular Biology Insights, Inc.) (see Supplemental Table 3.1). Primer specificity and efficiency (−3.4 > slope > −3.2) were confirmed using a test amplification run. Each individual sample was run in

triplicate under the following conditions: 50°C for 2 min; 95°C for 10 min; 95°C for 15 sec and 60°C for 1 min for 40 cycles. A dissociation curve was generated at the end of the amplification to ensure that a single product was amplified. PCR without template or template substituted with total RNA was used as a negative control to verify the experimental results. The threshold line was set in the linear region of the plots above the baseline noise, and threshold cycle (Ct) values were determined as the cycle number at which the threshold line crossed the amplification curve. Ovine *TUB* was used as the reference gene. The relative quantification of gene expression across treatments was evaluated by using the comparative CT method as previously described [142].

Table 3.1. Primer Sequences for Real-Time Quantitative PCR

Gene Symbol	**Gene Name**	**Accession Number**	**Primer Sequence 5' to 3'**
AGR2	Anterior Gradient Homolog 2	NM_001040500	For: CCTCTCTCCTGATGGCCAGTAT Rev: CAGTCAGGGATGGGTCAACAA
CDX2	Caudal Type Homeobox 2	XM_004012288.1	For: GGGCCCCAAGTGAAAACC Rev: ACGCTGGTGGTCGGTGTA
RUVBL2	Reptin	AF155138	For: GCTTGGTGTGCCGGAAAC Rev: CCCGCTTGATGTCATCCA

In Situ Hybridization Analysis

Localization of *AGR2* mRNAs in the ovine uterus and placentomes were determined by radioactive *in situ* hybridization as described previously [143]. Radiolabeled antisense and sense cRNA probes were generated by in vitro transcription using linearized partial plasmid cDNA templates, RNA polymerases and [α-^{35}S]UTP. Deparaffinized, rehydrated, and deproteinated uterine tissue sections were hybridized with radiolabeled antisense or sense cRNA probes. After hybridization, washing, and ribonuclease A digestions, slides were dipped in NTB-2 liquid photographic emulsion (Kodak, Rochester, NY) and exposed at 4C for 4 to 6 weeks. Slides were developed in Kodak D-19 developer, counterstained with Gill's hematoxylin (Fisher Scientific, Pittsburgh, PA) and then dehydrated through a graded series of alcohol to xylene. Coverslips were then affixed with Permount (Fisher). Images of representative fields were recorded under brightfield or darkfield illumination using a Nikon Eclipse 1000 photomicroscope (Nikon Instruments Inc., Lewisville, TX) fitted with a Nikon DXM1200 digital camera.

Western Blot Analysis

Protein content of concentrated flushes were determined using a Bradford protein assay (Bio-Rad, Hercules, CA) with BSA as the standard. Thirty micrograms of uterine flush proteins were denatured and separated by 15% SDS-PAGE and transferred to nitrocellulose as described previously[144]. Western blot analyses were conducted as described previously[145]. Blots were incubated with primary antibody overnight at 4 C, rinsed for 30 minutes at room temperature with TBST, incubated with the appropriate

peroxidase-conjugated secondary antibody for 1 hour at room temperature, and then rinsed again for 30 minutes at room temperature with TBDT. Immunoreactive proteins were detected using enhanced chemilluminescence (Amhersham Pharmacia Biotech) according to the manufacturer's recommendations. Immunoreactive AGR2 was detected using anti-AGR2 antibody produced in rabbit (Sigma-Aldrich, St. Louis.MO) at a 1:250 dilution.

Statistical Analysis

Regression analyses were conducted using the PROC-MIXED procedures of the Statistical Analysis System (SAS Institute, Cary, NC), as described previously [9]. 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 the 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. 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.

Results

Early Pregnancy

To determine temporal and spatial changes in expression of *AGR2* mRNAs during ovine pregnancy we conducted in situ hybridization on endometrial sections on days 10 through 16 of the estrous cycle and days 10 through 20 of pregnancy. *AGR2* mRNA is low to undetectable on day 10 of cyclicity and pregnancy. Its expression which is primarily localized to the LE and sGE increases to day 12 of cyclicity and pregnancy.

Interestingly, *AGR2* mRNA expression is greater on day 14 of pregnancy than the cycle and this continues to day 16 with expression detectable in the middle GE as well. *AGR2* mRNA expression remains abundant on days 18 and 20 of pregnancy, but is not present in the elongating conceptus on these days (Figure 3.2). To determine protein quantification during early pregnancy AGR2 (22 kDa) was detected in uterine flushings recovered from ewes on days 10, 12, 14, and 16 post estrus (Figure 3.3). AGR2 exhibits an effect by day and pregnancy status. AGR2 protein significantly increases on day 14 and 16 of pregnancy compared to cyclic ewes ($P<0.001$).

Because *AGR2* mRNA and protein expression were upregulated in the presence of an elongating conceptus we next investigated the effects of individual conceptus products on the regulation of *AGR2* mRNA expression. Results indicate that infusion of PG, CORT, or IFNT alone did not increase endometrial *AGR2* mRNA expression, however, when PG and CORT, CORT and IFNT, or PG and IFNT were administered in combination, *AGR2* mRNA expression increased to levels observed in control day 14 pregnant ewes ($P<0.05$) (Figure 3.4).

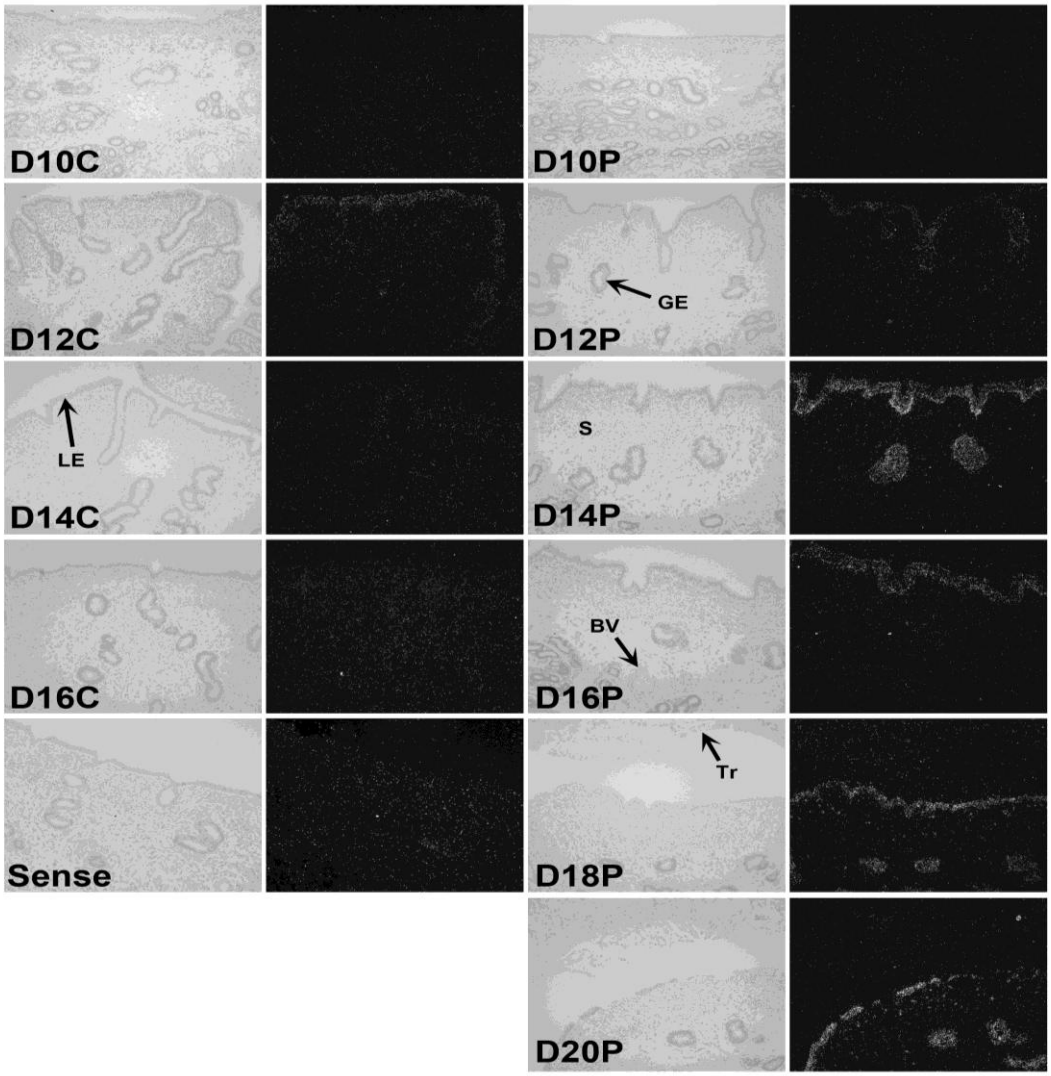
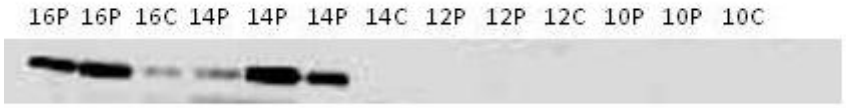


Figure 3.2. In situ localization of *AGR2* mRNA in the cyclic and early pregnant ovine uterus. Cross-sections of the uterus were hybridized with radiolabeled antisense or sense cRNA probes and digested with Rnase A. Protected transcripts were visualized by liquid emulsion autoradiography. Developed slides were counterstained lightly with hematoxylin, and photomicrographs recorded under bright-field or dark-field illumination. Legend: GE, glandular epithelium; LE, luminal epithelium; S, stroma; Tr, trophectoderm; BV, blood vessels.

A:



B:

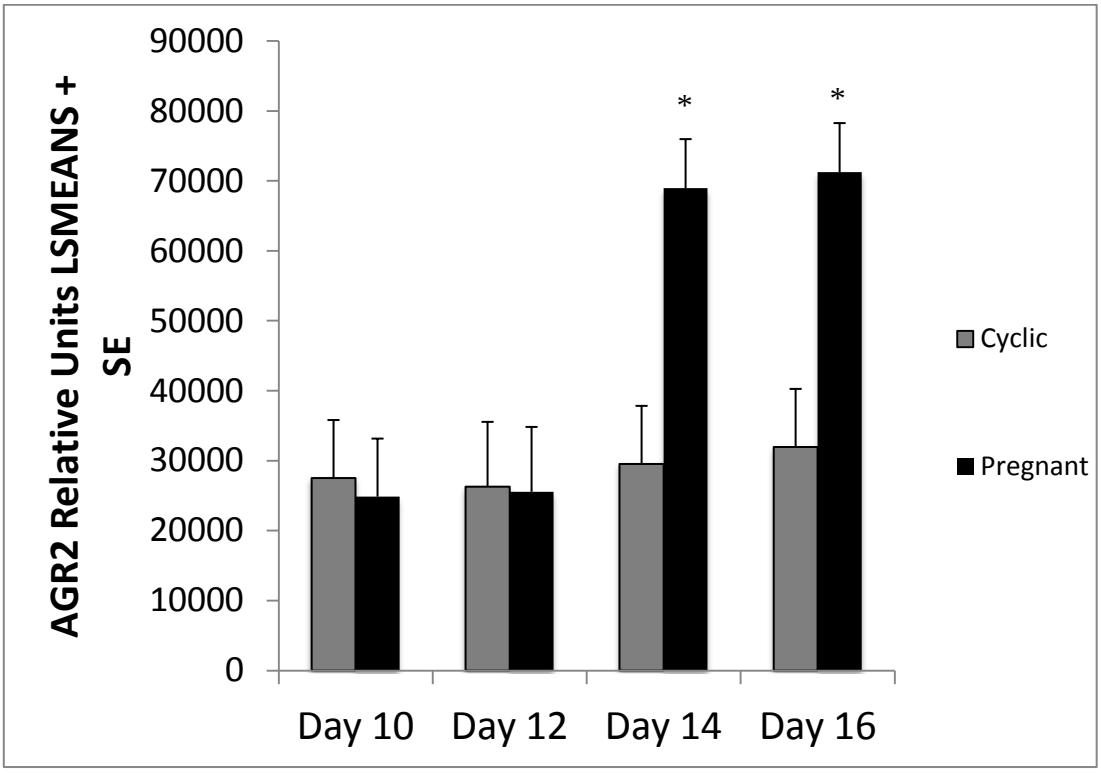


Figure 3.3. (A) Effect of day and pregnancy status on levels of AGR2 protein detected in uterine flushes. Flushes were recovered from ewes on days 10, 12, 14, and 16 post-estrus as measured by Western blot. Differences ($P < .001$) are denoted with an asterisk (*). (B) Quantification of blot density.

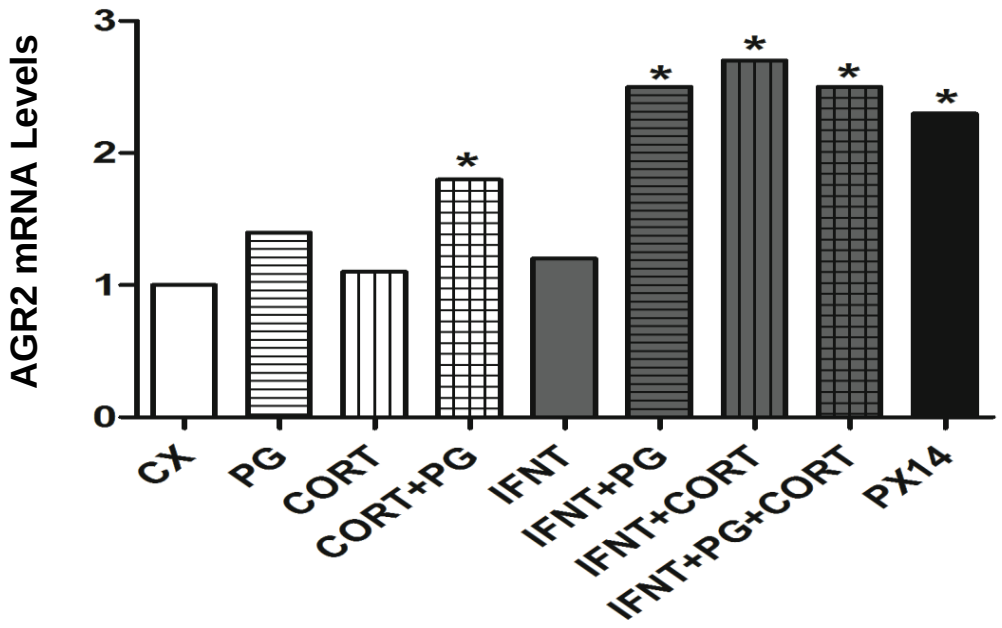


Figure 3.4. Effects of intrauterine treatment on expression of *AGR2* mRNA in the endometrium of cyclic sheep. Cyclic ewes received intrauterine infusions of vehicle as a CX, CORT (cortisol), PG (prostaglandin), CORT and PG, recombinant ovine IFNT, or IFNT and PG from day 10 to day 14 after estrus. Endometrial mRNA abundance was measured by real-time PCR and expressed as fold change relative to CX ewes. Differences ($P < .05$) are denoted with an asterisk (*) for the different treatments determined by orthogonal contrast.

Late Pregnancy

The role of AGR2 in ovine late pregnancy was also investigated. *In situ* hybridization was used to localize *AGR2* mRNA in the both the placentome and intercaruncular endometrium during pregnancy. During late gestation (study 3) *AGR2* mRNA was detected in the stratum compactum and GE of the uterine endometrium and this expression pattern remains present through Day 120 of pregnancy (Figure 3.5). In the placentome, real-time PCR confirmed *AGR2* expression throughout the time course of pregnancy (Day 50, 75, 100, and 125) in both adequately fed and nutrient restricted ewes (Figure 3.6). *In situ* hybridization showed *AGR2* mRNA is abundantly expressed in the caruncular crypts of the placentome (Figure 3.7).

Additionally, downstream targets of AGR2 were evaluated in the ovine placentome. Reptin, recently validated as an interacting protein with AGR2 in human cells plays a role in driving the stability of the AGR-reptin complex[110]. Reptin expression did not differ on Days 50 and 75 of gestation, however, Reptin expression was lower in placentomes from adequately fed ewes on Day 125 of pregnancy compared to Day 100. This can be compared to Reptin expression found in the placentomes from nutrient restricted ewes which remained elevated on Day 125 (Figure 3.8). Additionally, CDX2 (caudal related homeobox 2) is also found to be an interacting factor with AGR2. CDX2 is known to regulate trophectoderm differentiation as well as the trophoblast specific gene interferon tau (IFNT) in ruminants[117]. Thus, CDX2 was also evaluated in the ovine placentome throughout the described time course of pregnancy. *CDX2* expression was reduced in the ewes receiving 100% NRC compared to the 50% NRC

fed ewes on day 50 of pregnancy ($P<0.05$). Specifically, *CDX2* expression in placentomes of nutrient restricted ewes decreased on Day 125 compared to expression levels on Days 50 and 75. *CDX2* levels increased between days 50 to 100 and then decreased between days 100 to 125 (Figure 3.9).

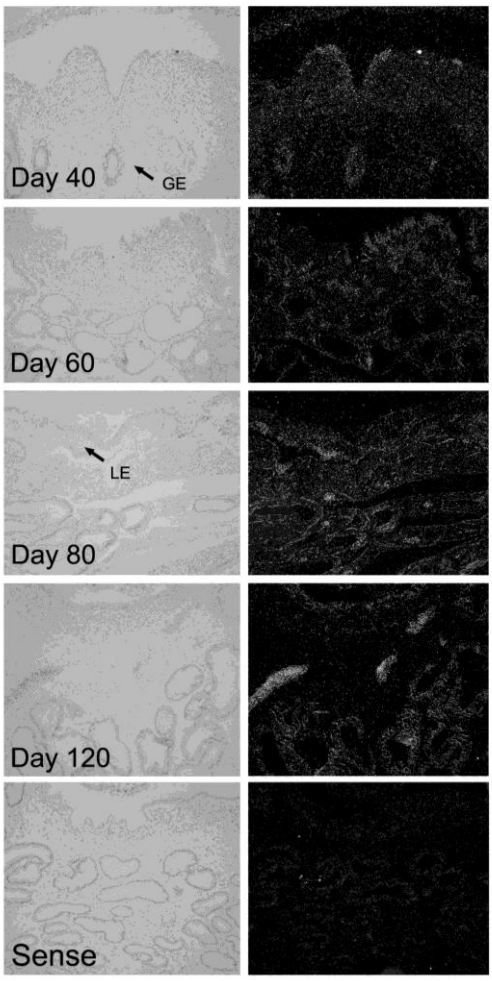


Figure 3.5. In situ localization of *AGR2* mRNA in the ovine uterus of pregnant ewes days of late pregnancy. Cross-sections of the uterus were hybridized with radiolabeled antisense or sense cRNA probes and digested with Rnase A. Protected transcripts were visualized by liquid emulsion autoradiography. Developed slides were counterstained lightly with hematoxylin, and photomicrographs recorded under bright-field or dark-field illumination. Legend: GE, glandular epithelium; LE, luminal epithelium

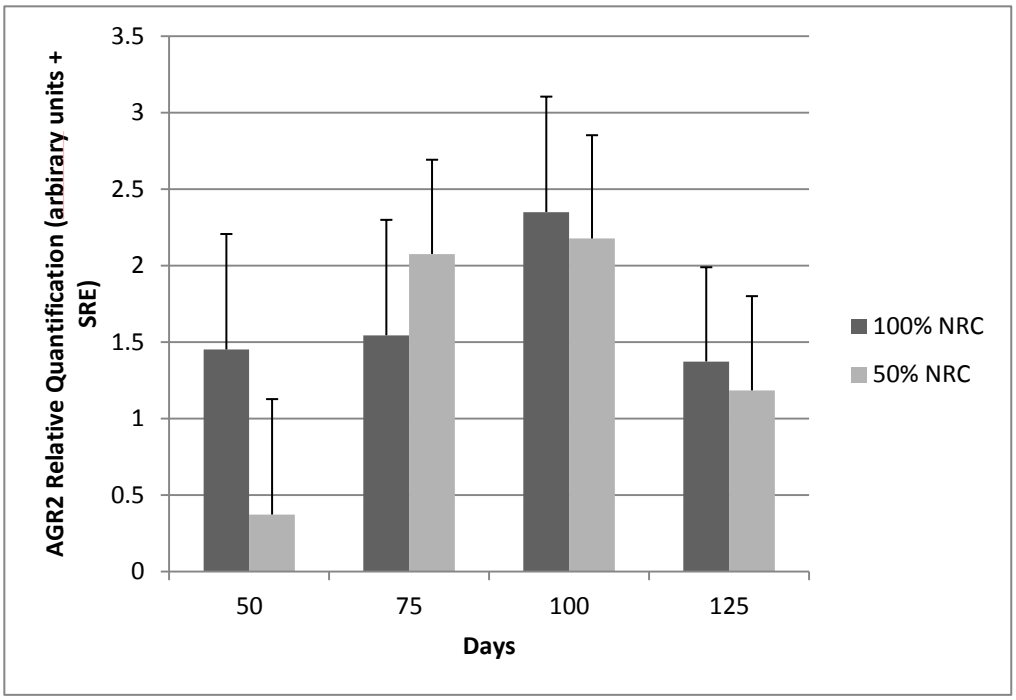


Figure 3.6. Steady state mRNA levels of *AGR2* in the ovine placentome throughout the time course of pregnancy. Ewes received either 100% NRC or 50% NRC on days 50, 75, 100, and 125 gestation. No significant changes were observed in AGR2 expression regardless of day and nutrient status.

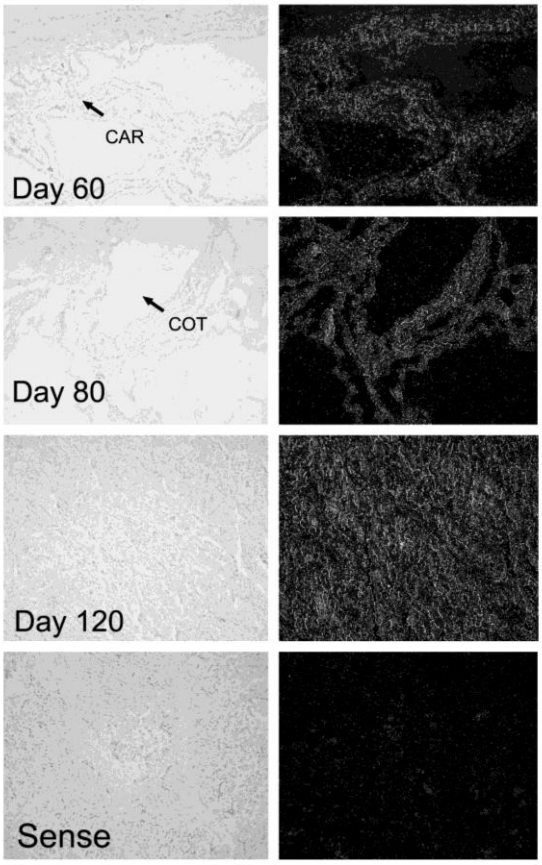


Figure 3.7. In situ localization of *AGR2* mRNA in the ovine placentome days of late pregnancy. Cross-sections of the placentome were hybridized with radiolabeled antisense or sense cRNA probes and digested with Rnase A. Protected transcripts were visualized by liquid emulsion autoradiography. Developed slides were counterstained lightly with hematoxylin, and photomicrographs recorded under bright-field or dark-field illumination. Legend: COT, Cotyledon; CAR, Caruncle.

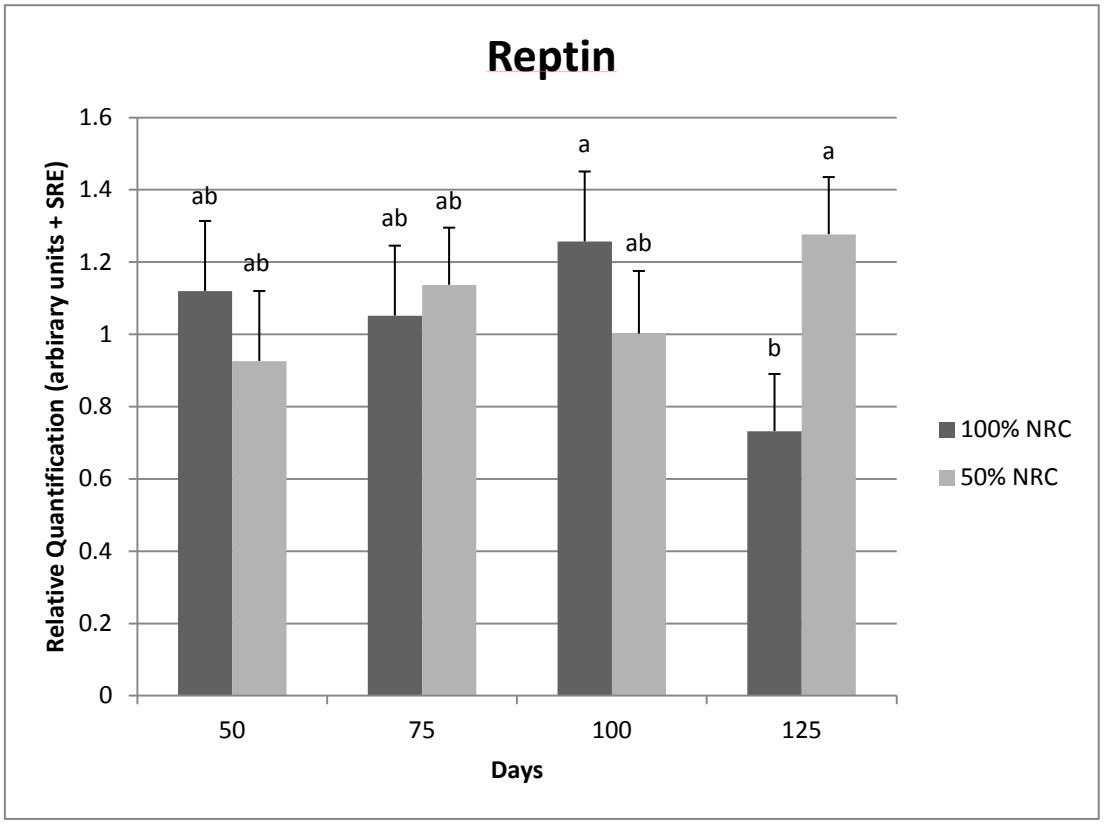


Figure 3.8. Steady state mRNA levels of *Reptin* in the ovine placentome throughout the time course of pregnancy. Ewes received either 100% NRC or 50% NRC on days 50, 75, 100, and 125 gestation. No significant changes were observed in Reptin expression on days 50 and 75. However, Reptin expression decreased in the adequately fed ewes on days 125 compared to day 100. Reptin expression remained elevated in the nutrient restricted ewes on day 125 gestation

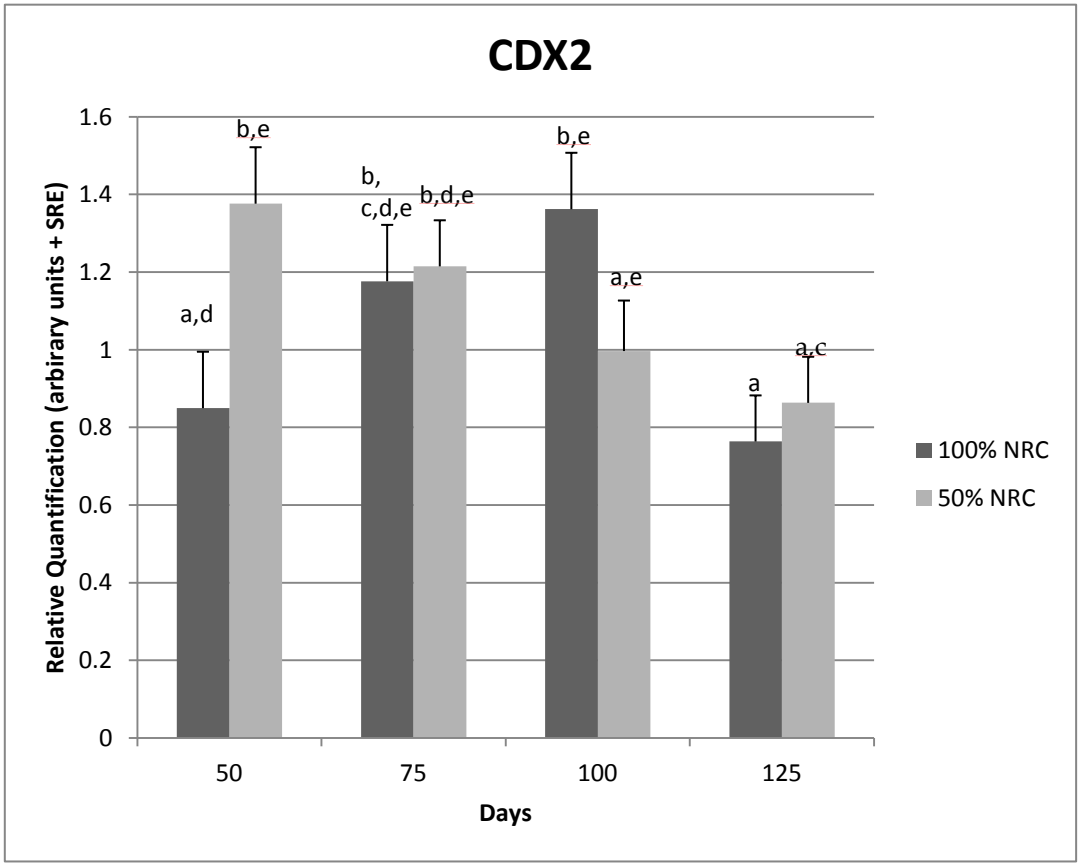


Figure 3.9. Steady state mRNA levels of *CDX2* in the ovine placentome throughout the time course of pregnancy. Ewes received either 100% NRC or 50% NRC on days 50, 75, 100, and 125 gestation. On day 50 CDX2 expression was significantly lower in the placentomes of the adequately fed ewes compared to the nutrient restricted ewes. Expression of CDX2 decreased on day 125 compared to expression levels seen on day 50 and 75 in the nutrient restricted ewes. Conversely, CDX2 expression increased from day 50 to 100 and decreased from day 100 to 125 in the 100% NRC fed ewes.

Using the model previously described by Dunlap et al. a study was completed to identify a population of IUGR and non-IUGR offspring from a similar cohort of nutrient-restricted ewes as a first step to assess adaptive mechanisms of placental nutrient transport [9]. This study tested the hypothesis that maternal nutrient restriction results in differential or adaptive placental transport of critical nutrients resulting in both IUGR and non-IUGR offspring[9]. Therefore, this study utilized an Affymetrix Bovine/Ovine Gene 1.0 ST array to capitalize on natural population variance in response to nutrient restriction and to identify novel factors regulating placental function and fetal growth. AGR2 was among the novel genes identified to be differentially expressed by the microarray. Steady state mRNA levels of *AGR2* were reduced ($P<0.05$) in placentomes from NR ewes producing non-IUGR fetuses compared to NR ewes producing IUGR fetuses (Figure 4.1). *In situ* hybridization showed localization of *AGR2* mRNA in the placentome. Control fed ewes as well as NR IUGR exhibited abundant expression of *AGR2* while *AGR2* expression lessens and is more diffuse in the NR non-IUGR placentome (Figure 4.2). Additionally genes known to interact with AGR2 were also evaluated using this research model. It was found that *Reptin* expression in the placentome at day 125 gestation was higher ($P<0.05$) in the NR non-IUGR compared to the NR IUGR (Figure 4.3). *CDX2* expression had a tendency to follow this same pattern in which expression is higher in the NR non-IUGR compared to the NR IUGR ($P<0.1$) (Figure 4.4).

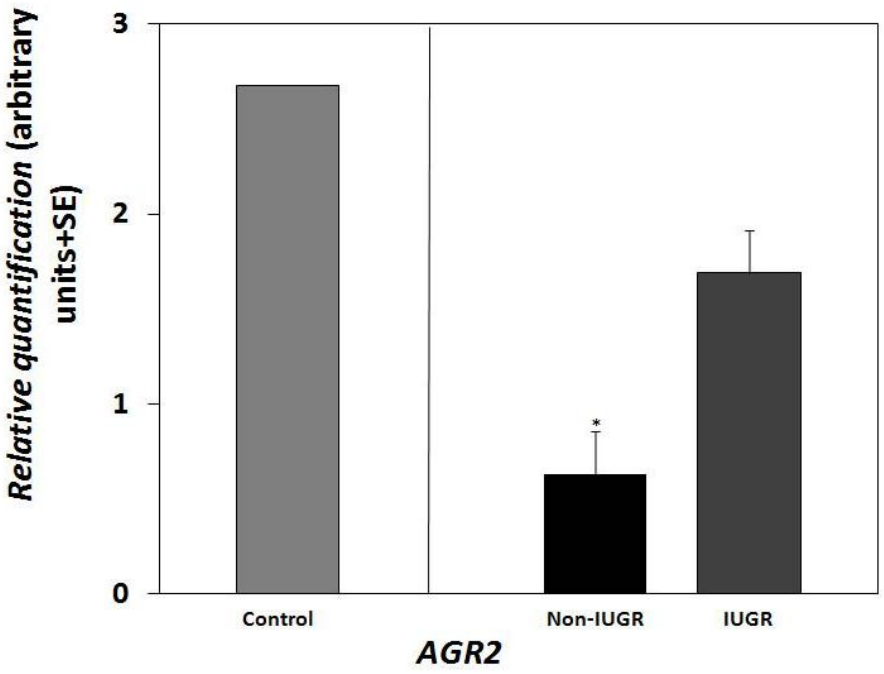


Figure 4.1. Steady state mRNA levels of *AGR2* in the ovine placentome of nutritionally manipulated ewes. Levels of *AGR2* were reduced($P<0.05$) in placentomes from ewes producing NR non-IUGR fetuses compared to placentomes from ewes producing NR IUGR fetuses.

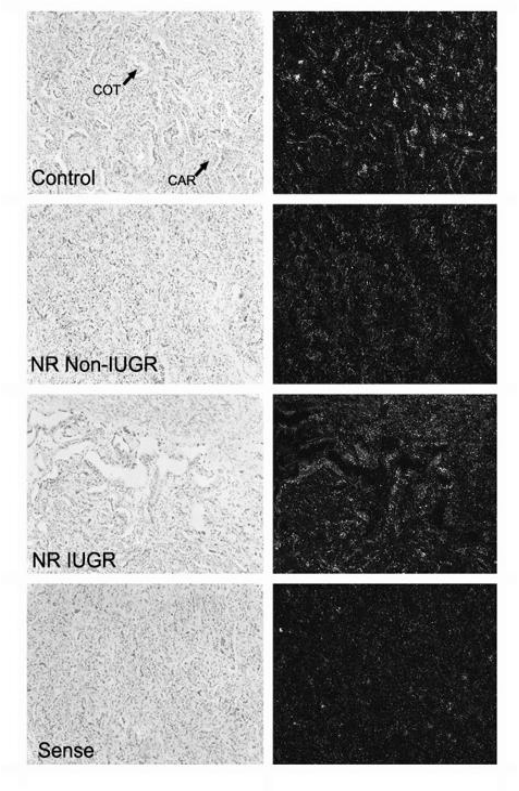


Figure 4.2. In situ localization of *AGR2* mRNA in the ovine placentome of nutritionally manipulated ewes. Cross-sections of the placentome were hybridized with radiolabeled antisense or sense cRNA probes and digested with Rnase A. Protected transcripts were visualized by liquid emulsion autoradiography. Developed slides were counterstained lightly with hematoxylin, and photomicrographs recorded under bright-field or dark-field illumination. Legend: COT, Cotyledon; CAR, Caruncle

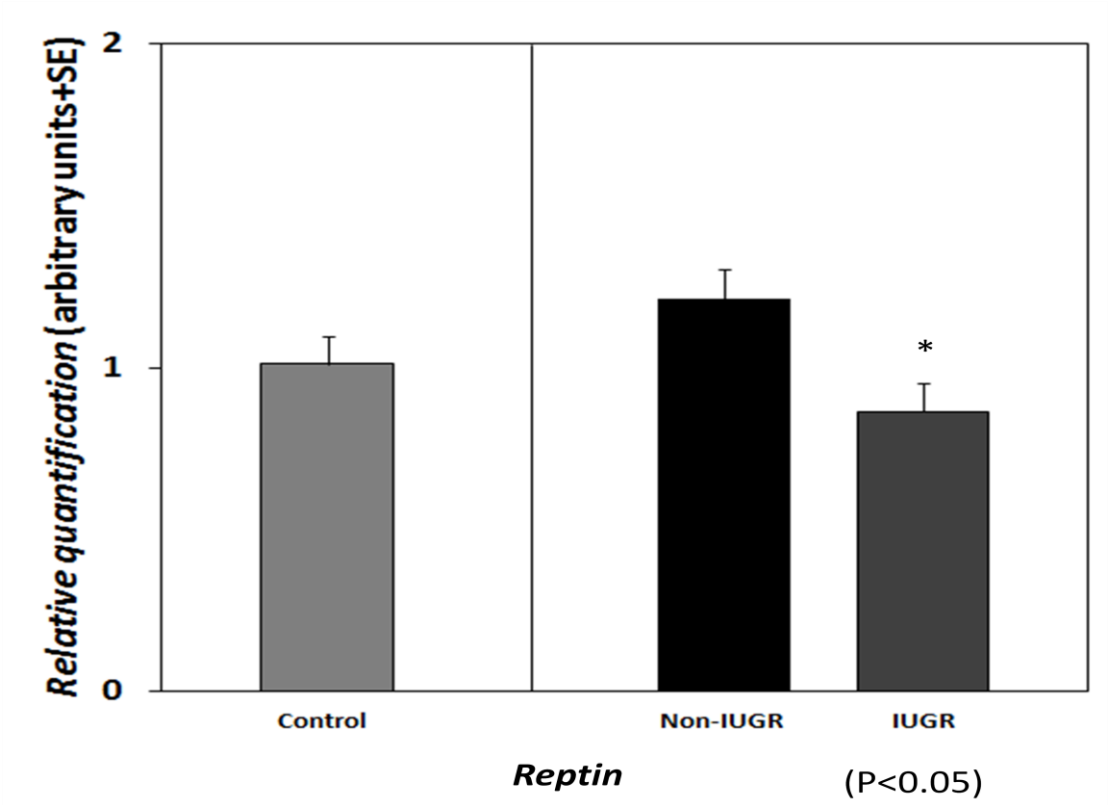


Figure 4.3. Steady state mRNA levels of *Reptin* in the ovine placentome of nutritionally manipulated ewes. Levels of *Reptin* were reduced($P<0.05$) in placentomes from ewes producing NR IUGR fetuses compared to placentomes from ewes producing NR Non-IUGR fetuses.

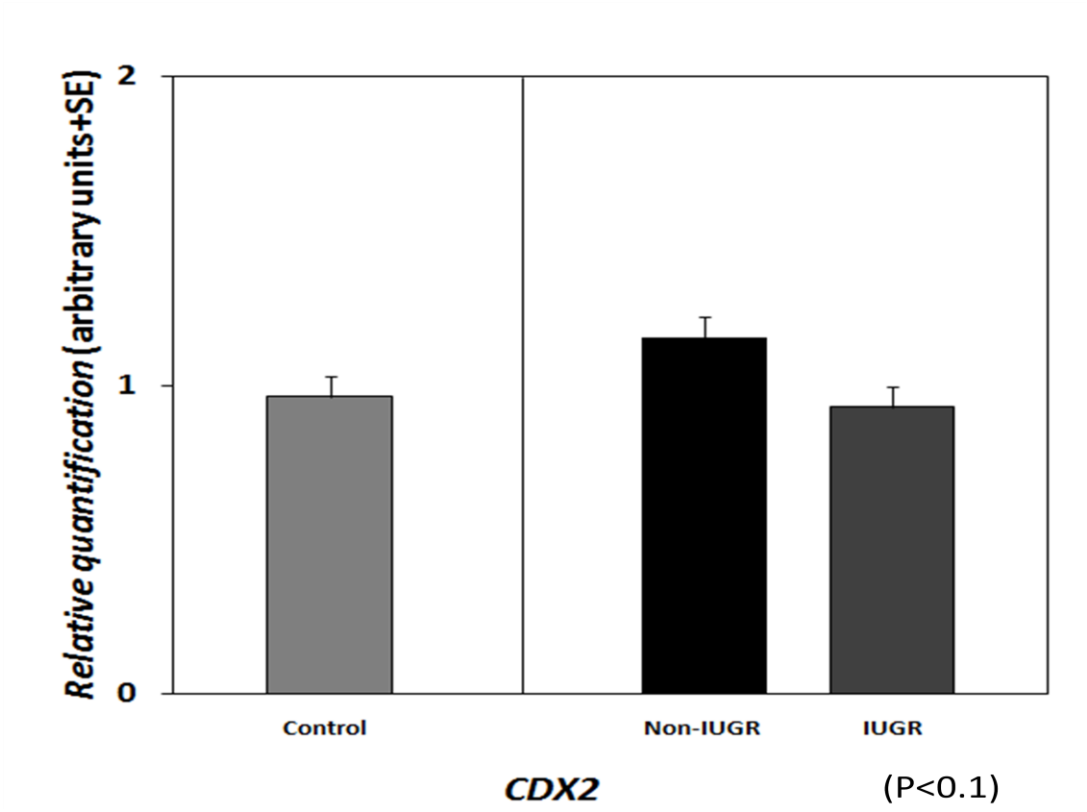


Figure 4.4. Steady state mRNA levels of *CDX2* in the ovine placentome of nutritionally manipulated ewes. Levels of *CDX2* had a tendency to be reduced (P<0.1) in placentomes from ewes producing NR IUGR fetuses compared to placentomes from ewes producing NR Non-IUGR fetuses.

Discussion

Results of the present study include examination of the AGR2 pathway in the ovine uterus and placenta throughout gestation in order to establish temporal and spatial expression. These studies provide a platform for which future mechanistic studies to examine function can be designed. Results demonstrate AGR2 to be hormonally regulated at the level of the CL and by conceptus derived products secreted into the uterine lumen suggesting a role in migration, proliferation and growth of the conceptus. Additionally, Hong et al., found AGR2 contributed to growth and angiogenesis in glioblastomas[146]. Recombinant AGR2 was found to induce human umbilical vein endothelial cell (HUVEC) migration and tube formation, this affect was abrogated by anti-AGR2 neutralizing antibodies[146]. Thus, these findings indicate AGR2 is a proliferative agent that stimulates angiogenesis. These known functions are critical for pregnancy success which suggests AGR2 could have similar actions on the conceptus. Further studies analyzing AGR2's role in growth and interaction with the conceptus are needed.

In order to determine spatial and temporal changes in AGR2 expression, representative photomicrographs of *in situ* hybridization in cyclic and pregnant uterine tissue indicate that *AGR2* mRNA expression in the LE, sGE, and middle GE increases from day 14 to 20 of early pregnancy. Interestingly, *AGR2* mRNA is not detected in the early elongating conceptus. Results further demonstrate that cyclic ewes receiving combined intrauterine infusions of conceptus derived hormones increases endometrial expression *AGR2* mRNA. Collectively, results of the present studies coupled with

previously published data indicate that endometrial *AGR2* expression is acutely controlled by both ovarian and conceptus derived hormones to alter the uterine milieu during early pregnancy. It is well established that blastocyst growth and development into an elongated conceptus is critical for pregnancy recognition and implantation in ruminants[147]. Migration ultimately allows for more equitable allocation of uterine resources and elongation ensures increased surface area of the uterus available to the conceptus for interactions that support pregnancy [148]. Furthermore AGR2 has garnered major scientific interest in regards to cancer and tumor development. Recent cell culture experiments have demonstrated that when cultured under serum depleted conditions and/or hypoxia, AGR2 was activated to promote angiogenesis, motility and invasion[121]. AGR2 thus may have similar roles in stimulating growth and development of the conceptus which is critical for pregnancy success. Elucidating the exact role of AGR2 in regulating and/or stimulating conceptus development is of critical importance.

Using the model previously described to investigate adaptive mechanisms of placental nutrient transport in both IUGR and non-IUGR offspring, AGR2 was found as a differentially expressed gene potentially necessary in adaptive placental development. AGR2 is induced by various stresses such as hypoxia, serum depletion and ER stress [149]. Ewes that experienced nutrient restriction induced stress and subsequently produced IUGR offspring showed elevated placentomal levels of AGR2. These findings indicate, NR ewes that produce normal fetal weight offspring may, through reduced

levels of AGR2, exhibit an adaptive placental response to maximize fetal growth in response to stress.

Although mainly localized in the endoplasmic reticulum, AGR2 has the ability to play other intracellular roles such as inducing EGF receptor ligand amphiregulin (AREG)[105], interacting with AAA+ protein Reptin [110], and has also been linked with the expression of the transcription factor caudal related homeobox 2 (CDX2)[100]. Recently, a protein named Reptin was identified as an AGR2 binding protein in a yeast two-hybrid screen and was validated as an interacting protein with AGR2 in human cells [110]. Reptin belongs to a family of proteins known as AAA+ATPases (ATPases associated with various cellular activity) [111]. AGR2 uses a divergent-binding loop to bind to Reptin and then Reptin uses two allosterically interacting ATP binding motifs to control its binding activity toward AGR2[110]. Real- time PCR confirmed *Reptin* expression in the placentome throughout the time course of pregnancy. However, its expression pattern differed from that observed for AGR2 indicating Reptin is not a downstream product of AGR2 signaling in the sheep placentome.

Additional studies have also linked AGR2 with the expression of the transcription factor caudal related homeobox 2 (CDX2). The induction of CDX2 expression by AGR2 stimulation is interesting from a reproductive context because CDX2 regulates trophectoderm differentiation as well as the trophoblast specific gene interferon tau (IFNT) in ruminants [116]. CDX2 is isolated to the trophectoderm and is essential for ensuring correct segregation of the Inner Cell Mass and Trophectoderm cell lineages in blastocysts as CDX2 ensures the repression of Oct4 and Nanog in the trophectoderm

[119]. Specifically, CDX2 has been detected in day 15 and 17 ovine conceptuses [150] and is involved in histone modifications of the IFNT gene [118]. Thus as a downstream target of AGR2, CDX2 may play a role in placental function and conceptus development. Interestingly, CDX2 expression mirrored the pattern of AGR2 expression in the adequately fed ewes. Expression pattern, however, was markedly different in the nutrient restrict (50% NRC) fed ewes, indicating CDX2 expression is most likely differentially regulated in the cases of gestational nutrient restriction. Results of the present study demonstrate localization for AGR2 during various stages of pregnancy in the ewe. Collectively, results suggest that AGR2 is regulated at the level of the CL and by conceptus derived products in early pregnancy. In nutrient scarce conditions a reduction in AGR2 may be necessary for enhanced placental function. Additionally, by analyzing genetic downstream targets of AGR2 we have found that Reptin and CDX2 may also play a role in stimulating placental function however it is not clear whether these genes are fully or partially regulated by AGR2. This study is foundational in establishing AGR2 as a relevant factor throughout gestation, still future research is needed to further evaluate the mechanisms by which AGR2 plays a role in ovine pregnancy.

CHAPTER IV

SUMMARY AND DIRECTION OF FUTURE RESEARCH

Extensive study has been conducted regarding AGR2's regulation of tumor development. Pregnancy can be likened to the hypoxic conditions necessary to facilitate tumor growth. However, to our knowledge, this study is the first to evaluate the expression and regulation of AGR2 in ovine pregnancy. Collectively, results suggest AGR2 is likely to play a role in conceptus development and endometrial function. In nutrient scarce conditions a reduction in the proliferative actions of AGR2 may be necessary for enhanced placental function. This novel finding has potential in further understanding the developmental events necessary for embryonic survival as well features that govern pregnancy success throughout gestation. Future studies will be needed to further evaluate the signaling cascades elicited by this gene and continue to define the role of AGR2 throughout pregnancy.

REFERENCES

1. Satterfield MC, Bazer FW, Spencer TE. Progesterone regulation of preimplantation conceptus growth and galectin 15 (LGALS15) in the ovine uterus. Biol Reprod 2006; 75:289-296.

2. Wu G, Bazer FW, Satterfield MC, Li X, Wang X, Johnson GA, Burghardt RC, Dai Z, Wang J, Wu Z. Impacts of arginine nutrition on embryonic and fetal development in mammals. Amino Acids 2013; 45:241-256.

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. 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.

6. 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.

7. 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.

8. 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.

9. Dunlap KA, Greff, S.M., McKnight, J.R., Keith, A.B., Satterfield, M.C. Adaptive response to maternal nutrient restriction alters nutrient availability and prevents growth restriction of the ovine f etus. In: Texas A&M University, College Station TX. Unpublished.

10. Barry JS, Anthony RV. The pregnant sheep as a model for human pregnancy. Theriogenology 2008; 69:55-67.

11. Wilcox AJ, Weinberg CR, O'Connor JF, Baird DD, Schlatterer JP, Canfield RE, Armstrong EG, Nisula BC. Incidence of early loss of pregnancy. N Engl J Med 1988; 319:189-194.
12. Diskin MG, Murphy JJ, Sreenan JM. Embryo survival in dairy cows managed under pastoral conditions. Anim Reprod Sci 2006; 96:297-311.

13. Dixon AB, Knights M, Winkler JL, Marsh DJ, Pate JL, Wilson ME, Dailey RA, Seidel G, Inskeep EK. Patterns of late embryonic and fetal mortality and association with several factors in sheep. J Anim Sci 2007; 85:1274-1284.

14. 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.

15. Spencer TE, Johnson GA, Bazer FW, Burghardt RC. Implantation mechanisms: insights from the sheep. Reproduction 2004; 128:657-668.

16. Guillomot M, Flechon JE, Wintenberger-Torres S. Conceptus attachment in the ewe: an ultrastructural study. Placenta 1981; 2:169-182.

17. Igwebuike UM. A review of uterine structural modifications that influence conceptus implantation and development in sheep and goats. Anim Reprod Sci 2009; 112:1-7.

18. Bazer FW, Spencer TE, Johnson GA, Burghardt RC, Wu G. Comparative aspects of implantation. Reproduction 2009; 138:195-209.

19. Chavatte-Palmer P, Guillomot M. Comparative implantation and placentation. Gynecol Obstet Invest 2007; 64:166-174.

20. Bazer FW, Burghardt RC, Johnson GA, Spencer TE, Wu G. Interferons and progesterone for establishment and maintenance of pregnancy: interactions among novel cell signaling pathways. Reprod Biol 2008; 8:179-211.

21. Bazer FW. Pregnancy recognition signaling mechanisms in ruminants and pigs. J Anim Sci Biotechnol 2013; 4:23.

22. Spencer TE, Bazer FW. Uterine and placental factors regulating conceptus growth in domestic animals. J Anim Sci 2004; 82 E-Suppl:E4-13.

23. Bazer FW, Wu G, Johnson GA, Kim J, Song G. Uterine histotroph and conceptus development: select nutrients and secreted phosphoprotein 1 affect mechanistic target of rapamycin cell signaling in ewes. Biol Reprod 2011; 85:1094-1107.

24. 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.

25. Wooding FB. The role of the binucleate cell in ruminant placental structure. J Reprod Fertil Suppl 1982; 31:31-39.

26. Burghardt RC, Johnson GA, Jaeger LA, Ka H, Garlow JE, Spencer TE, Bazer FW. Integrins and extracellular matrix proteins at the maternal-fetal interface in domestic animals. Cells Tissues Organs 2002; 172:202-217.

27. Spencer TE, Bazer FW. Temporal and spatial alterations in uterine estrogen receptor and progesterone receptor gene expression during the estrous cycle and early pregnancy in the ewe. Biol Reprod 1995; 53:1527-1543.

28. Carson DD, Bagchi I, Dey SK, Enders AC, Fazleabas AT, Lessey BA, Yoshinaga K. Embryo implantation. Dev Biol 2000; 223:217-237.

29. Johnson GA, Bazer FW, Jaeger LA, Ka H, Garlow JE, Pfarrer C, Spencer TE, Burghardt RC. Muc-1, integrin, and osteopontin expression during the implantation cascade in sheep. Biol Reprod 2001; 65:820-828.

30. Lasky LA, Singer MS, Dowbenko D, Imai Y, Henzel WJ, Grimley C, Fennie C, Gillett N, Watson SR, Rosen SD. An endothelial ligand for L-selectin is a novel mucin-like molecule. Cell 1992; 69:927-938.

31. Johnson GA, Burghardt RC, Bazer FW, Spencer TE. Osteopontin: roles in implantation and placentation. Biol Reprod 2003; 69:1458-1471.

32. Johnson GA, Spencer TE, Burghardt RC, Bazer FW. Ovine osteopontin: I. Cloning and expression of messenger ribonucleic acid in the uterus during the periimplantation period. Biol Reprod 1999; 61:884-891.

33. Lewis SK, Farmer JL, Burghardt RC, Newton GR, Johnson GA, Adelson DL, Bazer FW, Spencer TE. Galectin 15 (LGALS15): a gene uniquely expressed in the uteri of sheep and goats that functions in trophoblast attachment. Biol Reprod 2007; 77:1027-1036.

34. Simmons RM, Erikson DW, Kim J, Burghardt RC, Bazer FW, Johnson GA, Spencer TE. Insulin-like growth factor binding protein-1 in the ruminant uterus: potential endometrial marker and regulator of conceptus elongation. Endocrinology 2009; 150:4295-4305.

35. Wooding FB, Chambers SG, Perry JS, George M, Heap RB. Migration of binucleate cells in the sheep placenta during normal pregnancy. Anat Embryol (Berl) 1980; 158:361-370.

36. Wooding FB. Role of binucleate cells in fetomaternal cell fusion at implantation in the sheep. Am J Anat 1984; 170:233-250.

37. Wooding FB, Hobbs T, Morgan G, Heap RB, Flint AP. Cellular dynamics of growth in sheep and goat synepitheliochorial placentomes: an autoradiographic study. J Reprod Fertil 1993; 98:275-283.

38. 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.

39. 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.

40. Rawn SM, Cross JC. The evolution, regulation, and function of placenta-specific genes. Annu Rev Cell Dev Biol 2008; 24:159-181.

41. Power MLaS, Jay. The Evolution of the Human Placenta. Baltimore: The Johns Hopkins University Press; 2012.

42. Grazul-Bilska AT, Johnson ML, Borowicz PP, Baranko L, Redmer DA, Reynolds LP. Placental development during early pregnancy in sheep: effects of embryo origin on fetal and placental growth and global methylation. Theriogenology 2013; 79:94-102.

43. Senger PL. Pathways to Pregnancy and Parturition. Current Conceptions, Inc; 2012: 294-313.

44. Sammin D, Markey B, Bassett H, Buxton D. The ovine placenta and placentitis-A review. Vet Microbiol 2009; 135:90-97.

45. Reynolds LP, Redmer DA. Angiogenesis in the placenta. Biol Reprod 2001; 64:1033-1040.

46. 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.

47. 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.

48. Risau W. Mechanisms of angiogenesis. Nature 1997; 386:671-674.

49. 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.

50. Redmer DA, Milne JS, Aitken RP, Johnson ML, Borowicz PP, Reynolds LP, Caton JS, Wallace JM. Decreasing maternal nutrient intake during the final third of pregnancy in previously overnourished adolescent sheep: effects on maternal nutrient partitioning and feto-placental development. Placenta 2012; 33:114-121.

51. Meschia G, Cotter JR, Breathnach CS, Barron DH. The diffusibility of oxygen across the sheep placenta. Q J Exp Physiol Cogn Med Sci 1965; 50:466-480.

52. Klagsbrun M. Regulators of angiogenesis: stimulators, inhibitors, and extracellular matrix. J Cell Biochem 1991; 47:199-200.

53. Reynolds LP, Killilea SD, Redmer DA. Angiogenesis in the female reproductive system. FASEB J 1992; 6:886-892.

54. Folkman J, Klagsbrun M. Angiogenic factors. Science 1987; 235:442-447.

55. Gospodarowicz D. Biological activities of fibroblast growth factors. Ann N Y Acad Sci 1991; 638:1-8.

56. Zheng J, Bird IM, Melsaether AN, Magness RR. Activation of the mitogen-activated protein kinase cascade is necessary but not sufficient for basic fibroblast growth factor- and epidermal growth factor-stimulated expression of endothelial nitric oxide synthase in ovine fetoplacental artery endothelial cells. Endocrinology 1999; 140:1399-1407.

57. 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.

58. Gluckman PD, Beedle AS, Hanson MA, Low FM. Human growth: evolutionary and life history perspectives. Nestle Nutr Inst Workshop Ser 2013; 71:89-102.

59. Maulik N MG. Nutrition, epigenetic mechanisms, and human disease. New York: CRC Press; 2011.

60. 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.

61. Seki Y, Williams L, Vuguin PM, Charron MJ. Minireview: Epigenetic programming of diabetes and obesity: animal models. Endocrinology 2012; 153:1031-1038.

62. Low FM, Gluckman PD, Hanson MA. Developmental plasticity and epigenetic mechanisms underpinning metabolic and cardiovascular diseases. Epigenomics 2011; 3:279-294.

63. Evertts AG, Zee BM, Garcia BA. Modern approaches for investigating epigenetic signaling pathways. J Appl Physiol (1985) 2010; 109:927-933.

64. 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.

65. McEvoy TG, Robinson JJ, Sinclair KD. Developmental consequences of embryo and cell manipulation in mice and farm animals. Reproduction 2001; 122:507-518.

66. 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.

67. 2002. Available from http://www.marchofdimes.com/aboutus/680_2204.asp

68. Moore NW, Shelton JN. Egg Transfer in Sheep. Effect of Degree of Synchronization between Donor and Recipient, Age of Egg, and Site of Transfer on the Survival of Transferred Eggs. J Reprod Fertil 1964; 7:145-152.

69. Gootwine E. Variability in the rate of decline in birth weight as litter size increases in sheep. Animal Science 2005; 81:393-398.

70. Gootwine E, Spencer TE, Bazer FW. Litter-size-dependent intrauterine growth restriction in sheep. Animal 2007; 1:547-564.

71. Kawamura T, Gilbert RD, Power GG. Effect of cooling and heating on the regional distribution of blood flow in fetal sheep. J Dev Physiol 1986; 8:11-21.

72. Picciano MF. Pregnancy and lactation: physiological adjustments, nutritional requirements and the role of dietary supplements. J Nutr 2003; 133:1997S-2002S.

73. Paterson J, R. Funston, and D. Cash. Forage quality influences beef cow performance. Feedstuffs 2002; 19:11–12.

74. 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.

75. Council NR. Nutrient Requirements of Sheep. Washington, DC: Natl. Acad. Press; 1985.

76. Luther JS, Redmer DA, Reynolds LP, Wallace JM. Nutritional paradigms of ovine fetal growth restriction: implications for human pregnancy. Hum Fertil (Camb) 2005; 8:179-187.

77. McCrabb GJ, Hosking, B. J., & Egan, A. R. Changes in the maternal body and feto-placental growth following various lengths of feed restriction during mid-pregnancy in sheep. Australian Journal of Agricultural Research 1992; 43:1429 – 1440.

78. Ronnenberg AG, Wang X, Xing H, Chen C, Chen D, Guang W, Guang A, Wang L, Ryan L, Xu X. Low preconception body mass index is associated with birth outcome in a prospective cohort of Chinese women. J Nutr 2003; 133:3449-3455.

79. 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.

80. Osgerby JC, Wathes DC, Howard D, Gadd TS. The effect of maternal undernutrition on the placental growth trajectory and the uterine insulin-like growth factor axis in the pregnant ewe. J Endocrinol 2004; 182:89-103.

81. 2000. Available from: http://education.umn.edu/ceed/publications/factfind/ff1991a.htm.

82. Davis L, Edwards H, Mohay H, Wollin J. The impact of very premature birth on the psychological health of mothers. Early Hum Dev 2003; 73:61-70.

83. Roseboom TJ, Painter RC, van Abeelen AF, Veenendaal MV, de Rooij SR. Hungry in the womb: what are the consequences? Lessons from the Dutch famine. Maturitas 2011; 70:141-145.

84. Painter RC, Roseboom TJ, Bleker OP. Prenatal exposure to the Dutch famine and disease in later life: an overview. Reprod Toxicol 2005; 20:345-352.

85. Lumey LH. Reproductive outcomes in women prenatally exposed to undernutrition: a review of findings from the Dutch famine birth cohort. Proc Nutr Soc 1998; 57:129-135.

86. 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.

87. Nielsen MO, Kongsted AH, Thygesen MP, Strathe AB, Caddy S, Quistorff B, Jorgensen W, Christensen VG, Husted S, Chwalibog A, Sejrsen K, Purup S, et al. Late gestation undernutrition can predispose for visceral adiposity by altering fat distribution patterns and increasing the preference for a high-fat diet in early postnatal life. Br J Nutr 2013; 109:2098-2110.

88. George LA, Zhang L, Tuersunjiang N, Ma Y, Long NM, Uthlaut AB, Smith DT, Nathanielsz PW, Ford SP. Early maternal undernutrition programs increased feed intake, altered glucose metabolism and insulin secretion, and liver function in aged female offspring. Am J Physiol Regul Integr Comp Physiol 2012; 302:R795-804.

89. Hales CN, Barker DJ. The thrifty phenotype hypothesis. Br Med Bull 2001; 60:5-20.

90. Canani RB, Costanzo MD, Leone L, Bedogni G, Brambilla P, Cianfarani S, Nobili V, Pietrobelli A, Agostoni C. Epigenetic mechanisms elicited by nutrition in early life. Nutr Res Rev 2011; 24:198-205.

91. Torrens C, Hanson MA, Gluckman PD, Vickers MH. Maternal undernutrition leads to endothelial dysfunction in adult male rat offspring independent of postnatal diet. Br J Nutr 2009; 101:27-33.

92. Harrison M, Langley-Evans SC. Intergenerational programming of impaired nephrogenesis and hypertension in rats following maternal protein restriction during pregnancy. Br J Nutr 2009; 101:1020-1030.

93. Sathishkumar K, Elkins R, Yallampalli U, Yallampalli C. Protein restriction during pregnancy induces hypertension and impairs endothelium-dependent vascular function in adult female offspring. J Vasc Res 2009; 46:229-239.

94. Sohi G, Marchand K, Revesz A, Arany E, Hardy DB. Maternal protein restriction elevates cholesterol in adult rat offspring due to repressive changes in histone modifications at the cholesterol 7alpha-hydroxylase promoter. Mol Endocrinol 2011; 25:785-798.

95. 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.

96. Brychtova V, Vojtesek B, Hrstka R. Anterior gradient 2: a novel player in tumor cell biology. Cancer Lett 2011; 304:1-7.

97. Chevet E, Fessart D, Delom F, Mulot A, Vojtesek B, Hrstka R, Murray E, Gray T, Hupp T. Emerging roles for the pro-oncogenic anterior gradient-2 in cancer development. Oncogene 2013; 32:2499-2509.

98. Verma S, Salmans ML, Geyfman M, Wang H, Yu Z, Lu Z, Zhao F, Lipkin SM, Andersen B. The estrogen-responsive Agr2 gene regulates mammary epithelial proliferation and facilitates lobuloalveolar development. Dev Biol 2012; 369:249-260.

99. Wang Z, Hao Y, Lowe AW. The adenocarcinoma-associated antigen, AGR2, promotes tumor growth, cell migration, and cellular transformation. Cancer Res 2008; 68:492-497.

100. Gupta A, Dong A, Lowe AW. AGR2 gene function requires a unique endoplasmic reticulum localization motif. J Biol Chem 2012; 287:4773-4782.

101. Ivanova AS, Tereshina MB, Ermakova GV, Belousov VV, Zaraisky AG. Agr genes, missing in amniotes, are involved in the body appendages regeneration in frog tadpoles. Sci Rep 2013; 3:1279.

102. Sive HL, Hattori K, Weintraub H. Progressive determination during formation of the anteroposterior axis in Xenopus laevis. Cell 1989; 58:171-180.

103. Jessop CE, Watkins RH, Simmons JJ, Tasab M, Bulleid NJ. Protein disulphide isomerase family members show distinct substrate specificity: P5 is targeted to BiP client proteins. J Cell Sci 2009; 122:4287-4295.

104. Bu H, Schweiger MR, Manke T, Wunderlich A, Timmermann B, Kerick M, Pasqualini L, Shehu E, Fuchsberger C, Cato AC, Klocker H. Anterior gradient 2 and 3--two prototype androgen-responsive genes transcriptionally upregulated by androgens and by oestrogens in prostate cancer cells. FEBS J 2013; 280:1249-1266.

105. Dong A, Gupta A, Pai RK, Tun M, Lowe AW. The human adenocarcinoma-associated gene, AGR2, induces expression of amphiregulin through Hippo pathway co-activator YAP1 activation. J Biol Chem 2011; 286:18301-18310.

106. Akagi M, Yokozaki H, Kitadai Y, Ito R, Yasui W, Haruma K, Kajiyama G, Tahara E. Expression of amphiregulin in human gastric cancer cell lines. Cancer 1995; 75:1460-1466.

107. Bostwick DG, Qian J, Maihle NJ. Amphiregulin expression in prostatic intraepithelial neoplasia and adenocarcinoma: a study of 93 cases. Prostate 2004; 58:164-168.

108. Forsyth IA, Taylor JA, Keable S, Turvey A, Lennard S. Expression of amphiregulin in the sheep mammary gland. Mol Cell Endocrinol 1997; 126:41-48.

109. Zhang J, Ji JY, Yu M, Overholtzer M, Smolen GA, Wang R, Brugge JS, Dyson NJ, Haber DA. YAP-dependent induction of amphiregulin identifies a non-cell-autonomous component of the Hippo pathway. Nat Cell Biol 2009; 11:1444-1450.

110. Maslon MM, Hrstka R, Vojtesek B, Hupp TR. A divergent substrate-binding loop within the pro-oncogenic protein anterior gradient-2 forms a docking site for Reptin. J Mol Biol 2010; 404:418-438.

111. Grigoletto A, Lestienne P, Rosenbaum J. The multifaceted proteins Reptin and Pontin as major players in cancer. Biochim Biophys Acta 2011; 1815:147-157.

112. Jha S, Gupta A, Dar A, Dutta A. RVBs are required for assembling a functional TIP60 complex. Mol Cell Biol 2013; 33:1164-1174.

113. Rosenbaum J, Baek SH, Dutta A, Houry WA, Huber O, Hupp TR, Matias PM. The emergence of the conserved AAA+ ATPases Pontin and Reptin on the signaling landscape. Sci Signal 2013; 6:mr1.

114. Clodfelter KH, Miles GD, Wauthier V, Holloway MG, Zhang X, Hodor P, Ray WJ, Waxman DJ. Role of STAT5a in regulation of sex-specific gene expression in female but not male mouse liver revealed by microarray analysis. Physiol Genomics 2007; 31:63-74.

115. Blanc JF, Lalanne C, Plomion C, Schmitter JM, Bathany K, Gion JM, Bioulac-Sage P, Balabaud C, Bonneu M, Rosenbaum J. Proteomic analysis of differentially expressed proteins in hepatocellular carcinoma developed in patients with chronic viral hepatitis C. Proteomics 2005; 5:3778-3789.

116. Izumi N, Yamashita A, Iwamatsu A, Kurata R, Nakamura H, Saari B, Hirano H, Anderson P, Ohno S. AAA+ proteins RUVBL1 and RUVBL2 coordinate PIKK activity and function in nonsense-mediated mRNA decay. Sci Signal 2010; 3:ra27.

117. Ealy AD, Yang QE. Control of interferon-tau expression during early pregnancy in ruminants. Am J Reprod Immunol 2009; 61:95-106.

118. Sakurai T, Bai H, Konno T, Ideta A, Aoyagi Y, Godkin JD, Imakawa K. Function of a transcription factor CDX2 beyond its trophectoderm lineage specification. Endocrinology 2010; 151:5873-5881.

119. Strumpf D, Mao CA, Yamanaka Y, Ralston A, Chawengsaksophak K, Beck F, Rossant J. Cdx2 is required for correct cell fate specification and differentiation of trophectoderm in the mouse blastocyst. Development 2005; 132:2093-2102.

120. Nishioka N, Inoue K, Adachi K, Kiyonari H, Ota M, Ralston A, Yabuta N, Hirahara S, Stephenson RO, Ogonuki N, Makita R, Kurihara H, et al. The Hippo signaling pathway components Lats and Yap pattern Tead4 activity to distinguish mouse trophectoderm from inner cell mass. Dev Cell 2009; 16:398-410.

121. Zweitzig DR, Smirnov DA, Connelly MC, Terstappen LW, O'Hara SM, Moran E. Physiological stress induces the metastasis marker AGR2 in breast cancer cells. Mol Cell Biochem 2007; 306:255-260.

122. Hrstka R, Nenutil R, Fourtouna A, Maslon MM, Naughton C, Langdon S, Murray E, Larionov A, Petrakova K, Muller P, Dixon MJ, Hupp TR, et al. The pro-metastatic protein anterior gradient-2 predicts poor prognosis in tamoxifen-treated breast cancers. Oncogene 2010; 29:4838-4847.

123. Salmans ML, Zhao F, Andersen B. The estrogen-regulated anterior gradient 2 (AGR2) protein in breast cancer: a potential drug target and biomarker. Breast Cancer Res 2013; 15:204.

124. Jordan VC. Tamoxifen (ICI46,474) as a targeted therapy to treat and prevent breast cancer. Br J Pharmacol 2006; 147 Suppl 1:S269-276.

125. Innes HE, Liu D, Barraclough R, Davies MP, O'Neill PA, Platt-Higgins A, de Silva Rudland S, Sibson DR, Rudland PS. Significance of the metastasis-inducing protein AGR2 for outcome in hormonally treated breast cancer patients. Br J Cancer 2006; 94:1057-1065.

126. Zheng W, Rosenstiel P, Huse K, Sina C, Valentonyte R, Mah N, Zeitlmann L, Grosse J, Ruf N, Nurnberg P, Costello CM, Onnie C, et al. Evaluation of AGR2

and AGR3 as candidate genes for inflammatory bowel disease. Genes Immun 2006; 7:11-18.

127. Zhang JS, Gong A, Cheville JC, Smith DI, Young CY. AGR2, an androgen-inducible secretory protein overexpressed in prostate cancer. Genes Chromosomes Cancer 2005; 43:249-259.

128. Park K, Chung YJ, So H, Kim K, Park J, Oh M, Jo M, Choi K, Lee EJ, Choi YL, Song SY, Bae DS, et al. AGR2, a mucinous ovarian cancer marker, promotes cell proliferation and migration. Exp Mol Med 2011; 43:91-100.

129. Weinberg R. How a Tumor Is Like an Embryo. In: Bourzac K (ed.). MIT Technology Review: BIOMEDICINE NEWS; 2007.

130. Scheel C, Onder T, Karnoub A, Weinberg RA. Adaptation versus selection: the origins of metastatic behavior. Cancer Res 2007; 67:11476-11479; discussion 11479-11480.

131. Spencer TE, Bartol FF, Bazer FW, Johnson GA, Joyce MM. Identification and characterization of glycosylation-dependent cell adhesion molecule 1-like protein expression in the ovine uterus. Biol Reprod 1999; 60:241-250.

132. Dorniak P, Bazer FW, Spencer TE. Prostaglandins regulate conceptus elongation and mediate effects of interferon tau on the ovine uterine endometrium. Biol Reprod 2011; 84:1119-1127.

133. Dorniak P, Welsh TH, Jr., Bazer FW, Spencer TE. Endometrial HSD11B1 and cortisol regeneration in the ovine uterus: effects of pregnancy, interferon tau, and prostaglandins. Biol Reprod 2012; 86:124.

134. Van Heeke G, Ott TL, Strauss A, Ammaturo D, Bazer FW. High yield expression and secretion of the ovine pregnancy recognition hormone interferon-tau by Pichia pastoris. J Interferon Cytokine Res 1996; 16:119-126.

135. Ashworth CJ, Bazer FW. Changes in ovine conceptus and endometrial function following asynchronous embryo transfer or administration of progesterone. Biol Reprod 1989; 40:425-433.

136. Bazer FW, Spencer TE. Methods for studying interferon tau stimulated genes. Methods Mol Med 2006; 122:367-380.

137. 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.

138. 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.

139. 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.

140. Huang DW, Sherman BT, Lempicki RA. Systematic and integrative analysis of large gene lists using DAVID bioinformatics resources. Nature Protocols 2009; 4:44-57.

141. 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.

142. 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.

143. 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.

144. Johnson GA, Burghardt RC, Newton GR, Bazer FW, Spencer TE. Development and characterization of immortalized ovine endometrial cell lines. Biol Reprod 1999; 61:1324-1330.

145. Song G, Satterfield MC, Kim J, Bazer FW, Spencer TE. Gastrin-releasing peptide (GRP) in the ovine uterus: regulation by interferon tau and progesterone. Biol Reprod 2008; 79:376-386.

146. Hong XY, Wang J, Li Z. AGR2 expression is regulated by HIF-1 and contributes to growth and angiogenesis of glioblastoma. Cell Biochem Biophys 2013; 67:1487-1495.

147. Spencer TE, Johnson GA, Burghardt RC, Bazer FW. Progesterone and placental hormone actions on the uterus: insights from domestic animals. Biol Reprod 2004; 71:2-10.

148. Vallet JL, McNeel AK, Johnson G, Bazer FW. Triennial Reproduction Symposium: limitations in uterine and conceptus physiology that lead to fetal losses. J Anim Sci 2013; 91:3030-3040.

149. Ryu J, Park SG, Lee PY, Cho S, Lee do H, Kim GH, Kim JH, Park BC. Dimerization of pro-oncogenic protein Anterior Gradient 2 is required for the interaction with BiP/GRP78. Biochem Biophys Res Commun 2013; 430:610-615.

150. Imakawa K, Kim MS, Matsuda-Minehata F, Ishida S, Iizuka M, Suzuki M, Chang KT, Echternkamp SE, Christenson RK. Regulation of the ovine interferon-tau gene by a blastocyst-specific transcription factor, Cdx2. Mol Reprod Dev 2006; 73:559-567.