PROGESTERONE REGULATION OF ENDOMETRIAL

GENE EXPRESSION IN THE EARLY PREGNANT OVINE UTERUS

A Thesis

by

MEGAN A. MINTEN

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

MASTER OF SCIENCE

August 2011

Major Subject: Physiology of Reproduction

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Approved by:

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ABSTRACT

Progesterone Regulation of Endometrial Gene Expression

in the Early Pregnant Ovine Uterus. (August 2011)

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Co-Chairs of Advisory Committee: Dr. Thomas E. Spencer
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Establishment of pregnancy in ruminants requires blastocyst development to form an elongated filamentous conceptus that produces interferon tau (IFNT), the pregnancy recognition signal, and initiate implantation. Blastocyst growth and development is dependent upon secretions from the uterine endometrium. An early increase in post-ovulatory circulating levels of progesterone (P4) stimulates blastocyst growth and conceptus elongation in ruminants. Microarray analysis was used to identify candidate P4-regulated genes and regulatory networks in the endometrium that govern peri-implantation blastocyst/conceptus growth and development.

The first study was conducted to validate effects of P4 and/or pregnancy on expression of candidate genes identified by microarray analysis. The genes included: *ANGPTL3, CHGA, CLEC4E, CXCL14, EFNA1, EFNB1, FABP3, IFNG, IL6, LGALS3, PTH, RBP4, SLIT2, SLIT3,* and *VWF*. Early P4 treatment up-regulated *CXCL14* gene expression in Day 9 ovine endometrium compared to control endometrium, and *FABP3, IFNG, IL6* and *LGALS3* in Day 12 early P4-treated ovine endometrium. Expression of *ANGPTL3, CHGA, CXCL14, EFNA1, EFNB1, LGALS3* and *RBP4* was affected by day of pregnancy. Treatment of ewes with P4+RU486 (P4 receptor antagonist) reduced expression of *ANGPTL3, CHGA, EFNA1, EFNB1, FABP3, IFNG, IL6, LGALS3, RBP4*, and *SLIT2, SLIT3 and VWF* in comparison to Day 12 P4-treated endometrium.

The second study evaluated expression of genes identified by microarray analysis in endometrium from pregnant and cyclic ewes. Genes evaluated included those from the first study. *ANGPTL3*, *CHGA*, *CXCL14*, *EFNA1*, *EFNB1*, *IFNG*, *LGALS3*, *PTH*, *RBP4*, *SLIT2*, *SLIT3* and *VWF* were affected by day, status and/or their interaction between Days 10 and 16. Of note, *FABP3* increased 21-fold between Days 14 to 18 of pregnancy, and *IL6* increased 37-fold between Days 14 to 20 of pregnancy. *In situ* hybridization analysis detected *FABP3* mRNA in both luminal and superficial glandular epithelia of pregnant ewes and trophectoderm, whereas *IL6* mRNA was detected in immune cells within uterine luminal epithelium and glandular epithelium and trophectoderm.

Collectively, these results identify candidate genes encoding for biologically active molecules that regulate growth and development of the ovine conceptus during the peri-implantation period of pregnancy.

DEDICATION

To my dear Gramma,

I thought I would have more years with you but God called you home while I was away.

I dedicate this work to you—my pursuit of knowledge while I could've been with you,

when you needed me the most.

Love you always Gramma.

ACKNOWLEDGEMENTS

I would like to express my gratitude to my mentors, Drs. Thomas Spencer and Fuller Bazer, for their support and instruction throughout my time at Texas A&M. Also I would like to thank Dr. Satterfield for his assistance with my thesis work. I thank Drs. Jo-Ann Fleming and Kathrin Dunlap for their guidance in the lab and knowledge of techniques. To my grad student colleagues, moving to Texas was a diverse climate and social change for me, thanks for helping me adjust. I will cherish the times I was surrounded by good friends and strong spirits in Aggieland. Finally, my last thank you is to my parents. I am very grateful for all the support (and tough love) you have provided me. Thank you for finally dispersing the dairy cows. That change was the foundation to allowing me to explore new opportunities, thus leading me to research. I'm sorry I have traveled far from home but the world is a big place, I just want to see as much of it as possible and make an impact.

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CHAPTER I

INTRODUCTION

Successful establishment of pregnancy in ruminants involves complex communication between the conceptus (embryo and associated extraembryonic membranes) and mother. The pregnancy recognition signal from the ovine conceptus and other ruminant species is interferon tau (IFNT), a Type 1 IFN. Interferon tau exerts effects on the uterine epithelia to maintain a functional corpus luteum (CL) by silencing expression of estrogen receptor alpha (*ESR1*) and oxytocin receptor (*OXTR*) to prevent oxytocin-induced pulsatile release of luteolytic prostaglandin F2α (PGF) from the endometrium that causes functional and structural regression of the CL. The CL produces the hormone progesterone (P4), the required hormone of pregnancy that is critical to establishment of a maternal uterine environment conducive to conceptus growth, implantation, placentation and fetal development.

Progesterone and/or IFNT act on the endometrium to increase production of secretions that are termed histotroph and are vital for conceptus growth in ruminants. Histotroph is a complex mixture of enzymes, growth factors, cytokines, hormones, transport proteins, serum proteins, adhesion proteins, protease inhibitors, amino acids and other substances (Spencer *et al.* 2004b; Bazer *et al.* 2009). Evidence demonstrates that endometrial glands are necessary for blastocyst development into an elongated conceptus that can establish and maintain a pregnancy (Bartol *et al.* 1995; Gray *et al.* 2000a; Gray *et al.* 2001b). Furthermore, asynchronous embryo transfer studies demonstrated the temporal necessity of maternal hormonal influences on endometrial functions to produce histotroph for survival and growth of the conceptus during the protracted peri-implantation period of pregnancy in ruminants (Lawson *et al.* 1983; Pope 1988).

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This thesis follows the style of *Reproduction, Fertility and Development*.

Progesterone receptors (PGR) are down-regulated in response to continuous P4 exposure in uterine luminal (LE) and glandular epithelia (GE) by Day 11 and 13, respectively (Wathes and Hamon 1993; Spencer *et al.* 1995b). Down-regulation of PGR allows for a rapid increase in expression of ESR1 after Day 13 of the estrous cycle. The increase in *ESR1* expression precedes the increase of OXTR expression (Flint *et al.* 1986; Spencer *et al.* 1995a). Oxytocin (OXT) released in a pulsatile manner from the posterior pituitary and CL binds OXTR on uterine LE and superficial glandular epithelium (sGE) to initiate the secretion of luteolytic pulses of PGF (McCracken 1980; Flint *et al.* 1986). However IFNT, secreted by mononuclear trophectoderm cells of the elongating conceptus, acts in a paracrine manner to exert antiluteolytic effects by suppressing up-regulation of *ESR1*, thus preventing increases in *OXTR* gene expression in LE and sGE to abrogate development of the uterine luteolytic mechanism (Lamming *et al.* 1995; Spencer *et al.* 1995a). IFNT suppresses *ESR1* transcription through a pathway involving interferon regulatory factor two (*IRF2*), a transcriptional repressor in LE and sGE (Fleming *et al.* 2001). Thereby IFNT inhibits transcription of *ESR1* preceding transcription of *OXTR* consequently preventing OXT-induced release of luteolytic pulses of PGF. Thus, a functional CL is maintained to secrete progesterone throughout pregnancy. Ovarian P4 induces and conceptus IFNT stimulates a number of genes (Spencer *et al.* 2008; Bazer *et al.* 2010). Particularly, IFNT stimulates an increase in enzymes involved in prostaglandin (PG) synthesis and secretion. Recently, it was found that prostaglandin G/H synthase and cyclooxygenase 2 (PTGS2)-derived PGs mediate, in part, effects of P4 and IFNT on expression of endometrial genes regulating growth and development of the blastocyst and elongation of the conceptus (Dorniak *et al.* 2011).

Research with cattle and sheep found that the post-ovulatory rise in P4 after mating is correlated with conceptus growth and development (Garrett *et al.* 1988; Mann *et al.* 2006;

Satterfield *et al.* 2006). Ovarian P4 stimulates conceptus development through secretions from the endometrium (Bazer *et al.* 2008). In sheep, administration of exogenous P4 during metestrus accelerated growth of hatched blastocysts as evidenced by the presence of filamentous conceptuses on Day 12 in P4-treated ewes while only spherical to tubular blastocysts were recovered from uteri of control ewes on Day 12 of pregnancy (Satterfield *et al.* 2006). Additionally, IFNT in uterine flushings from Day 12 uteri was markedly higher in early P4-treated ewes.

Gene profiling of the endometrial transcriptome in pregnant ewes treated with exogenous P4 identified novel candidate genes and regulatory pathways governing preimplantation growth and development of the conceptus. The Database for Annotation, Visualization, and Integrated Discovery (DAVID) was used to identify functional groups of genes and biological processes in the endometrium associated with the growth and development of preimplantation blastocysts (Satterfield *et al.* 2009). Selected genes identified were associated with regulation of cell migration (*ANGPTL3*), extracellular space (*CHGA*), integral to membrane (*CLEC4E*), chemokine activity (*CXCL14*), protein binding and cellular differentiation (*EFNA1* and *EFNB1*), fatty acid binding (*FABP3*), interferon gamma precursor (*IFNG*), humoral immune response (*IL6*), plasma membrane (*LGALS3*), hormone activity (*PTH*), extracellular space and retinol binding (*RBP4*), extracellular space and receptor binding (*SLIT2* and *SLIT3*), and growth factor activity (*VWF*) (Satterfield *et al.* 2009).

Collectively, results of these studies indicate the role of P4 in inducing expression of molecules essential for the culmination of successful establishment of pregnancy. Knowledge of the endometrial gene profile during early pregnancy provides insight in gene pathways involved in blastocyst growth and conceptus development (Satterfield *et al.* 2009). Therefore, studies

were conducted to validate candidate P4-regulated genes and determine effects of the estrous cycle and pregnancy on expression of candidate genes in the endometrium.

CHAPTER II

LITERATURE REVIEW

Embryonic Development

Embryonic development begins with syngamy, the fusion of the male and female pronuclei resulting in a one cell embryo or zygote. The zygote develops by mitotic divisions, also referred to as cleavage divisions. Cleavage occurs within the zona pellucida, an extracellular matrix of glycoproteins. The cells comprising the embryo are referred to as blastomeres. Cytoplasmic mass does not increase during cleavage; therefore, there is an increase in the number of blastomeres with a fixed volume of cytoplasm. Around the 16- to 32-cell stage, blastomeres flatten on one another to form a solid, rounded ball of cells referred to as a morula. The surface microvilli of blastomeres also become asymmetrically polarized and undergo polarization. The combination of flattening and polarization of blastomeres is referred to as compaction. The outer blastomeres become more compacted than the inner cells. The peripheral cells form the cuboidal trophectoderm, which is covered by dense microvilli and allows for the accumulation of fluid by active ion transport to form a central cavity referred to as the blastocoele during cavitation. Gap junctions formed by the inner cells allows for communication between blastomeres and leads to the formation of the inner cell mass or embryonic disc. The blastocyst stage is achieved once an embryo has a recognizable blastocoele, inner cell mass and trophectoderm. The inner cell mass gives rise to the embryo proper that develops into the three germ layers (endoderm, mesoderm and ectoderm). The trophectoderm cells become highly attenuated and organized into a simple squamous epithelium which serves to translocate nutrients from the maternal environment into the blastocoele, and will later give rise to the chorion of the placenta (Zernicka-Goetz 2005).
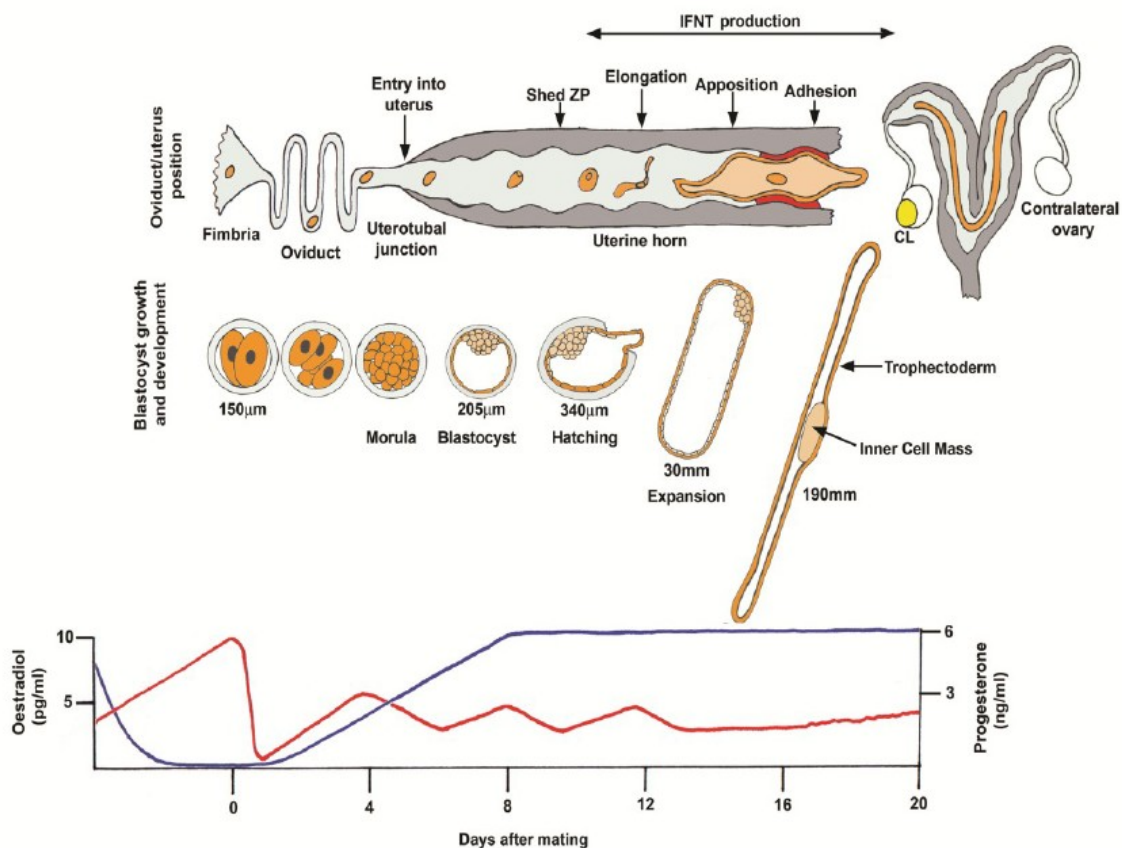
Figure 2.1. Early pregnancy events in sheep. This schematic describes relationships between development of the embryo and conceptus with respect to hormonal status and position within the maternal uterine environment. Embryos enter the uterus on Days 4-5 post-fertilization (Day 0), reach the blastocyst 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. 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 predominantly progesterone. Copied from (Spencer *et al.* 2007) and originally drawn by Dr. Greg A. Johnson, Texas A&M University.

Conceptus Development and Implantation in Ruminants

Fertilization of the oöcyte by sperm occurs within the ampullary-isthmic junction of the oviduct in ruminants (sheep, goat and cattle). Morula stage embryos enter the uterus on Days 4-5. By Day 6, blastocysts contain a blastocoele and inner cell mass surrounded by a monolayer of trophectoderm. The zona pellucida remains intact on Day 8 with numerous microvilli on the external surface of flattened trophectoderm and junctional complexes formed within the basal and lateral plasma membrane (Wintenberger-Torres and Flechon 1974; Spencer *et al.* 2004a). Endoderm cells migrate below the trophectoderm to form the extra-embryonic endoderm that lines the blastocoele. This ectoderm-endoderm double barrier separates the blastocoele from the external uterine milieu (Flechon *et al.* 2007). The endoderm differentiates into the visceral endoderm first and then the parietal endoderm. The endoderm reaches the equator of the blastocyst by Day 9 and the abembryonic pole by Day 10 (Wintenberger-Torres and Flechon 1974).

A diversity of implantation and placentation strategies exist among species however homology exists in the primary stages of establishing contact between the maternal and conceptus tissues (Spencer *et al.* 2004a). A comparative implantation scheme proposed by Guillomot and colleagues (Guillomot *et al.* 1981; Chavatte-Palmer and Guillomot 2007) has defined implantation as: 1) shedding of the zona pellucida; 2) pre-contact and blastocyst orientation; 3) apposition; 4) adhesion; and 5) endometrial invasion (however, endometrial invasion by the blastocyst does not occur in uteri of ruminants). The events of conceptus implantation are summarized in Figure 2.1. Expansion of the blastocoele and uterine and/or embryonic proteases results in the hatching of the blastocyst (approximately 300 cells) from the zona pellucida by Day 9 in sheep and Day 10 in cattle (Bindon 1969; Bindon 1971; Guillomot *et al.* 1988; Hafez 1993; Chavatte-Palmer and Guillomot 2007). After hatching and prior to

implantation, the ruminant blastocyst undergoes a rapid expansion that results in morphological changes and lengthening (Guillomot *et al.* 1988). By Day 10, the blastocyst measures 400-900 µm in diameter and contains approximately 3000 cells (Spencer *et al.* 2004a). In contrast to implantation of rodents, primates and humans, ruminants have a protracted peri-implantation period.

During the phase of pre-contact and orientation, the blastocyst transitions from spherical to tubular to filamentous forms. Trophectoderm elongation in sheep and cattle is initiated around Day 11 and Day 12, respectively (Guillomot *et al.* 2004; Blomberg *et al.* 2008). The parietal endoderm transitions to a multinucleated syncytium as it spreads from the embryonic disc to the elongating tips of the conceptus. Nuclear division, without cytokinesis, orients cells parallel to the long axis of the conceptus. The visceral endoderm retains features of a typical epithelium (Flechon *et al.* 2007). The trophectoderm transitions from flattened to cuboidal epithelia with high secretory activity evidenced by abundant endoplasmic reticulum (Wintenberger-Torres and Flechon 1974). By Day 13 in the sheep conceptus, the embryonic disc is raised above the elongated trophectoderm and the yolk sac is formed as an evagination of the foregut (Flechon *et al.* 1986; Wales and Cuneo 1989; Guillomot 1995). At Day 14, the approximately 10 cm filamentous conceptus appears to have a primitive streak and somites appear in the embryo soon thereafter (Wintenberger-Torres and Flechon 1974). The trophectoderm length increases 90-fold from Day 13 to 19 to resemble a long filament that will elongate from the uterine horn ipsilateral to the CL into the contralateral uterine horn by Day 17 (Rowson and Moor 1966; Wales and Cuneo 1989; Spencer *et al.* 2004a). During elongation, the conceptus trophectoderm secretes IFNT. The rate and timing of elongation of the trophectoderm is dependent upon the uterine environment. Blastocysts and trophoblastic vesicles do not

elongate *in vitro* unless transferred to the uterus because they require endometrial secretions and the uterine LE to elongate (Lawson *et al.* 1983; Flechon *et al.* 1986; Spencer *et al.* 2004a).

Apposition is defined as the first contact between the apical membranes of the trophectoderm and uterine LE (Spencer *et al.* 2004a). During this stage, the columnar trophectoderm is closely pressed against the endometrial cell apex which imprints on the trophectoderm surface (Guillomot *et al.* 1981; Wooding 1984; Chavatte-Palmer and Guillomot 2007). First cell contact begins around the embryonic region and spreads towards the tips of the elongating conceptus. Additionally, the trophectoderm attaches mainly to the caruncular (aglandular endometrium) LE and to the intercaruncular LE/sGE to a lesser extent (Chavatte-Palmer and Guillomot 2007). In areas of microvilli deficiency in both LE and trophectoderm, membranes are discontinuously apposed (Guillomot *et al.* 1981). Specialized structures are formed in the sheep and cattle trophectoderm (interestingly, not in goat trophectoderm) termed papillae, which are finger-like villi that extend into superficial ducts of uterine glands between Days 15 to 18 of pregnancy. The papillae may serve as anchoring pegs, especially in the papillae dense embryonic disc region where immobilization would be advantageous in maintaining a close contact with the nutrient supply (Wooding *et al.* 1982).

Adhesion begins around Day 16; however, it is loose and embryonic membranes can be dislodged by flushing the uterine lumen with saline (Boshier 1969) . Adhesion is the result of interlocking of the uterine microvilli and trophectoderm cytoplasmic projections (Guillomot *et al.* 1981). By Day 22, adhesion appears to be complete along the length of the conceptus in both caruncular and intercaruncular regions. The external surface of the uterine LE and trophectoderm is composed of a glycocalyx, which is a glycoprotein coat. The biochemistry of the glycocalyx is modified by the hormonal status of the maternal environment. During the luteal phase of continuous P4 exposure, *PGR* expression is down-regulated in concert with anti-

adhesive factors (Dharmaraj *et al.* 2010). MUC1 is particularly abundant on the microvilli and cilia that extend from the apical cell surface of the endometrial LE. MUC1 is thought to sterically interfere with adhesion molecules between the trophectoderm and uterine LE due to extensive glycosylation and extended extracellular structure (Burghardt *et al.* 2002). Down-regulation of MUC1 between Days 9 and 17 of pregnancy allows for the implantation cascade to occur in sheep (Johnson *et al.* 2001a). GLYCAM1 (glycosylation dependent cell adhesion molecule 1) (Spencer *et al.* 1999; Muniz *et al.* 2006), LGALS15 (lectin, galactoside-binding, soluble 15) (Lewis *et al.* 2007; Farmer *et al.* 2008) , SPP1 (secreted phosphoprotein 1 or osteopontin) (Johnson *et al.* 2003a; Johnson *et al.* 2003b; Dunlap *et al.* 2008), IGFBP1 (insulin-like growth factor binding protein-1) (Simmons *et al.* 2009), and integrins (Johnson *et al.* 2001a; Burghardt *et al.* 2002; Burghardt *et al.* 2009) function as bridging ligands in establishing attachment between uterine LE and trophectoderm (Bazer *et al.* 2010).

Mononucleate trophectoderm cells differentiate into giant binucleate cells (BNC) by nuclear division without cytokinesis resulting in polyploidy (Wooding 1984). BNC cells migrate in between mononuclear cells and likely fuse with individual LE cells to form multinucleated syncytial plaques (Wooding 1984; Chavatte-Palmer and Guillomot 2007; Spencer *et al.* 2008). Continued migration and presumably fusion enlarge the syncytium; however, these plaques appear to be limited to 20-25 nuclei in sheep. Syncytial plaques are linked by tight junctions and localize to caruncular regions to aid in formation of the placentome (Wooding 1984; Spencer *et al.* 2004a). Formation of syncytial plaques starts initially in caruncular regions adjacent to the embryo and extends toward the tips of the horns. Although BNC are inherently invasive, they do not cross the basal lamina of the LE (Spencer *et al.* 2004a). BNC formation has two main functions: 1) to form placentomes, and 2) to produce and secrete protein and steroid hormones (Wooding 1992). Wooding used phosphotungstic acid (PTA) staining to demonstrate granules

from BNC streaming down to the maternal interface suggesting exocytosis (Wooding 1984). Exocytosis of steroid and protein hormones includes pregnancy associated glycoproteins (PAGS), P4 and placental lactogen (chorionic somatomammotropin hormone 1, CSH1) which act on endometrial glands to stimulate their development and secretion of proteins (Wooding 1992; Spencer *et al.* 2007). BNC undergo migration and fusion throughout most of pregnancy. In sheep, more than 95% of the placentomal and interplacentomal regions of the fetomaternal interface is formed by hybrid syncytial plaques in the second half of pregnancy (Wooding 1992). The transformation of mononuclear trophectoderm into syncytial tissue could also contribute to the protection of the conceptus allograft from the maternal immune system (Chavatte-Palmer and Guillomot 2007).

Structure of Ruminant Fetomaternal Interface

The fetomaternal interface of ruminants is of three types between Days 18 and 24 of pregnancy; 1) fetomaternal hybrid tissue of multinucleated syncytial plaques; 2) an interlocking microvillar junction between the trophectoderm and mononuclear uterine epithelium; and 3) flat apposition of trophectoderm to uterine LE (Wooding 1984). Syncytial plaques develop in caruncular regions, which develop into placentomes that are specialized structures comprised of fetal cotyledons and maternal caruncles. The placentome structure is formed by the folding of the caruncular region thereby forming deep caruncular crypts which are penetrated by long, profusely branched cotyledonary villi (Igwebuike 2009). Thus the ruminant placenta is synepitheliochorial, being neither entirely syndesmochorial (without endometrial LE), nor completely epitheliochorial (with two apposed epithelia). An additional unique feature of the ruminant placenta (as well as that for pigs and horses) are interplacentomal areolae which develop as specialized areas for absorption of histotroph from the mouth of endometrial glands

(Spencer and Bazer 2004b). The developing conceptus, and later in gestation, the fetus, will continue to utilize histotrophic nutrition. Intercaruncular endometrial glands grow substantially in length and width throughout pregnancy (Wimsatt 1950). By Day 40, there is maximum juxtaposition of the maternal and fetal microvasculature (Cross *et al.* 2003). Placentomes provide a critical source of hematotrophic nutrition for the fetus during gestation due to the close proximity of the vasculature for the exchange of gases and micronutrients (Reynolds *et al.* 2010). The sheep placenta has approximately 90 to 100 placentomes evenly distributed throughout the fetomaternal interface (Hafez 1993). Placentation in ewes is essentially complete by approximately Days 50 to 60 (Guillomot 1995).

Effects of Interferon Tau and Prostaglandins

Interferon tau (IFNT), a Type I Interferon, shares high structural homology with interferons alpha (IFNA), beta (IFNB), delta (IFND) and omega (IFNW1) (Bazer *et al.* 2009). IFNT is unique to ruminants and has antiviral, antitumor, antiproliferative, immunomodulatory and therapeutic activities similar to other Type I IFNs (Pontzer *et al.* 1990; Bazer and Johnson 1991; Soos *et al.* 1995; Chon and Bixler 2010). The biological functions of IFNT in ruminants are in pregnancy recognition signaling to abrogate development of the luteolytic mechanism and as an inducer/stimulator of expression of genes critical for implantation and conceptus development.

Prostaglandins possess vasoactive, mitogenic and differentiating properties, and are implicated in various female reproductive functions (Wang and Dey 2005). Prostaglandins are generated from arachidonic acid by phospholipase A2 (PLA2) and PTGS2 enzymes into an unstable endoperoxide intermediate, PGH2. PGH2 is metabolized to five structurally active

related PGs including PGE2, PGD2, PGF, PGI2 and thromboxane A2 via cell specific isomerase and specific PG synthases.

Ruminants are polyestrous, spontaneous ovulators. Sheep and cattle have estrous lengths of 17-18 and 21 days, respectively. Following ovulation and during metestrus, 1-4 days after onset of estrus, theca and granulosa cells of the ovulatory follicle undergo luteinization under the influence of LH, resulting in a CL that secretes P4 with Day 4 of the estrous cycle and pregnancy marking the initial day of diestrus. The CL will reach maximum size on Day 7 and maintain high P4 secretion until approximately Day 14 of the estrous cycle. Progesterone maintains the myometrium in a quiescent state, promotes an endometrial environment conducive for pregnancy, and suppresses dominant follicles from ovulating during diestrus (Bazer *et al.* 1998). In addition to P4 preparing the uterus for pregnancy, P4 stimulates phospholipid stores for arachidonic acid availability in the endometrium, and IFNT increases activity of PTGS2 for synthesis of arachidonic acid to prostaglandins (Dorniak *et al.* 2011).

Progesterone receptors (PGR) in ovine uterine LE and GE are down-regulated under continuous P4 exposure on Days 11 and 13, respectively (Wathes and Hamon 1993; Spencer *et al.* 1995b). Down-regulation of P4 allows for a rapid increase in expression of ESR1 in uterine epithelia after Day 13 and then estradiol from ovarian follicles stimulates OXTR expression (Flint *et al.* 1986; Spencer *et al.* 1995a). Oxytocin from the posterior pituitary and CL, binds OXTR on the uterine LE and sGE to stimulate pulsatile release of luteolytic PGF (McCracken *et al.* 1984; Hixon and Flint 1987). PGF released from uterine LE and sGE occurs as a series of episodic surges lasting approximately one hour with about five pulses in 25 hours (Thorburn *et al.* 1973; Baird *et al.* 1976; McCracken *et al.* 1984). Sub-luteolytic pulses of PGF occur on Days 13 and 14 in both cyclic and pregnant ewes, but luteolytic pulses of PGF occur on Days 15 and 16 in cyclic ewes following an increase in circulating concentrations of estradiol (McCracken *et*

al. 1984; Bazer and Johnson 1991). Uterine PGF is released into the utero-ovarian vein and transferred locally to the ovarian artery via a counter-current exchange in the vascular utero-ovarian plexus resulting in delivery of high PGF concentrations to the CL (Heap *et al.* 1985; McCracken *et al.* 1999). Luteolysis by PGF is caused by decreased luteal blood flow, uncoupling of LH receptors from adenyl cyclase, activation of phospholipase C that stimulates protein kinase C to modify posttranslational processes of steroidogenesis and cholesterol availability, apoptosis, and infiltration of immune cells to the CL during serum decline of P4. The decreased secretion of P4 is the result of decreased luteal blood flow of nutrients and steroidogenic substrates, and decreased steroidogenic capacity of the luteal cells. Following PGF release, mRNA steroid conversion enzymes, cholesterol side chain cleavage enzyme complex and 3β-hydroxysteroid dehydrogenase, decline resulting in diminishing P4 levels followed by luteal cell loss (Niswender *et al.* 2000).

IFNT is synthesized and secreted by the mononuclear trophectoderm of the elongating conceptus between Days 10 and 21 of pregnancy with highest production on Days 12 to 16 of pregnancy in sheep (Roberts *et al.* 1999). IFNT acts in a paracrine manner on the endometrium to abrogate the luteolytic mechanism. The antiluteolysin acts on the endometrium to suppress *ESR1* transcription through the gene signaling pathway involving IRF2, a transcriptional repressor (Lamming *et al.* 1995; Spencer *et al.* 1995a; Fleming *et al.* 2001). IRF2 is specifically expressed in uterine LE/sGE beginning on Day 10 of pregnancy. IRF2 may mediate the effects of IFNT to silence *ESR1* transcription following the PGR loss from uterine epithelia; therefore, actions of estradiol from ovarian follicles are unable to induce *OXTR* gene expression resulting in the development of the uterine luteolytic cascade. Consequently, a functional CL secretes P4 to maintain pregnancy.

IFNT either induces or stimulates P4-induced and IFNT stimulated genes (ISG) in uterine LE/sGE that are important for uterine receptivity to implantation and conceptus development (Bazer *et al.* 2009). Interferon tau also stimulates expression of classical ISGs in extrauterine tissues and cells in the ewe (Yankey *et al.* 2001; Oliveira *et al.* 2008; Bazer *et al.* 2009). Type I IFNs induce cell signaling via the Janus-activated kinases (JAKs) and tyrosine kinase 2 (TYK2) pathways upon binding the heterodimer receptor complex (Darnell *et al.* 1994; Platanias 2005). Not all cell types of the endometrium express ISGs. IRF2, a transcriptional repressor, prevents the induction of ISG expression in ovine LE and sGE (Choi *et al.* 2001). Interestingly, nonclassical ISGs are expressed in the ovine uterine LE/sGE and are likely up-regulated via a nonclassical signaling pathway that does not involve STAT1, STAT2 and IRF9/ISGF3G (Spencer and Bazer 2002).

IFNT abrogates luteolysis by inhibiting up-regulation of *OXTR* but does not inhibit PGF production. Rather, IFNT up-regulates activity of PTGS2, the rate limiting enzyme of PG in the endometrium (Dorniak *et al.* 2011). Additionally, ovine conceptuses have been shown to release PTGS2 metabolites (Marcus 1981; Lewis and Waterman 1985). Changes in PTGS2 abundance is coincident with 11-beta-hydroxysteroid dehydrogenase (HSD11B1), an enzyme induced by P4 and stimulated by IFNT and PGs (Simmons *et al.* 2009; Dorniak *et al.* 2011). HSD11B1 modulates the actions of glucocorticoids by generating active cortisol from inactive cortisone and has a positive feedback stimulation of cortisol, PGs and proinflammatory cytokines (Michael *et al.* 2003). Glucocorticoids have beneficial effects that promote pregnancy, including stimulation of secretion of chorionic gonadotropin, suppression of uterine natural killer cells and promotion of trophoblast growth/invasion. However, exogenous glucocorticoids have potential negative aspects that might compromise pregnancy by inhibiting cytokine-prostaglandin signaling, restriction of trophoblast during implantation, induction of apoptosis

and inhibition of embryonic and placental growth (Michael and Papageorghiou 2008). PTGS2-derived PGs mediate effects of P4 to induce and IFNT to stimulate several epithelial genes (*CST3*, *GRP*, *HSD11B1*, *LGALS15*, and *IGFBP1*) that regulate conceptus elongation and implantation in the ovine uterus (Dorniak *et al.* 2011).

Effects of Progesterone

Progesterone is a critical component of reproductive processes encompassing ovarian dynamics, sexual behavior, pregnancy and lactation (Hammes and Levin 2007; Gellersen *et al.* 2009). More specifically to the uterus, P4 regulates gland morphogenesis, gland function, cell adhesion, implantation of the conceptus and maintenance of pregnancy (Rexroad 1984; Spencer and Bazer 2002; Spencer *et al.* 2004b; Spencer *et al.* 2007). Progesterone is a steroid hormone secreted by the CL, placenta and adrenal gland in sheep. Following ovulation, theca and granulosa cells undergo luteinization in response to LH, differentiating into small and large luteal cells, respectively (Smith *et al.* 1994). The ovulatory follicle will transition to a corpus hemorrhagicum (CH) temporarily, and then into the CL. The CL begins secreting P4 into the circulation by approximately Day 4, reach maximal size on Day 7 and maintain secretion if uninterrupted by luteolytic activity (Bazer *et al.* 1998). Progesterone is also secreted by the sheep placenta in sufficient quantities by Days 50 to 70 to maintain pregnancy and a second phase of increase between Days 90 to 120 of pregnancy (Ricketts and Flint 1980).

The actions of P4 are mediated primarily through nuclear PGR which is a modular protein consisting of a centrally located, highly conserved DNA-binding domain (DBD), a carboxyl-terminal ligand-binding domain (LBD) and a variable amino-terminal domain (Conneely and Jericevic 2002; Gellersen *et al.* 2009). There are two isoforms of PGR, A and B; PGRB differs from PGRA in that it is 164 nucleotides longer on the N-terminus. The PGRA and

PGRB isoforms are transcription factors that upon ligand binding recognize specific *cis*-acting hormone response elements usually located within the promoter region of genes to modulate gene expression. However, modulation of gene expression requires cofactors (coactivators or corepressors) to induce or inhibit transcription of the gene upon which response element PGR-A and/or PGR-B is targeting (Gellersen *et al.* 2009). Thus PGRs ability to interact with DNA in response to ligand binding and recruitment of cofactors is the mechanism by which gene expression and protein synthesis occur in P4-dependent genes. The P4 inducible gene network has been investigated to the greatest extent in mice and shown to encompass transcription and growth factors, homeobox genes and morphogens, peptide hormones, extracellular matrix and cell adhesion molecules, enzymes and protease inhibitors (Bagchi *et al.* 2003). Mouse models for PGRA null isoform have severe abnormalities in ovarian and uterine function leading to infertility; whereas PGRB null mice have impaired mammary functions. Thus PGRA is both necessary and sufficient to elicit P4-dependent reproductive responses necessary for female fertility, while PGRB is necessary for normal proliferative and differentiative responses of the mammary gland to P4 (Conneely and Jericevic 2002). Non-genomic PGRs have been investigated in certain tissues; however, their particular modes of action in female reproductive tissues remains elusive (Gellersen *et al.* 2009).

All mammalian uteri contain endometrial glands that synthesize and secrete or transport a complex array of proteins and related substances termed histotroph (Spencer and Bazer 2004b). In sheep, endometrial gland morphogenesis occurs postnatally after withdrawal from a P4 dominated prenatal environment at birth (Wiley *et al.* 1987; Gray *et al.* 2000a). Therefore, inappropriate exposure of ewe lambs to a synthetic, nonmetabolizable progestin results in a uterine gland knock out (UGKO) phenotype (Bartol *et al.* 1988; Gray *et al.* 2000b). The progestin epigenetically ablates development of uterine glands without disrupting development

of the myometrium and other Müllerian duct-derived female reproductive tract structures or the hypothalamic-pituitary-ovarian axis (Gray *et al.* 2001a). UGKO ewes lack uterine GE and have reduced LE surface area. The reduction of LE area results in insufficient OXTR to produce luteolytic PGF, thus UGKO ewes do not exhibit normal estrous cycles. However when administered exogenous PGF, the CL regresses and the ewes exhibit normal estrus behavior (Gray *et al.* 2000a). UGKO ewes are infertile and transfer of blastocysts into synchronized UGKO uteri also failed to establish a pregnancy (Gray *et al.* 2001b). Similarly, progestin treatment of neonatal heifers compromises uterine gland development and increases pregnancy failure in adult heifers (Bartol *et al.* 1995).

Failure of mated UGKO ewes to support pregnancy demonstrated the necessity of endometrial epithelia and their secretions for growth and elongation of the conceptus. Flushings from UGKO ewes on Days 6 and 9 post-mating contained developmentally normal blastocysts suggesting that compaction and blastocyst differentiation are not dependent upon endometrial glands and their secretions (Gray *et al.* 2001b). This is also evident in the success of *in vitro* produced blastocysts; however, sophisticated culture systems have failed to support development of embryos after hatching from the zona pellucida. Flushings from UGKO ewes on Day 14 post-mating contained severely growth retarded tubular conceptuses, approximately equivalent to morphology on Days 11 to 12 of pregnancy, and no detectable IFNT (Gray *et al.* 2001b). Restriction of ovine and porcine embryos to the oviductal environment, a milieu absent of endometrial glands and secretions, also results in failure of embryonic development beyond the blastocyst stage (Wintenberger-Torres 1956; Bazer 1975). Thus endometrial glands and their secretory products are not required for development of the embryo to the hatched blastocyst state, but are unequivocally required for development to an elongated conceptus (Gray *et al.* 2002).

Endometrial functions are influenced by the hormonal status of the female (Guillomot *et al.* 1988). Continuous exposure of the uterus to P4 induces expression of GE secretory products that are collectively termed histotroph (Brinsfield and Hawk 1974; Salamonsen *et al.* 1985). Histotroph includes, adhesion proteins, cytokines, enzymes, hormones, growth factors, protease inhibitors, serum proteins, transport proteins, nutrients, eicosanoids, ions and other molecules yet to be discovered (Spencer *et al.* 2004b; Bazer *et al.* 2009). A summary of factors known to comprise histotroph in sheep is presented in Table 2.1.

Table 2.1. Factors known to comprise endometrial histotroph in sheep during early pregnancy.

Factor		Days Present	Reference
Adhesion Molecules			
GLYCAM1	Glycosylation dependent cell adhesion molecule 1	13-19	(Spencer *et al.* 1999)
IGFBP1	Insulin-like growth factor binding protein 1	12-20	(Simmons *et al.* 2009)
LGALS15	Lectin, galactoside-binding, secreted 15	10-18	(Farmer *et al.* 2008)
SPP1	Secreted phosphoprotein 1 (osteopontin)	15-parturition	(Johnson *et al.* 1999a)
Cytokines			
B2M	Beta-2-microglobulin	10-20	(Choi *et al.* 2003)
CSF2	Colony stimulating factor (granulocyte macrophage)	17	(Imakawa *et al.* 1993)
CXCL10	Chemokine (C-X-C motif) ligand 10	14-20	(Imakawa *et al.* 2006)
LIF	Leukemia inhibitory factor	4-20	(Vogiagis *et al.* 1997)
MX1	Myxovirus (influenza virus) resistance 1	15-17	(Toyokawa *et al.* 2007)
OAS	2',5'-oligoadenylate synthetase	11-19	(Johnson *et al.* 2001b)
Enzymes			
CTSL	Cathepsin L	10-20	(Song *et al.* 2005)
Hormones			
GRP	Gastrin-releasing peptide	18-120	(Song *et al.* 2008)
STC1	Stanniocalcin 1	18-80, >120	(Song *et al.* 2006a)

Table 2.1 continued.

Factor		Days Present	Reference
Growth Factors			
FGF2	Fibroblast growth factor 2 (basic)	12-19	(Ocon-Grove *et al.* 2008)
IGF1	Insulin-like growth factor 1	3-22	(Ko *et al.* 1991)
IGF2	Insulin-like growth factor 2	10-16	(Ko *et al.* 1991)
TGFB	Transforming growth factor beta	12-20	(Imakawa *et al.* 1998)
Protease Inhibitors			
CST3	Cystatin C	10-20	(Song *et al.* 2006b)
TIMP1	Tissue metallopeptidase inhibitor 1	4-20	(Hampton *et al.* 1995)
TIMP2	Tissue metallopeptidase inhibitor 2	4-20	(Hampton *et al.* 1995)
UTMP	Uterine milk proteins (serpins)	15-80, >120	(Stewart *et al.* 2000)
Serum Proteins			
Endothelin		15-20	(Riley *et al.* 1994)
Transport Proteins			
ISG15	ISG15 ubiquitin-like modifier	13-25, >25-150	(Joyce *et al.* 2005)
IGFBP3	Insulin-like growth factor binding protein 3	12-20	(Simmons *et al.* 2009)
Nutrients			
Amino acids	Arg, Gln, Leu, Asp, Glu, Asn, His, β-Ala, Tyr, Trp, Met, Val, Phe, Ile, Lys, Cys, Pro, Glutathione (tripeptide)	10 < 16	(Gao *et al.* 2009)
Ions	Ca, Na, K	10 < 16	(Gao *et al.* 2009)
Carbohydrate	Glucose	10 < 16	(Gao *et al.* 2009)

Progesterone induces histotroph secretion in a temporal manner to best accommodate blastocyst growth and conceptus development. In embryo transfer, it is necessary for the donor and recipient uterine environments to be closely synchronized (Moore and Shelton 1964; Wilmut and Sales 1981). When asynchrony was studied, embryos placed in a physiologically advanced uterine environment grow faster than synchronously transferred embryos; however, these embryos were unable to secrete IFNT by the appropriate time to circumvent luteolysis (Lawson

et al. 1983). Additionally, placement of the transferred embryo in the uterine horn ipsilateral to the ovary with the CL, thus the horn with greatest P4 influence, increases pregnancy outcome (Sreenan *et al.* 1975). Progesterone induction of histotroph is indeed temporal and spatially regulated within the maternal environment.

Reproductive physiology of cattle has been negatively impacted over the past 50 years for the sake of improving production efficiencies (Archer *et al.* 1998; Lucy 2001). Early pregnancy wastage in beef cattle is estimated to be at least 25% in beef cattle and 45% or greater in dairy cattle (Diskin and Sreenan 1980; Thatcher *et al.* 2001). A majority of this loss (70-80%) occurs between Days 8 and 16 post-insemination (Sreenan and Diskin 1983; Diskin and Morris 2008). It is during this time frame that blastocyst growth to an elongated conceptus is crucial for producing IFNT, the pregnancy recognition signal. Endometrial secretion, essential for initiating and mediating changes in blastocyst growth and conceptus differentiation, is regulated by the quantity and timing of P4 release from the CL into the circulation (Geisert *et al.* 1992). In cattle, rapid blastocyst development and pregnancy establishment is positively correlated with increasing concentrations of circulating P4 (Sreenan and Diskin 1983; Mann and Lamming 1999).

Exogenous P4 administration has been the focus of research for the past seven decades in ameliorating the growing epidemic of embryonic mortality (Wiltbank *et al.* 1956; Johnson *et al.* 1958; Sreenan and Diskin 1983; Van Cleeff *et al.* 1991; Mann and Lamming 1999; Villarroel *et al.* 2004; Mann *et al.* 2006; Forde *et al.* 2010; Forde *et al.* 2011). Analysis of the research conducted over the years (presented in Table 2.2) suggests that exogenous P4 administration has moderate effects overall in enhancing pregnancy rates (Mann and Lamming 1999).

Table 2.2. Summary of studies conducted to investigate effects of P4 supplementation on pregnancy rate in cattle. Progesterone supplementation is presented for days post-mating and the duration of administration. Effects on pregnancy are the overall impact of P4 supplementation in comparison to control females, however no statistical comparisons are presented.

Status	Duration of P4 supplementation	Effect on Pregnancy	Reference
Repeat Breeding Heifers	At breeding	+30.0%	(Henrick 1953)
Dairy Heifers	3, 4 or 5	+30.1%	(Dawson 1954)
Dairy Cows selected for poor fertility	3-34	+11.9%	(Wiltbank *et al.* 1956)
Dairy Heifers and Cows	2-9	+32.3%	(Johnson *et al.* 1958)
Beef Heifers	10-20	+17.2%	(Sreenan and Diskin 1983)
Beef Heifers	5-Necropsy	+28.7%	(Sreenan and Diskin 1983)
Lactating Dairy Cows	10-20	+1.9%	(Sreenan and Diskin 1983)
Lactating Dairy Cows	5-12	+30.7%	(Robinson *et al.* 1989)
Lactating Dairy Cows	10-17	+29.3%	(Robinson *et al.* 1989)
Lactating Dairy Cows	5-11	+10.9%	(Walton *et al.* 1990)
Lactating Dairy Cows	13-21	+7.6%	(Stevenson and Mee 1991)
Dairy Heifers	7-13	+4.3%	(Van Cleeff *et al.* 1991)
Lactating Dairy Cows	6-19	+2.5%	(Villarroel *et al.* 2004)
Lactating Dairy Cows	4-11; 5-12	+1.7%	(Hanlon *et al.* 2005)
Lactating Dairy Cows	3.5-10	+12.8%	(Larson *et al.* 2007)

The results of these studies suggest that P4 supplementation is beneficial when it elicits an early post-ovulatory rise in P4. Concentration of P4 is not an absolute determinant of a successful pregnancy, but rather a factor which influences the probability of success or failure (Mann and Lamming 1999). Research suggests P4 supplementation after Day 6 post-mating yields poor results whereas P4 supplementation before Day 6 results in 10% improvement in pregnancy rate (Mann and Lamming 1999). Furthermore, if initial pregnancy rate was low (<50%), a greater improvement (19%) resulted from P4 supplementation; whereas, if initial pregnancy rate was high (>50%), there was no detectable benefit (Mann and Lamming 1999). It may be beneficial for the environment of the conceptus to be affected by exogenous P4 if enhanced conceptus development can be achieved and advancement of IFNT secretion can be

realized to signal for the establishment of pregnancy. This is especially true for the lactating dairy cow. Increased liver blood flow resulting from elevated feed intake in lactating dairy cows fed to meet their demanding nutritional requirements, have increased steroid metabolism (Sangsritavong *et al.* 2002).

From these studies it is clear that P4 supplementation has an effect on the developing blastocyst. The effect is indirect and a result of downstream P4-induced changes in endometrial gene expression resulting in changes in the composition of histotroph to which the developing embryo is exposed (Geisert *et al.* 1992; Bauersachs *et al.* 2006; Spencer *et al.* 2007; Spencer *et al.* 2008; Clemente *et al.* 2009). Current research being conducted regarding P4 supplementation is aimed at evaluating the transcriptome. In beef heifers, supplemental P4 was found to change the temporal transcriptional profile of the endometrium during different developmental stages of the conceptus which contributed to or consequently advanced conceptus development (Carter *et al.* 2008; Forde *et al.* 2009). Genes affected by P4 supplementation were associated with energy sources or contributors to histotroph (Forde *et al.* 2009).

Levels of P4 during the postovulatory rise have been demonstrated to be positively associated with enhanced conceptus development in sheep. In ewes, predisposed to either having short (15.9 ± 0.1 Days) or long (18.6 ± 0.4 days) estrous cycles, ewes with short cycles had greater concentrations of P4 on Days 2 to 4; but thereafter, concentrations of P4 were not statistically different between ewes with short or long estrous cycles (Nephew *et al.* 1991). Ewes with shorter estrous cycles had both advanced conceptus development and uterine environment by Day 13 as evidenced by the presence of filamentous conceptuses in uteri of ewes with short estrous cycles compared to no filamentous conceptus in uteri of ewes with longer estrous cycles. Greater amounts of protein and IFNT were present in uterine flushes from ewes with short estrous cycles suggesting an enhanced uterine environment (Nephew *et al.* 1991).

Progesterone supplementation during early pregnancy affects fetal growth. Administration of P4 from Days 1 to 3 in ewes was demonstrated to significantly increase fetal growth on Day 74 of pregnancy (Kleemann *et al.* 1994).

In a study conducted to identify genes regulating conceptus survival and elongation, Satterfield and colleagues (2006) developed a ewe model for advanced conceptus development by administering P4 from Day 1.5 post-mating to either Day 9 or Day 12. They found P4-treated ewes had blastocysts with diameters 220% greater than those for control ewes on Day 9 (Satterfield *et al.* 2006). Further, Day 12 control ewes had spherical to slightly tubular blastocysts in contrast to elongated and filamentous conceptuses of P4-treated ewes. In ewes treated with P4 from Days 1.5 to 8 and then treated Days 9 to 12 with RU486, a PGR antagonist, no blastocysts were recovered from the uterus. Early P4 treatment enhanced blastocyst growth and development post-hatching from the zona pellucida; however, the time of blastocyst hatching was unaltered (Satterfield *et al.* 2006).

In a subsequent study, Satterfield and colleagues (2009) used a microarray approach to identify the mechanisms by which P4 acts on the endometrium to advance conceptus development. The Database for Annotation, Visualization, and Integrated Discovery (DAVID) identified biological networks differentially regulated on Day 9 versus Day 12 of pregnancy, Day 9 P4-treated versus control, and Day 12 P4-treated versus control ewes (Satterfield *et al.* 2009). Of particular importance, transport and transporter activity, and factors which stimulate cellular proliferation, migration, and attachment were differentially expressed. Indeed, increases in expression of these genes would be expected as enhanced blastocysts growth requires an increase in composition and transport of histotroph, and conceptus development requires cellular proliferation and migration of trophectoderm cells at Day 12 of pregnancy.

Gene Profiles

The following information presented in this section of the Literature Review focuses on candidate genes identified by microarray analysis. Figure 2.2 summarizes regulation of candidate genes as found by Satterfield and authors (2009).
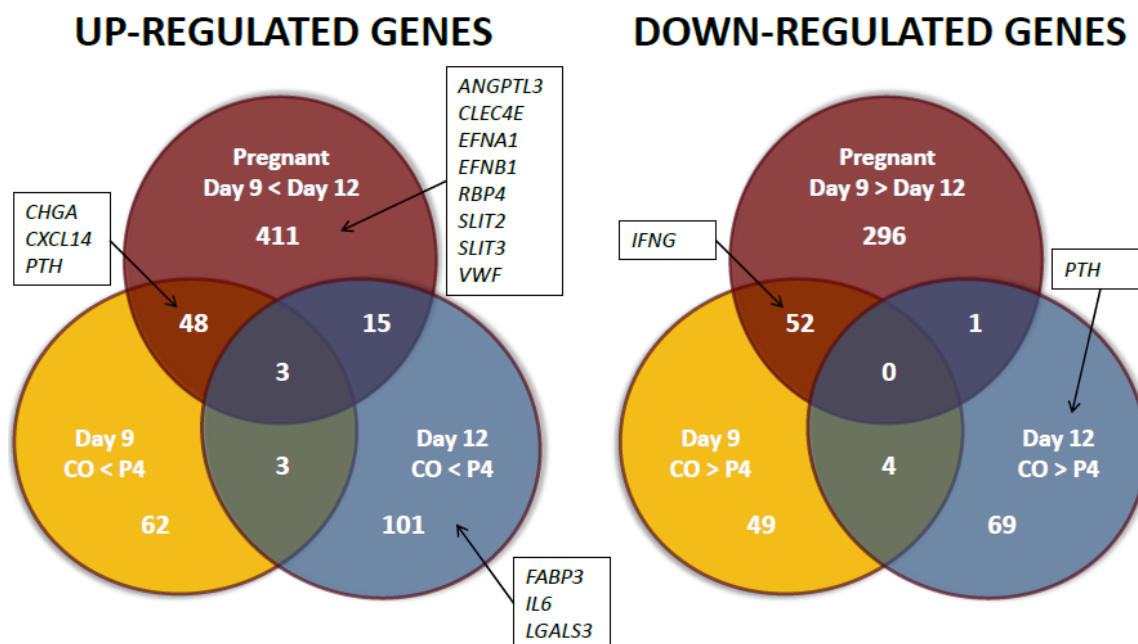
Figure 2.2. Progesterone regulated genes during early pregnancy in ovine endometrium. Venn diagram illustrating endometrial genes up-regulated and/or down-regulated between Days 9 and 12 of pregnancy and in response to early P4 treatment (Satterfield *et al*. 2009). Genes highlighted in the boxes were selected for further mRNA expression analysis. Adapted from (Satterfield *et al*. 2009).

ANGPTL3

Angiopoietin-like 3 (*ANGPTL3*) is structurally similar to the angiopoietin family, which function as secreted factors to facilitate in the cascade of angiogenesis, the proliferation of new blood vessels from pre-existing capillaries (Camenisch *et al*. 2002). Upon ligand of ANGPTL3, the two termini of the structure induce varying functions. The N-terminal coiled-coil domain

(CLD) increases plasma triglyceride levels in mice whereas the C-terminal fibrinogen-like domain (FLD) stimulates cell adhesion and migration (Gao *et al.* 2010). Overexpression of *ANGPTL3* in mice led to extremely high blood levels of triglycerides and cholesterol (Shan *et al.* 2009). Mostly produced in the liver and released into the circulation, ANGPLT3 stimulates adipose tissue lipolysis thereby raising free-fatty acid and glycerol levels in plasma (Lichtenstein and Kersten 2010). Lipolytic processing of triglyceride-rich lipoproteins is mediated by lipoprotein lipase (LPL) to generate intermediate-density lipoprotein, low-density lipoprotein and chylomicron remnants. LPL is tethered to capillary endothelium via heparin sulfate proteoglycans, and produced by adipocytes, cardiomyocytes and macrophages. Expression of LPL is governed by the peroxisome proliferator activated receptor alpha (PPARA) and liver X receptor alpha (LXRA) in the liver. Several modulators of LPL are known. Specifically, ANGPTL3 suppresses LPL activity. Consequently, uptake of fatty acids and cholesterol into tissues is decreased. *ANGPTL3* mRNA is markedly induced by activation of LXR that binds to a LXR response element in the *ANGPLT3* promoter. Whereas leptin and insulin down regulate *ANGPTL3* mRNA expression (Lichtenstein and Kersten 2010).

In epithelia, *ANGPLT3* has been implicated in cell adhesion and migration. Podocytes are highly specialized epithelial cells with a complex cellular organization consisting of a cell body, major processes and foot processes. Over expression of *ANGPTL3* in podocytes significantly promoted the migration and permeability of the epithelia (Gao *et al.* 2010). Migration and cell adhesion of *ANGPTL3* was conducted via integrin binding. The FLD of the *ANGPTL3* molecule does not bind Tie2, the angiopoietin family receptor, but rather integrin $\alpha_v\beta_3$ to stimulate endothelial cell adhesion and migration. Integrins are two-way signaling receptors responsible for the attachment of cells to the extracellular matrix (Camenisch *et al.* 2002). Integrin subunits α (v, 4, 5) and β (1, 3, 5) are constitutively expressed on the apical surfaces of

both conceptus trophectoderm and endometrial LE (Johnson *et al.* 2001a; Burghardt *et al.* 2002; Spencer *et al.* 2004a). *ANGPTL3* has not been evaluated in the uterus or during pregnancy.

CHGA

Chromogranin A (*CHGA*) gene is a precursor of biologically active peptides with endocrine, paracrine and autocrine functions. Functional peptides include vasostatin, β-granin, chromostatin, pancreastatin and parastatin (Hendy *et al.* 1995). The glycoprotein is acidic and hydrophilic, and involved in hormone packaging of secretory vesicles of many endocrine and neuroendocrine cells, and neurons. CHGA is concentrated and stored within secretory granules and released into the extracellular environment with co-resident hormones after an appropriate stimulus (Ratti *et al.* 2000). Hydrogen ion and calcium concentration can induce dramatic conformational changes of CHGA which contributes to the ability to sort peptide hormones and neurotransmitters, and package into secretory granules. CHGA synthesis opposes its action on biosynthesis of the resident hormone. In pituitary gonadotrophs, estradiol down-regulates *CHGA* gene expression and secretion, while enhancing luteinizing hormone (LH) and follicle stimulating hormone (FSH) synthesis and secretion. Thereby suppressing *CHGA* expression removes the inhibitory effect on the secretion of the resident LH and FSH to maximize secretion of estradiol (Hendy *et al.* 1995). An intracellular mechanism of CHGA is the regulation of granule biosynthesis by controlling the stayability and availability of granule proteins at the posttranslational level. Partial embryonic lethality occurred in *Chga* null mice. Surviving *Chga* null mice must possess compensatory mechanisms however they demonstrate decreased number of granules with elevated levels of corticosterone. This is likely attributed to the absence of *CHGA* down-regulating mechanism (Kim and Loh 2005). Information of *CHGA* in the uterus or during pregnancy is unknown.

CLEC4E

C-type lectin domain family 4, member E (*CLEC4E*) also termed Mincle (Macrophage-inducible C-type lectin) is a C-type lectin receptor expressed in activated macrophages. The C-type lectin receptor (CLR) family of calcium-dependent carbohydrate binding lectins is a member of the innate immune receptor family. Members also include toll-like receptors (TLR), nod-like receptor (NLR) and RIG-I-like receptors (RLR). CLEC4E is highly up-regulated after exposure to various stimuli such as inflammatory cytokines and TLR ligands. CLEC4E transduces activation signals through the immunoreceptor tyrosine-based activation motif (ITAM) containing adaptor protein, FcRγ, to induce secretion of pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNFα), chemokine (C-X-C motif) ligand (CXCL) 1, CXCL2 and interleukin-6 (IL6) to drive the recruitment of neutrophils (Crozat *et al.* 2009; Miyake *et al.* 2010). Inflammatory mediators are the first molecules released in the milieu after innate immune cell activation. Because they stimulate innate immune cells, they are considered the bridges between mechanisms of recognizing pathogens or altered self from self, and the induction of active effector functions (Crozat *et al.* 2009). Information of *CLEC4E* in the uterus or during pregnancy is unknown.

CXCL14

Chemokine (C-X-C motif) ligand 14 (*CXCL14*), also known as BRAK, is a member of the chemokine family that consists of approximately 50 ligands and 20 receptors (Meuter *et al.* 2007). Chemokines are implicated in many functions such as angiogenesis, hematopoiesis and organogenesis (Locati and Murphy 1999). *CXCL14* is expressed ubiquitously in normal epithelial tissues but shows significantly decreased expression in tumor cells (Starnes *et al.* 2006; Meuter *et al.* 2007). CXCL14 is a chemoattractant for dendritic cells, activated monocytes, neutrophils and natural killer cells under differing physiological conditions (Kurth *et al.* 2001;

Shellenberger *et al.* 2004; Shurin *et al.* 2005; Starnes *et al.* 2006; Kuang *et al.* 2009a; Salogni *et al.* 2009). CXCL14 is a potent mediator of tumor growth, invasion and metastasis, physiological occurrences which share similarities with trophectoderm invasion during implantation of invasive species (primates, humans, rodents) (Kirby 1965; Kuang *et al.* 2009a). Loss of *CXCL14* may allow tumor cells to escape mechanisms of immune surveillance and gain a selective advantage (Starnes *et al.* 2006). Several chemokine receptors have been tested for the ligand however the receptor of CXCL14 remains to be elucidated.

CXCL14 has been implicated in mice and human endometrial function during early pregnancy. In mice, CXCL14 regulated trophectoderm invasion during implantation, a process similar to tumor invasion. Biotinylated CXCL14 ligand was found to be bound by secondary trophectoderm giant cells of the ectoplacental cone at the fetomaternal interface (Kuang *et al.* 2009a). Dissimilar from other chemokines which could regulate trophectoderm migration in a similar method to that of leukocyte recruitment, CXCL14 does not play a promontory role and is shown to inhibit trophectoderm outgrowth and attachment (Kuang *et al.* 2009a). CXCL14 was found to down-regulate blastocyst and EPC matrix metalloproteinase (MMP) 2 and 9, proteases which are capable of degrading extracellular matrix. Thus the trophectoderm was unable to invade through the decidua to the maternal vasculature (Kuang *et al.* 2009a).

In women, *CXCL14* was expressed at the fetomaternal interface in first trimester implantation sites (Kuang *et al.* 2009b). CXCL14 was highly regulated in human midsecretory endometrium compared to early secretory endometrium. Furthermore, expression was high in cytotrophoblasts and moderate in decidualized stromal cells whereas no expression observed in extravillous trophoblast and syncytiotrophoblast, thereby expression may be regulated with trophoblast cell differentiation (Kuang *et al.* 2009b). During the menstrual cycle, *CXCL14* is expressed the highest during the P4 influenced secretory phase in uterine GE (Mokhtar *et al.*

2010). *CXCL14* was found to be transcriptionally regulated by P4 with at least two progesterone response elements (PRE) within the 2 kb promoter region of the gene (Mokhtar *et al.* 2010).

EFNA1/EFNB1

The Ephrin (EFN) families of ligands bind the EPH subfamily of receptor tyrosine kinases (RTK). EFN/EPH signaling mediate many biological processes, including developmental and tumor angiogenesis, and embryogenesis (Katoh and Katoh 2006). Ephrins are presented on the cell surface and attached to the membrane either through a glycosylphosphatidyl inositol (GPI) anchor or transmembrane domain. Thus, EFNs have been divided into two subclasses, class A (GPI anchor) and B (transmembrane domain), dependent upon which type of membrane interaction (Kuijper *et al.* 2007). Ligand and receptor binding is permissive. Specificity of binding may be linked to cell specific expression. EFN/EPH interaction does not promote cell proliferation during angiogenesis but rather mediate cell-cell attachment, cell-matrix contacts and cellular migration (Cheng *et al.* 2002). EFN and EPH molecules mediate cell-cell attachment by the cadherin family. Cadherins mediate cell interactions and have been implicated in embryo attachment during implantation (Singh and Aplin 2009). Stimulation of EFNB1 induces integrin-dependent cell attachment and dissociation from extracellular matrices (Cheng *et al.* 2002). EFNA1, an intermediate gene product of TNFα and vascular endothelial growth factor, has been proposed to induce endothelial cell migration during blood vessel assembly (Cheng *et al.* 2002). Several EFN and EPH molecules are over-expressed in tumor tissues and result in pathological angiogenesis (Kuijper *et al.* 2007). During embryogenesis, EFN/EPH signaling regulates cell movements that establish germ layers, tissue boundaries, and vascular and neural networking, including axon pathfinding (Duffy *et al.* 2006). EFNA1 is localized to extravillous trophoblast cells of the invasive human placenta, suggesting EFNA1 expression to be critical for the migrating trophoblast which penetrates the maternal

uterine epithelia and spiral artery endothelium to establish hematotrophic source of nutrition (Goldman-Wohl *et al.* 2004; Fujii *et al.* 2011).

FABP3

Fatty acid binding protein 3 (*FABP3*), muscle and heart (also known as mammary-derived growth inhibitor, MDGI) is a 15-kDa intracellular fatty acid binding protein. Fatty acid binding proteins bind hydrophobic ligands in a reversible manner and mediate fatty acid metabolism (Hanhoff *et al.* 2002). *FABP3* is proposed to function in solubilization, transportation and energy homeostasis of fatty acids (Nevo *et al.* 2010). *Fabp3* null mice display alterations in cardiac long chain polyunsaturated fatty acid (LCPUFA) uptake and esterification into triglycerides and phospholipids (Storch and Thumser 2010). Furthermore, arachidonic acid incorporation into triglycerides and phospholipids was decreased however phosphadtidylinositol and phosphatidyleserine were not affected, implying *FABP3* involvement in lipid-mediated signal transduction (Storch and Thumser 2010).

FABP3 has roles in growth inhibition and differentiation of the mammary gland from virgin to lactating state (Borchers *et al.* 1997). FABP3 content in virgin mammary gland was lower in comparison to late and lactating rat mammary gland. The induction of FABP3 was suggested to be mediated by lactogenic hormones (Borchers *et al.* 1997). In mice, FABP3 protein was up-regulated during cardiomyocyte differentiation and was associated with the inhibition of cardiomyocyte proliferation (Tang *et al.* 2004). Interestingly, *FABP3* expression was greater in human term syncytiotrophoblast that cytotrophoblasts, indicating differentiation as a regulator *FABP3* expression (Daoud *et al.* 2005). Additionally, the authors discovered LCPUFA linoleic acid efflux was greater in syncytiotrophoblasts and correlated with *FABP3* expression, thus increasing the amount of fatty acid transport via the placenta (Daoud *et al.* 2005). Fatty are required by the developing fetus to maintain fluidity, permeability and

conformation of membranes, as precursors of signaling compounds including prostaglandins, prostacylins, thromboxanes and leukotrienes, and as an energy source (Haggarty 2002). *FABP3* role during early pregnancy has not been elucidated however expression has been identified in horse blastocysts (Smits *et al.* 2011) and cattle endometrium during early pregnancy (Forde *et al.* 2011). During late gestation, *FABP*s are important in facilitating the transfer of fatty acids across membranes and intracellular channeling (Haggarty 2002). The deposit of LCPUFA to the fetus is rapid during growth and especially crucial for development of the brain (Duttaroy 2009).

IFNG

Interferon gamma (IFNG), a Type II IFN, plays important roles in activating innate and adaptive immune responses, inhibiting cell proliferation and inducing apoptosis (Boehm *et al.* 1997). IFNG activates gene expression via JAK/STAT1 pathway (Bazer *et al.* 2009). IFNG is produced primarily by natural killer (NK) cells (Murphy *et al.* 2009). Activation of the innate immune system by Type 1 IFNs and T-helper 1 (Th1) cells can be induced to synthesize and secrete IFNG (Murphy *et al.* 2009). NK cells increase at implantation sites to regulate spiral artery remodeling and decidualization during early pregnancy in mice and humans (Croy *et al.* 2006). Involvement of NK cells in ruminant pregnancy is not well understood (Hansen 2007). IFNG inhibits trophoblast invasion by down regulating expression of MMP2 and MMP9 (Hu *et al.* 2006; Lash *et al.* 2006). IFNG may play a role in preventing excessive invasion of trophoblast cells during implantation (Murphy *et al.* 2009). Of domestic species, IFNG is produced highest in pigs and is thought to have a synergistic role with IFN delta (IFND) in enhancing uterine receptivity by affecting polarity of uterine LE for remodeling (Bazer *et al.* 2009).

IL6

Interleukin 6 (*IL6*) is a pleiotrophic cytokine that is secreted as a 26-kDa soluble protein or complexed in a membrane-bound form with IL6 signal transducer (*IL6ST*, also known as gp130) (Taga and Kishimoto 1997; Kamimura *et al.* 2003; Robertson *et al.* 2010). IL6 is ubiquitous and has diverse roles in hematopoiesis and the immune system as a regulator of inflammatory response (Robertson *et al.* 2010). In the innate immune system, IL6 regulates the generation, recruitment, and functional phenotype of neutrophils, macrophages and dendritic cells (Romano *et al.* 1997; Mitani *et al.* 2000; Robertson *et al.* 2010). In the adaptive immune response, IL6 stimulates production of antibodies by B cells, and in T cells to play a role in the Th1/Th2 balance (Muraguchi *et al.* 1988; Rincon *et al.* 1997).

IL6 has been shown to be synthesized in the female reproductive tract and gestational tissues and appears to be involved in embryo implantation, placental development and the later stages of pregnancy (Robertson *et al.* 2010). *In vitro* culture of human primary cytotrophoblasts cells demonstrated IL6 to stimulate cell migration and invasion by activation of MMP2 and MMP9 and integrin interaction (Jovanovic and Vicovac 2009). IL6 deficient mice have reduced fertility and fewer viable implantation sites (Robertson *et al.* 2000). Furthermore, IL6 acts in late gestation to accelerate the events of labor and mediates as link between declining P4 and induction of uterine activation genes for parturition (Robertson *et al.* 2010).

A related cytokine, leukemia inhibiting factor (LIF) shares co-receptor IL6ST as IL6. LIF regulates the onset of uterine receptivity to blastocyst implantation and embryogenesis, and trophoblast giant cell differentiation in the placenta of mice (Vogiagis and Salamonsen 1999; Takahashi *et al.* 2008; Song *et al.* 2009). Upon LIF binding to its receptor, LIF recruits IL6ST as a co-receptor. The heterodimer complex activates JAK 1 or 2 and tyrosine kinase 2 (TYK2), and then STAT (particularly STAT3) to regulate gene expression (Auernhammer and Melmed

2000; Song *et al.* 2009). Uterine LIF has been implicated in endometrial function, and conceptus growth and development during peri-implantation in sheep (Song *et al.* 2009). Defective LIF production at the feto-maternal interface has been associated with pregnancy loss (Piccinni *et al.* 2001).

LGALS3

Lectin, galactoside-binding, soluble, 3 (*LGALS3*) is a member of the β-galactoside-binding lectins. *LGALS3* functions in cell growth (Barondes *et al.* 1994), apoptosis (Dumic *et al.* 2006), mRNA processing (Liu *et al.* 2002), metastasis (Takenaka *et al.* 2004), differentiation and angiogenesis of endothelial cells (Nangia-Makker *et al.* 2000) mediation of inflammation and leukocyte adhesion (Almkvist and Karlsson 2004) and chemoattractant for monocytes and macrophages (Sano *et al.* 2000). In humans, LGALS3 is expressed throughout the menstrual cycle with highest expression in the secretory phase in uterine GE (von Wolff *et al.* 2005). Furthermore, LGALS3 is expressed on both trophoblast, epithelium and decidualized stroma, implicating LGALS3 in cell adhesion during implantation (von Wolff *et al.* 2005). LGALS3 modulates cell adhesion by binding to several ligands including laminin, fibronectin and integrins after its secretion from epithelial cells (Andre *et al.* 1999; Hughes 1999). LGALS3 has been shown to be up-regulated in pathophysiological placentas (preeclamptic and intrauterine growth restricted) thus indicated to compensate for reduced trophoblast invasion based on results of LGALS3 role in initiating the adhesion of human breast and prostate carcinoma cells to endothelium (Jeschke *et al.* 2007). *Lgals3* null mutant mice show some effect on the survival or maintenance of neutrophils and macrophages at inflammatory sites and delayed phagocytosis (Poirier 2002; Sano *et al.* 2003; von Wolff *et al.* 2005). Galectins are thought to be optimizing molecules based on survival of *Lgals3* and closely related *Galectin-1* null mutant mice (Poirier 2002).

LGALS3 has been identified in ruminants. In sheep, LGALS3 is present in cotyledons during mid gestation and decreases at term (Iglesias *et al.* 1998). LGALS3 has been localized in the cow uterine epithelium and glands, and macrophages during early pregnancy (Kim *et al.* 2008).

PTH

Parathyroid hormone (PTH) contains an N-terminal conserved region that shares homology with PTH related peptide (PTHrP). PTH regulates the placental expression of genes involving calcium and other solute transfer that contribute to the regulation of placental calcium transfer (Simmonds *et al.* 2010). In the fetus, PTH is important for fetal calcium homeostasis. The fetal hypoparathyroid phenotype results in hypocalcemia, hypomagnesemia, hyperphospatemia, low amniotic fluid mineral content and reduced skeletal mineral content (Simmonds *et al.* 2010). PTH and PTHrP share a common receptor, Type 1 PTH receptor (PTH1R) to stimulate via an endocrine-like pathway, bone resorption and renal calcium resorption (Clemens *et al.* 2001). PTHrP has been implicated in uterine and placental tissues to play roles in regulating decidualization of stromal cells (Ferguson *et al.* 1998), modulating implantation and retention of the embryo (Williams *et al.* 1998), interaction of calcium binding and calcium sensing receptors to modulate trophoblastic outgrowth and differentiation (Nowak *et al.* 1999), and regulation of stretch and inhibition of oxytocin-stimulated activity during pregnancy in prevention of pre-term labor (Pitera *et al.* 1998; Clemens *et al.* 2001).

RBP4

Plasma retinol-binding protein 4, plasma (RBP4) is an intracellular transporter protein for retinol (Kanai *et al.* 1968). Retinoids have numerous effects on cell and tissue biology during embryonic development and throughout pregnancy (Thompson 1969; Bates 1983; Wellik and DeLuca 1995). RBP4 has been shown to be a major secretory product of the peri-

implantation domestic conceptus and shown to significantly affect litter size in pigs (Harney *et al.* 1993). Retinol influences the uniformity of embryos size and synchrony of development to improve the survival rate in pigs (Trout *et al.* 1991). Interestingly, RBP4 is a candidate gene for litter size in pigs (Harney *et al.* 1993; Rothschild *et al.* 2000). RBP4 also has roles as an adipocytokine which is synthesized by adipocytes or macrophages from adipose tissues. RBP4 concentrations have been reported to be higher in women with late-onset preeclampsia than in normal, late pregnancy controls and is thought to play a role in the pathophysiology of obese women with late-onset preeclampsia via increased insulin resistance (Masuyama *et al.* 2011).

SLIT2/SLIT3

Slit homolog 2 (*SLIT2*) and slit homolog 3 (*SLIT3*) are ligands for the roundabout (ROBO) transmembrane receptor. *SLIT-ROBO* interactions are implicated in the development of the nervous system by acting as guidance cues in developing organs (Hinck 2004; Andrews *et al.* 2007). Additionally, SLIT2 has been shown to block vascular endothelial growth factor and epidermal growth factor mediated migration of endothelial cells, and inhibits migration of leukocytes (Guan *et al.* 2003). Whereas, SLIT3 has been discovered to enhance monocyte migration and myeloid cell recruitment during inflammation, therefore SLIT3 promotes leukocyte migration (Geutskens *et al.* 2010).

SLIT2 and SLIT3 are expressed in endometrium throughout the menstrual cycle, with *SLIT3* significantly up-regulated in mid-secretory phase endometrium (Duncan *et al.* 2010). Both *SLIT2* and *SLIT3* mRNA was decreased in decidua tissue during early pregnancy. SLIT2 protein was shown to be expressed in uterine LE and GE, and to a lesser extent in stromal cells near LE. *SLIT2* expression was not affected by hormone steroids *in vitro* (Dickinson and Duncan 2010). The *SLIT-ROBO* pathway is also expressed in ovarian tissue. Cortisol negatively regulates *SLIT2* and *SLIT3* expression in primary cultures of luteal fibroblast-like

cells and luteinized granulosa cells (Dickinson and Duncan 2010). SLIT-ROBO pathway also hindered migration of these cells and increased apoptosis. Their activity is thought to promote luteolysis and SLIT-ROBO expression is hormonally regulated in the adult CL (Dickinson and Duncan 2010).

VWF

von Willebrand Factor (VWF) is a large multimeric plasma glycoprotein involved in normal homeostasis. VWF mediates platelet adhesion and acts as a carrier molecule for coagulation factor VIII, to protect from proteolytic degradation (McGrath *et al.* 2010). VWF is synthesized by megakaryocytes and stored within α-platelets, and by endothelial cells, stored within the Weibel-Palade bodies (Meyer *et al.* 1991). VWF unique localization to endothelial cells has resulted in the use of VWF as an endothelial cell marker for immunohistochemistry. However, VWF expression is heterogenous with higher expression occurring in venous than arterial vasculature (Zanetta *et al.* 2000). VWF expression has been shown to be up-regulated by fibroblast growth factor 2 and vascular endothelial growth factor during angiogenesis, thus indicating VWF expression as a detector of angiogenesis in tumor growth (Zanetta *et al.* 2000).

CHAPTER III

PROGESTERONE REGULATION OF ENDOMETRIAL

GENE EXPRESSION IN THE EARLY PREGNANT OVINE UTERUS

Introduction

The maternal environment to which the developing conceptus is exposed following hatching from the zona pellucida is physiologically crucial in survival and growth of the conceptus (embryo and associated extraembryonic membranes). In sheep, the morula stage embryo enters the uterus on Days 4 to 5 post-mating. The morula will develop into a blastocyst that contains an inner cell mass and blastocoele surrounded by a monolayer of trophectoderm (Guillomot 1995). On Day 10, the hatched blastocyst is spherical, 0.4 to 0.9 mm in diameter with approximately 3000 cells (Wintenberger-Torres and Flechon 1974). The blastocyst will transition from spherical to tubular to filamentous morphology. By Day 12, the tubular blastocyst is 10-22 mm in length, and elongated to 10 cm on Day 14 resembling a filamentous form (Wintenberger-Torres 1956). The trophectoderm increases 90-fold from Day 13 to 19 resulting in the elongation of the conceptus into the contralateral uterine horn of ovulation (Wales and Cuneo 1989). During elongation, the ruminant conceptus produces and secretes the maternal recognition of pregnancy signal, IFNT from the monolayer trophectoderm (Bazer *et al.* 1979; Spencer *et al.* 2008). IFNT inhibits development of the mechanism for release of luteolytic pulses of prostaglandin F2α from the uterine epithelia, thereby maintaining a functional corpus luteum (CL) and its production of progesterone (P4) required for pregnancy (Bazer *et al.* 1996). IFNT must be secreted by the elongating conceptus between Days 12 and 16 for a successful pregnancy outcome (Spencer and Bazer 2004a).

During early pregnancy under the continuous exposure of progesterone (P4) from the corpus luteum, the maternal environment synthesizes and secretes or transports a complex variety of substances, collectively termed histotroph, into the uterine lumen to nourish the developing embryo/conceptus. Histotroph is an array of adhesion proteins, cytokines, enzymes, hormones, growth factors, protease inhibitors, serum proteins, transport proteins, and other substances (Spencer *et al.* 2004b; Bazer *et al.* 2009). Endometrial glands and their secretions are vital to embryo/conceptus development in domestic species because of the protracted peri-implantation period (Bazer *et al.* 1979). Uterine gland knock out (UGKO) ewes contain a uterine milieu absent of endometrial glands and secretions along with reduced uterine LE due to epigenetic ablation by inappropriate exposure of progestin during neonate life (Gray *et al.* 2000b; Gray *et al.* 2001b). UGKO phenotype results in failure of embryonic development beyond the blastocyst stage thus demonstrating the importance of endometrial epithelia and their secretions on blastocyst development. Furthermore, studies of asynchrony between transferred embryos and the uterus have demonstrated the importance of the coordinate temporal relationships between endometrial secretions during early pregnancy and conceptus development (Pope 1988).

Satterfield et al. (2006) reported an ovine model of accelerated blastocyst development elicited by advancing the postovulatory rise in circulating levels of P4 during metestrus. Exogenous P4 administered to ewes from 36 h after onset of estrus had blastocysts on Day 9 with increased diameter and on Day 12, conceptuses were filamentous compared to spherical and tubular conceptuses in uteri of control (Satterfield *et al.* 2006). Their results indicate marked effects of early P4 supplementation on pre-implantation growth and development of blastocysts. Analyzing the endometrial transcriptome of P4 supplementation ewes by microarray, Satterfield et al. (2009) identified novel candidate genes and regulatory pathways governing P4 regulation

of peri-implantation blastocyst growth and conceptus development (Satterfield *et al.* 2009). The objective of this study was to validate P4 regulation of candidate genes by quantitative PCR in early P4-treated ovine endometrial tissues during early pregnancy.

Materials and Methods

Animals

Mature crossbred Suffolk ewes (*Ovis aries*) were observed daily for estrus in the presence of intact vasectomized rams and were used in experiments only after having exhibiting 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 in Research and Teaching and approved by the Institutional Animal Care and Use Committee of Texas A&M University.

Experimental Design

At estrus (Day 0), ewes were mated to an intact ram and assigned randomly to receive daily intramuscular injections beginning 36 hours after onset of estrus of either: corn oil (CO) through Day 9 (9CO, n =4) or through Day 12 (12CO, n = 5); 25 mg P4 in CO (Sigma Chemical, St. Louis, MO) through Day 9 (9P4, n=5) or through Day 12 (12P4, n = 5); or 25 mg P4 in CO through Day 8 and 75 mg mifepristone (RU486; Sigma Chemical Co.), a P4 receptor and glucorticoid receptor antagonist (Baulieu 1989), from Day 8 through Day 12 (P4+RU486; n=5), as described previously (Satterfield *et al.* 2006). Ewes were hysterectomized on either Day 9 or Day 12 of pregnancy. At hysterectomy, sections (~0.5 cm) from the midportion of each uterine horn ipsilateral to the CL were fixed in fresh 4% paraformaldehyde in PBS (pH 7.2). After 24 h, fixed tissues were changed to 70% ethanol (v/v) for 24 h, dehydrated through a graded series of alcohol to xylene, and then embedded in Paraplast-Plus (Oxford Labware, St. Louis, MO). The

remaining endometrium of the ipsilateral uterine horn was physically dissected from the myometrium, frozen in liquid nitrogen, and stored at -80°C for subsequent RNA extraction. No tissues from the contralateral uterine horn in monovulatory pregnant ewes were used for analysis.

RNA Isolation and Quantitative Real-Time PCR Analysis

Total cellular RNA was isolated from frozen ipsilateral endometrium using Trizol reagent (Gibco-BRL, Bethesda, MD) according to manufacturer's instructions. The quantity and quality of total RNA were determined by spectrometry and by denaturing agarose gel electrophoresis, respectively. Total RNA samples were digested with RNase-free DNase I and were cleaned up using the RNeasy MinElute Cleanup Kit (Qiagen, Valencia, CA).

Total RNA from each sample was reverse transcribed in a total reaction volume of 20 µl. Briefly, 5 µl RNA was combined with primer mix containing oligo (dT) primer (0.2 µg/µl), random hexamer primer (3 µg/µl) (Invitrogen, Carlsbad, CA) and incubated at 70°C for 10 minutes. A reverse transcriptase (RT) mix containing 5X first-stand buffer, RNasin, 100 mM dNTP, 100 mM DTT and SuperScript II RTase (Invitrogen, Carlsbad, CA) was added to the reaction and reverse transcription performed under following conditions: 42°C for 90 min and 95°C for 2 min. Complementary DNA (cDNA) preps were cleaned by phenol-choloroform extraction and acid-ethanol precipitation, resuspended in 20 µl of water and stored at -20°C. Control reactions in the absence of reverse transcriptase were prepared for each sample to detect genomic DNA contamination.

Quantitative PCR (qPCR) was performed using the ABI prism 7900HT system (Applied Biosystems, Foster, CA) with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster, CA) as specified by manufacturer. Specific oligonucleotide primers were designed and analyzed by Oligo 5 (Molecular Biology Insights, Inc). Forward and reverse primer sequences

for all genes analyzed are listed in Table 3.1. Primer specificity and efficiency (-3.2077 > threshold curve > -3.447) were confirmed using a test amplification run. Each individual sample was run in triplicate using the following conditions: $50^{\circ}C$ for 2 min, $95^{\circ}C$ for 10 min; and then $95^{\circ}C$ for 15 sec and $60^{\circ}C$ for 1 min for 40 cycles. A dissociation curve was generated to determine amplification of a single product. The threshold line was set at the linear region of the plots above the baseline noise, and threshold cycle (C_T) values were determined at the cycle number at which the threshold line crossed the amplification curve. The results are expressed in terms of change in C_T values (ΔC_T). To adjust for variations in the amount of cDNA, the mean C_T values for each gene were normalized against average C_T values for the reference gene (ovine alpha-tubulin, TUBA) as follows: ΔC_T = mean $C_{T\ specific\ gene}$ − mean $C_{T\ TUBA}$. Subtraction of an arbitrary constant from ΔC_T, generated $\Delta\Delta C_T$. Finally, the relative quantification (RQ) of each gene was calculated as $2^{-\Delta\Delta C_T}$.

Statistical Analysis

Relative quantification values were subjected to least-squares analysis of variance using the General Linear Models procedures of the Statistical Analysis System (SAS Institute, Cary, NC). $P < 0.05$ was regarded as significant. Data are presented as least squares means with overall standard errors.

Table 3.1. Candidate Entrez gene symbol, gene name, accession number and forward and reverse primer sequences for all genes.

Entrez Gene Symbol	Gene Name	Accession Number	Forward Primer	Reverse Primer
ANGPTL3	Angiopoietin-like 3	NM_001080345	GAAGAGCGACATCCAAACTG	GGTTAATTGGTCCTCCAGGT
CHGA	Chromogranin A	NM_181005	CCTCCCCAGCCCGAAATAC	TGTCTGCCTCCCTTGCTCT
CLEC4E	C-type lectin domain family 4, member E	XM_592701	GGCAATGGGTAGATGGTACA	CTTATGGTGGCACAGTCCTC
CXCL14	Chemokine (C-X-C motif) ligand 14	NM_001034410	GACCCAAGATCCGCTACAG	AACCATCTTCTCCTCGCAGT
EFNA1	Ephrin-A1	NM_001034292	GCAAAATCACTCACAGCCCT	GGTCATCTGCTGGAAGTCTC
EFNB1	Ephrin-B1	NM_001080299	CCGACAGCTTCTTCAACTCC	CAGCTTCAGTAGCAGGATGG
FABP3	Fatty acid binding protein 3, muscle and heart (MDGI)	NM_174313	AAATTCTCCTGGGGTCAGGT	GCCTTGGCTCTGCTTTATTG
IFNG	Interferon, gamma	NM_001009803	GCTCTGTGTGCTTTTGGGTT	GCTACATCTGGGTTACTTGC
IL6	Interleukin 6 (interferon, beta 2)	NM_001009392	GAGGGAAATCAGGAAACTGT	CTCGTTTGAGGACTGCATCT
LGALS3	Lectin, galactoside-binding, soluble, 3	NM_001102341	CTGGGGAAAGGAAGAAAGAC	GTCAGGTTCAACCAGCACTT
PTH	Parathyroid hormone	AF327654	GACTTACGGCGTCGGTTCTT	GCTCTGATTTCGGCTGTGTG
RBP4	Retinol binding protein 4, plasma	NM_001040475	TCCAGAAAGGAAACGATGAC	GGCAAACACGAAAGAGTAGC
SLIT2	Slit homolog 2	EF627036	CCAGATCACATTCCCCAGTA	GATCCCAGTAGCTTCCAACA
SLIT3	Slit homolog 3	EF627037	ACCAGTACGAGTGCCAGAAT	GTTGACGGTGATGAGCTTCT
TUBA	Tubulin, alpha	AF251146	GGTCTTCAAGGCTTCTTGGT	CATAATCGACAGAGAGGCGT
VWF	Von Willebrand Factor	XM_584169	GTGCTGTGACACATGTGAGG	CCTGGCAGTAGTGAATGTCC

Results

Table 3.2. Fold changes in mRNA levels of candidate genes in the ovine endometrium as determined by microarray and qPCR analyses. NC, no fold change observed for microarray comparison. Data presented as fold change; [a] $P<0.001$, [b] $P\leq0.05$ for the qPCR comparison.

Entrez Gene Symbol	Method	D12 CO vs. D9 CO	D9 P4 vs. D9 CO	D12 P4 vs. D12 CO
ANGPTL3	Microarray	2.8	NC	NC
	qPCR	2.1[a]	-1.1	-1.2
CHGA	Microarray	3.0	3.5	NC
	qPCR	4.3[b]	3.7	1.2
CLEC4E	Microarray	2.2	NC	NC
	qPCR	-1.5	1.2	1.1
CXCL14	Microarray	18.4	17.6	NC
	qPCR	15.5[b]	13.7[b]	1.0
EFNA1	Microarray	3.3	NC	NC
	qPCR	2.1[b]	1.3	1.1
EFNB1	Microarray	2.4	NC	NC
	qPCR	2.2[a]	1.4	-1.1
FABP3	Microarray	NC	NC	3.9
	qPCR	1.4	1.2	1.4[b]
IFNG	Microarray	-2.5	-3.6	NC
	qPCR	-1.1	-1.1	1.9[b]
IL6	Microarray	NC	NC	3.0
	qPCR	1.2	1.7	7.5[a]
LGALS3	Microarray	NC	NC	2.2
	qPCR	2.1[b]	1.6	1.5[b]
PTH	Microarray	4.5	3.2	-2.3
	qPCR	1.1	1.3	1.1
RBP4	Microarray	2.1	NC	NC
	qPCR	2.3[b]	1.6	-1.1
SLIT2	Microarray	2.2	NC	NC
	qPCR	2.3	1.6	1.3
SLIT3	Microarray	2.5	NC	NC
	qPCR	1.3	1.1	-1.1
VWF	Microarray	3.5	NC	NC
	qPCR	1.1	1.2	1.2

Figure 3.1. Effect of treatment in control (CO), progesterone (P4) or P4 and RU486 (P4+RU) endometrial mRNA. Relative quantification of A) *ANGPTL3*, B) *CHGA,* C) *CXCL14,* D) *EFNA1,* E) *EFNB1,* F) *FABP3*, G) *IFNG*, H) *IL6,* I) *LGALS3* and J) *RBP4* mRNA determined by qPCR. Comparisons made for day (†) and treatment (*), significance level, $P < 0.05$. Data presented as least-square mean RQ with standard error (SE).
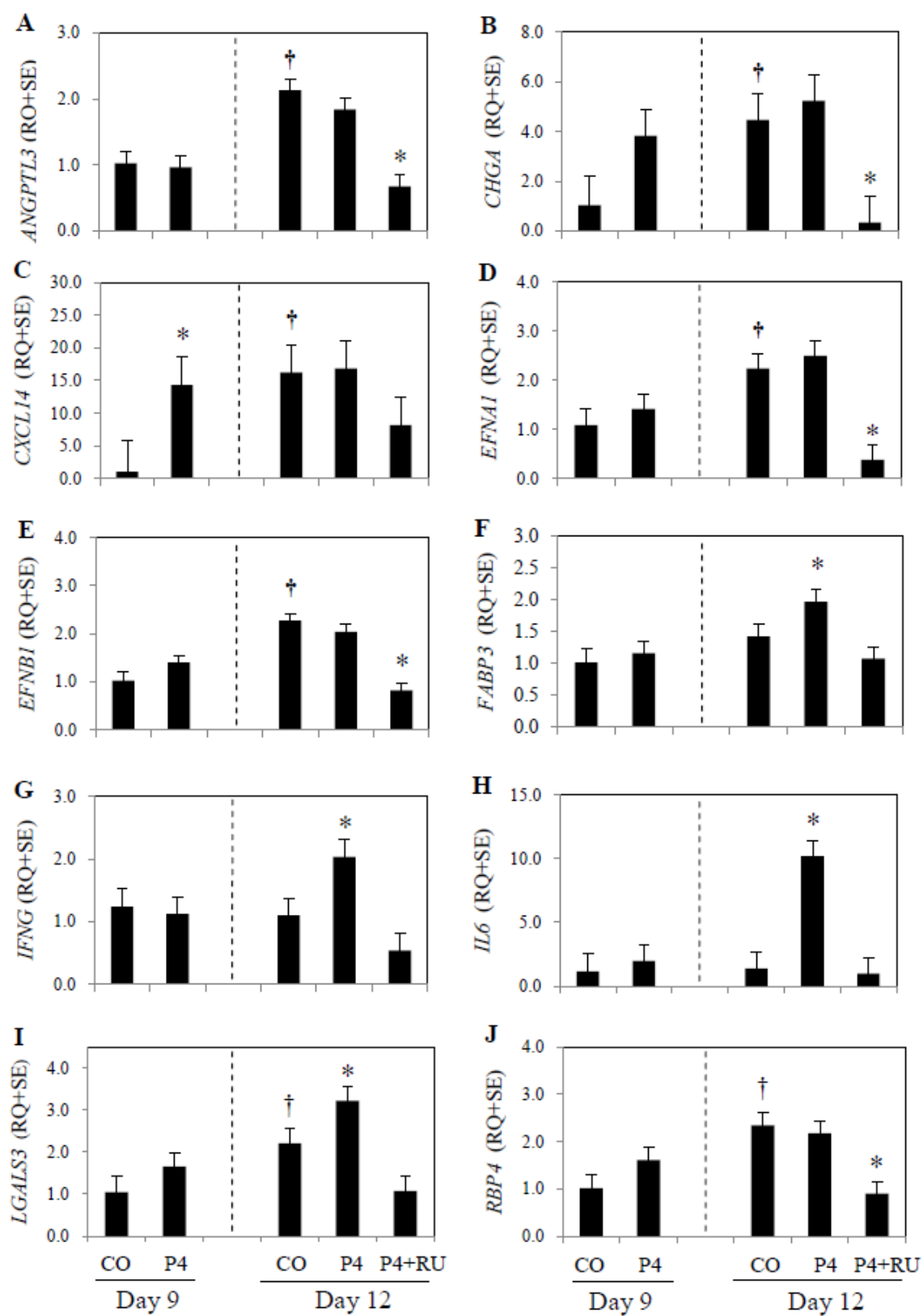
Microarray and qPCR data are summarized in Table 3.2. Quantitative PCR confirmed effects of P4 and day of pregnancy on expression of *ANGPTL3*, *CHGA, CXCL14*, *EFNA1, EFNB*, *FABP3*, *IL6*, *RBP4*, and *SLIT2* in the endometrium of the ovine uterus. Early P4 treatment or day did not affect (P > 0.10) expression of *CLEC4E, PTH, SLIT2, SLIT3* and *VWF* mRNAs. Those genes in which comparisons of day and/or treatment were significant are presented in Figure 3.1.

Gene Regulation by Day

Gene expression up-regulated from Day 9 to Day 12 of pregnancy in the ovine endometrium included *ANGPTL3*, *CHGA*, *CXCL14, EFNA1*, *EFNB1*, *LGALS3* and *RBP4*. The following fold increases occurred from Day 9 to Day 12 in control CO treatment endometrium: *ANGPTL3* mRNA increased (*P* < 0.001) 2.1-fold; *CHGA* mRNA increased (*P* < 0.05) 4.3-fold; *CXCL14* increased (*P* < 0.05) 15.5-fold; *EFNA1* increased (*P* < 0.05) 2.1-fold; *EFNB1* increased (*P* < 0.0001) 2.2-fold; *LGALS3* increased (*P* < 0.05) 2.1-fold; and *RBP4* increased (*P* < 0.01) 2.3-fold.

Gene Regulation by Treatment

CXCL14 mRNA increased (*P* = 0.05) 13.7-fold in P4-treated endometrium on Day 9 in comparison to control on Day 9. Gene mRNA levels were up-regulated in P4-treated endometrium on Day 12 in comparison to control Day 12 endometrium for *FABP3*, *IFNG*, *IL6* and *LGALS3*. *FABP3* mRNA increased (*P* = 0.05) 1.4-fold, *IFNG* mRNA increased (*P* < 0.05) 1.9-fold, *IL6* mRNA increased (*P* < 0.0001) 7.5-fold, and *LGALS3* increased (*P* < 0.05) 1.5-fold. Progesterone and RU486 treatment down-regulated mRNA of *ANGPTL3* 2.7-fold (*P* < 0.0001), *CHGA* 16.3-fold (*P* < 0.01), *EFNA1* 6.8-fold (*P* < 0.0001), *EFNB1* 2.5-fold (*P* < 0.0001), *FABP3* 1.8-fold (*P* < 0.01), *IFNG* 3.8-fold (*P* < 0.001), *IL6* 10.7-fold (*P* < 0.0001), *LGALS3* 3-

fold ($P < 0.001$), *RBP4* 2.4-fold ($P < 0.01$), *SLIT2* 3.7-fold ($P < 0.05$), *SLIT3* 4-fold ($P < 0.05$) and *VWF* 3-fold ($P < 0.01$) compared to Day 12 P4-treated endometrium.

Discussion

The present study validated candidate genes by qPCR analysis that were originally identified by microarray analysis to be implicated in effects of P4 on peri-implantation blastocyst growth and conceptus development. Similar mRNA expression by qPCR analysis that validated microarray data was observed for *ANGPTL3, CHGA, CXCL14, EFNA1, EFNB, FABP3, IL6, RBP4*, and *SLIT2*.

Results for *CLEC4E*, *PTH*, *SLIT2*, *SLIT3* and *VFW* from qPCR did not indicate any difference for day of pregnancy or treatment (CO vs. P4); however *SLIT2*, *SLIT3* and *VWF* were down-regulated in P4+RU endometrium compared to P4-treated endometrium on Day 12 of pregnancy. RU486 inhibits the function of both PGR and glucocorticoid receptors. Ewes that received P4+RU treatment were not exposed to continuous P4 thereby PGR was not down-regulated. *SLIT2*, *SLIT3* and *VWF* expression was not up-regulated by P4 treatment in either day however they were down-regulated in P4+RU treated endometrium. Flushings of the P4+RU treated uteri did not contain any conceptuses. Glucocorticoids inhibit both *SLIT* and receptor, *ROBO* expression in ovarian cells (Dickinson and Duncan 2010). The significant decrease of mRNA levels for *SLIT2* and *SLIT3* in P4+RU486 endometrium may be the result of RU486 binding the steroid response elements of these genes thereby affecting transcription of *SLIT2* and *SLIT3* (Duncan *et al.* 2010). *VWF* is unique to endothelial cells and is used as a marker for tumor angiogenesis, the generation of new vessels from pre-existing vasculature. Angiogenesis must occur in placental tissues to meet the hematotrophic needs of the fetus. Angiogenic molecules are not shown to be up-regulated in pregnancy until Days 20-30 (Grazul-Bilska *et al.*

2011). Therefore, it is discernible for *VWF* to not be affected by day or treatment in the scope of the present study.

ANGPTL3 was shown to be regulated by day according to microarray data. Quantitative PCR confirmed *ANGPTL3* regulation by day of pregnancy with greatest mRNA levels observed in Day 12 pregnant endometrium. Furthermore, P4+RU treatment down-regulated *ANGPTL3* mRNA. *ANGPTL3* induction occurs during the decline and loss of PGR (Spencer and Bazer 1995). Additionally, *ANGPTL3* was down-regulated in endometrium of P4+RU treatment, which still express LE and GE PGR. These results indicate ANGPTL3 to be regulated by P4, via down-regulation of PGR. ANGPTL3 is a secreted glycoprotein that has been shown to be expressed in human umbilical venous endothelial cells and human microvascular vein endothelial cells to stimulate cell adhesion and migration via integrin $\alpha_v\beta_3$ (Camenisch *et al.* 2002). *ANGPTL3* may play a role in the migration of trophectoderm and adhesion of the conceptus to uterine LE via integrin subunits α (v, 4, 5) and β (1, 3, 5) that are constitutively expressed on the apical surfaces of both conceptus trophectoderm and endometrial LE (Johnson *et al.* 2001a; Burghardt *et al.* 2002; Spencer *et al.* 2004a).

CHGA mRNA was regulated by day of pregnancy with greater levels detected in Day 12 control endometrium. These results are in agreement with microarray data. *CHGA* was also down-regulated in P4+RU treated endometrium. Thus, *CHGA* is indicated to be regulated by P4 via the loss of PGR. CHGA is a ubiquitous protein which is stored in secretory granules of the endocrine, exocrine and nervous systems, along with their respective hormones, enzymes, and neuropeptides and neurotransmitters to regulate secretory granule biosynthesis (Kim and Loh 2005). Under continuous P4 secretion, the endometrium produces and secretes histotroph to nourish the developing conceptus. CHGA may be implicated as a co-secretory granule product

of endometrial secretions, thus observing an increase from Day 9 to Day 12 would be anticipated with increased histotrophic production by the endometrium under continuous P4.

CXCL14 mRNA was regulated by early P4 treatment on Day 9 and by day of pregnancy, with greater mRNA levels in Day 12 control endometrium. Thus, *CXCL14* is regulated by P4. *CXCL14* is expressed in mice and humans during early pregnancy. In mice, *CXCL14* expression was induced at the embryo implantation site and expanded with decidualization. *In vitro* studies revealed CXCL14 as an inhibitory factor against trophoblast outgrowth by down regulating MMP2 and MMP9 activity. CXCL14 was suggested to balance the invasive ability of trophoblast against other promoting chemokines (Kuang *et al.* 2009a). Similar results were observed in women regarding suppression of MMP9 activity. CXCL14 was localized to villous cytotrophoblasts, moderately to decidualized stroma and very weakly in syncytiotrophoblasts and extravillous trophoblast during the first trimester (Kuang *et al.* 2009b). Additionally, *CXCL14* was found to be highly up-regulated during the mid-secretory phase of the menstrual cycle in women. Indeed, *CXCL14* contains progesterone response elements (Mokhtar *et al.* 2010). In pigs, *CXCL14* expression increased during conceptus elongation (Ross *et al.* 2009). Satterfield et al. (2009) reported *CXCL14* mRNA expressed exclusively in the endometrial LE and GE of P4-treated endometrium on Days 9 and 12 and Day 12 control endometrium. *CXCL14* may have implications in trophectoderm elongation by balancing the effects of promoting chemokines (Salamonsen *et al.* 2007). Therfore, CXCL14 may serve to maintain synchrony of the conceptus with the maternal environment in ovine during early pregnancy.

EFNA1 and *EFNB1* mRNA levels were both regulated by day with greater expression in Day 12 endometrium. Additionally, P4+RU treatment in Day 12 endometrium attenuated expression of *EFNA1* and *EFNB1*. These results indicate *EFNA1* and *EFNB1* to be regulated by P4. The *EFN* family is involved in migration and guidance of axons during embryogenesis and

angiogenesis, specifically mediating cell-cell contacts, cell adhesion to extracellular matrix and cell migration (Cheng *et al.* 2002). *EFNA1* is expressed in human uterine LE (Fujii *et al.* 2011). Authors evaluated *in vitro* actions of EFNA1, using human endometrial carcinoma cells, which show expression profiles of EFN molecules similar to normal uterine LE. EFNA1 was proposed to promote the intracellular dissociation of LE during implantation. EFNB1 modulates integrin-mediated cell attachment and migration during angiogenesis via $\alpha_v\beta_3$ and $\alpha_v\beta_5$ subunits (Huynh-Do *et al.* 2002).

FABP3 mRNA levels were increased in Day 12 P4-treated endometrium. Interestingly, *FABP3* is P4-regulated in bovine endometrium during early pregnancy and thought to contribute to histotroph composition (Forde *et al.* 2009). In the sheep, *FABP3* may be P4-regulated, however expression was not up-regulated in Day 9 P4-treated endometrium or in Day 12 CO endometrium. Increased *FABP3* mRNA level in Day 12 P4-treated endometrium is associated with IFNT secretion from the trophectoderm. Thus, FABP3 is likely induced by IFNT, as are a number of classical and non-classical IFN stimulated genes. The function of *FABP3* remains elusive however, it is proposed to function in intracellular solubilization, transportation and energy homeostasis of fatty acids (Nevo *et al.* 2010). Up-regulation of *FABP3* by conceptus factors, likely increases intracellular fatty acid trafficking thus making fatty acids readily available. Fatty acids during early pregnancy are important for synthesis of eicosanoids (PGs, tromboxanes and leukotrienes) (Mattos *et al.* 2000), maintaining cellular and organelle integrity (Duttaroy 2009), and mediating gene expression (Sampath and Ntambi 2005).

IFNG was up-regulated in Day 12 P4-treated endometrium. These results indicate *IFNG* to be regulated by IFNT from the conceptus. IFNG plays roles in diverse cellular processes, including activation of innate and adaptive immune responses, inhibiting cell proliferation, and inducing apoptosis (Boehm *et al.* 1997; Murphy *et al.* 2009). Interestingly, activation of IFNG

production in innate immune cells is typically preceded by signaling from type 1 IFN (Murphy *et al.* 2009). Thus IFNT (Type 1 IFN) produced by advanced filamentous conceptuses in Day 12 P4 treatment may be up-regulating endometrial *IFNG* mRNA.

Similar to cytokine *IFNG*, *IL6* was greatest in Day 12 P4-treated endometrium. Thus, *IL6* is also implicated as being induced by IFNT from the advanced conceptus in Day 12 P4-treated endometrium. In humans, IL6 is increased in mid-secretory phase, predominantly in LE and GE (Dimitriadis *et al.* 2005). IL6 is a pleiotrophic cytokine that belongs to the IL6 signal transducer receptor cytokine family which also includes LIF. Interestingly, LIF is regulated by P4 and IFNT in the ovine uterus (Song *et al.* 2009). LIF is implicated in conceptus growth and differentiation of the trophectoderm in sheep, based off observation of trophoblast giant cell differentiation in mice (Takahashi *et al.* 2008). *In vitro* studies of IL6 in human extravillous trophoblast cells demonstrate IL6 to stimulate cell migration and invasion by up-regulation of integrin subunits α_1, α_5 and β_1 and activation of MMP2 and MMP9 (Jovanovic and Vicovac 2009). Song et al. (2009) identified *IL6ST*, the co-receptor of *IL6* and *LIF* in sheep, thus this molecule may be involved in stimulating conceptus growth and migration in the ewe.

LGALS3 mRNA was up-regulated in Day 12 control endometrium and in P4-treated endometrium on Day 12. These results indicate LGALS3 to be induced by P4 and stimulated by IFNT from the trophectoderm. LGALS3 is expressed in cow LE and GE during early pregnancy (Kim *et al.* 2008). In humans, LGALS3 is highly expressed during the secretory phase of the menstrual cycle. During pregnancy LGALS3 is expressed on both trophoblast, epithelium and decidualized stroma (von Wolff *et al.* 2005). LGALS3 modulates cell adhesion by binding to several ligands including laminin, fibronectin and integrins after its secretion from epithelial cells (Andre *et al.* 1999; Hughes 1999). Family member, *LGALS15* is induced by P4 and stimulated by IFNT, of which *LGALS3* shares a similar expression profile (Satterfield *et al.*

2006). LGALS15 functions as a cell adhesion molecule that bridges integrins between uterine LE and trophectoderm (Farmer *et al.* 2008). *LGALS3* may serve in a similar fashion as an adhesion molecule in sheep.

RBP4 mRNA was up-regulated in Day 12 control endometrium compared to Day 9 control endometrium and down-regulated by P4+RU treatment, indicating RBP4 to be stimulated by P4. RBP4 binds retinol and has been shown to significantly affect litter size in pigs (Harney *et al.* 1993). Retinoids control cell growth, differentiation and death (Gomez *et al.* 2006). In pigs, the most advanced conceptuses secrete retinol binding protein (RBP) necessary for their development (Rothschild *et al.* 2000). However, less advanced conceptuses are vulnerable to rising levels of retinol and derivatives that may lead to embryonic toxicity. As a result, RBP4 has been identified as a candidate gene for litter size based on its transport and buffering ability (Rothschild *et al.* 2000). Previous research of RBP in cattle and sheep suggest RBP to be regulated by endocrine cues of P4 and estradiol (Harney *et al.* 1993).

Collectively, these results illustrate potential molecules involved in blastocyst growth and conceptus elongation. *ANGPTL3*, *CHGA*, *CXCL14*, EFNA1, *EFNB1*, and *RBP4* were regulated by P4 via the decline and loss of PGR from uterine LE and GE. *LGALS3* was induced by P4 and stimulated by IFNT, similar to ruminant specific family member *LGALS15*. *FABP3*, *IFNG* and *IL6* are indicated to be regulated by IFNT from the conceptus due to up-regulation of mRNA levels in Day 12 P4-treated endometrium. Further observations of these candidate genes during early pregnancy need to be assessed to generate expression profiles. *CXCL14* and *IL6* expressed the greatest mRNA levels in the present study of candidate genes analyzed. *In situ* hybridization reported *CXCL14* to be present within LE and GE (Satterfield *et al.* 2009). *CXCL14* mRNA expression in LE and GE along with observations made in mice (Kuang *et al.* 2009a), denotes *CXCL14* as a regulator of trophectoderm growth in sheep. Thus, this study has

identified a number of candidate endometrial genes whose products are proposed regulators of blastocyst growth and conceptus elongation, and mediate effects of ovarian P4 and/or the conceptus on endometrial function during early pregnancy.

CHAPTER IV

EFFECTS OF THE ESTROUS CYCLE AND EARLY PREGNANCY ON GENE

EXPRESSION IN THE ENDOMETRIUM OF THE OVINE UTERUS

Introduction

During peri-implantation, maternal environment and conceptus (embryo and associated extraembryonic membranes) interactions are important to the success in establishing and maintaining pregnancy. The maternal environment is influenced by continuous P4 exposure to synthesize and secrete a variety of substances, collectively termed histotroph. Histotroph is a complexity of adhesion proteins, cytokines, enzymes, hormones, growth factors, protease inhibitors, serum proteins, transport proteins, and other substances (Spencer *et al.* 2004b; Bazer *et al.* 2009). Endometrial glands and their secretions are required for elongation as evidenced by the uterine gland knock out (UGKO) ewe (Gray *et al.* 2000b; Gray *et al.* 2001b).

In sheep, the preimplantation embryo enters the uterus on Days 4 to 5 post-mating at the morula stage. The morula develops into a blastocyst which contains an inner cell mass and blastocoele surrounded by a monolayer of trophectoderm by Day 6 with shedding of the zona pellucida occurring between Days 8 and 9 (Guillomot 1995). By Day 10, the blastocyst measures 0.4-0.9 mm in diameter with approximately 3000 cells (Wintenberger-Torres and Flechon 1974). The hatched blastocyst will begin to elongate on Day 11, transitioning from spherical to tubular to filamentous morphology (Spencer *et al.* 2004a). On Day 14, the conceptus has elongated to approximately 10 cm in length and is filamentous in form. Elongation is concomitant with the onset of trophectoderm giant BNC differentiation (Wooding 1992). Binucleate cells form syncytial plaques with uterine LE in the caruncular regions to aid in the formation of the cotyledonary portion of the placentome. Additionally, binucleate cells

synthesize and secrete hormones, specifically chorionic somatomammotropin hormone 1 (CSH1 or placental lactogen) and P4 (Spencer *et al.* 2004a).

During elongation, the ruminant conceptus produces and secretes the maternal recognition of pregnancy signal, IFNT from the mononuclear trophectoderm (Bazer *et al.* 1979; Spencer *et al.* 2008). IFNT inhibits the luteolytic response to prostaglandin F2α from the uterine epithelia by preventing up-regulation of *ESR1*, thereby maintaining a functional corpus luteum (CL) and its production of progesterone (P4) required for pregnancy (Bazer *et al.* 1996). IFNT must be secreted by the elongating conceptus between Days 12 and 16 to be signaled to the maternal environment for a successful pregnancy outcome (Spencer and Bazer 2004a). The trophectoderm increases 90-fold from Day 13 to 19 resulting in the elongation of the conceptus into the contralateral uterine horn of ovulation (Wales and Cuneo 1989). Adhesion of the trophectoderm with the uterine LE begins on Day 16 and is completed around Day 22 (Boshier 1969; Spencer *et al.* 2004a).

Endometrial functions and epithelial secretions are largely regulated by P4, IFNT and prostaglandins (PG) during peri-implantation. Progesterone induces expression of genes while IFNT and PGs regulate their activity. Interferon stimulated genes (ISG) are expressed in a cell-specific manner within the endometrium to promote uterine receptivity (Bazer *et al.* 2009). Progesterone induces expression of prostaglandin G/H synthase and cyclooxygenase 2 (PTG2S) enzymes which converts arachidonic acid to PG between Days 10 and 12. PTGS2-derived PGs mediate, in part, the effects of P4 and IFNT on endometrial epithelial genes thought to be critical regulators of conceptus elongation (Dorniak *et al.* 2011).

Satterfield et al. (2006) reported a model of accelerated blastocyst development elicited by advancing the postovulatory rise in circulating levels of P4 following estrus. Their results indicated marked effects of early P4 supplementation on peri-implantation growth and

development of blastocysts by the presence of advanced stage blastocysts and filamentous conceptuses in uterine flushes from Days 9 and 12 of pregnancy, respectively. Analyzing the transcriptome of the early supplemented P4 endometrium by microarray, Satterfield et al. (2009) identified novel candidate genes and regulatory pathways of peri-implantation blastocyst growth and conceptus development governed by P4 regulation. Microarray and qPCR data of the early P4 treatment study demonstrated a number of genes to be up-regulated on Day 12, indicating induction of these genes to be associated with loss of PGR in uterine LE and GE. Therefore, the objective of this study was to analyze candidate genes by qPCR in cyclic and pregnant ovine endometrium to elucidate their expression in a broader scope during the estrous cycle and early pregnancy.

Materials and Methods

Animals

Mature crossbred Suffolk ewes (*Ovis aries*) were observed daily for estrus in the presence of intact vasectomized rams and were used in experiments only after having exhibiting two normal duration estrous cycles (16-18 Days). All experimental and surgical procedures were in compliance with the Guide for the Care and Use of Agriculture Animals in Research and Teaching and approved by the Institutional Animal Care and Use Committee of Texas A&M University.

Experimental Design

At estrus (Day 0), the ewes were mated to either an intact or vasectomized ram and then hysterectomized on either Day 10, 12, 14 or 16 of the estrous cycle or Day 10, 12, 14, 16, 18 or 20 of pregnancy (n=4-5 ewes per day and status) as described previously (Spencer *et al.* 1999). At hysterectomy, the uterus was flushed with 20 ml sterile saline. Pregnancy was confirmed on

Days 10-16 post-mating by the presence of a morphologically normal conceptus(es) in the uterine flushing. It was not possible to obtain uterine flushings from Day 18 and 20 pregnant uteri because the conceptus(es) had adhered to the uterine LE.

At hysterectomy, sections (~0.5 cm) from the midportion of each uterine horn ipsilateral to the CL were fixed in fresh 4% paraformaldehyde in PBS (pH 7.2). After 24 h, fixed tissues were changed to 70% ethanol (v/v) for 24 h, dehydrated through a graded series of alcohol to xylene, and then embedded in Paraplast-Plus (Oxford Labware, St. Louis, MO). The remaining endometrium of the ipsilateral uterine horn was physically dissected from the myometrium, frozen in liquid nitrogen, and stored at -80°C for subsequent RNA extraction. No tissues from the contralateral uterine horn in monovulatory pregnant ewes were used for analysis.

RNA Isolation and Quantitative Real-Time PCR Analysis

Total cellular RNA was isolated from frozen ipsilateral endometrium using Trizol reagent (Gibco-BRL, Bethesda, MD) according to manufacturer's instructions. The quantity and quality of total RNA were determined by spectrometry and by denaturing agarose gel electrophoresis, respectively. Total RNA samples were digested with RNase-free DNase I and were cleaned up using the RNeasy MinElute Cleanup Kit (Qiagen, Valencia, CA).

Total RNA from each sample was reverse transcribed in a total reaction volume of 20 µl. Briefly, 5 µl RNA was combined with primer mix containing oligo (dT) primer (0.2 µg/µl), random hexamer primer (3 µg/µl) (Invitrogen, Carlsbad, CA) and incubated at 70°C for 10 minutes. A reverse transcriptase (RT) mix containing 5X first-stand buffer, RNasin, 100 mM dNTP, 100 mM DTT and SuperScript II RTase (Invitrogen, Carlsbad, CA) was added to the reaction and reverse transcription performed under following conditions: 42°C for 90 min and 95°C for 2 min. Complementary DNA (cDNA) preps were cleaned by phenol-choloroform extraction and acid-ethanol precipitation, resuspended in 20 µl of water and stored at -20°C.

Control reactions in the absence of reverse transcriptase were prepared for each sample to detect genomic DNA contamination.

Quantitative PCR was performed using the ABI prism 7900HT system (Applied Biosystems, Foster, CA) with Power SYBR Green PCR Master Mix (Applied Biosystems, Foster, CA) as specified by manufacturer. Specific oligonucleotide primers were designed and analyzed by Oligo 5 (Molecular Biology Insights, Inc). Forward and reverse primer sequences for all genes analyzed are listed in Table 3.1. Primer specificity and efficiency (-3.2077 > threshold curve > -3.447) were confirmed using a test amplification run. Each individual sample was run in triplicate using the following conditions: 50°C for 2 min, 95°C for 10 min; and then 95°C for 15 sec and 60°C for 1 min for 40 cycles. A dissociation curve was generated to determine amplification of a single product. The threshold line was set at the linear region of the plots above the baseline noise, and threshold cycle (C_T) values were determined at the cycle number at which the threshold line crossed the amplification curve. The results are expressed in terms of change in C_T values (ΔC_T). To adjust for variations in the amount of cDNA, the mean C_T values for each gene were normalized against average C_T values for the reference gene (ovine alpha-tubulin, TUBA) as follows: ΔC_T = mean $C_{T\ specific\ gene}$ − mean $C_{T\ TUBA}$. Subtraction of an arbitrary constant from ΔC_T, generates $\Delta\Delta C_T$. Finally, the relative quantification (RQ) of each gene was calculated as $2^{-\Delta\Delta C_T}$.

In Situ Hybridization Analysis

Location of *FABP3* and *IL6* mRNAs in the ovine uterus were determined by radioactive in situ hybridization analysis using methods as previously described (Johnson *et al.* 1999b). Radiolabeled antisense and sense cDNA 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 digestion, slides were dipped in NTB-2 liquid photographic emulsion (Kodak, Rochester, NY), and exposed at 4°C for 1 week for *FABP3* and 3 weeks for *IL6*. After development and counterstaining, images of the representative fields were recorded under bright- and dark-field illumination.

Statistical Analysis

Relative quantification values were subjected to least-squares analysis of variance using the General Linear Models procedures of the Statistical Analysis System (SAS Institute, Cary, NC). Data from ewes on Days 10, 12, 14 and 16 were analyzed for the effects of day, status (cyclic or pregnant) and their interactions. A P value of 0.05 or less was considered significant. Data are presented as the least-squares means with overall standard errors.
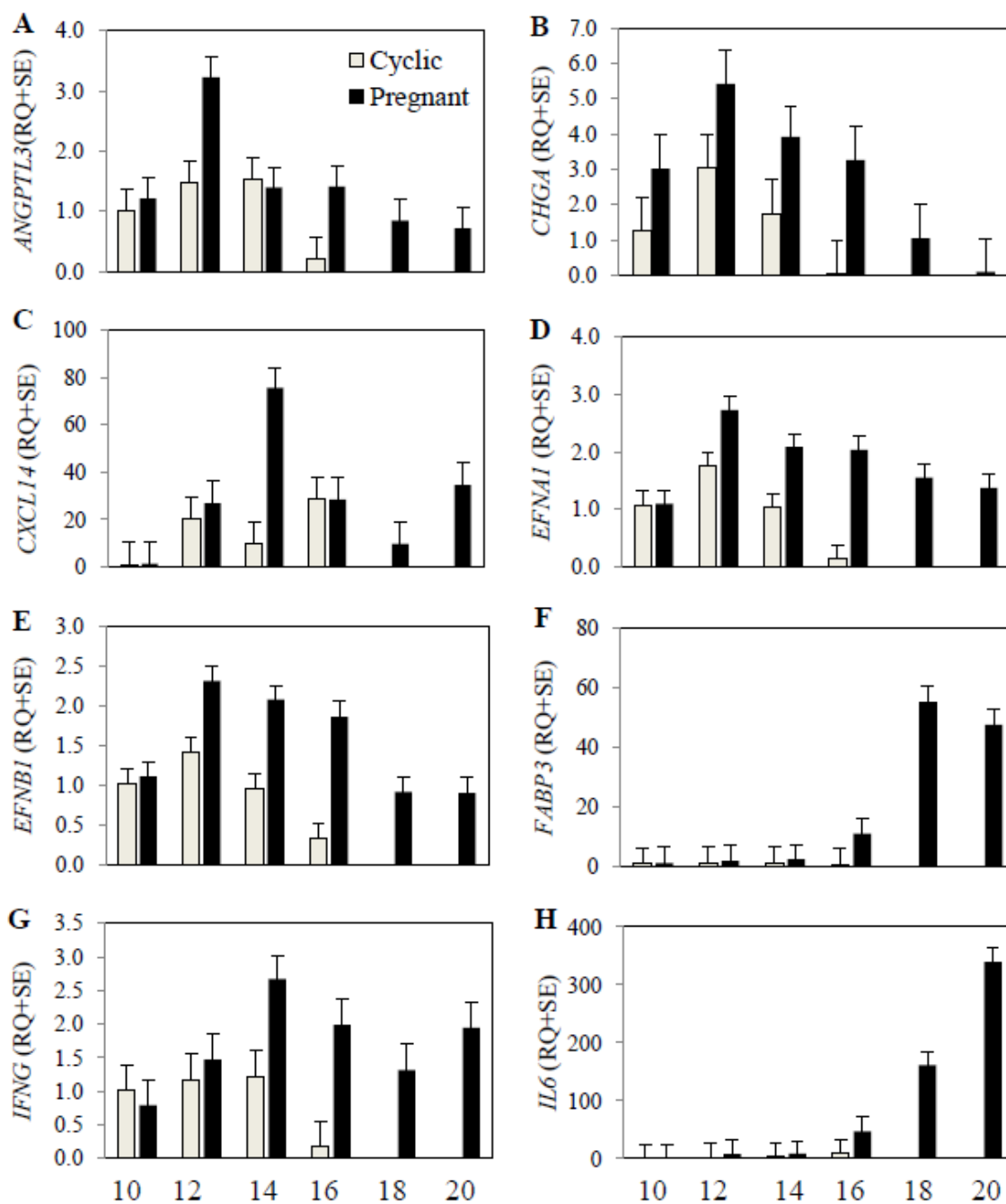
Results

Figure 4.1. mRNA levels of candidate genes in endometrium from cyclic and pregnant ewes as determined by qPCR. Relative quantification of A) *ANGPTL3*, B) *CHGA,* C) *CXCL14,* D) *EFNA1,* E) *EFNB1,* F) *FABP3*, G) *IFNG,* H) *IL6*, I) *LGALS3,* J) *PTH*, K) *RBP4*, L) *SLIT2*, M) *SLIT3*, and N) *VWF*. Data presented as least-square mean RQ with standard error (SE).
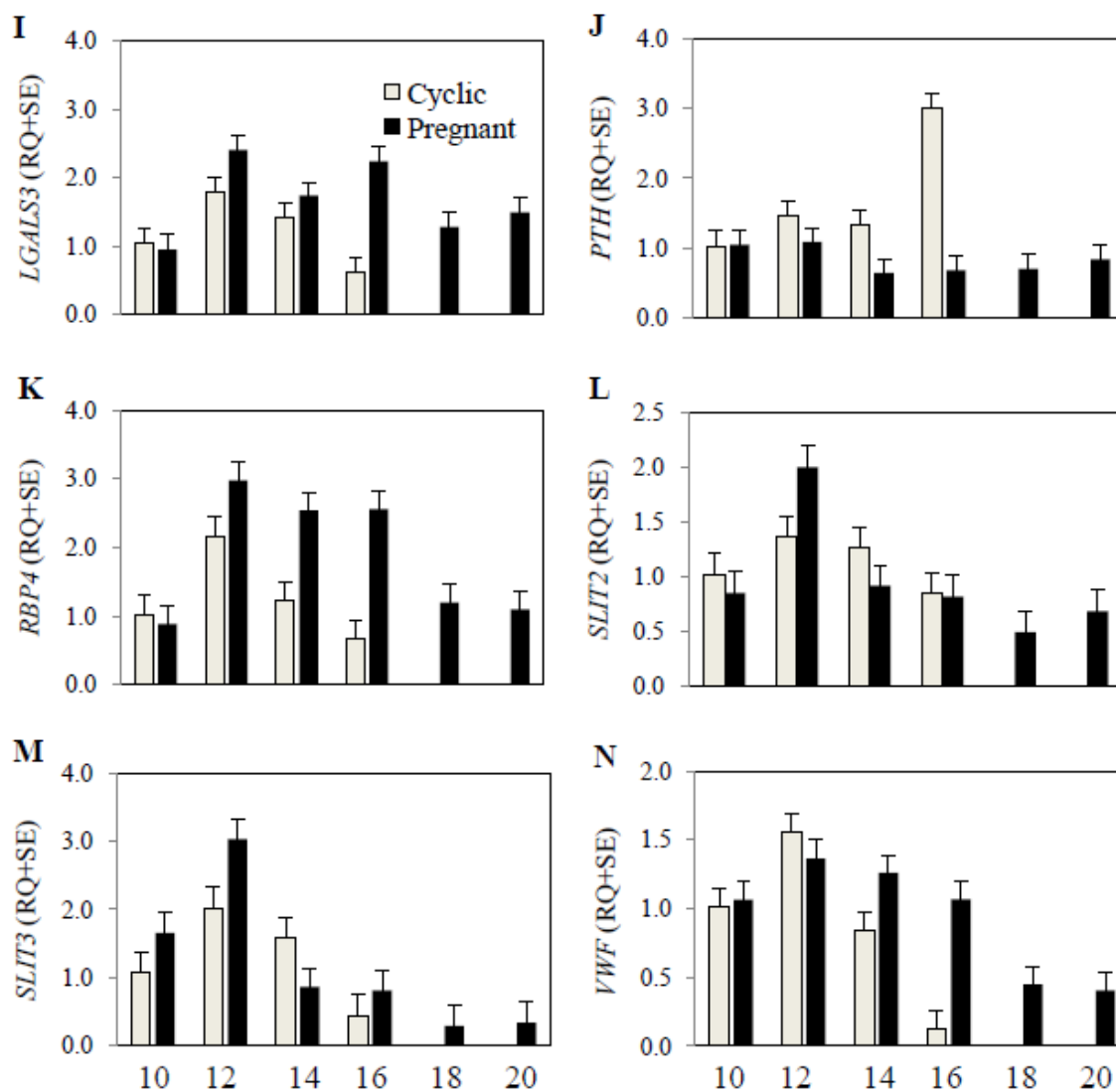
Figure 4.1 continued.

The present study analyzed microarray identified candidate genes in cyclic and pregnant endometrium. Genes affected by day, status and/or their interaction over Days 10 to 16, or highly up-regulated over Days 18 and 20 of pregnancy are presented in Figure 4.1. *CLEC4E* was not affected by day or status (cyclic or pregnant), thereby results are not presented.

Overall, *ANGPTL3* mRNA was greater (status, $P < 0.01$) in pregnant endometrium compared to cyclic during Days 10 to 16. mRNA increased ($P < 0.001$) 2.6-fold from Day 10 to

12 (day, *P* < 0.01). *ANGPTL3* mRNA in pregnant endometrium was 2.2-fold greater (P < 0.01) than cyclic endometrium mRNA levels on Day 12. Greatest *ANGPTL3* mRNA was observed in Day 12 pregnant endometrium. mRNA levels decreased (*P* < 0.001) 4.5-fold from Days 12 to 20 of pregnancy.

CHGA mRNA levels were greater in pregnant endometrium across Days 10 to 16 (status, P < 0.01). *CHGA* levels across days were similar for status until Day 16, which pregnant mRNA was 54-fold higher (*P* < 0.05) than cyclic endometrium. *CHGA* mRNA increased 1.8-fold from Day 10 to highest expression observed on Day 12. *CHGA* levels then decreased (*P* < 0.001) 60-fold from Day 12 to Day 20 of pregnancy.

CXCL14 mRNA levels were greater overall in pregnant endometrium from Days 10 to 16 (status, *P* < 0.01; day x status, *P* < 0.01). mRNA levels increased (*P* < 0.001) 56.7-fold from Day 10 to Day 14 of pregnancy. *CXCL14* expression decreased (*P* < 0.001) 2.6-fold after Day 14 in pregnant endometrium to cyclic levels on Day 16 (day, *P* < 0.01).

EFNA1 was greater in pregnant endometrium across Days 10 to 16 (status, *P* < 0.0001; day x status, *P* < 0.01). *EFNA1* mRNA increased (*P* < 0.001) 2.5-fold from Day 10 to 12 of pregnancy. *EFNA1* was highest in Day 12 endometrium for both cyclic and pregnant endometrium (day, *P* < 0.001). Pregnant endometrium *EFNA1* mRNA decreased (*P* < 0.001) 2-fold from Day 12 to Day 20 of pregnancy.

EFNB1 mRNA expression is similar to family member, *EFNA1* with greatest mRNA levels in pregnant endometrium (status, *P* < 0.001; day x status, *P* < 0.05). mRNA levels increased (*P* < 0.001) 2-fold from Day 10 to 12 in pregnant endometrium. Greatest expression was observed in Day 12 endometrium for both pregnant and cyclic endometrium (day, *P* < 0.01). *EFNB1* mRNA decreased (*P* < 0.001) 2.5-fold from Days 12 to 20 of pregnancy.

LGALS3 mRNA was greater overall in pregnant endometrium from Days 10 to 16 (status, $P < 0.001$). *LGALS3* increased ($P < 0.001$) 2.5-fold from Day 10 to 12 of pregnancy (day, $P < 0.001$). Cyclic mRNA levels decreased ($P < 0.001$) 3-fold from Day 12 to Day 16 of the estrous cycle whereas *LGALS3* mRNA remained high to Day 16 of pregnancy (day x status, $P < 0.01$). *LGALS3* decreased ($P < 0.05$) 1.5-fold from Day 16 to 20 of pregnancy.

RBP4 mRNA levels were greater overall in pregnant endometrium from Days 10 to 16 (status, $P < 0.001$; day x status, $P < 0.05$). *RBP4* increased ($P < 0.001$) 3.3-fold from Day 10 to Day 12 of pregnancy (day, $P < 0.001$). *RBP4* remained abundant throughout Days 12 to 16. *RBP4* mRNA then decreased ($P < 0.001$) 2.3-fold from Days 16 to 20 of pregnancy. *RBP4* mRNA decreased ($P < 0.001$) 3.2-fold from Day 12 to 16 of the estrous cycle.

SLIT2 mRNA levels were not affected by status ($P > 0.10$) during Days 10 to 16, however expression was differentiated over days (day, $P < 0.01$) with highest expression on Day 12 in both cyclic and pregnant endometrium. *SLIT3* mRNA levels were similar to *SLIT2*, with highest mRNA levels observed on Day 12 (day, $P < 0.001$) in both cyclic and pregnant endometrium.

VWF mRNA levels were greater overall in pregnant endometrium from Days 10 to 16 (status, $P < 0.01$; day x status, $P < 0.01$). *VWF* mRNA levels were similar Days 10 to 16 in pregnant endometrium, then decreased ($P < 0.01$) 2.6-fold from Day 16 to 20 of pregnancy. *VWF* mRNA levels decreased ($P < 0.001$) 12.9-fold from Day 12 to 16 of the estrous cycle.

PTH mRNA levels were greater overall in cyclic endometrium (status, $P < 0.001$). *PTH* mRNA levels remained low in pregnant endometrium whereas in cyclic endometrium, mRNA increased ($P < 0.001$) 3-fold from Days 10 to 16 of the estrous cycle.

FABP3 was indicated to be regulated by IFNT from the early P4 study in addition with *IFNG* and *IL6*. *FABP3* was not affected by day or status during Days 10 to 16. *FABP3* was up-regulated 21-fold from Day 14 to 18 of pregnancy. *In situ* hybridization analysis presented in Figure 4.2 identified *FABP3* mRNA in LE, sGE and trophectoderm on Days 18 and 20 of pregnancy (Figure 4.2).

IFNG mRNA was greater overall in pregnant endometrium from Days 10 to 16 (status, $P < 0.01$; day x status, $P < 0.05$). *IFNG* mRNA increased 3.4-fold from Day 10 to Day 14 of pregnancy and remained high. *IFNG* mRNA in pregnant endometrium on Day 14 was 2.2-fold greater ($P < 0.01$) that cyclic endometrium.

IL6 mRNA was not affected by day or status during Days 10 to 16. However, *IL6* mRNA was up-regulated 37-fold from Days 14 to 20. *In situ* hybridization analysis presented in Figure 4.3 identified *IL6* to be localized in immune cells within the LE on Day 18 and present at the fetomaternal interface on Day 20 of pregnancy.
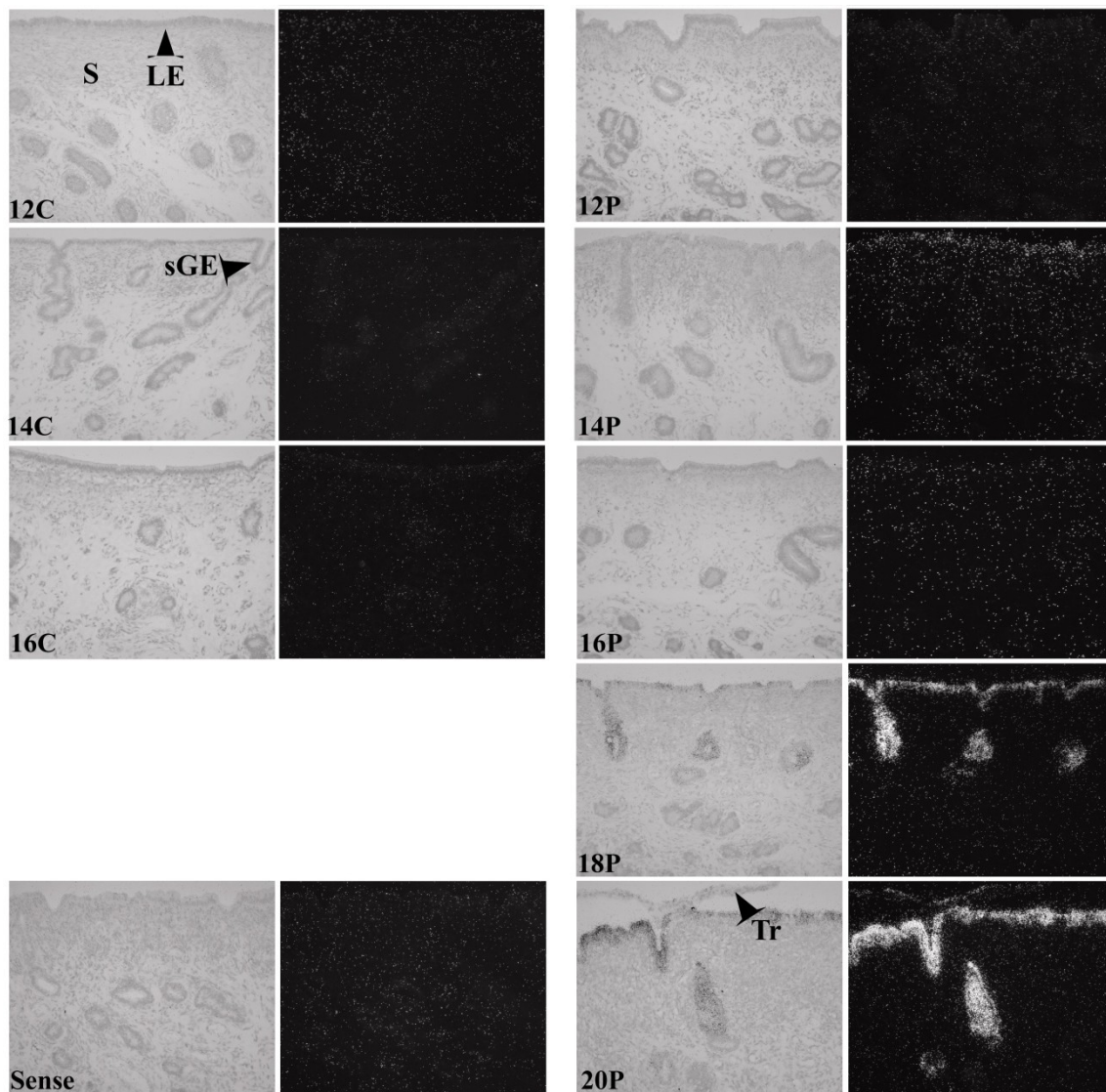
Figure 4.2. *In situ* hybridization analysis of *FABP3* mRNA in the uteri of cyclic and pregnant ewes. Cross sections of the uterine wall from cyclic (C) and pregnant (P) ewes were hybridized with radiolabeled antisense or sense *FABP3* cRNA probes. *FABP3* mRNA was present predominantly in the LE and sGE on Days 18 and Day 20 of pregnancy. *FABP3* mRNA was also present in the trophectoderm. In pregnant ewes, *FABP3* mRNA increased 21-fold between Days 14 to Day 18 and remained abundant on Day 20. LE, luminal epithelium; GE, glandular epithelium, S, stroma; Tr, trophectoderm. 15X magnification.
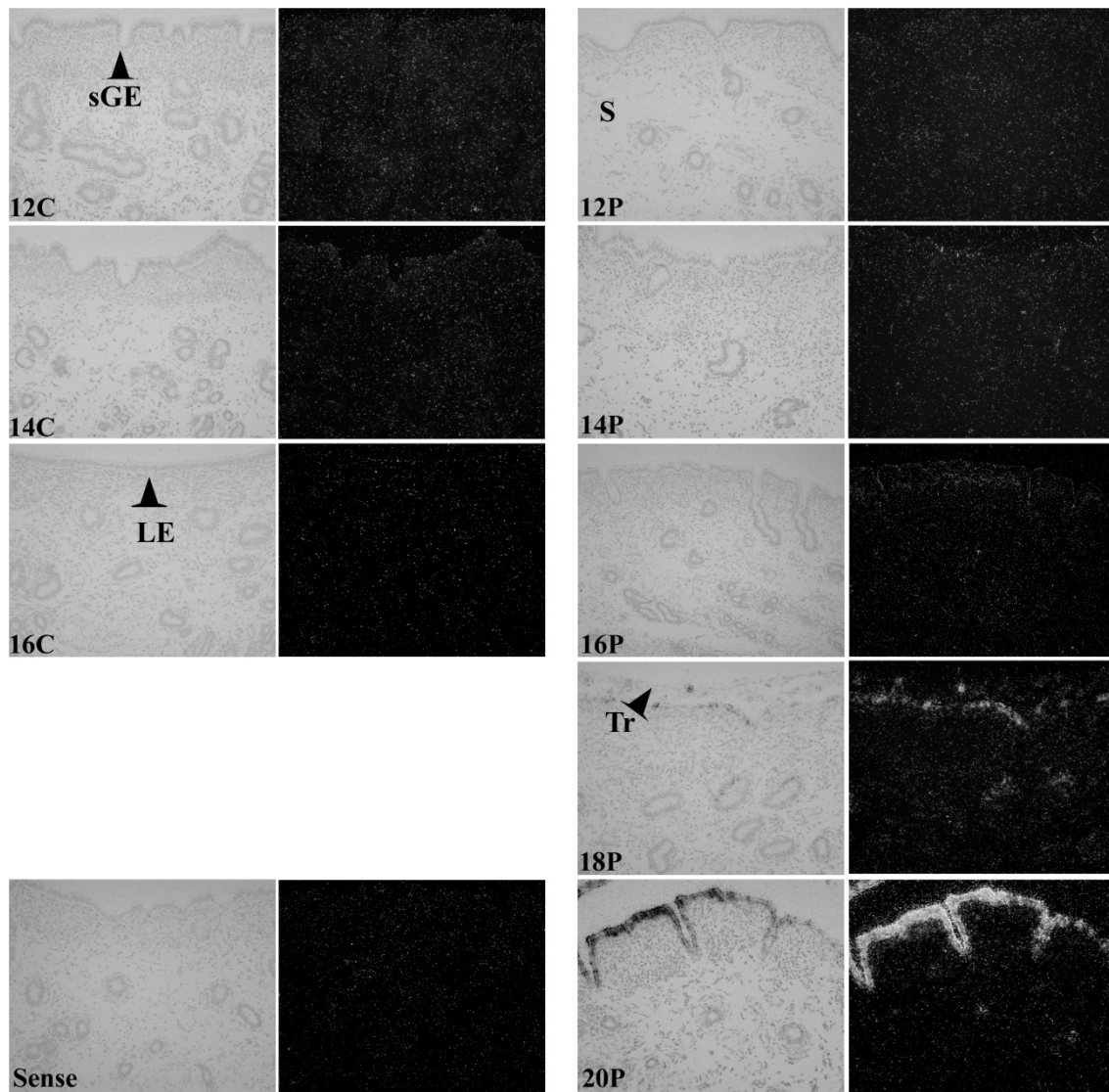
Figure 4.3. *In situ* hybridization analysis of *IL6* mRNA in the uteri of cyclic and pregnant ewes. Cross sections of the uterine wall from cyclic (C) and pregnant (P) ewes were hybridized with radiolabeled antisense or sense *IL6* cRNA probes. *IL6* mRNA localized to immune cells within the uterine LE on Day 18, and to LE and sGE on Day 20 of pregnancy. *IL6* was also present in the trophectoderm. In pregnant ewes, *IL6* increased 37-fold between Days 14 to Day 20. LE, luminal epithelium; GE, glandular epithelium, S, stroma; Tr, trophectoderm. 15X magnification.

Discussion

ANGPTL3 mRNA was up-regulated in pregnant endometrium on Day 12 to levels greater than cyclic endometrium. However, *ANGPTL3* expression did not remain abundant. Up-regulation of *ANGPTL3* from Day 10 to 12 is in association with the loss of PGR. These results are in agreement with the previous study conducted in early P4-treated ovine endometrium. Furthermore, *ANGPLT3* pregnant mRNA levels were greater than cyclic endometrium levels suggesting regulation by conceptus factors. Thus *ANGPTL3* may be induced by P4 and regulated by conceptus factors. ANGPTL3 stimulates cell adhesion and migration of human umbilical venous endothelial cells and human microvascular vein endothelial cells via integrin $\alpha_v\beta_3$ *in vitro* (Camenisch *et al.* 2002). ANGPTL3 may be involved in migration and adhesion during peri-implantation in sheep.

CHGA mRNA levels increased from Day 10 to 12, therefore indicating CHGA expression is stimulated by down-regulation of PGR. CHGA expression decreased after Day 12 in both cyclic and pregnant endometrium. CHGA is a ubiquitous protein that is co-secreted with hormones, enzymes, neuropeptides and neurotransmitters to regulate secretory granule biosynthesis. Stimulation of *CHGA* by P4 is concomitant with endometrial secretions regulated by P4. CHGA expression was overall greater in pregnant endometrium however pregnant mRNA levels were only significant on Day 16. CHGA may serve to regulate secretion of a co-resident product in histotroph.

CXCL14 mRNA increased 56.7-fold from Days 10 to 14 of pregnancy. *In situ* analysis of *CXCL14* mRNA in Days 10 to 20 pregnant endometrium showed expression in LE and GE, as observed in the early P4 samples reported by Satterfield et al. (2009). *CXCL14* mRNA was also expressed in deep GE (unpublished observations). CXCL14 inhibited trophoblast invasion by down regulating MMP2 and MMP9 in mice and humans, and may balance other invasive

promoting chemokines during implantation (Kuang *et al.* 2009a). Chemokines affect blastocyst development, migration and attachment (Hannan *et al.* 2006; Salamonsen *et al.* 2007). *CXCL14* may be involved in regulating depth of papillae, trophectoderm structures which protrude into glands to increase surface area for histotroph absorption according to observations of *CXCL14* expression in GE and deep GE. Increased *CXCL14* mRNA on Day 14 may serve as a temporal regulator of trophoblast elongation and to maintain synchrony between the conceptus and maternal environment.

EFNA1 and *EFNB1* mRNA levels were similar in expression profiles. mRNA levels increased from Day 10 to 12, indicating gene regulation by P4. Furthermore, pregnant endometrium mRNA levels were higher across Days 12 to 16, suggesting regulation by conceptus factors. EFNA1 is expressed in human LE and is proposed to promote the intracellular dissociation of the LE during implantation in humans (Fujii *et al.* 2011). EFNB1 modulates integrin-mediated cell attachment and migration during angiogenesis via $\alpha_v\beta_3$ and $\alpha_v\beta_5$ subunits (Huynh-Do *et al.* 2002) which are also expressed on the apical surface of uterine LE and trophectoderm in sheep (Johnson *et al.* 2001a). These molecules may have implications in uterine receptivity by modulating cell attachments and migration, specifically *EFNA1* and *EFNB1* may serve roles in the migration of BNC during syncytial formation. Placentation in sheep is not invasive in comparison to rodent and humans, however trophectoderm cells are thought to fuse with uterine LE to form multinucleated cells in formation of the synepitheliochorial placenta (Wooding 1984; Spencer *et al.* 2004a).

LGALS3 was higher in pregnant endometrium beginning Day 12 and throughout early pregnancy indicating *LGALS3* to be induced by P4 and regulated by IFNT, similar to family member *LGALS15* (Satterfield *et al.* 2006). In humans, LGALS3 is expressed in epithelia, decidualized stroma and trophoblast during early pregnancy (von Wolff *et al.* 2005). In sheep,

LGALS3 was expressed in mid-gestation placenta but decreased in term placenta (Iglesias *et al.* 1998). LGALS3 purified from mid-gestation placentae were discovered to increase lymphocyte proliferation response *in vitro*. Survival of double null *Lgals3* and closely related, *Lgals1* mice suggests these molecules are optimizing agents (Poirier 2002). LGALS3 functions in cell growth (Barondes *et al.* 1994), differentiation and angiogenesis of endothelial cells (Nangia-Makker *et al.* 2000), mediation of inflammation and leukocyte adhesion (Almkvist and Karlsson 2004), and chemoattract for monocytes and macrophages (Sano *et al.* 2000). These functions taken with LGALS3 expression at the fetomaternal interface during early pregnancy in women, delineate *LGALS3* to play a role during implantation. Furthermore, LGALS3 modulates cell adhesion by binding to several ligands including laminin, fibronectin and integrins after its secretion from epithelial cells (Andre *et al.* 1999; Hughes 1999). Up-regulation of *LGALS3* mRNA on Day 12 is in coordination with PGR down regulation and the onset of elongation in sheep. Thus *LGALS3* may be an optimizing molecule involved in trophectoderm elongation.

RBP4 mRNA was up-regulated in pregnant endometrium on Day 12, remained abundant to Day 16 and decreased on Days 18 and 20. RBP4 expression was greater in pregnant endometrium therefore indicating RBP4 is induced by P4 and regulated by conceptus factors. RBP4 is an intracellular transporter for retinol and derivatives (Kanai *et al.* 1968). Retinoids control cell growth, differentiation and death (Gomez *et al.* 2006). The ovine conceptus secretes RBP as early as Day 13 (Liu *et al.* 1992). *RBP4* expression in the endometrium during early pregnancy may be a protective feature. Conceptuses are vulnerable to rising levels of retinol and derivatives that may lead to embryonic toxicity of these compounds (Rothschild *et al.* 2000). The conceptus may not be able to produce adequate amount of RBP4 alone and the endometrium may expresses RBP4 as a protective measure during the time frame of embryogenesis, in which

retinoic acid serves as a morphogen, thereby ensuring homeostatic levels of retinoids in the conceptus environment (Liu *et al.* 1992).

*SLIT*s are chemorepellent molecules involved in axon guiding, cell migration and promoter of cell death (Dickinson and Duncan 2010). *SLIT2* and *SLIT3* were shown to be down-regulated in the deciduas of early pregnancy in women, with *SLIT2* expressed in LE and GE, and to a lesser extent in underlying stroma (Duncan *et al.* 2010). Both *SLIT2* and *SLIT3* mRNA was at the greatest level during the mid-secretory in women. In sheep endometrium, *SLIT2* and *SLIT3* had similar mRNA levels across cyclic and pregnant tissues with the exception of Day 12, which pregnant endometrium mRNA levels were greater. Observation of *SLIT2* in LE and sGE in human endometrium implicates these molecules to be involved in conceptus interactions. Additionally, increased cortisol as a result of *HSD11B1* expression is associated with reduced *SLIT/ROBO* expression in the ovary (Dickinson and Duncan 2010). *SLIT2* and *SLIT3* may be up-regulated by P4 but down-regulated by cortisol due to increased activity of HSD11B1. Down-regulation of *SLIT* expression would be optimal to promote cell migration and angiogenesis that is inhibited by *SLIT* expression (Guan *et al.* 2003).

VWF mRNA levels were similar between cyclic and pregnant endometrium on Day 12, although cyclic VWF mRNA decreased while pregnant VWF mRNA remained high to Day 18. *VWF* is uniquely expressed in endothelial cells and by megakaryocytes to mediate platelet adhesion at sites of vascular injury (McGrath *et al.* 2010). *VWF* is commonly used as an endothelial marker and detecting angiogenesis during tumor growth (Zanetta *et al.* 2000). Angiogenesis occurs in fetal and maternal placental tissues to develop a hematotrophic delivery system to supply the fetus with metabolic substrates via transplacental exchange (Grazul-Bilska *et al.* 2011). Angiogenic molecules are up-regulated after Day 20 (Grazul-Bilska *et al.* 2011),

beyond the scope of the present study. Thus *VWF* would not likely be differentiated expressed until later in pregnancy.

In contrast to the previous genes, *PTH* mRNA was greater in cyclic endometrium than pregnant endometrium. *PTH* plays a role in fetal calcium homeostasis (Simmonds *et al.* 2010) however no information of *PTH* during early pregnancy is known. With these results, and observations of no significant changes in the early P4 study suggests *PTH* does not play a major role during early pregnancy in sheep.

FABP3 mRNA levels increased 21-fold from Day 14 to Day 18 of pregnancy. From analysis in early P4-treated endometrium, *FABP3* was suggested to be regulated by IFNT. However *FABP3* up-regulation in pregnant endometrium during this study was concomitant with binucleate cell differentiation (Spencer *et al.* 2004a). Interestingly, FABP3 expression is up-regulated in differentiated tissues (Borchers *et al.* 1997; Tang *et al.* 2004) and human term syncytiotrophoblasts (Daoud *et al.* 2005). Additionally, syncytiotrophoblasts had a greater efflux of linoleic acid transport associated with increased *FABP3* expression. Interestingly, *FABP3* mRNA was localized to the fetomaternal interface Days 18 and 20 of pregnancy in sheep, concomitant with implantation. *FABP3* may serve to translocate fatty acids to the conceptus during implantation for cellular membrane development and precursor molecules for prostaglandin signaling.

Up-regulation of *FABP3* by conceptus factors, likely increases intracellular fatty acid trafficking thus making fatty acids readily available. Fatty acids during early pregnancy are important for synthesis of eicosanoids (PGs, tromboxanes and leukotrienes) (Mattos *et al.* 2000), maintaining cellular and organelle integrity (Duttaroy 2009), and mediating gene expression (Sampath and Ntambi 2005).

IFNG mRNA was up-regulated by Day 14 in pregnant endometrium at levels higher than cyclic endometrium. The increase of *IFNG* mRNA levels on Day 14 is concomitant with conceptus factors, such as IFNT. However, *IFNG* is not reported to be secreted by the ruminant trophectoderm as reported to occur by pig conceptuses (Bazer *et al.* 2008). Activation of IFNG production in innate immune cells is typically preceded by signaling from type 1 IFN (Murphy *et al.* 2009). In pigs, IFNG affects blastocyst attachment to LE by remodeling uterine epithelia to affect polarity, and stimulate PGE2 production (Bazer *et al.* 2008).

IL6 mRNA levels increased 37-fold from Days 14 to 20 of pregnancy. *In situ* analysis identified *IL6* mRNA in immune cells within uterine LE and sGE on Day 18 and 20 of pregnancy. Additionally, *IL6* mRNA was observed in trophectoderm. IL6 regulates various aspects of the immune response, acute phase reaction and hematopoiesis, and has functional redundancy with IL-11 and LIF (Dimitriadis *et al.* 2005). LIF has been implicated in the growth and differentiation of trophectoderm into giant BNC in sheep (Song *et al.* 2009). *In vitro* culture demonstrates IL6 to stimulate cell migration, invasion and integrin expression in human extravillous trophoblast cells. IL6 up-regulated α_1, α_5, β_1 proteins, thereby increasing cell migration (Jovanovic and Vicovac 2009). Placentas from preeclamptic pregnancies were found to produce less IL6 under normoxia compared to normal placenta, indicating endogenous IL6 is important for trophoblast invasion (Zhao *et al.* 2008). IL6 may serve to promote trophoblast migration in sheep as well as invasion of the uterine LE such as closely related molecule, LIF which is implicated in differentiation of the trophectoderm into giant BNC.

Collectively, the results from candidate genes identified by microarray in early P4-treated endometrium analyzed in cyclic and pregnant endometrium provide information on potential regulators governing blastocyst growth and conceptus elongation during peri-implantation. Potential migration and adhesion molecules include *ANGPTL3*, *EFNA1*, *EFNB1*

and *LGALS3*, of which appear to be P4-induced and regulated by conceptus factors. Angiogenic factors identified included *SLIT2*, *SLIT3* and *VWF*, however their expression was down-regulated during later days analyzed. *CHGA* and *RBP4* were up-regulated on Day 12 in concert with down-regulation of PGR, may have implications in granule secretion and transport of protein, respectively. *CXCL14*, indicated to regulate trophoblast outgrowth in sheep conceptus was up-regulated 7-fold on Day 14 of pregnancy in comparison to cyclic endometrium. Another cytokine, *IL6* increased 37-fold from Days 14 to 20 of pregnancy. *In situ hybridization* analysis demonstrated *IL6* mRNA to be localized to immune cells on Day 18 of pregnancy and at the fetomaternal interface on Day 20 of pregnancy. *IL6* may be involved in implantation and differentiation of the trophectoderm into BNC. *FABP3* mRNA was increased 21-fold from Days 14 to 18 of pregnancy and localized to uterine LE and sGE as well as trophectoderm. *FABP3* may serve as an intracellular transporter of fatty acids at the fetomaternal interface during conceptus development.

CHAPTER V

SUMMARY

In summary, the studies conducted analyzed genes identified by microarray that were proposed to govern peri-implantation blastocyst growth and conceptus development. These genes included *ANGPTL3*, *CHGA*, *CLEC4E*, *CXCL14*, *EFNA1*, *EFNB1*, *FABP3*, *IFNG, IL6*, *LGALS3*, *PTH*, *RBP4*, *SLIT2*, *SLIT3* and *VWF*. Analysis of these genes by qPCR was conducted in two studies. The first study indicates *ANGPTL3*, *CHGA*, *CXCL14*, *EFNA1*, *EFNB1*, *LGALS3* and *RBP4* to be regulated by P4 via loss of PGR based on observation of up-regulation of these genes in early P4-treated endometrium. *FABP3*, *IFNG* and *IL6* were up-regulated in Day 12 early P4-treated endometrium which contained elongated and filamentous conceptuses secreting IFNT, thus these genes are suggested to be regulated by conceptus factors such as IFNT and PTGS2-derived PGs.

The second study evaluated candidate genes in cyclic and pregnant endometrium. The following hypotheses are proposed of the candidate genes based on results of the second study. *ANGPTL3* is induced by P4, regulated by conceptus factors and involved in trophectoderm migration and adhesion. *CHGA* is induced by P4, regulated by conceptus factors and involved in granule secretion. *CXCL14* is induced by P4, regulated by conceptus factors to mediate trophectoderm growth. *EFNA1* and *ENFB1* are induced by P4, regulated by conceptus factors and involved in adhesion between the trophectoderm and uterine LE. *FABP3* is regulated by conceptus factors related to BNC differentiation and involved in intracellular transport of fatty acids at the fetomaternal interface. *IFNG* is regulated by IFNT and implicated in immune function at the fetomaternal interface. *IL6* is regulated by conceptus factors and involved in implantation by migration and differentiation of trophectoderm. *LGALS3* is induced by P4,

regulated by IFNT and implicated as an adhesion molecule. *RBP4* is induced by P4, regulated by conceptus factors and affects availability of retinoids to the conceptus.

Future studies should be directed at testing hypotheses of genes regulated by conceptus factors, including IFNT and PTGS2-derived PG mediation of gene expression. Protein localization for *CXCL14*, *FABP3* and *IL6* should be conducted to complement *in situ* hybridization results and confirm spatial and temporal expression. *In vitro* studies of *CXCL14*, *FABP3* and *IL6* are warranted to elucidate roles of trophectoderm growth and nutrient transport function during early pregnancy in ovine.

REFERENCES

Almkvist, J., and Karlsson, A. (2004). Galectins as inflammatory mediators. *Glycoconj. J.* **19**(7-9), 575-581.

Andre, S., Kojima, S., Yamazaki, N., Fink, C., Kaltner, H., Kayser, K., and Gabius, H.J. (1999). Galectins-1 and -3 and their ligands in tumor biology: non-uniform properties in cell-surface presentation and modulation of adhesion to matrix glycoproteins for various tumor cell lines, in biodistribution of free and liposome-bound galectins and in their expression by breast and colorectal carcinomas with/without metastatic propensity. *J. Cancer Res. Clin. Oncol.* **125**(8-9), 461-474.

Andrews, W.D., Barber, M., and Parnavelas, J.G. (2007). Slit-Robo interactions during cortical development. *J. Anat.* **211**(2), 188-198.

Archer, J.A., Arthur, P.F., Parnell, P.F., and van de Ven, R.J. (1998). Effect of divergent selection for yearling growth rate on female reproductive performance in angus cattle. *Livestock Prod. Sci.* **57**(1), 33-40.

Auernhammer, C.J., and Melmed, S. (2000). Leukemia-inhibitory factor-neuroimmune modulator of endocrine function. *Endocr. Rev.* **21**(3), 313-345.

Bagchi, I.C., Cheon, Y.P., Li, Q., and Bagchi, M.K. (2003). Progesterone receptor-regulated gene networks in implantation. *Front. Biosci.* **8**, s852-s861.

Baird, D.T., Land, R.B., Scaramuzzi, R.J., and Wheeler, A.G. (1976). Endocrine changes associated with luteal regression in the ewe: the secretion of ovarian oestradiol, progesterone and androstenedione and uterine prostaglandin F2alpha throughout the oestrous cycle. *J. Endocrinol.* **69**(2), 275-286.

Barondes, S.H., Cooper, D.N., Gitt, M.A., and Leffler, H. (1994). Galectins: structure and function of a large family of animal lectins. *J. Biol. Chem.* **269**(33), 20807-20810.

Bartol, F.F., Johnson, L.L., Floyd, J.G., Wiley, A.A., Spencer, T.E., Buxton, D.F., and Coleman, D.A. (1995). Neonatal exposure to progesterone and estradiol alters uterine morphology and luminal protein content in adult beef heifers. *Theriogenology* **43**(5), 835-844.

Bartol, F.F., Wiley, A.A., Coleman, D.A., Wolfe, D.F., and Riddel, M.G. (1988). Ovine uterine morphogenesis: effects of age and progestin administration and withdrawal on neonatal endometrial development and DNA synthesis. *J. Anim. Sci.* **66**, 3000-3009.

Bates, C.J. (1983). Vitamin A in pregnancy and lactation. *Proc. Nutr. Soc.* **42**(1), 65-79.

Bauersachs, S., Ulbrich, S.E., Gross, K., Schmidt, S.E., Meyer, H.H., Wenigerkind, H., Vermehren, M., Sinowatz, F., Blum, H., and Wolf, E. (2006). Embryo-induced transcriptome changes in bovine endometrium reveal species-specific and common molecular markers of uterine receptivity. *Reproduction* **132**(2), 319-331.

Baulieu, E.E. (1989). Contragestion and other clinical applications of RU 486, an antiprogesterone at the receptor. *Science* **245**(4924), 1351-1357.

Bazer, F.W. (1975). Uterine protein secretions: Relationship to development of the conceptus. *J. Anim. Sci.* **41**(5), 1376-1382.

Bazer, F.W., Burghardt, R.C., Johnson, G.A., Spencer, T.E., and Wu, G. (2008). Interferons and progesterone for establishment and maintenance of pregnancy: interactions among novel cell signaling pathways. *Reprod. Biol.* **8**(3), 179-211.

Bazer, F.W., Geisert, R.D., and Zavy, M.T. (1993) Fertilization, cleavage, and implantation. In 'Reproduction in Farm Animals.'. 6 edn. (Ed. ESE Hafez). pp. 188-212. (Lea & Febiger: Philadelphia).

Bazer, F.W., and Johnson, H.M. (1991). Type I conceptus interferons: maternal recognition of pregnancy signals and potential therapeutic agents. *Am. J. Reprod. Immunol.* **26**, 19-22.

Bazer, F.W., Ott, T.L., and Spencer, T.E. (1998). Endocrinology of the transition from recurring estrous cycles to establishment of pregnancy in subprimate mammals. In 'The Endocrinology of Pregnancy.' (Ed. FW Bazer). pp. 1-35. (Humana Press Inc.: Totowa, N.J.).

Bazer, F.W., Roberts, R.M., and Thatcher, W.W. (1979). Actions of hormones on the uterus and effect on conceptus development. *J. Anim. Sci.* **49 Suppl 2**, 35-45.

Bazer, F.W., Spencer, T.E., and Johnson, G.A. (2009). Interferons and uterine receptivity. *Semin. Reprod. Med.* **27**(1), 90-102.

Bazer, F.W., Spencer, T.E., and Ott, T.L. (1996). Placental interferons. *Am. J. Reprod. Immunol.* **35**(4), 297-308.

Bazer, F.W., Wu, G., Spencer, T.E., Johnson, G.A., Burghardt, R.C., and Bayless, K. (2010). Novel pathways for implantation and establishment and maintenance of pregnancy in mammals. *Mol. Hum. Reprod.* **16**(3), 135-152.

Bindon, B.M. (1969). Fate of the unfertilized sheep ovum. *J. Reprod. Fertil.* **20**(1), 183-184.

Bindon, B.M. (1971). Systematic study of preimplantation stages of pregnancy in the sheep. *Aust. J. Biol. Sci.* **24**(1), 131-147.

Blomberg, L., Hashizume, K., and Viebahn, C. (2008). Blastocyst elongation, trophoblastic differentiation, and embryonic pattern formation. *Reproduction* **135**(2), 181-195.

Boehm, U., Klamp, T., Groot, M., and Howard, J.C. (1997). Cellular responses to interferon-gamma. *Annu. Rev. Immunol.* **15**, 749-795.

Borchers, T., Hohoff, C., Buhlmann, C., and Spener, F. (1997). Heart-type fatty acid binding protein-involvement in growth inhibition and differentiation. *Prosta. Leuko. Essent. Fatty Acids* **57**(1), 77-84.

Boshier, D.P. (1969). A histological and histochemical examination of implantation and early placentome formation in sheep. *J. Reprod. Fertil.* **19**(1), 51-61.

Brinsfield, T.H., and Hawk (1974). Ultrastructure of sheep endometrial stromal cells after ovariectomy and hormone treatment. *Biol. Reprod.* **10**(1), 98-102.

Burghardt, R.C., Burghardt, J.R., Taylor, J.D., 2nd, Reeder, A.T., Nguen, B.T., Spencer, T.E., Bayless, K.J., and Johnson, G.A. (2009). Enhanced focal adhesion assembly reflects increased mechanosensation and mechanotransduction at maternal-conceptus interface and uterine wall during ovine pregnancy. *Reproduction* **137**(3), 567-582.

Burghardt, R.C., Johnson, G.A., Jaeger, L.A., Ka, H., Garlow, J.E., Spencer, T.E., and Bazer, F.W. (2002). Integrins and extracellular matrix proteins at the maternal-fetal interface in domestic animals. *Cells Tissues Organs* **172**(3), 202-217.

Camenisch, G., Pisabarro, M.T., Sherman, D., Kowalski, J., Nagel, M., Hass, P., Xie, M.H., Gurney, A., Bodary, S., Liang, X.H., Clark, K., Beresini, M., Ferrara, N., and Gerber, H.P. (2002). ANGPTL3 stimulates endothelial cell adhesion and migration via integrin alpha vbeta 3 and induces blood vessel formation in vivo. *J. Biol. Chem.* **277**(19), 17281-17290.

Carter, F., Forde, N., Duffy, P., Wade, M., Fair, T., Crowe, M.A., Evans, A.C., Kenny, D.A., Roche, J.F., and Lonergan, P. (2008). Effect of increasing progesterone concentration from Day 3 of pregnancy on subsequent embryo survival and development in beef heifers. *Reprod. Fertil. Dev.* **20**(3), 368-375.

Chavatte-Palmer, P., and Guillomot, M. (2007). Comparative implantation and placentation. *Gynecol. Obstet. Invest.* **64**(3), 166-174.

Cheng, N., Brantley, D.M., and Chen, J. (2002). The ephrins and Eph receptors in angiogenesis. *Cytokine Growth Factor Rev.* **13**, 75-85.

Choi, Y., Johnson, G.A., Burghardt, R.C., Berghman, L.R., Joyce, M.M., Taylor, K.M., Stewart, M.D., Bazer, F.W., and Spencer, T.E. (2001). Interferon regulatory factor-two restricts expression of interferon-stimulated genes to the endometrial stroma and glandular epithelium of the ovine uterus. *Biol. Reprod.* **65**(4), 1038-1049.

Choi, Y., Johnson, G.A., Spencer, T.E., and Bazer, F.W. (2003). Pregnancy and interferon tau regulate major histocompatibility complex class I and beta2-microglobulin expression in the ovine uterus. *Biol. Reprod.* **68**(5), 1703-1710.

Chon, T.W., and Bixler, S. (2010). Interferon-tau: current applications and potential in antiviral therapy. *J. Interferon & Cytokine Research* **30**(7), 477-485.

Clemens, T.L., Cormier, S., Eichinger, A., Endlich, K., Fiaschi-Taesch, N., Fischer, E., Friedman, P.A., Karaplis, A.C., Massfelder, T., Rossert, J., Schluter, K.D., Silve, C., Stewart, A.F., Takane, K., and Helwig, J.J. (2001). Parathyroid hormone-related protein and its receptors: nuclear functions and roles in the renal and cardiovascular systems, the placental trophoblasts and the pancreatic islets. *Br. J. Pharmacol.* **134**(6), 1113-1136.

Clemente, M., de La Fuente, J., Fair, T., Al Naib, A., Gutierrez-Adan, A., Roche, J.F., Rizos, D., and Lonergan, P. (2009). Progesterone and conceptus elongation in cattle: a direct effect on the embryo or an indirect effect via the endometrium? *Reproduction* **138**(3), 507-517.

Conneely, O.M., and Jericevic, B.M. (2002). Progesterone regulation of reproductive function through functionally distinct progesterone receptor isoforms. *Rev. Endocr. Metab. Disord.* **3**(3), 201-209.

Cross, J.C., Baczyk, D., Dobric, N., Hemberger, M., Hughes, M., Simmons, D.G., Yamamoto, H., and Kingdom, J.C. (2003). Genes, development and evolution of the placenta. *Placenta* **24**(2-3), 123-130.

Croy, B.A., van den Heuvel, M.J., Borzychowski, A.M., and Tayade, C. (2006). Uterine natural killer cells: a specialized differentiation regulated by ovarian hormones. *Immunol. Rev.* **214**, 161-185.

Crozat, K., Vivier, E., and Dalod, M. (2009). Crosstalk between components of the innate immune system: promoting anti-microbial defenses and avoiding immunopathologies. *Immunol. Rev.* **227**(1), 129-149.

Daoud, G., Simoneau, L., Masse, A., Rassart, E., and Lafond, J. (2005). Expression of cFABP and PPAR in trophoblast cells: effect of PPAR ligands on linoleic acid uptake and differentiation. *Biochim. Biophys. Acta.* **1687**(1-3), 181-194.

Darnell, J.E.J., Kerr, I.M., and Stark, G.R. (1994). Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. *Science* **264** (1415-1421).

Dawson, F.L.M. (1954). Progesterone in functional infertility of cattle. *Vet. Rec.* **66**, 324-326.

Dharmaraj, N., Wang, P., and Carson, D.D. (2010). Cytokine and progesterone receptor interplay in the regulation of MUC1 gene expression. *Mol. Endocrinol.* **24**(12), 2253-2266.

Dickinson, R.E., and Duncan, W.C. (2010). The SLIT-ROBO pathway: a regulator of cell function with implications for the reproductive system. *Reproduction* **139**(4), 697-704.

Dimitriadis, E., White, C.A., Jones, R.L., and Salamonsen, L.A. (2005). Cytokines, chemokines and growth factors in endometrium related to implantation. *Hum. Reprod. Update* **11**(6), 613-630.

Diskin, M.G., and Morris, D.G. (2008). Embryonic and early foetal losses in cattle and other ruminants. *Reprod. Domest. Anim.* **43 Suppl 2**, 260-267.

Diskin, M.G., and Sreenan, J.M. (1980). Fertilization and embryonic mortality rates in beef heifers after artificial insemination. *J. Reprod. Fertil.* **59**(2), 463-468.

Dorniak, P., Bazer, F.W., and Spencer, T.E. (2011). Prostaglandins regulate conceptus elongation and mediate effects of interferon tau on the ovine uterine endometrium. *Biol. Reprod.* **84**(6), 1119-1127.

Duffy, S.L., Steiner, K.A., Tam, P.P.L., and Boyd, A.W. (2006). Expression analysis of the Epha1 receptor tyrosine kinase and its high-affinity ligands Efna1 and Efna3 during early mouse development. *Gene Exp. Patterns* **6**, 719-723.

Dumic, J., Dabelic, S., and Flogel, M. (2006). Galectin-3: an open-ended story. *Biochim. Biophys. Acta.* **1760**(4), 616-635.

Duncan, W.C., McDonald, S.E., Dickinson, R.E., Shaw, J.L., Lourenco, P.C., Wheelhouse, N., Lee, K.F., Critchley, H.O., and Horne, A.W. (2010). Expression of the repulsive SLIT/ROBO pathway in the human endometrium and Fallopian tube. *Mol. Hum. Reprod.* **16**(12), 950-959.

Dunlap, K.A., Erikson, D.W., Burghardt, R.C., White, F.J., Reed, K.M., Farmer, J.L., Spencer, T.E., Magness, R.R., Bazer, F.W., Bayless, K.J., and Johnson, G.A. (2008). Progesterone and placentation increase secreted phosphoprotein one (SPP1 or osteopontin) in uterine glands and stroma for histotrophic and hematotrophic support of ovine pregnancy. *Biol. Reprod.* **79**(5), 983-990.

Duttaroy, A.K. (2009). Transport of fatty acids across the human placenta: a review. *Prog. Lipid. Res.* **48**(1), 52-61.

Farmer, J.L., Burghardt, R.C., Jousan, F.D., Hansen, P.J., Bazer, F.W., and Spencer, T.E. (2008). Galectin 15 (LGALS15) functions in trophectoderm migration and attachment. *FASEB J.* **22**(2), 548-560.

Ferguson, J.E., 2nd, Seaner, R.M., Bruns, D.E., Iezzoni, J.C., and Bruns, M.E. (1998). Expression and specific immunolocalization of the human parathyroid hormone/parathyroid hormone-related protein receptor in the uteroplacental unit. *Am. J. Obstet. Gynecol.* **179**(2), 321-329.

Flechon, J.E., Flechon, B., Degrouard, J., and Guillomot, M. (2007). Cellular features of the extra-embryonic endoderm during elongation in the ovine conceptus. *Genesis* **45**, 709-715

Flechon, J.E., Guillomot, M., Charlier, M., Flechon, B., and Martal, J. (1986). Experimental studies on the elongation of the ewe blastocyst. *Reprod. Nutr. Dev.* **26**(4), 1017-1024.

Fleming, J.A., Choi, Y., Johnson, G.A., Spencer, T.E., and Bazer, F.W. (2001). Cloning of the ovine estrogen receptor-alpha promoter and functional regulation by ovine interferon-tau. *Endocrinology* **142**(7), 2879-2887.

Flint, A.P., Leat, W.M., Sheldrick, E.L., and Stewart, H.J. (1986). Stimulation of phosphoinositide hydrolysis by oxytocin and the mechanism by which oxytocin controls prostaglandin synthesis in the ovine endometrium. *Biochem. J.* **237**(3), 797-805.

Forde, N., Beltman, M.E., Duffy, G.B., Duffy, P., Mehta, J.P., O'Gaora, P., Roche, J.F., Lonergan, P., and Crowe, M.A. (2011). Changes in the endometrial transcriptome during the bovine estrous cycle: effect of low circulating progesterone and consequences for conceptus elongation. *Biol. Reprod.* **84**(2), 266-278.

Forde, N., Carter, F., Fair, T., Crowe, M.A., Evans, A.C., Spencer, T.E., Bazer, F.W., McBride, R., Boland, M.P., O'Gaora, P., Lonergan, P., and Roche, J.F. (2009). Progesterone-regulated changes in endometrial gene expression contribute to advanced conceptus development in cattle. *Biol. Reprod.* **81**(4), 784-794.

Forde, N., Spencer, T.E., Bazer, F.W., Song, G., Roche, J.F., and Lonergan, P. (2010). Effect of pregnancy and progesterone concentration on expression of genes encoding for transporters or secreted proteins in the bovine endometrium. *Physiol. Genomics* **41**(1), 53-62.

Fujii, H., Fujiwara, H., Horie, A., Sato, Y., and Konishi, I. (2011). Ephrin A1 induces intercellular dissociation in Ishikawa cells: possible implication of the Eph-ephrin A system in human embryo implantation. *Hum. Reprod.* **26**(2), 299-306.

Gao, H., Wu, G., Spencer, T.E., Johnson, G.A., Li, X., and Bazer, F.W. (2009). Select nutrients in the ovine uterine lumen. I. Amino acids, glucose, and ions in uterine lumenal flushings of cyclic and pregnant ewes. *Biol. Reprod.* **80**(1), 86-93.

Gao, X., Xu, H., Liu, H., Rao, J., Li, Y., and Zha, X. (2010). Angiopoietin-like protein 3 regulates the motility and permeability of podocytes by altering nephrin expression in vitro. *Biochem. Biophys. Res. Commun.* **399**(1), 31-36.

Garrett, J.E., Geisert, R.D., Zavy, M.T., and Morgan, G.L. (1988). Evidence for maternal regulation of early conceptus growth and development in beef cattle. *J. Reprod. Fertil.* **84**(2), 437-446.

Geisert, R.D., Morgan, G.L., Short, E.C., Jr., and Zavy, M.T. (1992). Endocrine events associated with endometrial function and conceptus development in cattle. *Reprod. Fertil. Dev.* **4**(3), 301-305.

Gellersen, B., Fernandes, M.S., and Brosens, J.J. (2009). Non-genomic progesterone actions in female reproduction. *Hum. Reprod. Update* **15**(1), 119-138.

Geutskens, S.B., Hordijk, P.L., and van Hennik, P.B. (2010). The chemorepellent Slit3 promotes monocyte migration. *J. Immunol.* **185**(12), 7691-7698.

Goldman-Wohl, D., Greenfield, C., Haimov-Kochman, R., Ariel, I., Anteby, E.Y., Hochner-Celnikier, D., Farhat, M., and Yagel, S. (2004). Eph and ephrin expression in normal placental development and preeclampsia. *Placenta* **25**(7), 623-630.

Gomez, E., Caamano, J.N., Rodriguez, A., De Frutos, C., Facal, N., and Diez, C. (2006). Bovine early embryonic development and vitamin A. *Reprod. Domest. Anim.* **41 Suppl 2**, 63-71.

Gray, C.A., Bartol, F.F., Taylor, K.M., Wiley, A.A., Ramsey, W.S., Ott, T.L., Bazer, F.W., and Spencer, T.E. (2000a). Ovine uterine gland knock-out model: effects of gland ablation on the estrous cycle. *Biol. Reprod.* **62**(2), 448-456.

Gray, C.A., Bazer, F.W., and Spencer, T.E. (2001a). Effects of neonatal progestin exposure on female reproductive tract structure and function in the adult ewe. *Biol. Reprod.* **64**, 797-804.

Gray, C.A., Burghardt, R.C., Johnson, G.A., Bazer, F.W., and Spencer, T.E. (2002). Evidence that absence of endometrial gland secretions in uterine gland knockout ewes compromises conceptus survival and elongation. *Reproduction* **124**(2), 289-300.

Gray, C.A., Taylor, K.M., Bazer, F.W., and Spencer, T.E. (2000b). Mechanisms regulating norgestomet inhibition of endometrial gland morphogenesis in the neonatal ovine uterus. *Mol. Reprod. Dev.* **57**, 67-78.

Gray, C.A., Taylor, K.M., Ramsey, W.S., Hill, J.R., Bazer, F.W., Bartol, F.F., and Spencer, T.E. (2001b). Endometrial glands are required for preimplantation conceptus elongation and survival. *Biol. Reprod.* **64**(6), 1608-1613.

Grazul-Bilska, A.T., Johnson, M.L., Borowicz, P.P., Minten, M., Bilski, J.J., Wroblewski, R., Velimirovich, M., Coupe, L.R., Redmer, D.A., and Reynolds, L.P. (2011). Placental development during early pregnancy in sheep: cell proliferation, global methylation, and angiogenesis in the fetal placenta. *Reproduction* **141**(4), 529-540.

Guan, H., Zu, G., Xie, Y., Tang, H., Johnson, M., Xu, X., Kevil, C., Xiong, W.C., Elmets, C., Rao, Y., Wu, J.Y., and Xu, H. (2003). Neuronal repellent Slit2 inhibits dendritic cell migration and the development of immune responses. *J. Immunol.* **171**(12), 6519-6526.

Guillomot, M. (1995). Cellular interactions during implantation in domestic ruminants. *J. Reprod. Fertil. Suppl.* **49**, 39-51

Guillomot, M., Flechon, J.E., and Wintenberger-Torres, S. (1981). Conceptus attachment in the ewe: an ultrastructural study. *Placenta* **2**(2), 169-182.

Guillomot, M., Reinaud, P., Charpigny, G., and Martal, J. (1988). Embryo-uterine interactions during early stages of pregnancy in domestic mammals. *Reprod. Nutr. Dev.* **28**(6B), 1629-1648.

Guillomot, M., Turbe, A., Hue, I., and Renard, J.P. (2004). Staging of ovine embryos and expression of the T-box genes Brachyury and Eomesodermin around gastrulation. *Reproduction* **72**, 491-501.

Haggarty, P. (2002). Placental regulation of fatty acid delivery and its effect on fetal growth--a review. *Placenta* **23 Suppl A**, S28-38.

Hammes, S.R., and Levin, E.R. (2007). Extranuclear steroid receptors: nature and actions. *Endocr. Rev.* **28**(7), 726-741.

Hampton, A.L., Butt, A.R., Riley, S.C., and Salamonsen, L.A. (1995). Tissue inhibitors of metalloproteinases in endometrium of ovariectomized steroid-treated ewes and during the estrous cycle and early pregnancy. *Biol. Reprod.* **53**(2), 302-311.

Hanhoff, T., Lucke, C., and Spener, F. (2002). Insights into binding of fatty acids by fatty acid binding proteins. *Mol. Cell. Biochem.* **239**(1-2), 45-54.

Hanlon, D.W., Davidson, P.J., Hittmann, A.R., and Joe, A.K. (2005). Supplementing previously treated anestrous dairy cows with progesterone does not increase first-service conception rate. *Theriogenology* **63**(1), 239-245.

Hannan, N.J., Jones, R.L., White, C.A., and Salamonsen, L.A. (2006). The chemokines, CX3CL1, CCL14, and CCL4, promote human trophoblast migration at the feto-maternal interface. *Biol. Reprod.* **74**(5), 896-904.

Hansen, P.J. (2007). Regulation of immune cells in the uterus during pregnancy in ruminants. *J. Anim. Sci.* **85**(13 Suppl), E30-31.

Harney, J.P., Ott, T.L., Geisert, R.D., and Bazer, F.W. (1993). Retinol-binding protein gene expression in cyclic and pregnant endometrium of pigs, sheep, and cattle. *Biol. Reprod.* **49**(5), 1066-1073.

Heap, R.B., Fleet, I.R., and Hamon, M. (1985). Prostaglandin F-2 alpha is transferred from the uterus to the ovary in sheep by lymphatic and blood vascular pathways. *J. Reprod. Fertil.* **74**(645-656).

Hendy, G.N., Bevan, S., Mattei, M.G., and Mouland, A.J. (1995). Chromogranin A. *Clin. Invest. Med.* **18**(1), 47-65.

Henrick, J.B. (1953). Clinical observations of progesterone therapy in repeat breeding heifers. *Vet. Med.* **48**, 489-490.

Hinck, L. (2004). The versatile roles of "axon guidance" cues in tissue morphogenesis. *Dev. Cell* **7**(6), 783-793.

Hixon, J.E., and Flint, A.P. (1987). Effects of a luteolytic dose of oestradiol benzoate on uterine oxytocin receptor concentrations, phosphoinositide turnover and prostaglandin F-2 alpha secretion in sheep. *J. Reprod. Fertil.* **79**(2), 457-467.

Hu, Y., Dutz, J.P., MacCalman, C.D., Yong, P., Tan, R., and von Dadelszen, P. (2006). Decidual NK cells alter in vitro first trimester extravillous cytotrophoblast migration: a role for IFN-gamma. *J. Immunol.* **177**(12), 8522-8530.

Hughes, R.C. (1999). Secretion of the galectin family of mammalian carbohydrate-binding proteins. *Biochim. Biophys. Acta.* **1473**(1), 172-185.

Huynh-Do, U., Vindis, C., Liu, H., Cerretti, D.P., McGrew, J.T., Enriquez, M., Chen, J., and Daniel, T.O. (2002). Ephrin-B1 transduces signals to activate integrin-mediated migration, attachment and angiogenesis. *J. Cell. Sci.* **115**(Pt 15), 3073-3081.

Iglesias, M.M., Rabinovich, G.A., Ambrosio, A.L., Castagna, L.F., Sotomayor, C.E., and Wolfenstein-Todel, C. (1998). Purification of galectin-3 from ovine placenta: developmentally regulated expression and immunological relevance. *Glycobiology* **8**(1), 59-65.

Igwebuike, U.M. (2009). A review of uterine structural modifications that influence conceptus implantation and development in sheep and goats. *Anim. Reprod. Sci.* **112**(1-2), 1-7.

Imakawa, K., Helmer, S.D., Nephew, K.P., Meka, C.S., and Christenson, R.K. (1993). A novel role for GM-CSF: enhancement of pregnancy specific interferon production, ovine trophoblast protein-1. *Endocrinology* **132**(4), 1869-1871.

Imakawa, K., Imai, M., Sakai, A., Suzuki, M., Nagaoka, K., Sakai, S., Lee, S.R., Chang, K.T., Echternkamp, S.E., and Christenson, R.K. (2006). Regulation of conceptus adhesion by endometrial CXC chemokines during the implantation period in sheep. *Mol. Reprod. Dev.* **73**(7), 850-858.

Imakawa, K., Ji, Y., Yamaguchi, H., Tamura, K., Weber, L.W., Sakai, S., and Christenson, R.K. (1998). Co-expression of transforming growth factor beta and interferon tau during peri-implantation period in the ewe. *Endocr. J.* **45**(4), 441-450.

Jeschke, U., Mayr, D., Schiessl, B., Mylonas, I., Schulze, S., Kuhn, C., Friese, K., and Walzel, H. (2007). Expression of galectin-1, -3 (gal-1, gal-3) and the Thomsen-Friedenreich (TF) antigen in normal, IUGR, preeclamptic and HELLP placentas. *Placenta* **28**(11-12), 1165-1173.

Johnson, G.A., Bazer, F.W., Jaeger, L.A., Ka, H., Garlow, J.E., Pfarrer, C., Spencer, T.E., and Burghardt, R.C. (2001a). Muc-1, integrin, and osteopontin expression during the implantation cascade in sheep. *Biol. Reprod.* **65**(3), 820-828.

Johnson, G.A., Burghardt, R.C., Bazer, F.W., and Spencer, T.E. (2003a). Osteopontin: roles in implantation and placentation. *Biol. Reprod.* **69**(5), 1458-1471.

Johnson, G.A., Burghardt, R.C., Joyce, M.M., Spencer, T.E., Bazer, F.W., Pfarrer, C., and Gray, C.A. (2003b). Osteopontin expression in uterine stroma indicates a decidualization-like differentiation during ovine pregnancy. *Biol. Reprod.* **68**(6), 1951-1958.

Johnson, G.A., Burghardt, R.C., Spencer, T.E., Newton, G.R., Ott, T.L., and Bazer, F.W. (1999a). Ovine osteopontin: II. Osteopontin and alpha(v)beta(3) integrin expression in the uterus and conceptus during the periimplantation period. *Biol. Reprod.* **61**(4), 892-899.

Johnson, G.A., Spencer, T.E., Hansen, T.R., Austin, K.J., Burghardt, R.C., and Bazer, F.W. (1999b). Expression of the interferon tau inducible ubiquitin cross-reactive protein in the ovine uterus. *Biol. Reprod.* **61**(1), 312-318.

Johnson, G.A., Stewart, M.D., Gray, C.A., Choi, Y., Burghardt, R.C., Yu-Lee, L.Y., Bazer, F.W., and Spencer, T.E. (2001b). Effects of the estrous cycle, pregnancy, and interferon tau on 2',5'-oligoadenylate synthetase expression in the ovine uterus. *Biol. Reprod.* **64**(5), 1392-1399.

Johnson, K.R., Ross, R.H., and Fourt, D.L. (1958). Effect of progesterone administration on reproductive efficiency. *J. Anim. Sci.* **17**, 386-390.

Jovanovic, M., and Vicovac, L. (2009). Interleukin-6 stimulates cell migration, invasion and integrin expression in HTR-8/SVneo cell line. *Placenta* **30**(4), 320-328.

Joyce, M.M., White, F.J., Burghardt, R.C., Muniz, J.J., Spencer, T.E., Bazer, F.W., and Johnson, G.A. (2005). Interferon stimulated gene 15 conjugates to endometrial cytosolic proteins and is expressed at the uterine-placental interface throughout pregnancy in sheep. *Endocrinology* **146**(2), 675-684.

Kamimura, D., Ishihara, K., and Hirano, T. (2003). IL-6 signal transduction and its physiological roles: the signal orchestration model. *Rev. Physiol. Biochem. Pharmacol.* **149**, 1-38.

Kanai, M., Raz, A., and Goodman, D.S. (1968). Retinol-binding protein: the transport protein for vitamin A in human plasma. *J. Clin. Invest.* **47**(9), 2025-2044.

Katoh, Y., and Katoh, M. (2006). Comparative integromics on Ephrin family. *Oncol. Rep.* **15**(5), 1391-1395.

Kim, M., Kim, S., Kim, H., Joo, H.G., and Shin, T. (2008). Immunohistochemical localization of galectin-3 in the reproductive organs of the cow. *Acta. Histochem.* **110**(6), 473-480.

Kim, T., and Loh, Y.P. (2005). Chromogranin A: a surprising link between granule biogenesis and hypertension. *J. Clin. Invest.* **115**(7), 1711-1713.

Kirby, D.R.S. (1965) The "invasiveness" of the trophoblast. In 'The early conceptus, normal and abnormal.' (Ed. WW Park) pp. 68-73. (University of St. Andrews Press: Edinburgh).

Kleemann, D.O., Walker, S.K., and Seamark, R.F. (1994). Enhanced fetal growth in sheep administered progesterone during the first three days of pregnancy. *J. Reprod. Fertil.* **102**(2), 411-417.

Ko, Y., Lee, C.Y., Ott, T.L., Davis, M.A., Simmen, R.C., Bazer, F.W., and Simmen, F.A. (1991). Insulin-like growth factors in sheep uterine fluids: concentrations and relationship to ovine trophoblast protein-1 production during early pregnancy. *Biol. Reprod.* **45**(1), 135-142.

Kuang, H., Chen, Q., Fan, X., Zhang, Y., Zhang, L., Peng, H., Cao, Y., and Duan, E. (2009a). CXCL14 inhibits trophoblast outgrowth via a paracrine/autocrine manner during early pregnancy in mice. *J. Cell Physiol.* **221**, 448-457.

Kuang, H., Chen, Q., Zhang, L., Peng, H., Ning, L., Cao, Y., and Duan, E. (2009b). The cytokine gene CXCL14 restricts human trophoblast cell invasion by suppressing gelatinase activity. *Endocrinology* **150**(12), 5596-5605.

Kuijper, S., Turner, C.J., and Adams, R.H. (2007). Regulation of angiogenesis by Eph-Ephrin interactions. *Trends. Cardiovasc. Med.* **17**, 145-151.

Kurth, I., Willimann, K., Schaerli, P., Hunziker, T., Clark-Lewis, I., and Moser, B. (2001). Monocyte selectivity and tissue localization suggests a role for breast and kidney-expressed chemokine (BRAK) in macrophage development. *J. Exp. Med.* **194**, 855-861.

Lamming, G.E., Wathes, D.C., Flint, A.P., Payne, J.H., Stevenson, K.R., and Vallet, J.L. (1995). Local action of trophoblast interferons in suppression of the development of oxytocin and oestradiol receptors in ovine endometrium. *J. Reprod. Fertil.* **105**(1), 165-175.

Larson, S.F., Butler, W.R., and Currie, W.B. (2007). Pregnancy rates in lactating dairy cattle following supplementation of progesterone after artificial insemination. *Anim. Reprod. Sci.* **102**(1-2), 172-179.

Lash, G.E., Otun, H.A., Innes, B.A., Kirkley, M., De Oliveira, L., Searle, R.F., Robson, S.C., and Bulmer, J.N. (2006). Interferon-gamma inhibits extravillous trophoblast cell invasion by a mechanism that involves both changes in apoptosis and protease levels. *FASEB J.* **20**(14), 2512-2518.

Lawson, R.A., Parr, R.A., and Cahill, L.P. (1983). Evidence for maternal control of blastocyst growth after asynchronous transfer of embryos to the uterus of the ewe. *J. Reprod. Fertil.* **67**(2), 477-483.

Lewis, G.S., and Waterman, R.A. (1985). Metabolism of arachidonic acid in vitro by ovine conceptuses recovered during early pregnancy. *Prostaglandins* **30**(2), 263-283.

Lewis, S.K., Farmer, J.L., Burghardt, R.C., Newton, G.R., Johnson, G.A., Adelson, D.L., Bazer, F.W., and Spencer, T.E. (2007). Galectin 15 (LGALS15): a gene uniquely expressed in the uteri of sheep and goats that functions in trophoblast attachment. *Biol. Reprod.* **77**(6), 1027-1036.

Lichtenstein, L., and Kersten, S. (2010). Modulation of plasma TG lipolysis by Angiopoietin-like proteins and GPIHBP1. *Biochim. Biophys. Acta.* **1801**(4), 415-420.

Liu, F.T., Patterson, R.J., and Wang, J.L. (2002). Intracellular functions of galectins. *Biochim. Biophys. Acta.* **1572**(2-3), 263-273.

Liu, K.H., Gao, K.X., Baumbach, G.A., and Godkin, J.D. (1992). Purification and immunolocalization of ovine placental retinol-binding protein. *Biol. Reprod.* **46**(1), 23-29.

Locati, M., and Murphy, P.M. (1999). Chemokines and chemokine receptors: biology adn clinical relevance in inflammation and AIDS. *Annu. Rev. Med.* **50**, 425-440.

Lucy, M.C. (2001). Reproductive loss in high-producing dairy cattle: where will it end? *J. Dairy Sci.* **84**(6), 1277-1293.

Mann, G.E., Fray, M.D., and Lamming, G.E. (2006). Effects of time of progesterone supplementation on embryo development and interferon-tau production in the cow. *Vet. J.* **171**(3), 500-503.

Mann, G.E., and Lamming, G.E. (1999). The Influence of Progesterone During Early Pregnancy in Cattle. *Reprod. in Domest. Anim.* **34**(3-4), 269-274.

Marcus, G.J. (1981). Prostaglandin formation by the sheep embryo and endometrium as an indication of maternal recognition of pregnancy. *Biol. Reprod.* **25**(1), 56-64.

Masuyama, H., Inoue, S., and Hiramatsu, Y. (2011). Retinol-binding protein 4 and insulin resistance in preeclampsia. *Endocr. J.* **58**(1), 47-53.

Mattos, R., Staples, C.R., and Thatcher, W.W. (2000). Effects of dietary fatty acids on reproduction in ruminants. *Rev. Reprod.* **5**(1), 38-45.

McCracken, J.A. (1980). Hormone receptor control of prostaglandin F2 alpha secretion by the ovine uterus. *Adv. Prosta. Thromb. Res.* **8**, 1329-1344.

McCracken, J.A., Custer, E.E., and Lamsa, J.C. (1999). Luteolysis: a neuroendocrine-mediated event. *Physiol. Rev.* **79**(2), 263-323.

McCracken, J.A., Schramm, W., and Okulicz, W.C. (1984). Hormone receptor control of pulsatile secretion of PGF2alpha from the ovine uterus during luteolysis and its abrogation in early pregnancy. *Anim. Reprod. Sci.* **7**(31-55).

McGrath, R.T., McRae, E., Smith, O.P., and O'Donnell, J.S. (2010). Platelet von Willebrand factor--structure, function and biological importance. *Br. J. Haematol.* **148**(6), 834-843.

Meuter, S., Schaerli, P., Roos, R.S., Brandau, O., Bosl, M.R., Andrian, U.R.v., and Moser, B. (2007). Murine CXCL14 is dispensable for dendritic cell function and localization within peripheral tissues. *Mol. Cell. Biol.* **27**(3), 983-992.

Meyer, D., Pietu, G., Fressinaud, E., and Girma, J.P. (1991). von Willebrand factor: structure and function. *Mayo Clin. Proc.* **66**(5), 516-523.

Michael, A.E., and Papageorghiou, A.T. (2008). Potential significance of physiological and pharmacological glucocorticoids in early pregnancy. *Hum. Reprod. Update* **14**(5), 497-517.

Michael, A.E., Thurston, L.M., and Rae, M.T. (2003). Glucocorticoid metabolism and reproduction: a tale of two enzymes. *Reproduction* **126**(4), 425-441.

Mitani, H., Katayama, N., Araki, H., Ohishi, K., Kobayashi, K., Suzuki, H., Nishii, K., Masuya, M., Yasukawa, K., Minami, N., and Shiku, H. (2000). Activity of interleukin 6 in the differentiation of monocytes to macrophages and dendritic cells. *Br. J. Haematol.* **109**, 288-295.

Miyake, Y., Ishikawa, E., Ishikawa, T., and Yamasaki, S. (2010). Self and nonself recognition through C-type lectin receptor, Mincle. *Self Nonself* **1**(4), 310-313.

Mokhtar, N.M., Cheng, C.W., Cook, E., Bielby, H., Smith, S.K., and Charnock-Jones, D.S. (2010). Progestin regulates chemokine (C-X-C motif) ligand 14 transcript level in human endometrium. *Mol. Hum. Reprod.* **16**(3), 170-177.

Moore, N.W., and Shelton, J.N. (1964). 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.* **7**, 145-152.

Muniz, J.J., Joyce, M.M., Taylor, J.D., 2nd, Burghardt, J.R., Burghardt, R.C., and Johnson, G.A. (2006). Glycosylation dependent cell adhesion molecule 1-like protein and L-selectin expression in sheep interplacentomal and placentomal endometrium. *Reproduction* **131**(4), 751-761.

Muraguchi, A., Hirano, T., Tang, B., Matsuda, T., Horii, Y., Nakajima, K., and Kishimoto, T. (1988). The essential role of B cell stimulatory factor 2 (BSF-2/IL-6) for the terminal differentiation of B cells. *J. Exp. Med.* **167**, 332-344.

Murphy, S.P., Tayade, C., Ashkar, A.A., Hatta, K., Zhang, J., and Croy, B.A. (2009). Interferon gamma in successful pregnancies. *Biol. Reprod.* **80**(5), 848-859.

Nangia-Makker, P., Honjo, Y., Sarvis, R., Akahani, S., Hogan, V., Pienta, K.J., and Raz, A. (2000). Galectin-3 induces endothelial cell morphogenesis and angiogenesis. *Am. J. Pathol.* **156**(3), 899-909.

Nephew, K.P., McClure, K.E., Ott, T.L., Dubois, D.H., Bazer, F.W., and Pope, W.F. (1991). Relationship between variation in conceptus development and differences in estrous cycle duration in ewes. *Biol. Reprod.* **44**(3), 536-539.

Nevo, J., Mai, A., Tuomi, S., Pellinen, T., Pentikainen, O.T., Heikkila, P., Lundin, J., Joensuu, H., Bono, P., and Ivaska, J. (2010). Mammary-derived growth inhibitor (MDGI) interacts with integrin alpha-subunits and suppresses integrin activity and invasion. *Oncogene* **29**(49), 6452-6463.

Niswender, G.D., Juengel, J.L., Silva, P.J., Rollyson, M.K., and McIntush, E.W. (2000). Mechanisms controlling the function and life span of the corpus luteum. *Physiol. Rev.* **80**, 1-29.

Nowak, R.A., Haimovici, F., Biggers, J.D., and Erbach, G.T. (1999). Transforming growth factor-beta stimulates mouse blastocyst outgrowth through a mechanism involving parathyroid hormone-related protein. *Biol. Reprod.* **60**(1), 85-93.

Ocon-Grove, O.M., Cooke, F.N., Alvarez, I.M., Johnson, S.E., Ott, T.L., and Ealy, A.D. (2008). Ovine endometrial expression of fibroblast growth factor (FGF) 2 and conceptus expression of FGF receptors during early pregnancy. *Domest. Anim. Endocrinol.* **34**(2), 135-145.

Oliveira, J.F., Henkes, L.E., Ashley, R.L., Purcell, S.H., Smirnova, N.P., Veeramachaneni, D.N., Anthony, R.V., and Hansen, T.R. (2008). Expression of interferon (IFN)-stimulated genes in extrauterine tissues during early pregnancy in sheep is the consequence of endocrine IFN-tau release from the uterine vein. *Endocrinology* **149**(3), 1252-1259.

Piccinni, M.P., Scaletti, C., Vultaggio, A., Maggi, E., and Romagnani, S. (2001). Defective production of LIF, M-CSF and Th2-type cytokines by T cells at the fetomaternal interface is associated with pregnancy loss. *J. Reprod. Immunol.* **52**, 35-43.

Pitera, A.E., Smith, G.C., Wentworth, R.A., and Nathanielsz, P.W. (1998). Parathyroid hormone-related peptide (1 to 34) inhibits in vitro oxytocin-stimulated activity of pregnant baboon myometrium. *Am. J. Obstet. Gynecol.* **179**(2), 492-496.

Platanias, L.C. (2005). Mechanisms of type-I- and type-II-interferon-mediated signalling. *Nat. Rev. Immunol.* **5**, 375-386.

Poirier, F. (2002). Roles of galectins in vivo. *Biochem. Soc. Symp.* (**69**), 95-103.

Pontzer, C.H., Ott, T.L., Bazer, F.W., and Johnson, H.M. (1990). Localization of an antiviral site on the pregnancy recognition hormone, ovine trophoblast protein 1. *Proc. Natl. Acad. Sci. USA* **87**, 5945-5949.

Pope, W.F. (1988). Uterine asynchrony: a cause of embryonic loss. *Biol. Reprod.* **39**(5), 999-1003.

Ratti, S., Curnis, F., Longhi, R., Colombo, B., Gasparri, A., Magni, F., Manera, E., Metz-Boutigue, M.H., and Corti, A. (2000). Structure-activity relationships of chromogranin A in cell adhesion. Identification of an adhesion site for fibroblasts and smooth muscle cells. *J. Biol. Chem.* **275**(38), 29257-29263.

Rexroad, C.E., Jr. (1984). Steroid receptors in the myometrium during pregnancy in the ewe. *J. Anim. Sci.* **58**(5), 1278-1284.

Reynolds, L.P., Borowicz, P.P., Caton, J.S., Vonnahme, K.A., Luther, J.S., Buchanan, D.S., Hafez, S.A., Grazul-Bilska, A.T., and Redmer, D.A. (2010). Uteroplacental vascular development and placental function: an update. *Int. J. Dev. Biol.* **54**(2-3), 355-366.

Ricketts, A.P., and Flint, A.P. (1980). Onset of synthesis of progesterone by ovine placenta. *J. Endocrinol.* **86**(2), 337-347.

Riley, S.C., Butt, A.R., Doughton, B.W., Li, S.X., Zheng, S.H., Findlay, J.K., and Salamonsen, L.A. (1994). Endothelin in the ovine uterus during the oestrous cycle and early pregnancy. *J. Reprod. Fertil.* **100**(2), 451-459.

Rincon, M., Anguita, J., Nakamura, T., Fikrig, E., and Flavell, R.A. (1997). Interleukin (IL)-6 directs the differentiation of IL-4 producing CD4+ T cells. *J. Exp. Med.* **185**, 461-469.

Roberts, R.M., Ealy, A.D., Alexenko, A.P., Han, C.S., and Ezashi, T. (1999). Trophoblast interferons. *Placenta* **20**, 259-264.

Robertson, S.A., Christiaens, I., Dorian, C.L., Zaragoza, D.B., Care, A.S., Banks, A.M., and Olson, D.M. (2010). Interleukin-6 is an essential determinant of on-time parturition in the mouse. *Endocrinology* **161**(9), 3996-4006.

Robertson, S.A., O'Connell, A., and Ramsey, A. (2000). The effect of interleukin-6 deficiency on implantation, fetal development and parturition in mice. *Proc. Aust. Soc. Reprod. Biol.* **31**, 97.

Robinson, N.A., Leslie, K.E., and Walton, J.S. (1989). Effect of treatment with progesterone on pregnancy rate and plasma concentrations of progesterone in Holstein cows. *J. Dairy Sci.* **72**(1), 202-207.

Romano, M., Sironi, M., Toniatti, C., Polentarutti, N., Fruscella, P., Ghezzi, P., Faggioni, R., Luini, W., van Hinsbergh, V., Sozzani, S., Bussolino, F., Poli, V., Ciliberto, G., and Mantovani, A. (1997). Tole of IL-6 and its soluble receptor in induction of chemokines and leukocytes recruitment. *Immunity* **6**, 315-325.

Ross, J.W., Ashworth, M.D., Stein, D.R., Couture, O.P., Tuggle, C.K., and Geisert, R.D. (2009). Identification of differential gene expression during porcine conceptus rapid trophoblastic elongation and attachment to uterine luminal epithelium. *Physiol. Genomics* **36**(3), 140-148.

Rothschild, M.F., Messer, L., Day, A., Wales, R., Short, T., Southwood, O., and Plastow, G. (2000). Investigation of the retinol-binding protein 4 (RBP4) gene as a candidate gene for increased litter size in pigs. *Mamm. Genome* **11**(1), 75-77.

Rowson, L.E., and Moor, R.M. (1966). Development of the sheep conceptus during the first fourteen days. *J. Anat.* **100**, 777-785.

Salamonsen, L.A., Hannan, N.J., and Dimitriadis, E. (2007). Cytokines and chemokines during human embryo implantation: roles in implantation and early placentation. *Semin. Reprod. Med.* **25**(6), 437-444.

Salamonsen, L.A., Wai, S.O., Doughton, B., and Findlay, J.K. (1985). The effects of estrogen and progesterone in vivo on protein synthesis and secretion by cultured epithelial cells from sheep endometrium. *Endocrinology* **117**(5), 2148-2159.

Salogni, L., Musso, T., Bosisio, D., Mirolo, M., Jala, V.R., Haribabu, B., Locati, M., and Sozzani, S. (2009). Activin A induces dendritic cell migration through the polarized release of CXC chemokine ligands 12 and 14. *Blood* **113**(23), 5848-5856.

Sampath, H., and Ntambi, J.M. (2005). Polyunsaturated fatty acid regulation of genes of lipid metabolism. *Annu. Rev. Nutr.* **25**, 317-340.

Sangsritavong, S., Combs, D.K., Sartori, R., Armentano, L.E., and Wiltbank, M.C. (2002). High feed intake increases liver blood flow and metabolism of progesterone and estradiol-17beta in dairy cattle. *J. Dairy Sci.* **85**(11), 2831-2842.

Sano, H., Hsu, D.K., Apgar, J.R., Yu, L., Sharma, B.B., Kuwabara, I., Izui, S., and Liu, F.T. (2003). Critical role of galectin-3 in phagocytosis by macrophages. *J. Clin. Invest.* **112**(3), 389-397.

Sano, H., Hsu, D.K., Yu, L., Apgar, J.R., Kuwabara, I., Yamanaka, T., Hirashima, M., and Liu, F.T. (2000). Human galectin-3 is a novel chemoattractant for monocytes and macrophages. *J. Immunol.* **165**(4), 2156-2164.

Satterfield, M.C., Bazer, F.W., and Spencer, T.E. (2006). Progesterone regulation of preimplantation conceptus growth and galectin 15 (LGALS15) in the ovine uterus. *Biol. Reprod.* **75**(2), 289-296.

Satterfield, M.C., Song, G., Kochan, K.J., Riggs, P.K., Simmons, R.M., Elsik, C.G., Adelson, D.L., Bazer, F.W., Zhou, H., and Spencer, T.E. (2009). Discovery of candidate genes and pathways in the endometrium regulating ovine blastocyst growth and conceptus elongation. *Physiol. Genomics* **39**(2), 85-99.

Shan, L., Yu, X.C., Liu, Z., Hu, Y., Sturgis, L.T., Miranda, M.L., and Liu, Q. (2009). The angiopoietin-like proteins ANGPTL3 and ANGPTL4 inhibit lipoprotein lipase activity through distinct mechanisms. *J. Biol. Chem.* **284**(3), 1419-1424.

Shellenberger, T.D., Wang, M., Gujrati, M., Jayakumar, A., Strieter, R.M., Burdick, M.D., Ioannides, C.G., Efferson, C.L., El-Naggar, A.K., Roberts, D., Clayman, G.L., and Frederick, M.J. (2004). BRAK/CXCL14 is a potent inhibitor of angiogenesis and a chemotactic factor for immature dendritic cells. *Cancer Res.* **64**(22), 8262-8270.

Shurin, G.V., Ferris, R.L., Tourkova, I.L., Perez, L., Lokshin, A., Balkir, L., Collins, B., Chatta, G.S., and Shurin, M.R. (2005). Loss of new chemokine CXCL14 in tumor tissue is associated with low infiltration by dendritic cells (DC), while restoration of human CXCL14 expression in tumor cells causes attraction of DC both in vitro and in vivo. *J. Immunol.* **174**(9), 5490-5498.

Simmonds, C.S., Karsenty, G., Karaplis, A.C., and Kovacs, C.S. (2010). Parathyroid hormone regulates fetal-placental mineral homeostasis. *J. Bone Miner. Res.* **25**(3), 594-605.

Simmons, R.M., Erikson, D.W., Kim, J., Burghardt, R.C., Bazer, F.W., Johnson, G.A., and Spencer, T.E. (2009). Insulin-like growth factor binding protein-1 in the ruminant uterus: potential endometrial marker and regulator of conceptus elongation. *Endocrinology* **150**(9), 4295-4305.

Singh, H., and Aplin, J.D. (2009). Adhesion molecules in endometrial epithelium: tissue integrity and embryo implantation. *J. Anat.* **215**, 3-13.

Smith, M.F., McIntush, E.W., and Smith, G.W. (1994). Mechanisms associated with corpus luteum development. *J. Anim. Sci.* **72**(7), 1857-1872.

Smits, K., Goossens, K., Van Soom, A., Govaere, J., Hoogewijs, M., and Peelman, L.J. (2011). In vivo-derived horse blastocysts show transcriptional upregulation of developmentally important genes compared with in vitro-produced horse blastocysts. *Reprod. Fertil. Dev.* **23**(2), 364-375.

Song, G., Bazer, F.W., Wagner, G.F., and Spencer, T.E. (2006a). Stanniocalcin (STC) in the endometrial glands of the ovine uterus: regulation by progesterone and placental hormones. *Biol. Reprod.* **74**(5), 913-922.

Song, G., Satterfield, M.C., Kim, J., Bazer, F.W., and Spencer, T.E. (2008). Gastrin-releasing peptide (GRP) in the ovine uterus: regulation by interferon tau and progesterone. *Biol. Reprod.* **79**(2), 376-386.

Song, G., Satterfield, M.C., Kim, J., Bazer, F.W., and Spencer, T.E. (2009). Progesterone and interferon tau regulate leukemia inhibitory factor receptor and IL6ST in the ovine uterus during early pregnancy. *Reproduction* **137**(3), 553-565.

Song, G., Spencer, T.E., and Bazer, F.W. (2005). Cathepsins in the ovine uterus: regulation by pregnancy, progesterone, and interferon tau. *Endocrinology* **146**(11), 4825-4833.

Song, G., Spencer, T.E., and Bazer, F.W. (2006b). Progesterone and interferon-tau regulate cystatin C in the endometrium. *Endocrinology* **147**(7), 3478-3483.

Soos, J.M., Subramaniam, P.S., Hobeika, A.C., Schiffenbauer, J., and Johnson, H.M. (1995). The IFN pregnancy recognition hormone IFN-tau blocks both development and superantigen reactivation of experimental allergic encephalomyelitis without associated toxicity. *J. Immunol.* **155**(5), 2747-2753.

Spencer, T.E., Bartol, F.F., Bazer, F.W., Johnson, G.A., and Joyce, M.M. (1999). Identification and characterization of glycosylation-dependent cell adhesion molecule 1-like protein expression in the ovine uterus. *Biol. Reprod.* **60**(2), 241-250.

Spencer, T.E., and Bazer, F.W. (1995). 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.* **53**(6), 1527-1543.

Spencer, T.E., and Bazer, F.W. (2002). Biology of progesterone action during pregnancy recognition and maintenance of pregnancy. *Front. Biosci.* **7**, d1879-1898.

Spencer, T.E., and Bazer, F.W. (2004a). Conceptus signals for establishment and maintenance of pregnancy. *Reprod. Biol. Endocrinol.* **2**, 49.

Spencer, T.E., and Bazer, F.W. (2004b). Uterine and placental factors regulating conceptus growth in domestic animals. *J. Anim. Sci.* **82 E-Suppl**, E4-13.

Spencer, T.E., Becker, W.C., George, P., Mirando, M.A., Ogle, T.F., and Bazer, F.W. (1995a). Ovine interferon-tau inhibits estrogen receptor up-regulation and estrogen-induced luteolysis in cyclic ewes. *Endocrinology* **136**(11), 4932-4944.

Spencer, T.E., Becker, W.C., George, P., Mirando, M.A., Ogle, T.F., and Bazer, F.W. (1995b). Ovine interferon-tau regulates expression of endometrial receptors for estrogen and oxytocin but not progesterone. *Biol. Reprod.* **53**(3), 732-745.

Spencer, T.E., Johnson, G.A., Bazer, F.W., and Burghardt, R.C. (2004a). Implantation mechanisms: insights from the sheep. *Reproduction* **128**(6), 657-668.

Spencer, T.E., Johnson, G.A., Bazer, F.W., Burghardt, R.C., and Palmarini, M. (2007). Pregnancy recognition and conceptus implantation in domestic ruminants: roles of progesterone, interferons and endogenous retroviruses. *Reprod. Fertil. Dev.* **19**(1), 65-78.

Spencer, T.E., Johnson, G.A., Burghardt, R.C., and Bazer, F.W. (2004b). Progesterone and placental hormone actions on the uterus: insights from domestic animals. *Biol. Reprod.* **71**(1), 2-10.

Spencer, T.E., Sandra, O., and Wolf, E. (2008). Genes involved in conceptus-endometrial interactions in ruminants: insights from reductionism and thoughts on holistic approaches. *Reproduction* **135**(2), 165-179.

Sreenan, J.M., Beehan, D., and Mulvehill, P. (1975). Egg transfer in the cow: factors affecting pregnancy and twinning rates following bilateral transfers. *J. Reprod. Fertil.* **44**(1), 77-85.

Sreenan, J.M., and Diskin, M.G. (1983). Early embryonic mortality in the cow: its relationship with progesterone concentration. *Vet. Rec.* **112**(22), 517-521.

Starnes, T., Rasila, K.K., Robertson, M.J., Brahmi, Z., Dahl, R., Christopherson, K., and Hromas, R. (2006). The chemokine CXCL14 (BRAK) stimulates activated NK cell migration: implications for the downregulation of CXCL14 in malignancy. *Exp. Hemato.* **34**, 1101-1105.

Stevenson, J.S., and Mee, M.O. (1991). Pregnancy rates of Holstein cows after postinsemination treatment with a progesterone-releasing intravaginal device. *J. Dairy Sci.* **74**(11), 3849-3856.

Stewart, M.D., Johnson, G.A., Gray, C.A., Burghardt, R.C., Schuler, L.A., Joyce, M.M., Bazer, F.W., and Spencer, T.E. (2000). Prolactin receptor and uterine milk protein expression in the ovine endometrium during the estrous cycle and pregnancy. *Biol. Reprod.* **62**(6), 1779-1789.

Storch, J., and Thumser, A.E. (2010). Tissue-specific functions in the fatty acid-binding protein family. *J. Biol. Chem.* **285**(43), 32679-32683.

Taga, T., and Kishimoto, T. (1997). Gp130 and the interleukin-6 family of cytokines. *Annu. Rev. Immunol.* **15**, 797-819.

Takahashi, Y., Takahashi, M., Carpino, N., Jou, S.T., Chao, J.R., Tanaka, S., Shigeyoshi, Y., Parganas, E., and Ihle, J.N. (2008). Leukemia inhibitory factor regulates trophoblast giant cell differentiation via Janus kinase 1-signal transducer and activator of transcription 3-suppressor of cytokine signaling 3 pathway. *Mol. Endocrinol.* **22**, 1673-1681.

Takenaka, Y., Fukumori, T., and Raz, A. (2004). Galectin-3 and metastasis. *Glycoconj. J.* **19**(7-9), 543-549.

Tang, M.K., Kindler, P.M., Cai, D.Q., Chow, P.H., Li, M., and Lee, K.K. (2004). Heart-type fatty acid binding proteins are upregulated during terminal differentiation of mouse cardiomyocytes, as revealed by proteomic analysis. *Cell Tissue Res.* **316**(3), 339-347.

Thatcher, W.W., Guzeloglu, A., Mattos, R., Binelli, M., Hansen, T.R., and Pru, J.K. (2001). Uterine-conceptus interactions and reproductive failure in cattle. *Theriogenology* **56**(9), 1435-1450.

Thompson, J.N. (1969). Vitamin A in development of the embryo. *Am. J. Clin. Nutr.* **22**(8), 1063-1069.

Thorburn, G.D., Cox, R.I., Currie, W.B., Restall, B.J., and Schneider, W. (1973). Prostaglandin F and progesterone concentrations in the utero-ovarian venous plasma of the ewe during the oestrous cycle and early pregnancy. *J. Reprod. Fertil. Suppl.* **18**, 151-158.

Toyokawa, K., Carling, S.J., and Ott, T.L. (2007). Cellular localization and function of the antiviral protein, ovine Mx1 (oMx1): I. Ovine Mx1 is secreted by endometrial epithelial cells via an 'unconventional' secretory pathway. *Am. J. Reprod. Immunol.* **57**(1), 13-22.

Trout, W.E., McDonnell, J.J., Kramer, K.K., Baumbach, G.A., and Roberts, R.M. (1991). The retinol-binding protein of the expanding pig blastocyst: molecular cloning and expression in trophectoderm and embryonic disc. *Mol. Endocrinol.* **5**(10), 1533-1540.

Van Cleeff, J., Drost, M., and Thatcher, W.W. (1991). Effects of postinsemination progesterone supplementation on fertility and subsequent estrous responses of dairy heifers. *Theriogenology* **36**(5), 795-807.

Villarroel, A., Martino, A., BonDurant, R.H., Deletang, F., and Sischo, W.M. (2004). Effect of post-insemination supplementation with PRID on pregnancy in repeat-breeder Holstein cows. *Theriogenology* **61**(7-8), 1513-1520.

Vogiagis, D., Fry, R.C., Sandeman, R.M., and Salamonsen, L.A. (1997). Leukaemia inhibitory factor in endometrium during the oestrous cycle, early pregnancy and in ovariectomized steroid-treated ewes. *J. Reprod. Fertil.* **109**(2), 279-288.

Vogiagis, D., and Salamonsen, L.A. (1999). Review: The role of leukaemia inhibitory factor in the establishment of pregnancy. *J. Endocrinol.* **160**(2), 181-190.

von Wolff, M., Wang, X., Gabius, H.J., and Strowitzki, T. (2005). Galectin fingerprinting in human endometrium and decidua during the menstrual cycle and in early gestation. *Mol. Hum. Reprod.* **11**(3), 189-194.

Wales, R.G., and Cuneo, C.L. (1989). Morphology and chemical analysis of the sheep conceptus from the 13th to the 19th day of pregnancy. *Reprod. Fertil. Dev.* **1**(1), 31-39.

Walton, J.S., Halbert, G.W., Robinson, N.A., and Leslie, K.E. (1990). Effects of progesterone and human chorionic gonadotrophin administration five days postinsemination on plasma and milk concentrations of progesterone and pregnancy rates of normal and repeat breeder dairy cows. *Can. J. Vet. Res.* **54**(3), 305-308.
Wang, H., and Dey, S.K. (2005). Lipid signaling in embryo implantation. *Prosta. Other Lipid Mediat.* **77**(1-4), 84-102.

Wathes, D.C., and Hamon, M. (1993). Localization of oestradiol, progesterone and oxytocin receptors in the uterus during the oestrous cycle and early pregnancy of the ewe. *J. Endocrinol.* **138**(3), 479-492.

Wellik, D.M., and DeLuca, H.F. (1995). Retinol in addition to retinoic acid is required for successful gestation in vitamin A-deficient rats. *Biol. Reprod.* **53**(6), 1392-1397.

Wiley, A.A., Bartol, F.F., and Barron, D.H. (1987). Histogenesis of the ovine uterus. *J. Anim. Sci.* **64**, 1262-1269.

Williams, E.D., Major, B.J., Martin, T.J., Moseley, J.M., and Leaver, D.D. (1998). Effect of antagonism of the parathyroid hormone (PTH)/PTH-related protein receptor on decidualization in rat uterus. *J. Reprod. Fertil.* **112**(1), 59-67.

Wilmut, I., and Sales, D.I. (1981). Effect of an asynchronous environment on embryonic development in sheep. *J. Reprod. Fertil.* **61**(1), 179-184.

Wiltbank, J.N., Hawk, H.W., Kidder, H.E., Black, W.G., Ulberg, L.C., and Casida, L.E. (1956). Effect of progesterone therapy on embryo survival in cows of lowered fertility. *J. Dairy. Sci.* **39**(456-461)

Wimsatt, W.A. (1950). New histological observations on the placenta of the sheep. *Am. J. Anat*. **87**, 391-436.

Wintenberger-Torres, S. (1956). Les rapports entre l'oeuf en segmentation et le tractus maternel chez la brebis. *Proc. Third Int. Congr. Anim. Reprod., Cambridge* Section **I**, 62.

Wintenberger-Torres, S., and Flechon, J.E. (1974). Ultrastructural evolution of the trophoblast cells of the pre-implantation sheep blastocyst from day 8 to day 18. *J. Anat.* **118**(Pt 1), 143-153.

Wooding, F.B. (1984). Role of binucleate cells in fetomaternal cell fusion at implantation in the sheep. *Am. J. Anat.* **170**(2), 233-250.

Wooding, F.B., Staples, L.D., and Peacock, M.A. (1982). Structure of trophoblast papillae on the sheep conceptus at implantation. *J. Anat.* **134**(Pt 3), 507-516.

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

Yankey, S.J., Hicks, B.A., Carnahan, K.G., Assiri, A.M., Sinor, S.J., Stellflug, J.N., Stellflug, J.N., and Ott, T.L. (2001). Expression of the antiviral protein Mx in peripheral blood mononuclear cells of pregnant and bred, non-pregnant ewes. *J. Endocrinol.* **170**(2), R7-11.

Zanetta, L., Marcus, S.G., Vasile, J., Dobryansky, M., Cohen, H., Eng, K., Shamamian, P., and Mignatti, P. (2000). Expression of Von Willebrand factor, an endothelial cell marker, is up-regulated by angiogenesis factors: a potential method for objective assessment of tumor angiogenesis. *Int. J. Cancer* **85**(2), 281-288.

Zernicka-Goetz, M. (2005). Cleavage pattern and emerging asymmetry of the mouse embryo. *Nat. Rev. Mol. Cell Biol.* **6**(12), 919-928.

Zhao, S., Gu, Y., Dong, Q., Fan, R., and Wang, Y. (2008). Altered interleukin-6 receptor, IL-6R and gp130, production and expression and decreased SOCS-3 expression in placentas from women with pre-eclampsia. *Placenta* **29**(12), 1024-1028.

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