

**PROGESTERONE REGULATION OF ENDOMETRIAL FACTORS  
SUPPORTING CONCEPTUS GROWTH AND DEVELOPMENT  
IN THE OVINE UTERUS**

A Dissertation

by

MICHAEL CAREY SATTERFIELD

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

May 2008

Major Subject: Physiology of Reproduction

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**ABSTRACT**

Progesterone Regulation of Endometrial Factors Supporting Conceptus Growth and  
Development in the Ovine Uterus. (May 2008)

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Progesterone is unequivocally required for the establishment and maintenance of pregnancy in all mammals studied. Its known functions are complex and encompass global changes in gene expression. Therefore, studies were conducted to characterize the effects of progesterone on expression of genes for endometrial factors having roles in conceptus growth, implantation and establishment of pregnancy.

The first study characterized the effect of an artificially induced early increase in circulating progesterone on conceptus growth and development and regulation of expression of galectin-15 (LGALS15), a recently identified protein secreted by the ovine uterine luminal epithelium (LE). Exogenous progesterone beginning on Day 1.5 post-mating accelerated conceptus development on Days 9 and 12. On Day 12 the conceptus was functionally and morphologically advanced to produce greater quantities of interferon tau (IFNT) than blastocysts from control ewes. Further, the endometrium responded to early progesterone and IFNT with early expression of cathepsin L (CTSL), radical S-adenosyl methionine domain containing 2 (RSAD2), and LGALS15 within the endometrium.

The second study identified structural changes within the luminal epithelium which could alter the flux of factors into and out of the uterine lumen to maintain

appropriate fetal/maternal communication. In this study, progesterone reduced quantities of proteins associated with both tight and adherens junctions during the elongation period. IFNT subsequently increased these proteins after conceptus elongation.

The third and fourth studies identified progesterone-regulated genes which have been implicated as having importance to implantation in sheep, mouse, and human. WNT signaling was transiently downregulated by progesterone, while members of several growth factor families are upregulated including insulin-like growth factor binding proteins (IGFBPs) 1 and 3, hepatocyte growth factor (HGF) and fibroblast growth factor 7 (FGF7), which may enhance conceptus growth.

Collectively, these studies assess the role of progesterone in altering gene uterine expression to establish a favorable environment for conceptus development. The long-term goals of these studies are to establish biomarkers of receptivity to conceptus development and implantation, enhance our understanding of gene and pathway regulation in early pregnancy loss, and identify genes which may be targeted in therapeutic strategies to improve reproductive success in humans and animals.

**DEDICATION**

To my sons,

Hunter Lee Satterfield

&

Walker Riley Satterfield

With persistence comes reward.

## ACKNOWLEDGEMENTS

My gratitude to those who have guided me along the way to reach this achievement can not be expressed adequately. Specifically, my father and mother were always supportive in the attainment of my goals. To Grandmother, Mams, and Paw Paw who in their own ways influenced me to be a better person. My sisters and brother who were always there to support and, of course, express sibling sarcasm or skepticism. I should also recognize my Aunt Lea who lent much needed encouragement to persist through and finish even when times were difficult and my drive was lacking.

In my professional career I have been fortunate to be surrounded by wonderful people who have a strong desire to succeed in whatever they do. To my mentors; Dr Fuller Bazer for providing a shining example of the face of science always willing to hear new ideas yet always prepared with scientific literature to challenge your thoughts. To Dr. Tom Spencer, first for taking a chance on a young Texan with no research training and a strong desire to ask questions and supporting him in his education into science and second for taking a personal interest in his success to the next level. A great group of committee members, professors, and past and present lab members round out the list of people to whom I am greatly indebted for providing the wonderful experience I had during my graduate studies. Thank you!

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## CHAPTER I

### INTRODUCTION

Establishment of pregnancy requires a complex dialogue between the maternal endometrium and the developing conceptus (embryo/fetus and associated extraembryonic membranes). Progesterone, from the corpus luteum, orchestrates a myriad of temporal and spatial changes in the endometrium to establish a dynamic and optimal uterine microenvironment for conceptus growth and development. The conceptus must in turn signal its presence to the endometrium to maintain pregnancy and continue production of progesterone by the corpus luteum. In ruminant species, the elongating conceptus secretes large quantities of IFNT (interferon tau) which is the pregnancy recognition signal (Roberts et al. 1999; Spencer and Bazer 2004; Spencer et al. 2007b). IFNT acts on the luminal (LE) and superficial glandular (GE) of the maternal endometrium to inhibit transcription of the *estrogen receptor alpha (ESR1)* gene that indirectly blocks upregulation of the oxytocin receptor (OXTR) (Fleming et al. 2001; Fleming et al. 2006; Spencer et al. 1995a). Blockade of the OXTR abrogates the mechanisms for oxytocin-induced release of luteolytic prostaglandin F<sub>2α</sub> (PGF) to prevent luteolysis and maintain the corpus luteum for production of progesterone.

Conceptus development during this period is supported by uterine secretions, collectively referred to as histotroph, which are primarily regulated by progesterone (Bazer 1975; Bazer et al. 1979b; Fazleabas et al. 1994; Kane et al. 1997; Roberts and Bazer 1988). Studies of the uterine gland knockout ewe (UGKO) established that secretions of the endometrial epithelia are essential for peri-implantation conceptus elongation and survival (Gray et al. 2002; Gray et al. 2001c). The endometrial LE and GE are the main sources of uterine secretions. Histotroph is comprised of a number of factors including ions, amino acids, sugars, growth factors, enzymes, adhesion proteins, cytokines, hormones, and transport proteins.

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This dissertation follows the style of Molecular Reproduction and Development.

Regulation of these factors or factors responsible for their transport into the uterine lumen are hypothesized to be either directly or indirectly under the control of progesterone.

Studies characterizing temporal and spatial alterations in gene expression during the peri-implantation period in rodents and primates have established a time sensitive window of receptivity of the maternal endometrium to implantation by the blastocyst (Achache and Revel 2006; Dey et al. 2004; Fazleabas et al. 1999; Paria et al. 2000). Those species undergo a rapid implantation process characterized by nidation of the blastocyst into the uterine stroma and the initiation of a decidual response, which is easily observed and characterized by expression of gene markers of decidualization. Alternatively, implantation in domesticated species of livestock is relatively superficial with the blastocyst remaining within the uterine lumen. The timing of implantation in domestic livestock species is more protracted and involves transformation of the spherical blastocyst into an elongated, filamentous conceptus prior to apposition, adhesion, and attachment, a process which takes several days following elongation to the filamentous form (Guillomot 1995; Guillomot et al. 1993). Despite gross developmental differences between livestock, humans and rodents, the establishment of a window of receptivity and the importance of reciprocal cross-talk is equally important across these species. Asynchronous embryo transfer experiments in sheep illustrated the requirement for the embryo and uterus to exhibit developmental synchrony within 2 days to maintain pregnancy (Rowson and Moor 1966b). Later studies added to this knowledge and further demonstrated that the blastocyst is capable of accelerating or decelerating its growth pattern in response to the stage of the uterus in an attempt to maintain appropriate synchrony (Lawson and Cahill 1983; Lawson et al. 1983). Further investigation illustrated that progesterone is responsible for establishing the maternal uterine microenvironment, thereby indirectly regulating conceptus development in sheep (Kleemann et al. 2001; Kleemann et al. 1994), as well as cattle (Garrett et al. 1988b).

Collectively, available studies established a fundamental role for maternal progesterone in initiating conceptus/maternal communications to orchestrate

implantation and maintain a successful pregnancy. Therefore, studies were conducted to identify endometrial changes in response to progesterone which may be developmental hallmarks for blastocyst growth and development as well as the initiation of implantation in sheep.

## CHAPTER II

### LITERATURE REVIEW

#### *Early Conceptus Development and Implantation in Sheep*

Implantation in sheep and other ruminants is classified as synepitheliochorial (Wooding 1992). Approximately 24-30 h after onset of estrus (Day 0) the ovarian follicle(s) ovulates in response to a surge of Luteinizing Hormone from the anterior pituitary. The unfertilized ova enter the oviduct and are fertilized by sperm at the ampullary-isthmic junction. Following fertilization, the single cell embryo, encased in the mucopolysaccharide zona pellucida, undergoes multiple mitotic divisions within the oviduct resulting in a 32-64 cell morula embryo that enters the uterus around Day 4 (Figure 2.1) (Bindon 1971; Chang and Rowson 1965; Rowson and Moor 1966a). The embryo continues to develop within the zona pellucida and undergoes compaction which segregates individual cells within the embryo (termed morula at this stage) and is believed to be important for determining the developmental fate of each cell. Compaction results in the formation of the inner cell mass, which gives rise to the fetus, and the trophoblast, that forms the placenta. At this time, formation of adherens, tight, and gap junctions between adjacent cells initiate complex cell-cell communication and mediate changes in cell shape and attachment, resulting in the formation of a fluid-filled blastocoel which marks the transformation of the morula to a blastocyst (Bindon 1971; Chang and Rowson 1965; Rowson and Moor 1966a; Ziomek and Johnson 1980). The blastocoel is surrounded by trophoblast cells while the inner cell mass comprises a smaller group of cells localized to a single pole of the embryo. The blastocoel continues to increase in volume resulting in stretching of the zona pellucida and, in response to enzymes from the trophectoderm, hatching of the blastocyst occurs around Day 8. The spherical hatched blastocyst of 200-400  $\mu\text{m}$  expands in diameter until around Day 10.



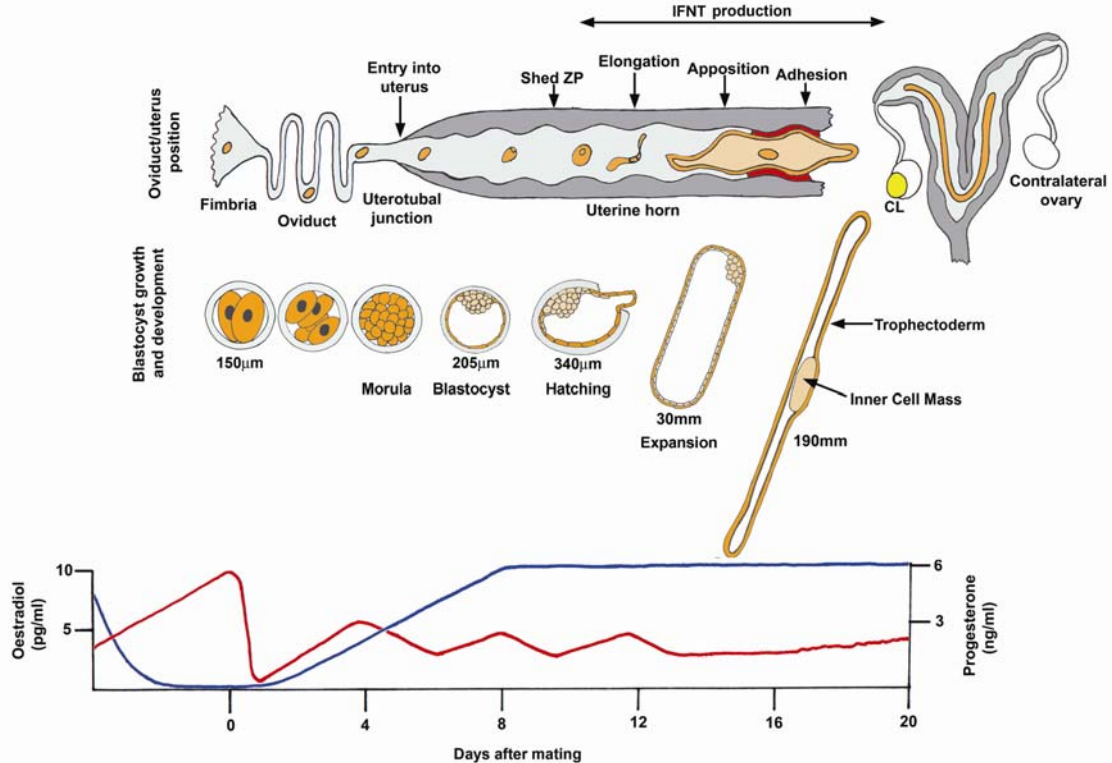


FIG. 2.1. Early pregnancy events in sheep. The embryo enters the uterus on Day 4 after mating (Day 0 = estrus/mating) at the morula stage of development and then develops into a blastocyst on Day 6. Between Days 8 and 9, the blastocyst sheds the zona pellucida by enzymatic lysis by uterine- and/or blastocyst-derived enzymes including proteases and glycosidases. After Day 10, the blastocyst elongates into a tubular and then into a filamentous conceptus that appears to be immobilized in the uterine lumen in close association with the endometrial luminal epithelium (LE) followed by unstable adhesion. Between Days 16 and 22, the trophoblast begins to adhere firmly to LE by interdigitation between uterine epithelial microvilli and projections of the trophoblast cells, and/or penetration of trophoblast papillae into the superficial duct of uterine glands (sGE). During this time, the trophoblast giant cells migrate, appose, and fuse to the apical surface of endometrial LE to form syncytial plaques. Eventually, as a part of synepitheliochorial placentation in sheep, syncytial plaques cover the caruncular surface and aid in formation of placentome formed by fusion of placental cotyledons and endometrial caruncles. Adapted from (Spencer et al. 2007a) and originally drawn by Dr. Greg A. Johnson.

The blastocyst then undergoes a morphological transition from a spherical (<1 mm) to tubular and then elongated filamentous conceptus by Day 14 reaching 10 cm or more in length by Day 14 and 25 cm or more by Day 17 (Guillomot et al. 1993)

Blastocyst elongation is a uterine dependent event as neither blastocysts or trophoblastic vesicles elongate *in vitro*, but do so when transferred into the uterus (Flechon et al. 1986; Heyman et al. 1984). Elongation of blastocysts results in the developmentally regulated synthesis and secretion of the pregnancy recognition signal, IFNT (Farin et al. 1989; Gray et al. 2002; Guillomot et al. 1990), which acts in a paracrine manner on the endometrium to inhibit development of the luteolytic mechanism required for pulsatile release of prostaglandin F<sub>2</sub> $\alpha$  from the uterine epithelium, thereby maintaining the continued secretion of progesterone from the ovarian corpus luteum (Spencer et al. 2007b; Thatcher et al. 1989). IFNT is produced exclusively by the mononuclear trophoctoderm cells of sheep conceptuses from Days 10-25. Secretion of IFNT is directly related to the stage of development of the blastocyst or length of the filamentous conceptus. In addition to pregnancy recognition, IFNT induces or enhances the expression of a number of genes within the endometrium that are hypothesized to further support pregnancy (Hansen et al. 1999; Spencer et al. 2007b). A subpopulation of mononuclear trophoblast cells become binucleated by Day 16 and begin to migrate and form a syncytium with uterine LE (Wooding 1992). Trophoblast binucleate and multinucleated syncytiotrophoblast cells produce placental hormones such as placental lactogen (CSH1) and pregnancy associated glycoproteins (PAG) which also alter endometrial gene expression to support pregnancy (Spencer and Bazer 2002; Spencer et al. 1999b; Spencer et al. 2004b; Wooding 1992).

The phases of implantation in ruminant species include: (1) shedding of the zona pellucida; (2) precontact and blastocyst orientation; (3) apposition; (4) adhesion; and (5) endometrial invasion albeit limited in comparison to invasive implanting species (Figure 2.2) (Guillomot 1995). During the elongation period, apposition of the conceptus trophoctoderm and endometrial LE is followed by attachment and interdigitation of cytoplasmic projections of trophoctoderm cells and microvilli of the LE (Guillomot

1995; Guillomot et al. 1981). This interdigitation ensures firm adhesion of the fetal maternal interface in both caruncular and intercaruncular areas by Day 16 of pregnancy. By Day 18, the interdigitation between the conceptus and endometrium is extensive and impairs the ability to recover the conceptus intact from the uterine lumen via surgical flushing. Importantly, progesterone is the predominant circulating hormone during this developmental process and is believed to be instrumental in regulating uterine support of this process (Spencer and Bazer 2002; Spencer et al. 2007b; Spencer et al. 2004b).

### ***Progesterone Actions in Early Pregnancy***

Successful pregnancy in mammals unequivocally requires the actions of progesterone. Following ovulation of the dominant follicle, the follicular theca and granulosa cells differentiate into small and large luteal cells, respectively (Leymarie and Martal 1993). This transformation marks formation of the corpus luteum (CL) which begins secreting large quantities of progesterone into the circulation to support pregnancy. In the sheep, the corpus luteum begins secreting progesterone around Day 3 of pregnancy with a steady increase to Day 7 at which times luteal phase progesterone concentrations are maximal. Progesterone is maintained at high levels throughout pregnancy. In sheep, the chorion produces sufficient quantities of P4 by Day 60 of gestation to maintain pregnancy even in ovariectomized ewes (Casida and Warwick 1945; Ricketts and Flint 1980).

Progesterone support of pregnancy is predominantly via actions on the uterus which both accommodate and nurture the developing fetus to term. The actions of progesterone in support of pregnancy are multifaceted and encompass roles in uterine gland morphogenesis and function, cell adhesion, derivation of factors supporting conceptus development, implantation, and pregnancy recognition; all of which are essential components for early pregnancy success (Spencer and Bazer 2002; Spencer et al. 2004a; Spencer et al. 2007a; Spencer et al. 2007b; Spencer et al. 2004b).

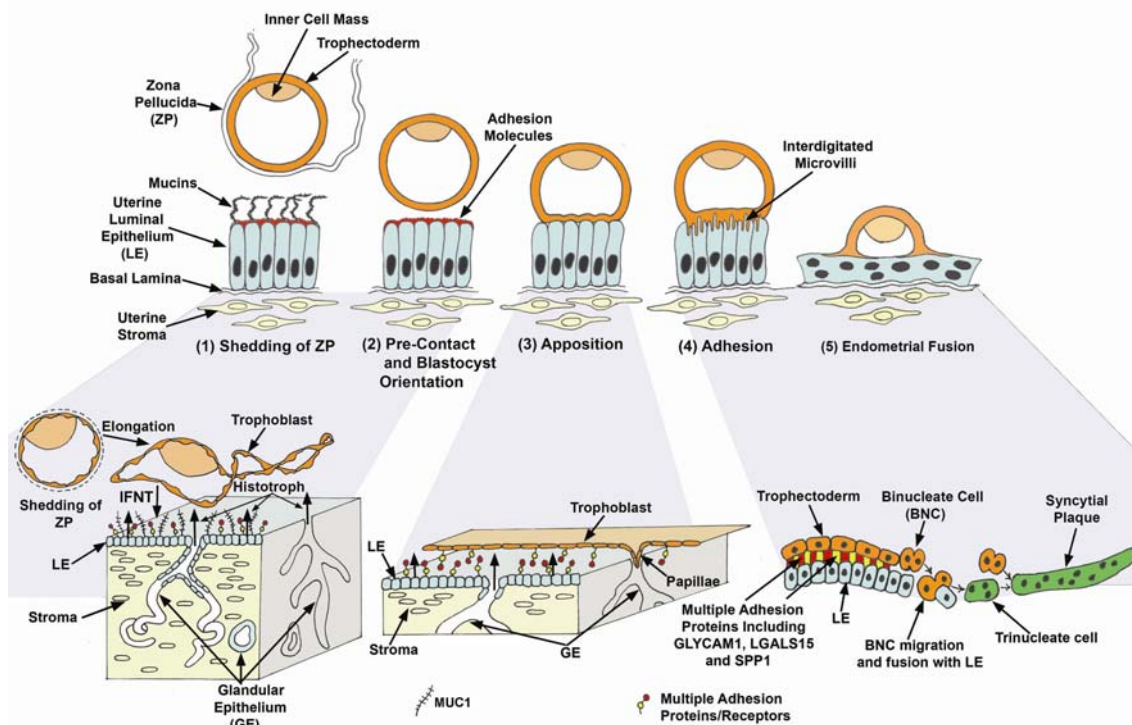


FIG. 2.2. The phases of blastocyst implantation in sheep. Shedding of the zona pellucida (Phase 1): The embryo enters the uterus on Day 4. The blastocyst is formed on Day 6 and the zona pellucida is shed on Day 8 or 9 due to blastocyst growth and uterine and/or trophectoderm enzymes. After Day 10, the blastocyst elongates and develops into a tubular and then into a filamentous conceptus. Precontact and blastocyst orientation (Phase 2): Between Days 9 and 14, there is no definitive cellular contact between conceptus trophectoderm and endometrial epithelium, but the blastocyst appears to be positioned and immobilized in the uterus. During this time, elongation of the blastocyst plays and production of IFNT are coordinate in the sheep uterus. Apposition (Phase 3): The conceptus trophectoderm associates closely with the endometrial LE followed by unstable adhesion. In ruminants, the trophoblast develops finger-like villi or papillae that penetrate into opening of uterine glands into the uterine lumen. This event has been hypothesized to anchor the peri-attachment conceptus and allow it to absorb histotroph from uterine glands. Adhesion (Phase 4): On Day 16, the trophoblast begins to adhere firmly to the endometrial LE. The interdigitation of the trophectoderm and endometrial LE occurs in both the caruncular and intercaruncular areas of the endometrium. During this time, the mononuclear trophectoderm cells differentiate into trophoblast giant binucleate cells. Adapted from (Spencer et al. 2007a) and originally drawn by Dr. Greg A. Johnson.

The actions of progesterone are mediated primarily by the nuclear progesterone receptor (PGR) (Bagchi et al. 2003; Conneely et al. 2002). Nuclear steroid receptors exist in a modular structure that has distinct functional domains including an activation function domain, a ligand binding domain, a DNA binding domain, and a hinge region (Bain et al. 2007). In addition, steroid receptors contain a relatively less well defined N-terminal region. It is variation in this region of the PGR that gives rise to different isoforms. Indeed, null mutation of the *Pgr* gene in mice renders them infertile, and they exhibit multiple reproductive defects including impaired ovulation, uterine hyperplasia, and failed decidualization (Lydon et al. 1995). Wild-type embryos transferred into progesterone receptor knockout (PRKO) females failed to implant indicating that the implantation failure is not due to an embryonic defect but rather a uterine defect. In mice and humans, a single gene yields multiple isoforms of the PGR (Kastner et al. 1990; McDonnell et al. 1994). Information regarding the presence of multiple isoforms of PGR in ruminant species is limited. Analysis of the bovine oviduct using a mouse antibody that recognizes both PGR-A and PGR-B identified two putative bands hypothesized to be PGR-A and PGR-B, although the biological significance of this finding has not been determined (Ulbrich et al. 2003). In mice, selective deletion of PGR-A isoform resulted in fertility defects linked to failed decidualization that were milder than deletion of both isoforms suggesting functional differences between the PGR isoforms *in vivo* (Mulac-Jericevic et al. 2000). Experiments were then designed to determine expression of a number of known progesterone regulated genes in these mutant mice. Gene expression of *calcitonin (Calca)*, *histidine decarboxylase (Hdc)*, and *amphiregulin (Areg)* was increased in uterine epithelia by progesterone during the receptive phase of implantation (Das et al. 1995; Paria et al. 1998; Zhu et al. 1998). *Lactoferrin (Ltf)*, a secretory product of the epithelia induced by estrogen and inhibited by progesterone (McMaster et al. 1992), was also studied. PRKO mice exhibited dysregulation of each of these genes in response to loss of P4 signaling. In contrast, specific mutation, characterized by the PRAKO, only exhibited loss of *Calca* and *Areg* expression while expression of *Hdc* and *Ltf* was unaffected (Mulac-Jericevic et al. 2000).

Further, microarray analysis of human breast cancer cells identified differential gene regulation by distinct PGR isoforms (Richer et al. 2002). Collectively, these findings demonstrate that the different PGR isoforms maintain functional independence in regards to regulating transcription of specific genes involved in pregnancy.

Interestingly, in all mammalian species studied to date, both LE and GE of uteri exhibit loss of PGR prior to becoming receptive to implantation and establishment of pregnancy (Geisert et al. 1994; Lessey et al. 1988; Robinson et al. 2001; Spencer and Bazer 1995; Tan et al. 1999). Loss of PGR results from downregulation of the *PGR* gene by continuous exposure of the endometrium to progesterone. In sheep, 8 to 10 days of continuous progesterone exposure results in loss of PGR protein from LE (Figure 2.3) (Spencer and Bazer 1995; Wathes and Hamon 1993). Loss of PGR protein in the GE ensues following an additional 2 days of P4 exposure. Despite loss of PGR within the epithelia, the endometrial stroma continues to express PGR throughout pregnancy. Maintenance of PGR within the stroma suggests that the direct actions of progesterone are mediated on and via this cell type. Interestingly, a number of changes in gene expression and uterine function observed in the epithelia are directly linked to the actions of P4 (Spencer et al. 2007b). The absence of PGR within these cell types is a developmental conundrum which has resulted in two distinct hypotheses. First, these changes may be mediated in response to P4 by unknown factors from the PGR positive stroma which act in a paracrine manner on the adjacent epithelium to modulate P4 actions (i.e., a progestamedin(s)). Alternatively, loss of PGR may be sufficient to initiate a developmental cascade which alters gene expression within the epithelia to promote differentiated functions.

Recently, a family of membrane progesterone receptors, which have been shown to mediate rapid non-genomic cellular changes in response to P4, were identified (Ashley et al. 2006; Karteris et al. 2006; Zhu et al. 2003a; Zhu et al. 2003b). At least one of these receptors has been identified in the sheep uterus; however, the role these receptors in pregnancy are unknown (Ashley et al. 2006). Importantly, blockade of P4 signaling through its classical nuclear receptor using an antagonist specific to the nuclear

receptor results in pregnancy loss (Baird 2002; Roblero and Croxatto 1991), illustrating the importance of nuclear receptor signaling in the maintenance of pregnancy. Collectively, the mechanisms by which P4 mediates its actions may be as diverse as the roles of P4 in establishment and maintenance of pregnancy.

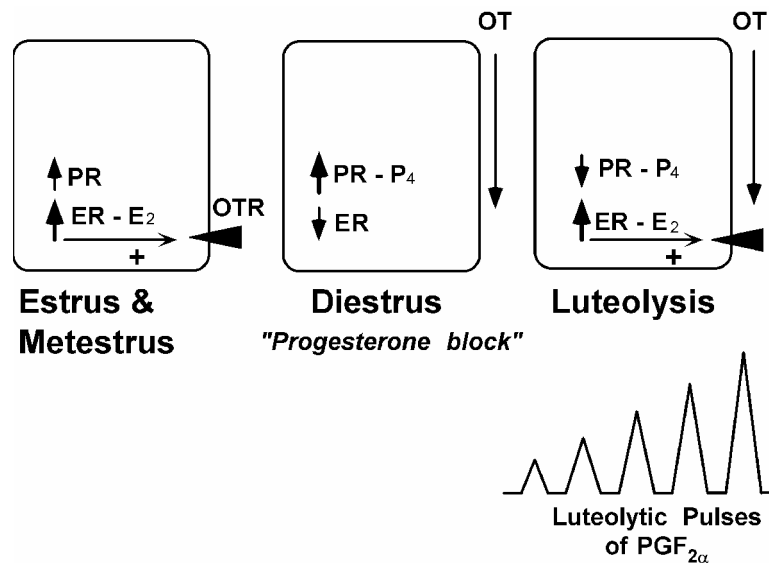


FIG. 2.3. Schematic illustrating regulation of hormone receptor expression in the ovine uterine endometrial epithelium during the estrous cycle. During estrus and metestrus, OXTR are present on uterine epithelia, because estrogen levels are high and increase expression of ESR1 and OXTR. The PGR is present, but low circulating levels of progesterone result in insufficient numbers of activated PGR to suppress ESR1 and OXTR synthesis. During diestrus, endometrial ESR1 and estradiol in plasma are low, and progesterone levels begin to increase with formation of the CL. Progesterone acts through its receptor to maintain the “progesterone block” to ESR1 and OXTR synthesis for 8 to 10 days. During late diestrus (~Days 11 to 12 of the cycle), progesterone negatively autoregulates PGR gene expression, which allows for increases in ESR1 and OXTR synthesis. The increase in OXTR expression is facilitated by increasing secretion of estrogen by ovarian follicles and then decreasing secretion of progesterone. The pulsatile release of oxytocin from the CL and posterior pituitary activates OXTR on endometrial epithelia and induces release of luteolytic pulses of PGF to regress the CL. Legend: E2, estrogen; ESR1, estrogen receptor alpha; OXT, oxytocin; OXTR, oxytocin receptor; P4, progesterone; PGF, prostaglandin F2 alpha; PGR, progesterone receptor. Adapted from (Spencer and Bazer 2002).

### ***Endometrial Glands as a Source of Histotroph***

Mammalian uteri contain a vast network of uterine glands which synthesize and secrete or transport a complex array of molecules collectively referred to as histotroph. These molecules include growth factors, ions, amino acids, nutrient transport proteins, hormones, enzymes, proteases, protease inhibitors, glucose, cytokines, lymphokines, and many other factors (Bazer 1975; Carson et al. 2000; Gray et al. 2001a; Roberts and Bazer 1988). Histotroph emanates from both the LE and GE; and each respective cell type is unique in contributing factors to the uterine milieu. The importance of these secretions has been evidenced in both primate and subprimate species as regulators of conceptus development and survival, pregnancy recognition, and implantation/placentation (Burton et al. 2002; Gray et al. 2002; Gray et al. 2001c). Interestingly, in species exhibiting delayed implantation, alterations in uterine secretions have been implicated in regulation of this process (Zhu et al. 1998). The utilization of knockout technology in mice has identified several factors including leukemia inhibitory factor (Lif) and Calca that are secreted exclusively from the uterine glands and are required for uterine receptivity and implantation (Stewart et al. 1992; Zhu et al. 1998). The prolonged nature of pre-implantation conceptus development in domesticated species of livestock such as the pig, cow, horse, and sheep suggests that endometrial support of conceptus development mediated primarily via histotroph is of critical importance in these species.

Studies of the uterine gland knockout ewe (UGKO) revealed an essential role for uterine secretions in early conceptus development and initiation of pregnancy recognition signals (Gray et al. 2002; Gray et al. 2001c). The UGKO ewe model is produced by the continuous administration of a synthetic, non-metabolizable progestin to neonatal ewe lambs from birth to postnatal Day 56 (Gray et al. 2000a). This inappropriate progestin exposure permanently ablates, epigenetically, development of endometrial glands from the LE. UGKO ewes have no apparent defect in development of the myometrium or other Müllerian duct-derived structures in the female reproductive tract or function of the hypothalamic-pituitary-ovarian axis (Gray et al. 2001b; Gray et al.



2000b). The adult UGKO endometrium is devoid of glands and has a markedly reduced LE surface area. UGKO ewes exhibit defects in that they are unable to experience normal estrous cycles or to support early embryonic development exhibited by failure of the blastocyst to elongate. The defect in blastocyst development is manifest following hatching from the zona pellucida as blastocysts transferred from normal synchronous ewes into UGKO recipients also experience implantation failure (Gray et al. 2001c). Further, early conceptus development prior to Day 9 of pregnancy, was not affected by a lack of uterine histotroph (Gray et al. 2002; Gray et al. 2001c) suggesting that development to the blastocyst stage does not require uterine secretions. Following hatching from the zona pellucida, around Day 8, the blastocyst is in direct contact with contents of the uterine lumen. A lack of glandular secretions results in failure of blastocysts to develop and elongate normally, experience reduced secretion of IFNT to induce interferon stimulated genes important for pregnancy, and ultimately conceptus demise by Day 14 of pregnancy. Uterine development and pregnancy success in adult cows is also compromised following neonatal progestin treatment of heifers (Bartol et al. 1995).

Analyses of molecular components in uteri of UGKO ewes indicated that failure of blastocysts to elongate was not due to alterations in expression of steroid hormones, the anti-adhesive factor mucin 1 (MUC1), integrins on the surface of LE, or IFNT responsiveness of the endometrium (Gray et al. 2000a; Gray et al. 2002). However, uterine luminal contents recovered from UGKO ewes contained low to undetectable amounts of galectin-15 (LGALS15), glycosylated cell adhesion molecule 1 (GLYCAM1), and secreted phosphoprotein 1 (SPP1, also known as osteopontin), which collectively act as adhesion molecules and are hypothesized to regulate apposition and attachment of trophoblast to the LE, an action likely required for elongation of the blastocyst (Gray et al. 2006; Gray et al. 2004; Gray et al. 2002). Undoubtedly, other essential unidentified molecules in histotroph are reduced in flushings from UGKO ewes and may contribute to the infertile phenotype of these ewes.

Indeed, histotroph is a complex fluid comprised of not only proteins, but also lipids, amino acids, and sugars, complicating the task of identifying specific components. Gene expression analyses may be used to identify candidate genes in endometria encoding proteins possibly present in histotroph. However, genetic approaches do not identify histotrophic components of non-endometrial origin such as those from serum or conceptus derived factors. Two-dimensional gel electrophoresis of uterine fluids from a number of species isolated a number of proteins, however, efforts to establish their identity has remained limited (Ashworth and Bazer 1989a; Ashworth and Bazer 1989b; Fazleabas et al. 1988; Kayser et al. 2006; MacLaughlin et al. 1986). In human uterine fluid samples, 24 proteins were identified throughout the menstrual cycle as unique compared to those found in serum (MacLaughlin et al. 1986). In pigs, two-dimensional gel electrophoresis followed by a more sensitive labeling technique, identified 280 total proteins of which 132 were affected by pregnancy, day, or the interaction of day and pregnancy (Kayser et al. 2006). In sheep, Ashworth and Bazer (Ashworth and Bazer 1989b) identified 30 endometrial proteins present in uterine luminal fluid. Of these, 13 proteins were absent on Day 8 but present on Day 10 indicating changes in secretory function of the endometrium with longer exposure to progesterone. Recent work, utilized proteomic approaches and mass spectrometry to identify components of uterine histotroph or components of the endometrial proteome, have yielded limited findings despite the availability of improved protein sequence databases (TABLE 2.1). In addition to IFNT, the primary secretory product of the developing conceptus, these studies identified aldose reductase, cytoplasmic actin, and transferrin as proteins of conceptus origin present in uterine luminal fluid (Lee et al. 1998b). Berendt et al. (Berendt et al. 2005) reported an increase in four endometrial proteins associated with pregnancy on Day 18 in cattle including Rho GDP dissociation inhibitor beta; 20 alpha-hydroxysteroid dehydrogenase, soluble NADP<sup>+</sup>-dependent isocitrate dehydrogenase 1, and acyl-CoA-binding protein. Analyses of non-protein components of histotroph in pigs identified distinct changes in amino acids, lipids, and sugars that occur in the uterine lumen as well as fetal fluids as pregnancy progresses (Geisert et al. 1982; Kwon

et al. 2003a; Kwon et al. 2003b). In humans, glucose and amino acids are important for pre-implantation embryo development (Devreker et al. 2000; Leese 1995). Both amino acids and their transporters have been implicated in blastocyst implantation and differentiation of the trophoblast through modulation of nutrient-sensing pathways (Martin and Sutherland 2001; Martin et al. 2003). In mice and humans, lipids and their metabolites regulate aspects of endometrial function and placental development (Schaiff et al. 2006; Schaiff et al. 2007). Revisiting classical two-dimensional studies with modern protein identification techniques should soon enhance understanding of the functional significance of individual components of histotroph and their effects on blastocyst development and implantation. Upon identification, null mutagenesis studies in mice, similar to those used to implicate *Lif* and *Calca* in implantation, or the utilization of antisense knockdown technologies in other species will enhance functional understanding of the roles of these proteins *in vivo*.

TABLE 2.1. Components of uterine histotroph in sheep and cattle<sup>a</sup>.

| <b>Name</b> | <b>Component</b>                        | <b>Cycle</b>    | <b>Pregnancy</b> | <b>Bovine</b> | <b>Reference</b>                            |
|-------------|---|-----------------|------------------|---------------|---|
| B2M         | Beta2-microglobulin                     | ND <sup>a</sup> | 16               | ND            | (Vallet et al. 1991)                        |
| SPP1        | Secreted phosphoprotein 1 (Osteopontin) | ND              | 15-Term          | Yes           | (Johnson et al. 1999b)                      |
| GRP         | Gastrin-releasing Peptide               | 16              | 17-20, 115-145   | Yes           | (Giraud et al. 1993; Whitley et al. 1998)   |
| ISG15       | Interferon Stimulated Gene 15           |                 | 13-19            | Yes           | (Johnson et al. 1999c)                      |
| GLYCAM1     | Glycosylated Cell Adhesion Molecule-1   | 1-11            | 13-19            | ND            | (Spencer et al. 1999a)                      |
|             | Endothelin                              | 12-16           | 15-20            | ND            | (Riley et al. 1994)                         |
| UTMP        | Uterine Milk Proteins (Serpins)         | 13-15           | 15-150           | Yes           | (Moffatt et al. 1987a; Stewart et al. 2000) |
| LGALS15     | Galectin 15                             | 10-14           | 10-18            | No            | (Gray et al. 2004)                          |
| CTSL        | Cathepsin L                             | 10-12           | 10-20            | ND            | (Song et al. 2005)                          |
| CST3        | Cystatin C                              | 12              | 12-20            | ND            | (Song et al. 2006b)                         |
| STC1        | Stanniocalcin One                       | 10-16           | 10-120           | ND            | (Song et al. 2006a)                         |
| CXCL10      | Chemokine Ligand 10                     |                 | 14-20            | ND            | (Imakawa et al. 2006; Nagaoka et al. 2003)  |
| IGF1        | Insulin-like Growth Factor-1            | 3-16            | 3-22             | Yes           | (Cann et al. 1998; Ko et al. 1991)          |

<sup>a</sup> ND = Not determined

TABLE 2.1. Cont.

| <b>Name</b>      | <b>Component</b>                                    | <b>Cycle</b> | <b>Pregnancy</b> | <b>Bovine</b> | <b>Reference</b>       |
|------------------|---|--------------|------------------|---------------|------------------------|
| IGF2             | Insulin-like Growth Factor-2                        | 10-16        | 10-16            | Yes           | (Ko et al. 1991)       |
| IGFBP2<br>IGFBP3 | Insulin-Like Growth Factor Binding Proteins 2 &3    | 12-15        | ND               | Yes           | (Peterson et al. 1998) |
| TGFB             | Transforming Growth Factor Beta1-3                  | 13-16        | 14-30            | Yes           | (Dore et al. 1996)     |
| CSF2             | Granulocyte-Macrophage Colony Stimulating Factor    |              | 17               | ND            | (Imakawa et al. 1993)  |
| LIF              | Leukemia Inhibitory Factor                          | 4-16         | 4-20             | ND            | (Vogiagis et al. 1997) |
| PAF              | Platelet Activating Factor                          | 14-15        |                  | ND            | (Battye et al. 1996)   |
| FGF2             | Fibroblast Growth Factor 2                          | ND           | ND               | Yes           | (Michael et al. 2006)  |
| TIMP1<br>TIMP2   | Tissue Inhibitor of Matrix metalloproteinases 1 & 2 | 4-16         | 4-20             | ND            | (Hampton et al. 1995)  |
| CXCL6            | Granulocyte Chemotactic Protein-2                   | ND           | ND               | Yes           | (Teixeira et al. 1997) |
|                  | Beta-N-acetylglucosaminidase                        | 13           | 7-18             | ND            | (Roberts et al. 1976)  |
| GLB1             | Beta-D-galactosidase                                | 13-15        | 13-15            | ND            | (Roberts et al. 1976)  |

TABLE 2.1. Cont.

| <b>Name</b> | <b>Component</b>                | <b>Cycle</b> | <b>Pregnancy</b> | <b>Bovine</b> | <b>Reference</b>       |
|-------------|---------------------------------|--------------|------------------|---------------|------------------------|
| NAGA        | Beta-N-acetylgalactos-aminidase | 13           | 7-18             | ND            | (Roberts et al. 1976)  |
|             | Alpha-L-fucosidase              | 13           | 17-18            | ND            | (Roberts et al. 1976)  |
|             | Alpha-D-glucosidase             |              | 13-15            | ND            | (Roberts et al. 1976)  |
| OAS1        | 2'-5'-oligo(A)synthetase        | ND           | 16               | ND            | (Mirando et al. 1991)  |
|             | Glucose                         |              | 140              | Yes           | (Bazer et al. 1979a)   |
|             | Prostaglandin F2-alpha          | 13-15        | 30-144           | Yes           | (Moffatt et al. 1987b) |
|             | Prostaglandin E2                | 14           |                  | Yes           | (Vallet et al. 1988)   |
|             | Amino acids                     |              | 30-140           | Yes           | (Kwon et al. 2003a)    |
| MX1         | Mx1                             | 15           | 15-17            | Yes           | (Toyokawa et al. 2007) |

## ***Adhesion Molecules in Implantation***

### ***Mucin 1 (MUC1)***

In the uterus, the pre-implantation blastocyst approaches a glycocalyx, including mucins, which are large transmembrane glycoproteins expressed at the apical surface of epithelia in the reproductive tracts of a number of species (Brayman et al. 2004). The extracellular domain of MUC1 contains a large amount of glycans (Aplin and Hey 1995) with the core protein of 120-220 kDa and can reach over 400kDa with glycosylation. Given that mucins contain a large number of glycans recognizable by blastocysts or secreted animal lectins, they may be involved in the initial apposition phase of implantation (Aplin and Hey 1995; Brayman et al. 2004). Expression of MUC1 and MUC4 on the uterine LE is hypothesized to block accessibility of trophoblast integrin receptors to their ligands for cell-cell and cell-extracellular matrix adhesion, both of which are necessary for the initial phases of implantation (Burghardt et al. 2002; Carson et al. 2000). In sheep, removal of MUC1 from the LE correlates with loss of the PGR (Figure 2.4) (Johnson et al. 2001a) and may be required for the exposure of other glycoproteins involved in trophoblast adhesion. Immunoreactive MUC1 in the LE decreases from Days 9-17 of pregnancy in ewes (Johnson et al. 2001a). In contrast, total MUC1 expression increases in the LE of rabbits and humans during uterine receptivity; however, MUC1 is locally reduced at the site(s) of implantation (Brayman et al. 2004; Carson et al. 2000). Regardless of the mechanism, loss of MUC1 is hypothesized to be necessary to expose other glycoproteins involved in adhesion between the trophoblast and LE.

### ***Integrins***

Integrins encompass a family of heterodimeric intrinsic transmembrane glycoprotein receptors that regulate cell differentiation, motility, and adhesion (Giancotti and Ruoslahti 1999). In the uterus, integrins play a pivotal role in extracellular matrix (ECM) interactions to transduce cell signals in both the epithelia and trophoblast

(Burghardt et al. 2002; Johnson et al. 2001a; Johnson et al. 2003). Integrins likely function in implantation to induce cytoskeletal reorganization, stabilize adhesion, and mediate cell migration, proliferation, and differentiation (Giancotti and Ruoslahti 1999). Altered integrin expression correlates with causes of infertility (Lessey 1998), null mutations of several integrins results in peri-implantation lethality (Hynes 1996), and functional inhibition of specific integrins decreases the number of implantation sites (Hynes 1996). In sheep, the integrin subunits  $\alpha$  (v, 4, and 5) and  $\beta$  (1, 3, and 5) are constitutively expressed on the apical surfaces of uterine LE and conceptus trophoderm during the peri-implantation period (Johnson et al. 2001a), and are not changed in a spatial or temporal manner in response to hormones or conceptus derived signals. However, alterations in temporal and spatial expression of integrin ligands during this period support a role for integrins in regulating conceptus attachment and adhesion in sheep. In other species, such as the pig, mouse, and humans, the putative window of implantation is framed by interactions between specific integrins and ECM proteins (Burghardt et al. 2002; Carson et al. 2000; Lessey 2002).

#### *Glycosylated Cell Adhesion Molecule (GLYCAM1)*

GLYCAM1 is a sulfated glycoprotein secreted by endothelial cells that mediates leukocyte-endothelial cell adhesion (Lasky et al. 1992) and has a functional role as a carbohydrate ligand for the lectin domain of leukocyte cell-surface selectin (SELL or L-selectin) in the lymphoid system (Rosen 1993). Ligation of SELL by GLYCAM1 activates  $\beta$ 1 and  $\beta$ 2 integrins and promotes firm adhesion to fibronectin (Giblin et al. 1997; Hwang et al. 1996). In humans, trophoblast SELL is believed to mediate interactions with the LE that may be important for establishment of pregnancy (Genbacev et al. 2003). The expression pattern of GLYCAM1 in the ovine uterus suggests that it may be a mediator of implantation (Spencer et al. 1999a). GLYCAM1 expression increases in the endometrial LE and superficial (sGE) between Days 1 and 5 and then decreases between Days 11 and 15 in cyclic ewes. In contrast, GLYCAM1 is low between Days 11 and 13 of pregnancy, increases on Day 15 and is abundant by



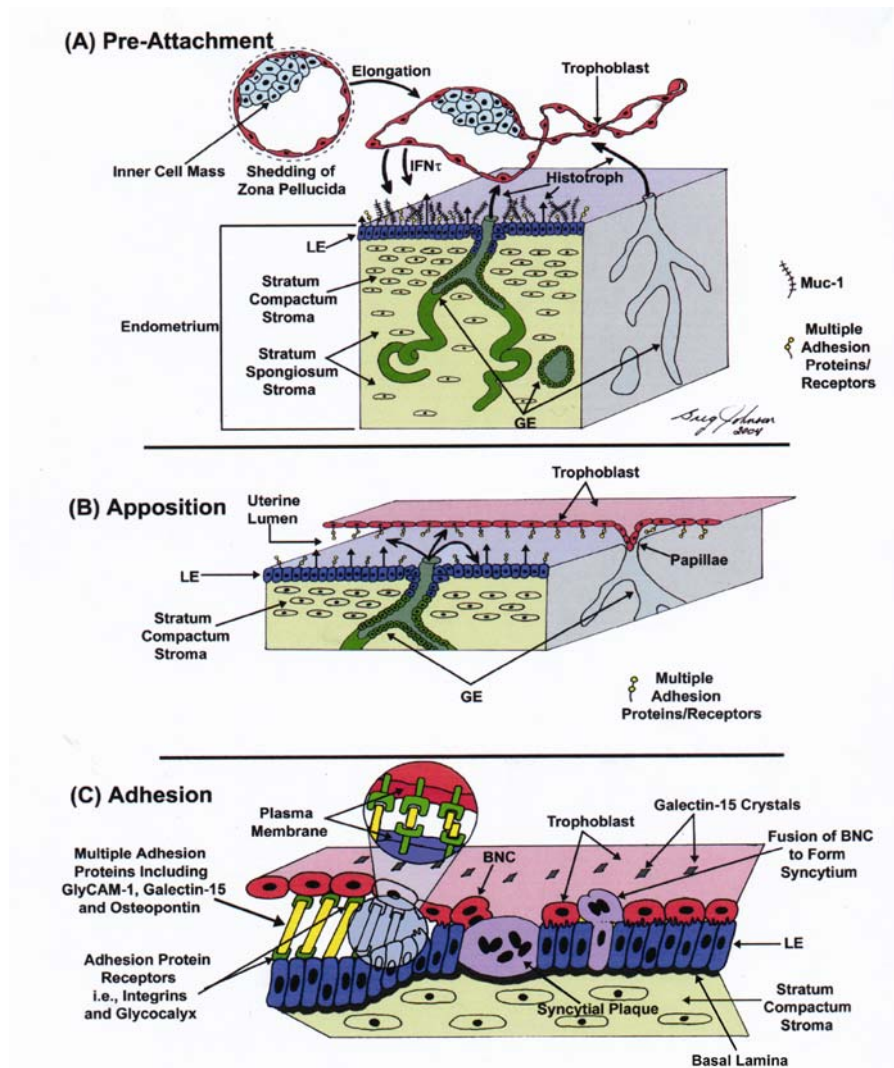


FIG. 2.4. Schematic illustration of the pre-attachment, apposition, and adhesion stages of superficial implantation in sheep. Preattachment (A) involves shedding of the zona pellucida, followed by conceptus expansion, as well as precontact and blastocyst orientation. MUC1 expression prevents contact with integrin subunits and/or carbohydrate receptors. Apposition (B) involves a decrease in MUC1 expression by the LE and extension of trophoblast papillae into the mouth of uterine glands to aid in access to histotroph and elongation of the conceptus. Adhesion (C) between the apical surfaces of the conceptus trophoblast and LE is mediated by uterine secretory proteins such as SPP1 (osteopontin), GLYCAM1, LGALS15, binding to receptors. Trophoblast binucleate cells fuse with the LE cells to form syncytial plaques, but are not invasive beyond this single cell layer (Spencer et al. 2004a).

Days 17 and 19 (Spencer et al. 1999a). In uterine flushings, GLYCAM1 increases from Days 11 and 13 to Days 15 and 17. GLYCAM1 is also detected in conceptus trophoblast from Days 13-19. Therefore, GLYCAM1 is hypothesized to be a secretory product of the maternal endometrium that mediates conceptus-maternal interactions during implantation in sheep.

#### *Galectin 15 (LGALS15)*

Galectins are a family of proteins that contain a conserved carbohydrate recognition domain that binds  $\beta$ -galactosides. Binding results in the cross-linking of glycoproteins and glycolipid receptors on the cell surface to initiate a biological response (Yang and Liu 2003). LGALS15 was initially characterized as a P4 modulated 14-kDa protein located in crystalline inclusion bodies in uterine epithelia and conceptus trophoderm (Kazemi et al. 1990).

Functional studies of other galectins identified fundamental processes intrinsic to implantation including roles in cell growth, differentiation, apoptosis, cell adhesion, chemoattraction, and migration (Yang and Liu 2003) which support the hypothesis that LGALS15 is involved in blastocyst implantation in sheep (Spencer et al. 2004a). Indeed, LGALS15 stimulates proliferation of ovine trophoderm cells and also acts as an anti-apoptotic agent in response to staurosporine induced apoptosis (Farmer et al. 2007). Additionally, LGALS15 stimulated trophoderm cell migration and attachment by binding integrins through its conserved RGD sequence (Farmer et al. 2007; Lewis et al. 2007). Collectively, results indicate that LGALS15 may play a role in mediating blastocyst elongation and implantation.

#### *Secreted Phosphoprotein 1 (SPP1)*

Secreted phosphoprotein 1, also known as osteopontin, is an acid phosphorylated glycoprotein molecule that is detected and secreted from the epithelia of a number of tissues (Butler et al. 1996). SPP1 is a component of the extracellular matrix and binds to specific integrin heterodimers via its conserved RGD sequence to promote cell adhesion,

spreading and migration (Butler et al. 1996). In sheep, the uterine glands synthesize and secrete SPP1 during the peri-implantation period, a time when the conceptus is developing a firm adhesion to the uterine LE (Johnson et al. 1999a; Johnson et al. 1999b). Synthesis of SPP1 by the glands is dependent upon progesterone as administration of a PGR antagonist ablates *SPP1* mRNA expression (Johnson et al. 2000). SPP1 then binds integrin heterodimers expressed by the trophectoderm and uterine LE to stimulate morphological changes of the conceptus trophectoderm as well as induce adhesion between the LE and trophectoderm essential for implantation and placentation.

### ***Epithelial Junctional Complexes***

Regulation of epithelial organization, structure, and subsequent function is modulated by two forms of junctional complexes: tight junctions and adherens junctions. Tight junctions are located on the plasma membrane and facilitate cellular polarity, cell-cell contact, and adhesion (Figure 2.5). Tight junctions function as barriers that regulate the passage of ions, water, and molecules through the paracellular space. In addition, tight junctions maintain the proper distribution of proteins and lipids within domains of the plasma membrane (Gonzalez-Mariscal et al. 2003). Proteins known to be associated with the formation of tight junctions are classified into one of three families of molecules: junctional adhesion molecules (JAM), occludins (OCLN), and claudins (CLDNs).

Transepithelial paracellular permeability can be regulated by both OCLN and CLDNs (Tsukita et al. 2001). Zona occludens contain members of a submembranous class of proteins [tight junction proteins (TJP)] that bind both CLDNs and OCLN (Wittchen et al. 1999) and function as a scaffold to bring structurally diverse proteins into close proximity at tight junctions (Gonzalez-Mariscal et al. 2000a). The amount of OCLN and TJP1 present in a tissue is inversely related to the permeability of that tissue (Gonzalez-Mariscal et al. 2000b). Regulation of tight-junction-dependent transepithelial paracellular permeability is mediated by multiple factors including calcium (Gonzalez-

Mariscal et al. 1985; Gonzalez-Mariscal et al. 1990), growth factors, kinases, and second messengers (Balda et al. 1991), as well as hormones including P4, prolactin, and placental lactogen (Nguyen et al. 2001).

Adherens junctions formed by classical cadherin/catenin complexes mediate epithelial organization and function. E-cadherin (CDH1) facilitates cell to cell adhesion within epithelia through homodimeric attachment to other CDH1 molecules on adjacent cells. These cadherins are first bound by intracytoplasmic Beta-catenin (CTNNB1) (McCrea and Gumbiner 1991; McCrea et al. 1991). Alpha-Catenin (CTNNA1) is subsequently recruited to the complex (Aberle et al. 1994) and binds to the actin cytoskeleton to facilitate cellular organization and shape (Rimm et al. 1995). Stable cell to cell adhesion is mediated by the phosphorylation state of CTNNB1 (Lilien and Balsamo 2005). Homodimeric CDH1 interactions as well as other cell adhesion molecules have also been implicated in the relative invasive capabilities of tumors (Cowin et al. 2005; Okegawa et al. 2002).

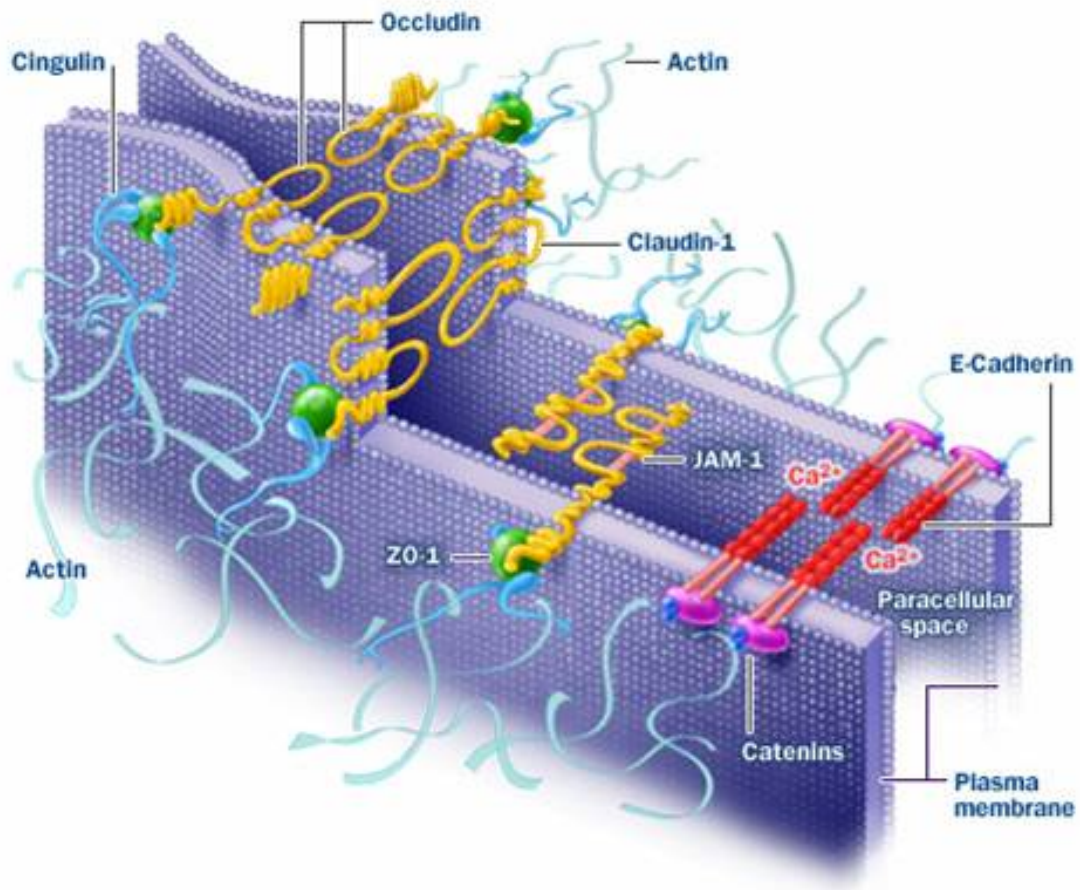


FIG. 2.5. Schematic illustration of junctional complexes mediating epithelial cell-cell interactions. Tight junctions are formed from a large family of proteins including occludins, claudins, and junctional adhesion molecules bound intracellularly to zonula occludens proteins which then stabilize the junctional complex via interaction with the actin cytoskeleton. Tight junctions mediate cell polarity and paracellular permeability which is the trafficking of molecules between adjacent epithelial cells. Adherens junctions are formed across adjacent epithelial cells via calcium dependent homodimeric interactions of E-cadherin proteins. Intracellular E-cadherin is bound by beta catenin which recruits and binds alpha catenin to form a cadherin/catenin complex. This complex binds the actin cytoskeleton and intermediate filaments to regulate cell shape and polarity. (Schematic from Natestech Pharmaceutical Company Inc. [http://www.natestech.com/natestech/junctions\\_biology](http://www.natestech.com/natestech/junctions_biology))

### ***Factors in Endometrial Function & Embryo Development***

#### *Cathepsin L (CTSL)*

Cathepsins are peptidases that functionally degrade extracellular matrix, catabolize intracellular proteins, and process prohormones to regulate uterine receptivity for implantation and trophoblast invasion (Afonso et al. 1997). Specifically, CTSL is induced by P4 in LE and sGE of the ovine endometrium between Days 10 and 12 of both the estrous cycle and pregnancy and further increased by IFNT in pregnant ewes (Song et al. 2005). Protease activity mediated by cathepsins is balanced by their inhibitor, cystatin C, which is also regulated by P4 and IFNT in pregnancy (Song et al. 2006b). A balance of proteolytic activity is likely required to modify the glycocalyx on endometrial LE and trophectoderm during the apposition and adhesion phases of implantation (Carson et al. 2000).

#### *Wingless-type Mouse Mammary Tumor Virus Integration Site (WNT) Family*

WNT genes are homologous to the *Drosophila* segment polarity gene wingless (*wg*). In humans and mice, the WNT family encodes a group of 19 highly conserved secreted glycoproteins that regulate cell and tissue growth and differentiation as well as components of junctional complexes (Davies 2002; Davies and Fisher 2002; Robinson et al. 2000). WNTs associate with their extracellular surface receptors, frizzled (FZD), in both an autocrine and paracrine manner to mediate intracellular signal transduction pathways (Dale 1998). The ten FZDs are a family of seven transmembrane G protein-coupled receptors that possess an extracellular cysteine-rich domain for WNT binding (Liu et al. 1999; Wang et al. 1996). FZD then binds to low-density lipoprotein receptor-related proteins 5 and 6 (LRP5/6) which serve as coreceptors (Pinson et al. 2000). Another family of proteins, termed secreted FZD-related proteins (SFRPs) produced by six different genes, exert inhibitory effects on WNT signaling by competing with WNT ligands for the FZD receptor and forming a nonfunctional complex with FZDs in a dominant-negative manner (Bafico et al. 1999; Kawano and Kypta 2003). WNT

signaling is also antagonized by four Dickkopf (DKK) genes which encode secreted proteins that bind the LRP coreceptor (Nusse 2001). In the canonical WNT signaling pathway, FZD receptors inhibit glycogen synthase kinase 3 beta (GSK3B), resulting in stabilization of the transcriptional regulator CTNNB1 (Logan and Nusse 2004; Pandur et al. 2002b). Nuclear CTNNB1 interacts with transcription factors, most notably members of the transcription factor 7 (T-cell specific, HMG-box) (TCF7/LEF) family, to regulate transcription of genes such as *JUN* (c-Jun) (Mann et al. 1999), *LEF1/TCF7* (Roose et al. 1999), *MET* (c-Met) (Boon et al. 2002), and *MSX2* (msh homeobox homolog 2) (Hussein et al. 2003; Willert et al. 2002). The noncanonical or planar cell polarity pathways, activated by *WNT5A* and *WNT11*, mediate cell polarity, cell movements during gastrulation, and other processes modifying the actin cytoskeleton via small GTPases of the Rho family, such as Rho, Rac, and Cdc42 (Huelsenken and Birchmeier 2001; Kuhl et al. 2000; Pandur et al. 2002a; Yamanaka et al. 2002). Rac activation stimulates c-Jun N-terminal kinase (JNK) activity (Boutros et al. 1998; Habas et al. 2003; Veeman et al. 2003). JNK activation plays essential roles in organogenesis by regulating cell survival, apoptosis, and proliferation (Nishina et al. 2004).

During implantation, WNTs play a critical role in coordinating uterine-conceptus interactions required for implantation in mice, and perhaps other mammals (Mohamed et al. 2005). In sheep, *WNT7A* was the first gene known to be induced by IFNT between Days 12 and 16 of pregnancy specifically within LE and sGE (Kim et al. 2003). A recent study cataloged members of the WNT signaling family and a number of downstream target genes present in the ovine endometrium and identified roles for WNT signaling in regulation of conceptus growth and development (Hayashi et al. 2007). In addition to actions on the conceptus, WNTs are hypothesized to elicit paracrine and/or autocrine actions on the LE and sGE to regulate expression of target genes implicated in uterine receptivity and implantation, including *LGALS15*, *CST3*, and *CTSL*. Known alterations in uterine epithelial architecture may also be regulated by WNT signaling as limited results illustrate regulation of several tight junction associated proteins by members of the WNT family (Mankertz et al. 2004; Miwa et al. 2001).

### *Insulin-Like Growth Factor Family*

The insulin-like growth factor (IGF) family consists of IGF1, IGF2, IGF1 receptor (IGF1R) and IGF2 receptor (IGF2R), and 7 high affinity IGF binding proteins (IGFBPs 1-7) which modulate IGF activity (Wang and Chard 1999). IGF1 and IGF2 possess both mitogenic and differentiative properties and have long been implicated in early embryonic and placental development in multiple species including humans, rodents, pigs, and domesticated ruminants (Baker et al. 1993; D'Ercole 1987; DeChiara et al. 1990; Gluckman et al. 1992; Wathes et al. 1998; Zhou and Bondy 1992). The IGF1R demonstrates high affinity for IGF1 and moderate affinity for IGF2 and is the primary functional receptor associated with pregnancy (Wang and Chard 1999). Confounding clear understanding of the roles of IGFs are the actions of the IGFBPs which can both enhance or retard IGF actions. IGFBPs bind IGFs with high affinity although relative affinity is altered by phosphorylation state of the IGFBP. Further, wide arrays of proteolytic molecules cleave IGFBPs from IGF yielding free IGF to act on adjacent cells expressing IGF1R. These molecules include, matrix metalloproteinases (MMPs), kallikriens, cathepsins, pregnancy associated plasma protein-A (PAPP-A), calpain, and serine proteases (Bunn and Fowlkes 2003; Ghosh et al. 2005; Rajah et al. 1995). Collectively, the mechanisms regulating IGF actions involve the availability of receptors, the presence and phosphorylation state of IGFBPs, and the activity of available proteases to regulate local concentrations of free IGF.

Gene mutagenesis studies in mice indicated critical roles for both *Igf1* and *Igf2* in embryonic, placental, and fetal growth (Baker et al. 1993; DeChiara et al. 1990; Liu et al. 1993). Further, IGF1 null-mice are infertile. In pigs, proteolytic cleavage of IGFBPs to yield free IGF has been associated with the elongation period of conceptus development (Lee et al. 1998a). Studies in the cow revealed the presence of mRNAs encoding IGFBPs, 1, 2, 3, 4, and 5 within the endometrium during early conceptus elongation (Keller et al. 1998). Early embryonic and fetal development in cows can be stimulated by components of the growth hormone-IGF system (Moreira et al. 2002a; Moreira et al. 2002b). Administration of bovine somatotropin (bST) that enhances pregnancy rates



in lactating dairy cows, possibly via alterations in the IGF system, increased pregnancy rate, conceptus length and IFNT secretion on Day 17 (Bilby et al. 2006a; Bilby et al. 2006b). These effects were accompanied by increased concentrations of IGF1 in serum. Functional results elucidating a clear role of the IGF system in post-hatching blastocyst development in either ewes or cows are not available.

#### *Stromal Cell-Derived Growth Factors*

Actions of P4 on the endometrial epithelium in the absence of a functional PGR are a reproductive conundrum limiting the understanding of the process of implantation. Fibroblast growth factor 7 (FGF7), FGF10, and hepatocyte growth factor (HGF), are secreted by stromal cells and act via receptors on adjacent epithelial cells. Available results indicate that stromal cells of the primate uterus express FGF7 in response to P4 (Koji et al. 1994; Rubin et al. 1995). FGF7 and FGF10 both act via a specific isoform of the FGF receptor 2 (FGFR2<sub>IIIb</sub>), while the receptor for HGF is a proto-oncogene *c-met* (MET). HGF is expressed by fibroblasts and smooth muscle cells of the uterus, placenta, and ovaries in rodents, humans, sheep and horses (Chen et al. 2000a) although its regulation by P4 has not been determined. HGF expression is increased by estrogen in the primate uterus and mouse ovary indicating that steroid hormones regulation of gene expression. Both FGF7 and HGF are powerful mitogens on epithelial cells stimulating proliferation, migration, and differentiation. Stromal FGF10 has critical roles in development of lung, brain, and limbs, as mice lacking *Fgf10* fail to develop (Sekine et al. 1999).

In sheep, FGF10 mRNA is detected at moderate levels throughout the stroma during the estrous cycle and early pregnancy. In contrast, FGF7 mRNA is localized in the media intima of uterine blood vessels consistent with expression within the spiral arteries of the primate endometrium (Brenner et al. 1997). The distinct spatial expressions of these growth factors suggest non-redundant functions in regulating uterine biology and conceptus development. HGF is expressed and temporally regulated in the adult ovine uterus although hormonal regulation has not been elucidated (Chen et

al. 2000a). In addition to the uterine LE, both MET and FGFR2 are expressed in conceptus trophectoderm suggesting that HGF, FGF7, and FGF10 may stimulate conceptus development directly as well as modulating epithelial functions.

### ***Progesterone Regulation of Preimplantation Blastocyst Growth and Elongation***

Progesterone stimulates and/or maintains endometrial functions necessary for conceptus growth, implantation, placentation, and development throughout pregnancy (Bazer et al. 1979b; Spencer and Bazer 2002; Spencer et al. 2004b). Circulating concentrations of progesterone affect not only blastocyst growth, but also its survival during early pregnancy (Mann et al. 2006; Mann 1999). In cattle, rapid blastocyst development and ultimately successful establishment of pregnancy correlates positively with increasing circulating levels of progesterone (Mann and Lamming 2001; Sreenan and Diskin 1983). Low circulating concentrations of progesterone during the early luteal phase of both heifers and ewes are associated with growth-retarded conceptuses which secrete reduced amounts of IFNT (Mann and Lamming 2001; Nephew et al. 1991). Indeed, administration of early exogenous progesterone from Days 2 to 5 or Days 5 to 9 enhanced conceptus size and development in beef heifers on Day 14 (Garrett et al. 1988b) and in dairy cattle on Day 16, respectively (Mann et al. 2006). It is hypothesized that these developmental changes in response to progesterone are mediated by the stimulatory effects of progesterone on endometrial function and result in increased blastocyst growth and survival. Indeed, endometrial secretions were increased on Day 5 in explant cultures from heifers receiving early exogenous progesterone (Garrett et al. 1988b). A similar phenomenon has been observed in sheep with the early administration of progesterone during metestrus and early diestrus (Kleemann et al. 1994). The effects of early progesterone treatment were still detected at Day 74 of pregnancy with increased fetal weights (Kleemann et al. 2001). Genetic regulation of factors responsible for the accelerated development of the blastocyst has not been identified. A number of progesterone stimulated genes have been identified in ovine endometria which are expressed in uterine epithelia that paradoxically lose progesterone receptor protein

immediately prior to induction of these genes and initiation of morphogenesis of spherical blastocysts into filamentous conceptuses (Spencer et al. 2004b).

### ***Luteal Insufficiency and Efficacy of P4 Supplementation in Enhancing Pregnancy***

Low fertility in dairy cattle has reached epidemic proportions throughout the world with a 20-30% reduction in conception rates over the past 5 decades (Lucy 2001). These findings correlate with an increase in calving intervals and near doubling of the number of artificial inseminations (AI) per conception. Low concentrations of circulating progesterone has been implicated in early pregnancy failure (Mann 1999) although other factors such as excess urea in blood may also play a role in reduced fertility (Larson et al. 1997). The reduction in circulating P4 and increase in urea in blood are linked to extreme alterations in metabolic state of the modern dairy cow which has resulted in a doubling of milk production per cow since the 1950s. Abnormal luteal function and reduced pregnancy rates are correlated with milk yield, dry matter intake, total digestible nutrients, and digestible crude protein in dairy cattle (Hommeida et al. 2004). Similar findings in sheep have identified a negative correlation between feed intake and concentrations of P4 in blood (Parr et al. 1987; Parr et al. 1993a). The reduction in P4 is believed to be due to increased hepatic blood flow and the clearance of P4 by the liver (Parr et al. 1993b). Correlative data has indicated that pregnancy success in cattle is improved with an early and rapid rise in circulating P4 between Days 4 and 5 (Mann and Lamming 2001; Stronge et al. 2005). A delayed rise in P4 after Day 5 resulted in retarded conceptus growth and little or no production of IFNT. In addition, administration of early exogenous progesterone beginning on Day 1.5 post-estrus increased conceptus length by 10-fold on Day 14 (Garrett et al. 1988b). In light of these observations, and in response to the need to improve conception rates and prevent increased costs due to reproductive losses, researchers have attempted to improve pregnancy rates by supplementing high producing dairy cattle with exogenous P4. These studies have aimed to determine the efficacy of supplementation for enhancing

pregnancy rates and establish the appropriate timing of P4 supplementation for maximal effect (Table 2.2).

Multiple factors limit the ability to interpret and integrate the above findings into a unified strategy for improving pregnancy rates; including parity, production status, timing of supplementation, and numbers of animals per study. In addition, the modern dairy is evolving rapidly and comparisons of results from studies in different eras present challenges. A general interpretation of the findings summarized in Table 2.2 suggests that for P4 supplementation to be beneficial to pregnancy, it must be applied to mature, lactating cows, during early stages of diestrus (Days 4-9). Researchers have begun to assess effects of P4 supplementation on conceptus development during the peri-implantation period to elucidate mechanisms of P4 action. Indeed, P4 supplementation from Days 5-9, but not Days 12-16 increased trophoblast length and IFNT production by the conceptus on Day 16 (Mann et al. 2006). Early exogenous progesterone in sheep also advanced conceptus develop (Kleemann et al. 1994); however, P4 supplementation during the early luteal phase yielded varied results suggesting either no effect (Diskin and Niswender 1989; Kenyon et al. 2005) or improved pregnancy rates (Parr et al. 1987). The improvement in pregnancy rates was associated with overfed ewes that had reduced circulating progesterone prior to exogenous treatment. Diskin et al. (Diskin and Niswender 1989) suggested that supplemental P4, at least in sheep, may not improve pregnancy rates under normal conditions when fertility is normally high. Nonetheless, P4 supplementation is a potential strategy for improving pregnancy rates in both sheep and cattle with reduced fertility, necessitating further research into mechanisms by which pregnancy rates may be increased. Enhanced conceptus development is hypothesized to result from increased secretory activity of the uterine endometrium, providing the conceptus a uterine milieu rich in factors required for development and survival.

The aim of the present studies were to employ molecular biology and functional genomics approaches to identify endometrial factors that enhance conceptus growth and

development in response to P4 as a first step toward improving rates of early embryo survival and pregnancy success in ruminants.

TABLE 2.2. Summary of studies evaluating the efficacy and timing of supplementing cattle with P4 in an attempt to improve pregnancy rate.

| <b>Parity/<br/>Status</b> | <b>Duration<br/>of P4<br/>Supp.</b> | <b>Effect on<br/>Pregnancy<br/>Rate<sup>a</sup></b>                    | <b>Day of Preg<br/>Determination</b> | <b>Reference</b>             |
|---------------------------|-------------------------------------|--|--------------------------------------|------------------------------|
| Heifers<br>and Cows       | D2-9                                | Increase   | 150                                  | (Johnson 1958)               |
| Cows                      | D5                                  | Increase   |                                      | (Wiltbank<br>1956)           |
| Beef Heifer<br>Dairy Cows | D5<br>D10                           | Increase.<br>No effect   |                                      | (Sreenan and<br>Diskin 1983) |
| Lactating<br>cows         | D5-12<br>D10-17                     | Increase<br>Increase   | 55                                   | (Robinson et<br>al. 1989)    |
| Heifers                   | D1-8<br>D2-9                        | Decrease<br>Decrease   | 40-51                                | (Van Cleeff et<br>al. 1996)  |
| Heifers                   | D7-13                               | No effect  | 45                                   | (Van Cleeff et<br>al. 1991)  |
| Anestrous<br>cows         | D4-11<br>D5-12                      | No effect  | >42                                  | (Hanlon et al.<br>2005)      |
| Lactating<br>cows         | D3.5-10                             | Increase   | >40                                  | (Larson et al.<br>2007)      |
| Cows                      | D5-17                               | No effect<br>overall<br>(Increase. in<br>young late<br>lactation cows) | 40-47                                | (Villarroel et<br>al. 2004)  |
| Cows                      | D10-17                              | No effect  | 20-25                                | (Mann et al.<br>2001)        |

**CHAPTER III**  
**PROGESTERONE REGULATION OF PREIMPLANTATION CONCEPTUS**  
**GROWTH AND GALECTIN 15 (LGALS15) IN THE OVINE UTERUS\***

***Introduction***

Maternal support of conceptus (embryo/fetus and associated membranes) growth and development is critical for pregnancy recognition signaling and implantation (Ashworth and Bazer 1989b; Gray et al. 2000a). In sheep, morula-stage embryos enter the uterus on Days 4 to 5 and the blastocyst, formed by Day 6, contains a blastocoele or central cavity surrounded by a monolayer of trophoderm (Guillomot 1995; Spencer et al. 2004a). After hatching from the zona pellucida on Day 8, blastocysts develop into a tubular form by Day 11, elongate to 10 cm or more in length by Day 14, and reach 25 cm or more in length by Day 17. Factors supporting growth of pre- and peri-implantation blastocysts and elongating conceptuses in the uterine lumen are obtained primarily from secretions of the uterus and selective transport from maternal blood that are collectively referred to as histotroph. This hypothesis is supported by results from studies of asynchronous uterine transfer of embryos and trophoblast vesicles (Flechon et al. 1986; Lawson et al. 1983) and from studies of uterine gland knockout (UGKO) ewes (Gray et al. 2002; Gray et al. 2001c). The endometrial luminal (LE) and glandular (GE) epithelia are the main sources of uterine secretions or histotroph.

During early pregnancy, endometrial functions are regulated primarily by progesterone from the corpus luteum and hormones from the placenta (Bazer 1975; Bazer et al. 1979b; Roberts and Bazer 1988; Spencer et al. 2004b). In sheep, pregnancy recognition and establishment involves elongation of spherical blastocysts to filamentous conceptuses between Days 12 and 16 and production of interferon tau (IFNT) by the conceptus (Roberts et al. 1999; Spencer and Bazer 2004). IFNT is antiluteolytic and acts

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on the endometrium to inhibit development of the luteolytic mechanism, thereby maintaining corpus luteum (CL) function and ensuring continued production of P4 (Spencer et al. 1996). Progesterone acts on the uterus to stimulate and maintain endometrial functions necessary for conceptus growth, implantation, placentation and development to term (Bazer 1975; Bazer et al. 1979b; Spencer and Bazer 2002; Spencer et al. 2004b). The concentrations of P4 in early pregnancy clearly affect embryonic survival during early pregnancy (Mann 1999). Increasing concentrations of P4 from Days 2 to 5 or Days 5 to 9 enhanced conceptus development and size on Day 14 in heifers (Garrett et al. 1988a) and Day 16 in cows (Mann et al. 2006), while animals with lower concentrations of P4 in the early luteal phase had retarded embryonic development (Mann and Lamming 2001; Nephew et al. 1991) and decreased production of interferon tau (IFNT) from bovine conceptuses (Mann and Lamming 2001). In both lactating dairy cows and heifers, there is a strong positive association between early luteal phase concentrations of P4 in plasma and embryonic survival (Larson et al. 1997; Starbuck 1999; Villa-Godoy et al. 1988). Indeed, P4 supplementation of cattle after artificial insemination increased embryonic survival (Johnson 1958; Mann 1999; Sreenan and Diskin 1983; Van Cleeff et al. 1991; Wiltbank 1956). However, the mechanisms through which pre-implantation P4 regulates blastocyst survival and growth are not well investigated, but presumably are mediated by secretions from the endometrium. In sheep, P4 acts on the endometrium to induce a number of genes that encode for proteins secreted into the uterine lumen, including galectin 15 (*LGALS15*) and secreted phosphoprotein one (*SPP1* or osteopontin) (Gray et al. 2004; Johnson et al. 2000). These proteins are hypothesized to regulate conceptus survival, growth, and adhesion during implantation (Johnson et al. 2003; Spencer et al. 2004a).

This study tested the hypotheses that pre-implantation conceptus growth and survival in the ovine uterus can be stimulated by progesterone and involves increases in expression of specific endometrial genes. The objectives were to determine effects of early progesterone on blastocyst growth and endometrial expression of secreted proteins. Results indicate that P4 stimulation of blastocyst growth is manifest after shedding the

zona pellucida and is associated with earlier expression of *LGALS15* by endometrial epithelia.

## ***Materials and Methods***

### *Animals*

Mature Suffolk-type ewes (*Ovis aries*) were observed for onset of estrus (Day 0) in the presence of a vasectomized ram and used in experiments only after exhibiting at least two estrous cycles of normal duration (16–18 Days). 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*

**Study One** At estrus, ewes (n = 5) were mated to intact Suffolk rams and received daily injections of 25 mg progesterone (P4) in corn oil (CO) vehicle from Days 1.5 to 6. On Day 6, the uterine lumen was flushed with 20 ml sterile saline and morphology of the blastocyst(s) examined by light microscopy.

**Study Two** At estrus, ewes were mated to intact Suffolk rams and assigned randomly to receive daily i.m. injections from Days 1.5 to 9 of either corn oil vehicle (CO; n = 6) or 25 mg progesterone (P4; n = 6). All ewes were hysterectomized on Day 9. The uterine lumen was flushed with 20 ml sterile saline. If pregnant, the morphology of the blastocyst(s) was examined by light microscopy, fixed in 4% (wt/vol) paraformaldehyde in PBS (pH 7.2), and diameter measured and images recorded using a Nikon SMZ800 microscope with camera.

At hysterectomy, sections (~0.5 cm) from the mid-portion of each uterine horn ipsilateral to the CL were fixed in fresh 4% paraformaldehyde. After 24 h, fixed tissues were changed to 70% (vol/vol) ethanol 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 was physically dissected from myometrium, frozen in



liquid nitrogen, and stored at  $-80^{\circ}\text{C}$  for subsequent RNA or protein extraction. In monovulatory pregnant ewes, uterine tissue samples were marked as either contralateral or ipsilateral to the ovary bearing the CL. No tissues from the contralateral uterine horn were used for further analysis.

**Study Three** At estrus (Day 0), ewes were mated to intact Suffolk rams and assigned randomly to receive daily intramuscular (i.m.) injections of either: (a) corn oil vehicle from Days 1.5 to 12 (CO,  $n = 8$ ); (b) 25 mg progesterone (P4; Sigma Chemical Co., St. Louis, MO) from Days 1.5 to 12 (P4,  $n = 7$ ); or (c) 25 mg progesterone (P4; Days 1.5 to 8,  $n = 5$ ) and then P4 and 75 mg RU486 (Sigma), a progesterone receptor (PGR) antagonist, from Days 8 to 12 (P4+RU). Blood samples were collected daily from CO- and P4-treated ewes beginning on Day 0 via jugular venipuncture. All ewes were hysterectomized on Day 12, and the uterine lumen flushed with 20 ml sterile saline. If pregnant, the morphology of the blastocyst(s) was examined by light microscopy, fixed in 4% paraformaldehyde, and images of blastocysts captured using a Nikon SMZ800 microscope with camera. The volume of the uterine flush was recorded, clarified by centrifugation (3000  $g$  for 30 min at  $4^{\circ}\text{C}$ ), aliquoted and frozen at  $-80^{\circ}\text{C}$ . The uteri were then processed as described for study 2.

#### *RNA Isolation*

Total cellular RNA was isolated from frozen ipsilateral endometrium of pregnant ewes only (Studies Two and Three) using Trizol reagent (Gibco-BRL, Bethesda, MD) according to manufacturer's recommendations. The quantity and quality of total RNA were determined by spectrometry and denaturing agarose gel electrophoresis, respectively.

#### *Slot Blot Hybridization Analysis*

Steady-state levels of *LGALS15*, *CTSL*, and *RSAD2* mRNA in endometria were assessed by slot blot hybridization using methods described previously (Spencer et al. 1999c). Briefly, radiolabeled antisense and sense cRNA probes were generated by *in*

*in vitro* transcription using linearized plasmid templates containing partial cDNAs, RNA polymerases, and [ $\alpha$ -<sup>32</sup>P]-UTP. Denatured total endometrial RNA (20  $\mu$ g) from each ewe in Studies One and Two was hybridized with radiolabeled cRNA probes. To correct for variation in total RNA loading, a duplicate RNA slot blot membrane was hybridized with radiolabeled antisense 18S cRNA (pT718S; Ambion, Austin, TX). Following washing, the blots were digested with ribonuclease A and radioactivity associated with slots was quantified using a Typhoon 8600 MultiImager (Molecular Dynamics, Piscataway, NJ). Data are expressed as relative units (RU).

#### *In situ Hybridization Analysis*

Location of *CTSL*, *RSAD2*, *LGALS15*, *SPP1* and *SERPIN* (ovine uterine serine proteinase inhibitor or uterine milk protein) mRNAs in the ovine uterus were determined by radioactive *in situ* hybridization analysis using methods described previously (Spencer et al. 1999c). Radiolabeled antisense and sense cRNA probes were generated by *in vitro* transcription using linearized partial plasmid cDNA templates, RNA polymerases, and [ $\alpha$ -<sup>35</sup>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 5 Days. Slides were developed in Kodak D-19 developer, counterstained with Gill's hematoxylin (Fisher Scientific, Fairlawn, NJ), and then dehydrated through a graded series of alcohol to xylene. Coverslips were then affixed with Permount (Fisher). Images of representative fields were recorded under brightfield or darkfield illumination using a Nikon Eclipse 1000 photomicroscope (Nikon Instruments Inc., Lewisville, TX) fitted with a Nikon DXM1200 digital camera.

#### *Immunohistochemistry*

Immunocytochemical localization of PGR and LSGAL15 protein in the ovine uterus was performed using methods described previously (Dunphy et al. 2000; Gray et al. 2004). PGR protein was detected using a primary mouse monoclonal antibody

against human PGR (MA1-411; Affinity Bioreagents, Golden, CO) at a final concentration of 0.25 µg/ml and a Vectastain ABC anti-mouse kit (Vector Laboratories, Inc., Burlingame, CA). LGALS15 protein was detected using rabbit anti-ovine galectin 15 antibody (kindly provided by Dr. Els Meeusen, Australia) (Dunphy et al. 2000) at a final dilution of 1:10000 and a Vectastain ABC anti-rabbit kit. For both PGR and LGALS15, antigen retrieval was performed using boiling citrate buffer as described previously (Taylor et al. 2000). Negative controls included substitution of the primary antibody with mouse IgG (PGR) or rabbit IgG (LGALS15). Immunoreactive protein was visualized using diaminobenzidine tetrahydrochloride (Sigma) as the chromagen. Sections were dehydrated and coverslipped affixed with Permount.

#### *Western Blot Analyses*

Protein content of concentrated flushes was determined using a Bradford protein assay (Bio-Rad, Hercules, CA) with BSA as the standard. Uterine flush proteins (30 µg) were denatured and separated by 15% SDS-PAGE for both IFNT (Study 1) and LGALS15 (Studies 1 and 2). Western blot analyses were conducted as described previously (Spencer et al. 1999a) using enhanced chemiluminescence detection. Immunoreactive IFNT was detected using primary rabbit anti-ovine IFNT serum at a 1:5000 dilution. Rabbit anti-ovine IFNT serum was generated by immunizing rabbits with recombinant ovine IFNT (Van Heeke et al. 1996).

#### *Slot Blot Quantification of Proteins in Uterine Flushings*

Uterine flushings (30 µg for IFNT and 20 µg for LGALS15) were diluted to 200 µl final volume with Tris buffered saline (TBS). Nitrocellulose membranes (Schleicher and Shuell), presoaked with TBS, were loaded into the slot blot apparatus backed by Whatman filter paper. The wells were subsequently washed with 200 µl TBS prior to addition of the diluted sample, and then washed again with 200 µl TBS. The membrane was allowed to air dry and then blocked in 5% (wt/vol) milk/TBS-tween (TBST) for 1 h at room temperature. The membrane was incubated in either primary rabbit anti-ovine IFNT serum at 1:1000 dilution (30 µg protein per slot) or rabbit anti-ovine LGALS15

serum (Dunphy et al. 2000) at 1:2500 dilution (20 µg protein per slot) in 2.5% milk/TBST overnight at 4°C. The blot was then washed for 30 min in TBST followed by incubation with goat anti-rabbit IgG horseradish peroxidase conjugate at 1:20000 diluted in 2.5% milk/TBST for 1 h at room temperature. The blot was washed again for 30 min in TBST and immunoreactive proteins were detected using enhanced chemiluminescence. Blots were imaged and quantified using a Typhoon 8600 Variable Mode Imager (Amersham Biosciences Corp., Piscataway, NJ). The total amounts of IFNT and LGALS15 protein were calculated based on the amount of uterine flush protein loaded into each well, the concentration of protein in the uterine flush, and the recovered volume of the uterine flush recorded at surgery.

#### *Radioimmunoassay*

Blood samples were allowed to clot for 1 h at room temperature. Serum was then collected following centrifugation (3000 x g for 30 min at 4°C) and stored at -20°C for hormone analysis. Concentrations of progesterone in serum were determined according to manufacturer's specifications using an antiserum highly specific for progesterone (DSL-3900 ACTIVE Progesterone Coated-Tube Radioimmunoassay Kit, Diagnostic Systems Laboratories, Webster, TX). The RIA used rabbit anti-progesterone immunoglobulin coated tubes and iodinated progesterone. The primary anti-serum cross-reacts 6.0%, 2.5%, 1.2%, 0.8%, 0.48%, and 0.1% with 5 $\alpha$ -Pregnane-3,20-dione, 11-Deoxycorticosterone, 17  $\alpha$ -Hydroxyprogesterone, 5 $\beta$ -Pregnane-3,20-dione, 11-Deoxycortisol, and 20 $\alpha$ -Dihydroprogesterone, respectively. The progesterone standard curve (1.0–190.8 nmol/L) was provided in the assay kit. The intra-assay variation was 10.1%. Assay results were calculated using the AssayZap Version 3.1 program (Biosoft, Ferguson, CA).

#### *Photomicroscopy*

Photomicrographs of *in situ* hybridization and immunocytochemistry slides were taken using a Nikon Eclipse E1000 photomicroscope (Nikon Instruments, Melville, NY). Digital images were captured using a Nikon DXM 1200 digital camera and assembled

using Adobe Photoshop 7.0 (Adobe Systems, Seattle, WA). Images of embryos were captured using a Nikon SMZ800 microscope with camera.

### *Statistical Analyses*

Data from radioimmunoassay, slot blot hybridization, and protein slot blot analyses were subjected to least-squares analysis of variance using the General Linear Models procedures of the Statistical Analysis System (SAS Institute, Cary, NC). Slot blot hybridization data were corrected for differences in sample loading by using the 18S rRNA data as a covariate. Protein slot blot data was corrected for differences in conceptus number by using them as a covariate. Data are presented as the least-squares means (LSM) with overall standard error (SEM).

## ***Results***

### *Circulating Progesterone*

Circulating concentrations of progesterone in serum from jugular venous blood in Study 3 were determined by radioimmunoassay (Figure 3.1). Progesterone increased (linear,  $P < 0.01$ ) after Day 3 and reached maximal levels by Day 11 in CO ewes. Exogenous treatment of ewes with P4 beginning at 36 h postmating increased concentrations of P4 in serum after Day 1, and they remained higher in P4 than CO ewes to Day 12 ( $P < 0.0001$ , Day  $\times$  Treatment).

### *Early Progesterone Enhances Blastocyst Development*

In all studies, ewes assigned to the early P4 treatment group received P4 beginning 36 h postmating (Day 1.5). In Study 1, embryos recovered on Day 6 from P4 ewes were morphologically classified as either morulae or blastocysts, with 4 of 5 possessing an intact zona pellucida (data not shown). In Study 2, hatched blastocysts were recovered on Day 9 from both CO- and P4-treated ewes (Figure 2) and blastocyst diameters were 220% greater ( $P < 0.02$ ) for those from P4- ( $636 \pm 64 \mu\text{m}$ ) compared to

CO- ( $282 \pm 64 \mu\text{m}$ ) treated ewes. In Study 3, blastocysts recovered from CO-treated ewes were spherical to slightly tubular in morphology on Day 12 (Figure 3.2), whereas those recovered from P4-treated ewes were elongated and filamentous. No blastocysts were recovered from ewes receiving P4+RU treatment. Therefore, early P4 treatment enhanced blastocyst growth and development after hatching from the zona pellucida, but did not affect time of blastocyst hatching.

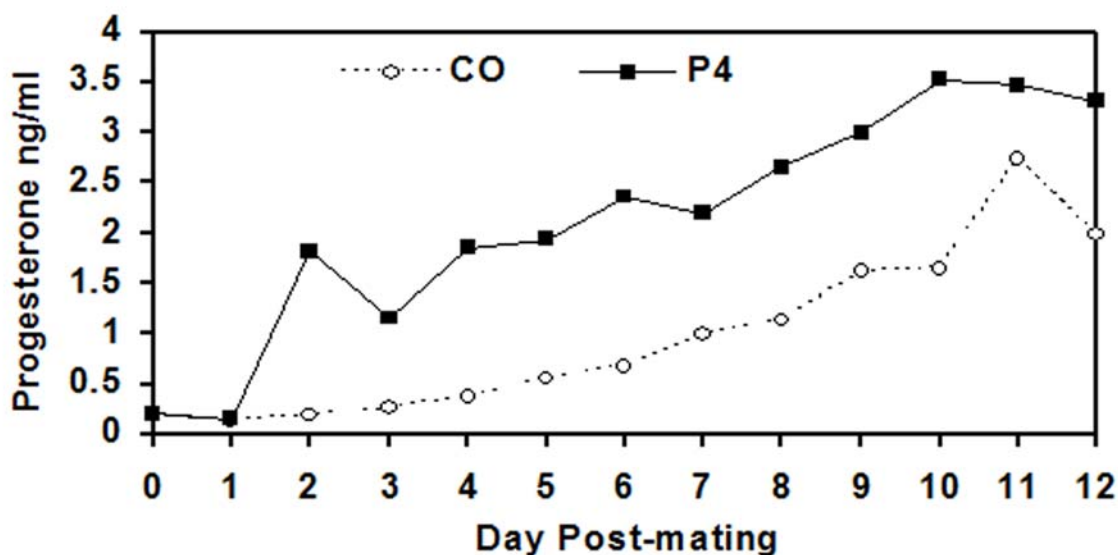


FIG. 3.1. Concentrations of progesterone in serum of ewes treated daily with either corn oil (CO) vehicle or progesterone (P4) from 36 h post-mating to Day 12. Concentrations of progesterone were higher in P4 than CO ewes after Day 1 (Day  $\times$  treatment,  $P < 0.01$ ). Data are presented as LSM [ with overall SE – There is no information here on SEM, so you need to either put in SEM bars or just indicate that the graph is based on LSM].

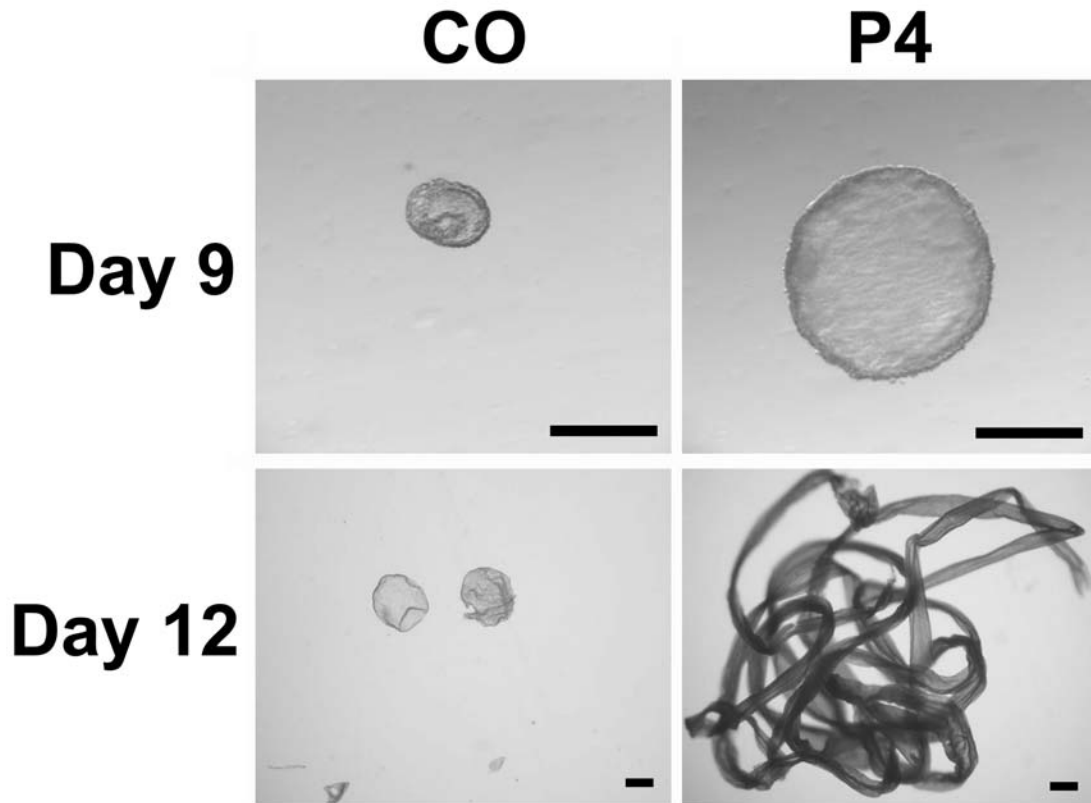


FIG. 3.2. Effects of early exogenous progesterone treatment on blastocyst morphology on Days 9 and 12 of pregnancy. In Study Two, ewes were treated daily with corn oil (CO) vehicle or progesterone (P4) from 36 h post-mating. On Day 9, blastocyst diameter was greater ( $P<0.02$ ) for ewes receiving P4 ( $636\pm64\ \mu\text{m}$ ) than CO ( $282\pm64\ \mu\text{m}$ ). In Study Three, ewes were treated daily with CO or P4 from 36 h post-mating to Day 12 or P4 from 36 h post-mating to Day 8 and then P4 and RU486 (P4+RU) from Days 8 to 12. Elongated and filamentous conceptuses were recovered from P4-treated ewes, while only spherical to early tubular blastocysts were recovered from CO-treated ewes. No blastocysts were recovered from P4+RU treated ewes. The magnification bar represents  $400\ \mu\text{m}$  in the representative photomicrographs.

*Immunolocalization of PGR Protein*

PGR protein was most abundant in endometrial LE and GE with lower levels in the stroma of Day 9 CO-treated ewes (Figure 3.3). For P4-treated ewes on Day 9 (Study 2), PGR protein was markedly lower in the endometrial cells, particularly in the epithelia. In Day 12 CO-treated ewes (Study 3), PGR protein was either very low or not detectable in the nuclei of the endometrial LE. In P4-treated ewes, PGR abundance was markedly reduced as compared with CO ewes, particularly in the endometrial LE and GE. In P4+RU-treated ewes, PGR protein was higher in endometrial stroma and GE, but not different in the LE.

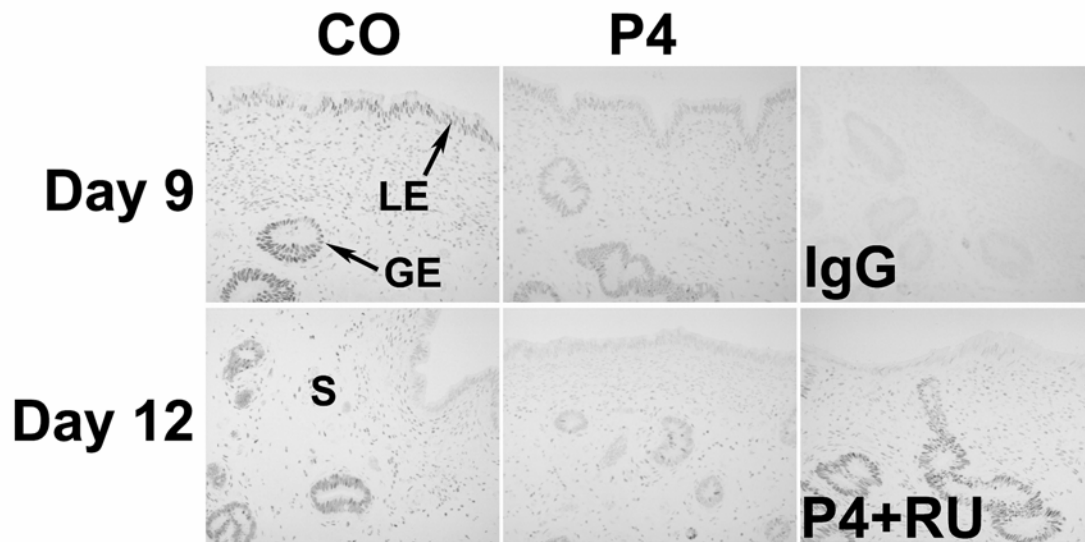


FIG. 3.3. Effects of corn oil (CO), progesterone (P4) or P4 and RU486 on expression of progesterone receptor (PGR) protein in endometria from Day 9 (Study Two) and Day 12 (Study Three) ewes. Immunoreactive PGR protein was detected using a mouse monoclonal antibody against human PGR. For the IgG control, normal mouse IgG was substituted for the primary antibody. Sections were not counterstained. Legend: LE, luminal epithelium; GE, glandular epithelium; S, stroma. All photomicrographs are shown at the same width of field (420  $\mu$ m).



### *IFNT and IFNT-stimulated Genes*

As illustrated in Figure 3.4, immunoreactive IFNT (17 kDa) was detected in uterine flushings from all P4-treated ewes on Day 12, but was markedly lower or absent in uterine flushings from Day 12 CO ewes. IFNT was not detected in uterine flushings from either CO or P4 ewes on Day 9 (Study 2) or on Day 12 if ewes received P4+RU treatment (Study 3). As determined by protein slot blot analyses, relative total amounts of IFNT protein in uterine flushings from Day 12 ewes in Study 3 were approximately 155-fold greater ( $P < 0.0002$ ) in P4 ( $12,126 \pm 2,763$  relative units) compared with CO ewes ( $77 \pm 10$  relative units).

Cathepsin L (*CTSL*) is expressed only in endometrial LE and superficial GE in response to both P4 and IFNT (Gray et al. 2006; Song et al. 2005). Endometrial *CTSL* mRNA levels were not different ( $P > 0.10$ ) on Day 9 in Study 2. In Study 3, endometrial *CTSL* mRNA levels were about 2-fold higher ( $P < 0.05$ ) in P4 than CO ewes and about 4-fold higher in P4 than P4+RU ewes on Day 12 (Figure 3.5A). As expected, *CTSL* mRNA was detected only in the endometrial LE and superficial GE of uteri from CO and P4 ewes in both studies (Figure 5C).

Radical S-adenosyl methionine domain containing 2 (*RSAD2*), also known as viperin, is an IFNT-stimulated gene (Gray et al. 2006). *RSAD2* mRNA was not detected in endometria from Day 9 ewes in Study 3 (Figure 3.5C). Endometrial *RSAD2* mRNA levels were about 9-fold higher ( $P < 0.05$ ) in endometria of P4 versus CO- or P4- versus P4+RU-treated ewes on Day 12 in Study 3 (Figure 3.5B). *In situ* hybridization analysis localized *RSAD2* mRNA primarily to the stratum compactum stroma of the endometrium in Day 12 P4-treated ewes (Figure 3.5C). Thus, IFNT-stimulated genes are only induced in the endometrium from P4-treated Day 12 ewes with an elongated and filamentous conceptus that secretes IFNT.

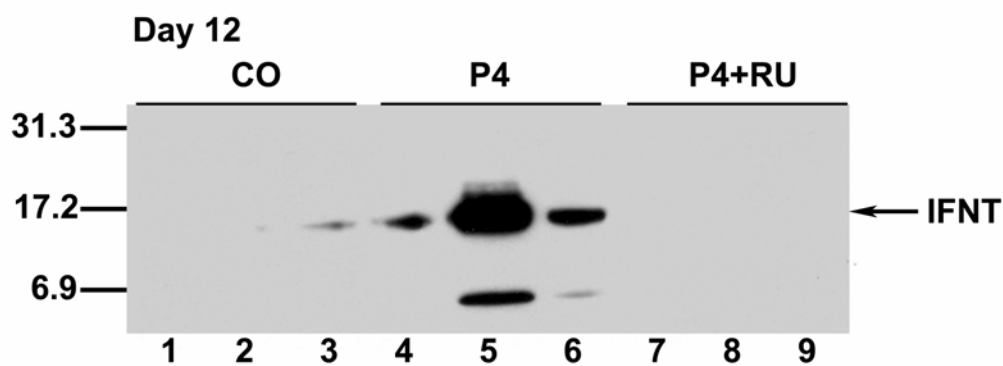


FIG. 3.4. Western blot analysis of IFNT in uterine flushings from Day 12 ewes. In Study One, ewes were treated daily with CO or P4 from 36 h post-mating to Day 12 or P4 from 36 h post-mating to Day 8 and then P4 and RU486 (P4+RU) from Days 8 to 12. Proteins in uterine flushings were analyzed by 15% SDS-PAGE (30  $\mu$ g/lane) and western blot analysis using rabbit anti-ovine IFNT serum. IFNT was detected in uterine flushings from all ewes treated with P4. Representative results from 3 ewes per treatment are presented.

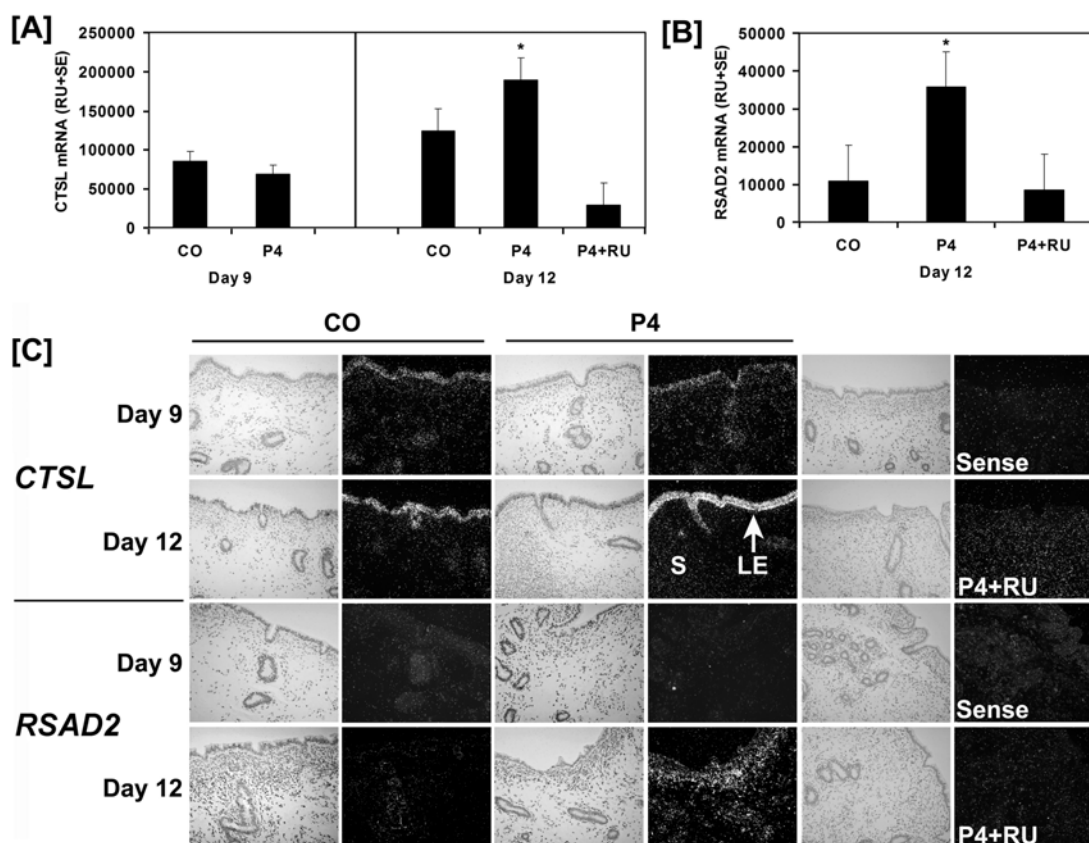


FIG. 3.5. Effects of treatments with corn oil (CO), progesterone (P4) or P4 and RU486 on endometrial *CTSL* and *RSAD2* mRNA. [A] Steady-state levels of *CTSL* mRNA in endometria from Day 9 (Study Two) and Day 12 (Study One) ewes, as determined by slot blot analysis, increased only in endometria of P4-treated ewes on Day 12 (\*,  $P < 0.05$ ). [B] Steady-state levels of *RSAD2* mRNA in the endometrium of Day 12 ewes (Study One) increased only in endometria from P4-treated ewes on Day 12 (\*,  $P < 0.05$ ). [C] *In situ* localization of *CTSL* and *RSAD2* mRNAs in endometria of Day 9 (Study Two) and Day 12 ewes (Study One) are presented. Cross-sections of uteri were hybridized with radiolabeled antisense or sense ovine cRNA probes for each respective gene. Legend: LE, luminal epithelium; S, stroma; sGE, superficial glandular epithelium. All representative photomicrographs are shown at the same width of field (630  $\mu\text{m}$ ).

*LGALS15, SPP1, and Uterine SERPIN*

*LGALS15*, *SPP1*, and *SERPIN* are P4-induced genes expressed in endometrial epithelia during early pregnancy (Gray et al. 2004; Ing et al. 1989; Johnson et al. 2000). In Study 2, steady-state levels of *LGALS15* mRNA were about 3-fold higher ( $P < 0.01$ ) in endometria of P4- compared with CO-treated ewes on Day 9 (Figure 3.6A). Similarly, endometrial *LGALS15* mRNA levels were approximately 2-fold higher ( $P < 0.01$ ) in P4- compared with CO-treated ewes on Day 12 in Study 3 (Figure 3.6A). Further, *LGALS15* mRNA was approximately 59-fold and 136-fold lower ( $P < 0.01$ ) in endometria of P4+RU-treated ewes compared with ewes receiving CO or P4, respectively. As expected, *LGALS15* mRNA was present in LE and superficial GE of endometrium from Day 9 P4 and Day 12 CO and P4-treated ewes, but absent in endometria from Day 9 CO ewes and Day 12 ewes that received P4+RU treatment (Figure 3.6B). The melanocytes present in the sub-epithelial stroma of Day 12 CO ewes were not positive for *LGALS15* mRNA, but rather diffract light and appeared white under darkfield conditions.

*LGALS15* protein was localized predominantly to endometrial LE and superficial GE in uteri from Day 9 and Day 12 ewes (Figure 3.7A). In Day 9 ewes (Study 2), *LGALS15* protein was more abundant at the apical surface of the endometrial LE in P4- compared with CO-treated ewes (denoted by arrowhead). *LGALS15* protein was abundant in endometrial LE and superficial GE of uteri from Day 12 in CO- and P4-treated ewes (Study 2). *LGALS15* protein was also detected in LE and superficial GE of endometria from Day 12 P4+RU-treated ewes, but the immunoreactive protein was concentrated more toward the apical surface of the epithelia rather than uniformly distributed throughout the epithelia as in Day 12 CO- and P4-treated ewes. The relative amount of *LGALS15* protein in uterine flushings was determined in both studies. For Day 9 ewes, total *LGALS15* protein in uterine flushings was not different ( $P > 0.10$ ) in P4- compared with CO-treated ewes (Figure 3.7B); however, *LGALS15* protein tended ( $P = 0.06$ ) to be more abundant in uterine flushings from P4- compared with CO-treated ewes on Day 12 as well as in uterine flushings from ewes receiving P4 compared with

ewes treated with P4+RU (Figure 3.7C). *SPP1* (osteopontin) and *SERPIN* mRNAs were not detected in endometria of any ewes in either Study 1 or Study 2 (data not shown).

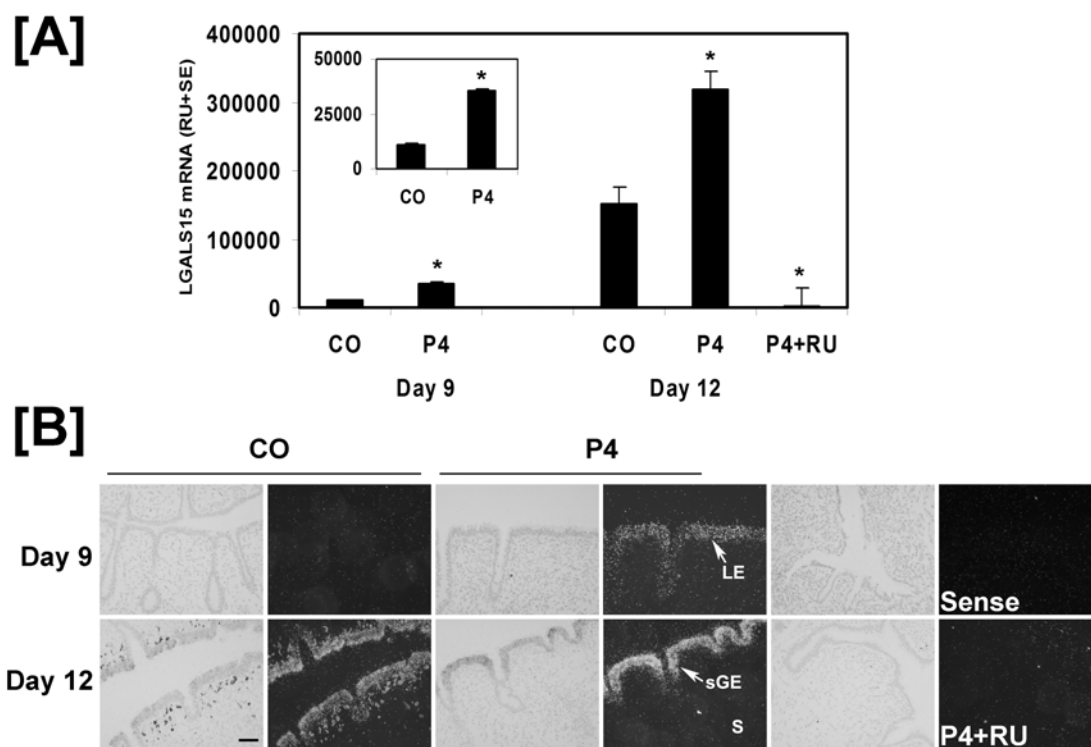


FIG. 3.6. Effects of treatments with corn oil (CO), progesterone (P4) or P4 and RU486 on endometrial *LGALS15* mRNA. [A] Steady-state levels of *LGALS15* mRNA in endometria from ewes on Day 9 (Study Two) and Day 12 (Study One), determined by slot blot analyses, indicated increased expression in endometria of P4-treated ewes on Days 9 and 12 (\*,  $P < 0.01$ ) and lower ( $P < 0.001$ ) expression in endometria of P4+RU-treated ewes compared to P4- or CO-treated ewes [B] *In situ* localization of *LGALS15* mRNA in endometria from Day 9 (Study Two) and Day 12 ewes (Study One). Cross-sections of uteri were hybridized with radiolabeled antisense or sense ovine cRNA probes for *LGALS15*. Legend: LE, luminal epithelium; S, stroma; sGE, superficial glandular epithelium. The black melanocytes in the stratum compactum of Day 12 endometria do not express *LGALS15* mRNA, but appear white in a darkfield photomicrograph. All representative photomicrographs are shown at the same width of field (420  $\mu\text{m}$ ).

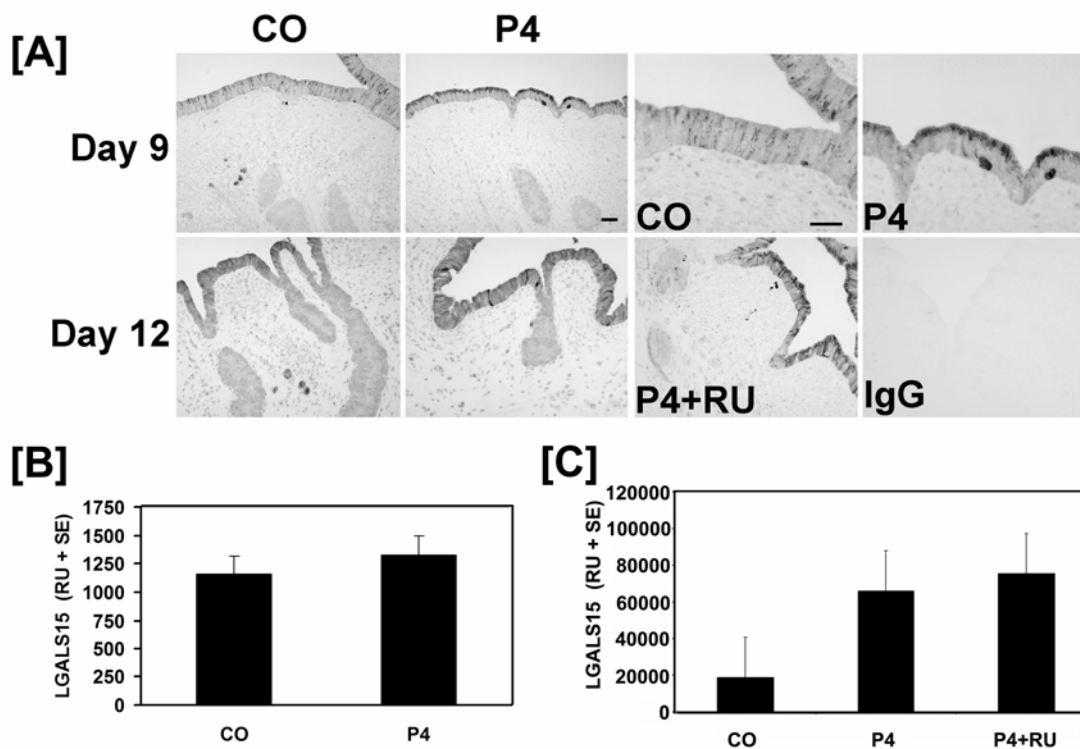


FIG. 3.7. Effects of treatment of ewes with corn oil (CO), progesterone (P4) or P4 and RU486 on endometrial LGALS15 protein in the uterus. [A] Immunolocalization of LGALS15 protein in endometria from Day 9 (Study Two) and Day 12 (Study Three) ewes revealed an increase in apical distribution in the luminal epithelium (LE) of P4-treated ewes from Day 9 (denoted by arrowhead). For the IgG control, normal rabbit IgG was substituted for the primary antibody. Representative photomicrographs are shown at the same width of field (420  $\mu\text{m}$ ) with the exception of the higher magnification (210  $\mu\text{m}$  width of field) images of Day 9 CO and P4 endometrial LE (upper righthand corner). Sections were not counterstained. Legend: LE, luminal epithelium; sGE, superficial ductal glandular epithelium; S, stroma. [B] Total amounts of LGALS15 protein in uterine flushings from Day 9 ewes (Study Two) were not different between CO and P4-treated ewes. [C] LGALS15 protein, in relative units (RU) with overall SE, in uterine flushings from Day 12 ewes (Study Three) tended to be higher ( $P < 0.06$ ) for ewes treated with P4 or P4+RU compared to CO.

### ***Discussion***

Advancement of development of conceptuses at varying time points by administration of early exogenous progesterone has been described for both cattle (Garrett et al. 1988b; Mann et al. 2006) and sheep (Kleemann et al. 2001; Kleemann et al. 1994). In the present study, early exogenous P4 treatment accelerated growth of hatched blastocysts as evidenced by increased blastocyst diameter on Day 9 and the presence of elongated and filamentous conceptuses on Day 12 in uteri from P4-treated ewes. Indeed, blockade of P4 actions by administration of RU486 from Days 8 to 12 resulted in loss of all embryos. These results indicate an unequivocal requirement for P4 actions for maintenance of pregnancy in sheep, as in other mammals. Importantly, hatching of the blastocyst from the zona pellucida was not accelerated by early exogenous P4 and it did not prevent transport of embryos from the oviduct into the uterus. Therefore, early P4 effects are manifest after hatching of the blastocyst from the zona pellucida, which normally occurs on Day 8 (Spencer et al. 2004a). Thus, the zona pellucida may act as a barrier to embryotrophic factors in uterine histotroph, and hatching is required for them to either gain access to the blastocyst or for the blastocyst to respond in terms of blastocyst expansion on Day 9 or early transition from spherical to filamentous conceptuses by Day 12.

Advancement of blastocyst growth coincided with functional development in the present study, because blastocysts from early P4-treated ewes secreted greater quantities of IFNT on Day 12 than blastocysts recovered from CO-treated ewes. In fact, IFNT production by the blastocyst is developmentally regulated and affected by uterine factors (Farin et al. 1990; Robinson et al. 2006). Further evidence for effects of IFNT was induction of *RSAD2* in the stroma and increased *CTSL* and *LGALS15* in LE and sGE of endometria of P4-treated ewes on Day 12. *CTSL*, *LGALS15*, and *RSAD2* are IFNT-stimulated genes in the ovine uterus (Gray et al. 2006; Gray et al. 2004; Song et al. 2005). Thus, timely communication between maternal endometrium and conceptus development allowed maintenance of synchrony and establishment of pregnancy in the present study. Since early exposure of the uterus to P4 accelerates development of the

luteolytic mechanism, it is essential that P4 stimulate development of the conceptus so that it secretes sufficient quantities of IFNT to abrogate development of the luteolytic mechanism (Spencer and Bazer 2004; Spencer et al. 2004a). In dairy cattle, an early increase in circulating concentrations of P4 was highly correlated with increased production of IFNT by the conceptus as well as decreased quantities of circulating prostaglandin metabolite, suggesting that the luteolytic mechanism is inhibited by advanced blastocysts (Mann et al. 2006; Mann and Lamming 2001).

Our working hypothesis is that the stimulatory effects of P4 on blastocyst survival and growth are mediated by specific effects of P4 on the endometrium. During the pre-implantation period, P4 is essential for blastocyst survival and acts through receptors present in all endometrial cell types. Continuous exposure of the endometrium to P4 for 8 to 10 days down-regulates PGR expression in endometrial epithelia (Spencer et al. 1995b), so that PGR protein is not detectable in endometrial LE and GE in ewes after Days 11 and 13 of pregnancy, respectively (Spencer and Bazer 1995; Wathes and Hamon 1993). The paradigm of loss of PGR in uterine epithelia immediately before implantation is common across mammals (Carson et al. 2000; Spencer et al. 2004b). Thus, several genes induced by P4 in LE and superficial GE are implicated in conceptus implantation, including *LGALS15*, *CST3*, and *CTSL*, and, in GE, expression of genes including *SPP1*, *SERPINS*, and *STC1* requires loss of epithelial cell PGR (Gray et al. 2006; Gray et al. 2004; Johnson et al. 2000; Song et al. 2006a; Song et al. 2005; Song et al. 2006b; Spencer et al. 1999b). Loss of epithelial PGR may reprogram patterns of gene expression in the endometrial epithelia.

One of the most abundant P4-regulated genes in sheep uteri is *LGALS15* (Gray et al. 2004; Gray et al. 2005). Galectin proteins have a conserved carbohydrate recognition domain that binds beta-galactosides, thereby cross-linking glycoproteins as well as glycolipid receptors on the surface of cells and initiating biologic responses that include cell adhesion, growth, and differentiation (Cooper 2002; Yang and Liu 2003). In cyclic and pregnant ewes, induction of *LGALS15* mRNA occurs between Days 10 and 12 in endometrial LE and superficial GE (Gray et al. 2004), which is associated with the



decline and loss of PGR from those epithelia (Spencer and Bazer 1995; Wathes and Hamon 1993). Further, P4 induction of *LGALS15* mRNA in endometrial LE and superficial GE is inhibited by a PGR antagonist (Gray et al. 2006; Gray et al. 2004). Of particular importance, *LGALS15* is a novel gene expressed only by LE and superficial GE in response to intrauterine infusions of IFNT in progestinized ewes (Gray et al. 2006; Gray et al. 2004). In the present study, *LGALS15* mRNA was induced by early exogenous P4 in Study 2 (Day 9) and stimulated by IFNT in Sstudy 3 (Day 12). Further, administration of RU486, a PGR antagonist, ablated *LGALS15* mRNA expression in endometrial epithelia which supports evidence that *LGALS15* is induced by P4 (via down-regulation of the PGR) and further stimulated by IFNT from the developing conceptus.

In the present study, LGALS15 protein was higher at the apical surface of endometrial LE of P4-treated ewes on Day 9 and tended to be more abundant in uterine flushings from P4-treated ewes on Day 12. Although secreted LGALS15 protein was not greater in the uterine flush from P4 ewes on Day 9, it was more concentrated near the apical surface of the LE of endometria. It is likely that a factor from the conceptus during trophoblast outgrowth and elongation stimulates the secretion of proteins, such as LGALS15, from the endometrial epithelia. Nonetheless, stimulation of blastocyst growth and development in response to early exogenous P4 treatment was strongly associated with increases in endometrial LGALS15 mRNA in Studies 2 and 3 and LGALS15 protein in uterine flushes in Study 3 (Day 12). Available results indicate that LGALS15 protein is synthesized and secreted by endometrial LE and superficial GE into the uterine lumen, where it is absorbed by conceptus trophoctoderm (Gray et al. 2004). Indeed, LGALS15 protein is detectable on the surface of the trophoctoderm and within crystalline structures found inside the trophoctoderm (Gray et al. 2004; Gray et al. 2005). Thus, the amount of LGALS15 protein present in the uterine lumen of Day 12 P4-treated ewes may have been greater than for CO ewes, except that the elongated and filamentous conceptuses from P4-treated ewes may have imbibed more of the secreted protein. In P4+RU-treated ewes, LGALS15 mRNA was not present in the endometrium, but

LGALS15 protein was detected in endometrial LE and abundant in uterine flushings. The crystalline nature of LGALS15 protein may be responsible for low levels of immunoreactive protein in LE which do not express *LGALS15*. Further, LGALS15 is a secreted lectin that presumably binds glycoconjugates that are part of the extracellular matrix and rather stable, potentially accounting for the low levels of LGALS15 protein observed in cells not expressing the gene. *SPP1* and uterine *SERPIN* are well-characterized progesterone-induced genes that appear specifically in the endometrial glands between Days 14 to 16 (*SPP1*) and 16 to 18 (*SERPIN*) of pregnancy (Johnson et al. 2003; Stewart et al. 2000). These genes were not responsible for effects of progesterone on pre-implantation blastocyst growth and development, because *SPP1* and uterine *SERPIN* mRNAs were not detected in endometria from any ewes in the present studies.

Collectively, these results illustrate that rate of development of blastocysts in utero is stimulated by actions of P4 on the uterus and support the hypothesis that P4 acts on the endometrium to down-regulate expression of PGR in epithelia and to induce expression of specific genes that encode secreted proteins, such as LGALS15, that stimulate blastocyst growth and development. Indeed, a large number of undefined components of histotroph, including other secreted proteins, amino acids, sugars and ions, may also be regulated by progesterone during the peri-implantation period of pregnancy to stimulate conceptus growth and development (Bazer 1975; Martin et al. 2003). Results of the present study allow further development of the ovine model in which early exogenous P4 treatment advances conceptus development to allow it to maintain synchrony with the endometrium, as evidenced by advanced onset of IFNT production by the conceptus and induction of IFNT-stimulated genes in the endometrium that are hypothesized to be required for conceptus implantation and establishment and maintenance of pregnancy. Future studies will use genomic and proteomic approaches to identify the mechanisms by which progesterone acts on the endometrium to enhance blastocyst survival and growth after hatching from the zona pellucida.

**CHAPTER IV**  
**TIGHT AND ADHERENS JUNCTIONS IN THE OVINE UTERUS:**  
**DIFFERENTIAL REGULATION BY PREGNANCY AND PROGESTERONE\***

***Introduction***

Establishment and maintenance of pregnancy in mammals involves dynamic changes in the uterine epithelia that are regulated by steroid hormones, cytokines, and growth factors. These changes establish receptivity of the uterine luminal epithelium (LE) to the developing embryo, differentiated functions of glandular epithelium (GE) required for subsequent secretion of uterine histotroph, and protection of the developing semiallogeneic conceptus (embryo/fetus and associated extra-embryonic membranes) from the maternal immune system (Burghardt et al. 2002; Carson et al. 2000; Johnson et al. 2001a; Kirby et al. 1964; Muggleton-Harris and Johnson 1976; Spencer et al. 2004a). In domestic animals, the implantation cascade is characterized by preattachment elongation of the blastocyst followed by apposition, adhesion, and attachment of the trophectoderm to the LE (Guillomot et al. 1993). Uterine-dependent conceptus elongation ensues in response to cues from the endometrial LE and GE in the form of histotroph (Gray et al. 2001c) and possibly through stromal and serum-derived factors that bypass the epithelia via trafficking through the paracellular space into the uterine lumen to act directly on the conceptus.

Regulation of epithelial organization, structure, and subsequent function is modulated by two forms of junctional complexes, tight junctions, and adherens junctions. Tight junctions are located on the plasma membrane and facilitate cellular polarity, cell-cell contact, and adhesion. Tight junctions function as barriers that regulate the passage of ions, water, and molecules through the paracellular space. In addition, tight junctions maintain the proper distribution of proteins and lipids within domains of

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the plasma membrane (Gonzalez-Mariscal et al. 2003). A growing class of proteins is classified into one of three families of molecules known to be associated with the formation of tight junctions. These proteins are: junctional adhesion molecules, occludins (OCLN), and claudins (CLDNs). Transepithelial paracellular permeability can be regulated by both OCLN and CLDNs (Tsukita et al. 2001). Zona occludens contain members of a submembranous class of proteins [tight junction proteins (TJP)] that bind both CLDNs and OCLN (Wittchen et al. 1999) and function as a scaffold to bring structurally diverse proteins into close proximity at tight junctions (Gonzalez-Mariscal et al. 2000a). The amount of OCLN and TJP1 protein present in a tissue is related inversely to the permeability of that tissue (Gonzalez-Mariscal et al. 2000b). Regulation of tight-junction-dependent transepithelial paracellular permeability is mediated by multiple factors including calcium (Gonzalez-Mariscal et al. 1985; Gonzalez-Mariscal et al. 1990), growth factors, kinases, and second messengers (Balda et al. 1991), as well as hormones including progesterone (P4), prolactin, and placental lactogen (Nguyen et al. 2001).

Adherens junctions formed by classical cadherin/catenin complexes mediate epithelial organization and function. E-cadherins (CDH1) facilitate cell to cell adhesion within epithelia through homodimeric attachment to other CDH1 molecules on adjacent cells. These cadherins are first bound by intracytoplasmic  $\beta$ -catenin (CTNNB1) (McCrea and Gumbiner 1991; McCrea et al. 1991).  $\alpha$ -Catenin (CTNNA1) is subsequently recruited to the complex (Aberle et al. 1994) and binds to the actin cytoskeleton to facilitate cellular organization and shape (Rimm et al. 1995). Stable cell to cell adhesion is mediated by the phosphorylation state of CTNNB1 (for review see Ref. (Lilien and Balsamo 2005)). Homodimeric CDH1 interactions as well as other cell adhesion molecules have also been implicated in the relative invasive capabilities of tumors (for review see Refs. (Cowin et al. 2005; Okegawa et al. 2002)). During establishment of pregnancy, CDH1 and CTNNB1 may be important for maintaining the LE in a receptive state for implantation and blastocyst trophectoderm integrity during its rapid elongation to form a filamentous conceptus. Our working hypotheses are that dynamic changes

occur in assembly of tight junctions and adherens junctions in the ovine endometrium during the peri-implantation period of pregnancy to change paracellular permeability, and that assembly of these junctional complexes is regulated by hormones of pregnancy. As a first step in testing this hypothesis, we determined effects of pregnancy and P4 on tight junction and adherens junction components in the ovine uterine endometrium.

### ***Materials and Methods***

#### *Animals*

Mature ewes (*Ovis aries*) were observed for estrus (designated as Day 0) in the presence of a vasectomized ram and used in experiments only after exhibiting at least two estrous cycles of normal duration (16–18 Day). 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*

**Study One** At estrus (Day 0), ewes were mated to either an intact or vasectomized ram as described previously (Spencer et al. 1999a) and then hysterectomized (n = 5 ewes/Day) on either Day 10, 12, 14, or 16 of the estrous cycle or Day 10, 12, 14, 16, 18, or 20 of pregnancy. Pregnancy was confirmed on Day 10–16 after mating by the presence of a morphologically normal conceptus in the uterus. At hysterectomy, several sections (~0.5 cm) from the midportion of each uterine horn ipsilateral to the corpus luteum were fixed in fresh 4% paraformaldehyde in PBS (pH 7.2). After 24 h, fixed tissues were changed to 70% ethanol for 24 h and then dehydrated and embedded in Paraplast-Plus (Oxford Labware, St. Louis, MO). Several sections (1–1.5 cm) from the middle of each uterine horn were embedded in Tissue-Tek OCT compound (Miles, Oneonta, NY), frozen in liquid nitrogen vapor, and stored at –80°C. The remaining endometrium was physically dissected from myometrium, frozen in liquid nitrogen, and stored at –80°C for subsequent RNA or protein extraction. In

monovulatory pregnant ewes, uterine tissue samples were marked as either contralateral or ipsilateral to the ovary bearing the corpus luteum. No tissues from the contralateral uterine horn were used in this study.

**Study Two** At estrus (Day 0), ewes were mated to intact rams and then assigned randomly to receive daily im injections from Days 1.5 to 9 of either: 1) corn oil vehicle (CO; n = 6) or 2) 25 mg P4 (Sigma Chemical Co., St. Louis, MO; n = 6) in CO vehicle as described previously (Satterfield et al. 2006). All ewes were hysterectomized on Day 9. Uteri were processed as described in Study 1.

**Study Three** At estrus (Day 0), ewes were mated to intact rams as described previously (Satterfield et al. 2006) and then assigned randomly to receive daily intramuscular (im) injections of either: 1) CO vehicle from Days 1.5 to 12 (CO, n = 8); 2) 25 mg P4 (Sigma Chemical Co.) from Days 1.5 to 12 (P4, n = 7); or 3) 25 mg P4 (Days 1.5 to 8, n = 5) followed by 75 mg RU486 (Sigma Chemical Co.), a P4 receptor antagonist, from Days 8 to 12 (P4 + RU). All ewes were hysterectomized on Day 12, and uteri were processed as described for Study 1.

### *Immunohistochemistry*

Immunohistochemical localization of immunoreactive proteins in the ovine uteri were performed as described previously (Spencer et al. 1999c). Rabbit antibodies against tight-junction-associated proteins were purchased from Zymed Laboratories (San Francisco, CA) including: CLDN1 (no. 51–9000) used at a final concentration of 2.5 µg/ml, CLDN2 (no. 51–6100) used at a final concentration of 1 µg/ml, CLDN3 (no. 34–1700) used at 0.1 µg/ml, CLDN4 (no. 36–4800) used at 1 µg/ml, OCLN (no. 71–1500) used at 1 µg/ml, and TJP2 (no. 71–1400) used at 1 µg/ml. A Vectastain ABC antirabbit kit was used for detection of the aforementioned proteins after antigen retrieval with boiling citrate buffer as described previously (Taylor et al. 2000). Negative controls included substitution of the primary antibody with rabbit IgG at the same concentration.

Immunoreactive CTNNB1 protein was detected using an anti- $\beta$ -catenin (610153) antibody (BD Biosciences) at a final dilution of 1:250. Mouse IgG was substituted for the primary antibody as a negative control. A Vectastain ABC antimouse kit was used for detection of CTNNB1 protein after antigen retrieval with boiling citrate buffer. Immunoreactive proteins were visualized using diaminobenzidine tetrahydrochloride (Sigma Chemical Co.) as the chromagen. Sections were subsequently dehydrated and coverslips were affixed with Permount.

#### *Immunofluorescence Analyses*

Frozen sections (8  $\mu$ m) of uteri embedded in OCT compound were cut with a cryostat and mounted on Superfrost/Plus microscope slides (Fisher Scientific, Pittsburgh, PA). Using methods described previously (Choi et al. 2001; Johnson et al. 2001b), sections were fixed in  $-20^{\circ}\text{C}$  methanol, permeabilized with 0.3% Tween 20 in 0.02 M PBS, blocked in antibody dilution buffer (two parts 0.02 M PBS, 1.0% BSA, and 0.3% Tween 20, and one part glycerol) containing 10% normal goat serum, and incubated overnight at  $4^{\circ}\text{C}$  with anti-E-cadherin (CDH1) rabbit antiserum (no. 07-697; Upstate Cell Signaling Solutions, Lake Placid, NY) at a dilution of 1:350 or nonimmune rabbit serum at the same concentration. Immunoreactive protein was detected using a fluorescein-conjugated goat antirabbit IgG (Molecular Probes, Eugene, OR). Sections were then rinsed and overlaid with a coverslip and Prolong Antifade mounting reagent (Molecular Probes).

#### *RNA Isolation*

Total cellular RNA was isolated from frozen endometrium from the uterine horn ipsilateral to the corpus luteum (Studies 2 and 3) using TRIzol reagent (Life Technologies, Inc., Bethesda, MD) according to the manufacturer's recommendations. The quantity and quality of total RNA was determined by spectrometry and denaturing agarose gel electrophoresis, respectively.

### *Slot Blot Hybridization Analysis*

Steady-state levels of *CDHI* and *CTNNB1* mRNA in endometria were assessed by slot blot hybridization as described previously (Spencer et al. 1999c). Briefly, radiolabeled antisense cRNA probes were generated by *in vitro* transcription using linearized plasmid templates containing partial cDNAs, RNA polymerases, and [ $\alpha$ -<sup>32</sup>P]UTP. Denatured total endometrial RNA (20  $\mu$ g) from each ewe in Studies 2 and 3 was hybridized with radiolabeled cRNA probes. To correct for variation in total RNA loading, a duplicate RNA slot blot membrane was hybridized with radiolabeled antisense 18S cRNA (pT718S; Ambion, Austin, TX). After washing, the blots were digested with ribonuclease A, and radioactivity associated with slots was quantified using a Typhoon 8600 MultiImager (Molecular Dynamics, Piscataway, NJ). Data are expressed as relative units.

### *In Situ Hybridization Analysis*

Location of *CDHI* and *CTNNB1* mRNAs in the ovine uterus was determined by radioactive *in situ* hybridization analysis as described previously (Spencer et al. 1999c). Radiolabeled antisense and sense cRNA probes were generated by *in vitro* transcription using linearized partial plasmid cDNA templates, RNA polymerases, and [ $\alpha$ -<sup>35</sup>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 10 Day. Slides were developed in Kodak D-19 developer, counterstained with Gill's hematoxylin (Fisher Scientific, Pittsburgh, PA), and then dehydrated through a graded series of alcohol to xylene. Coverslips were then affixed with Permount (Fisher Scientific).

### *Photomicroscopy*

Photomicrographs of *in situ* hybridization and immunocytochemistry slides were taken using a Nikon Eclipse E1000 photomicroscope (Nikon Instruments, Melville, NY). Digital images were captured using a Nikon DXM 1200 digital camera with ACT-1



software and assembled using Adobe Photoshop 7.0 (Adobe Systems, Seattle, WA). Fluorescence images of representative fields were recorded using a Zeiss Axioplan2 microscope (Carl Zeiss, Thornwood, NY) with a Axiocam HR digital camera and Axiovision 4.3 software and assembled as previously described (Johnson et al. 1999a).

### *Statistical Analyses*

Data from slot blot hybridization analyses were subjected to least-squares ANOVA using the General Linear Models procedures of the Statistical Analysis System (SAS Institute, Cary, NC). Slot blot hybridization data were corrected for differences in sample loading by using the 18S rRNA data as a covariate. Data are presented as the least-squares means with overall SE.

### **Results**

#### *Tight-Junction-Associated Proteins in Ovine Endometrium (study 1)*

Immunoreactive TJP2 and OCLN proteins were abundant in endometrial LE and GE on Day 10 of the cycle and pregnancy (Figure 4.1). In both cyclic and pregnant ewes, TJP2 and OCLN proteins were markedly decreased in the LE on Day 12. In cyclic ewes, TJP2 and OCLN protein remained low in LE and GE on Day 14 and then increased on Day 16; however, in pregnant ewes, TJP2 and OCLN proteins increased in both LE and GE on Day 14 and remained abundant in those epithelia on Day 16. Immunoreactive TJP2 and OCLN proteins were also observed in the conceptus trophoctoderm from Day 18 and 20 pregnant ewes.

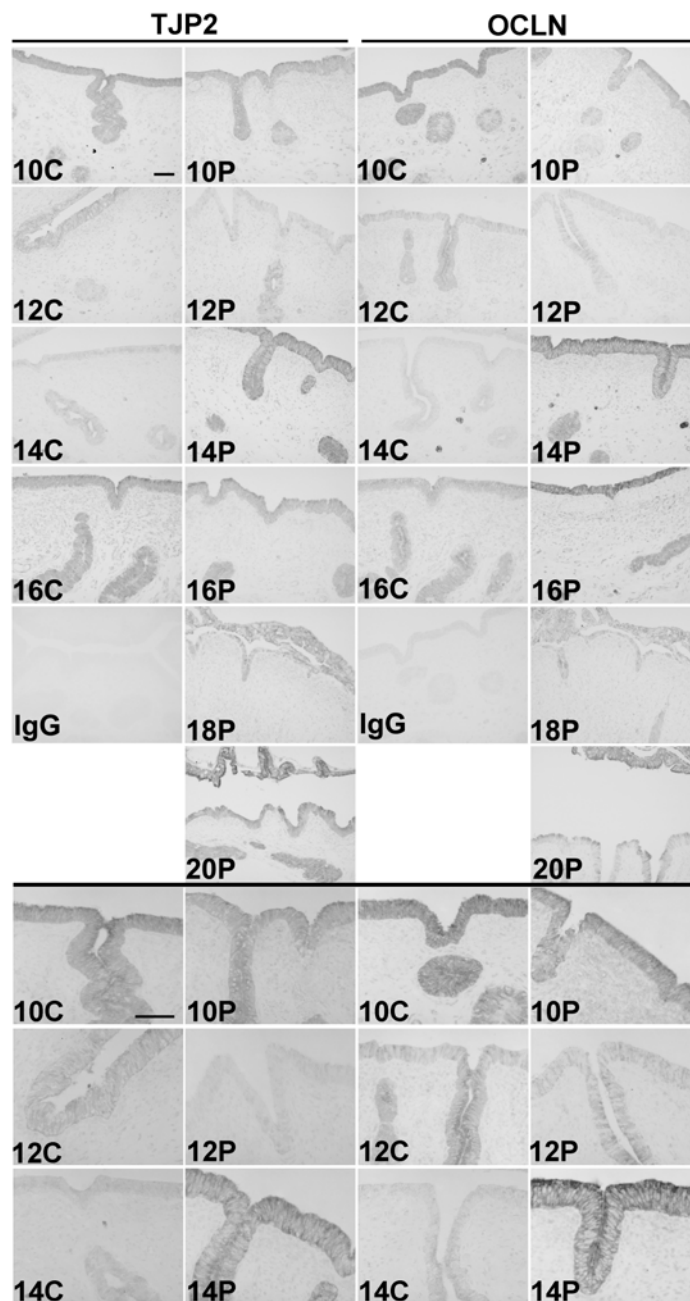


FIG. 4.1. Immunolocalization of TJP2 and OCLN proteins in the ovine endometrium during the estrous cycle and pregnancy (Study One). Immunoreactive proteins were detected using specific rabbit polyclonal antibodies against the respective proteins. For the IgG control, normal rabbit IgG was substituted for the primary antibody. Sections were not counterstained. Sections in lower panels are from areas of upper panels shown at higher magnification. Legend: LE, luminal epithelium; GE, glandular epithelium; S, stroma; Tr, trophoctoderm. Bar = 10  $\mu$ m.

Temporal and spatial alterations in the CLDN proteins were detected in uteri of cyclic and pregnant ewes (Figure 4.2). In cyclic ewes, immunoreactive CLDN1 protein decreased in the LE from Days 10 to 14 of the cycle and then increased on Day 16. There was also a decrease in CLDN1 protein in the LE between Days 10 to 12 of pregnancy, but CLDN1 protein was more abundant in the LE beginning on Days 14 to 18 and there was a marked increase in CLDN1 protein in both LE and GE on Day 20 of pregnancy. CLDN1 protein was also detected in the trophectoderm of conceptuses on both Days 18 and 20 of pregnancy.

The patterns of change in abundance of CLDN2, CLDN3, and CLDN4 proteins were similar in uteri of cyclic and pregnant ewes (Figure 4.2). The abundance of these proteins in LE and GE decreased between Day 10–12 in both cyclic and pregnant ewes. The remainder of the estrous cycle was characterized by low levels of CLDN2, CLDN3, and CLDN4 proteins on Day 14 followed by an increase on Day 16. However, in pregnant ewes, there was an increase in abundance of CLDN2, CLDN3, and CLDN4 proteins on both Days 14 and 16. A more punctate expression pattern for these proteins was observed on Days 18 and 20 of pregnancy for CLDN2 and CLDN4 while CLDN3 protein abundance increased throughout the LE and GE. CLDN2, CLDN3, and CLDN4 proteins were detected in trophectoderm of conceptuses on both Days 18 and 20 of pregnancy. For all of the proteins analyzed in this study, no differences were detected in their abundance in LE of either intercaruncular or caruncular endometrium.

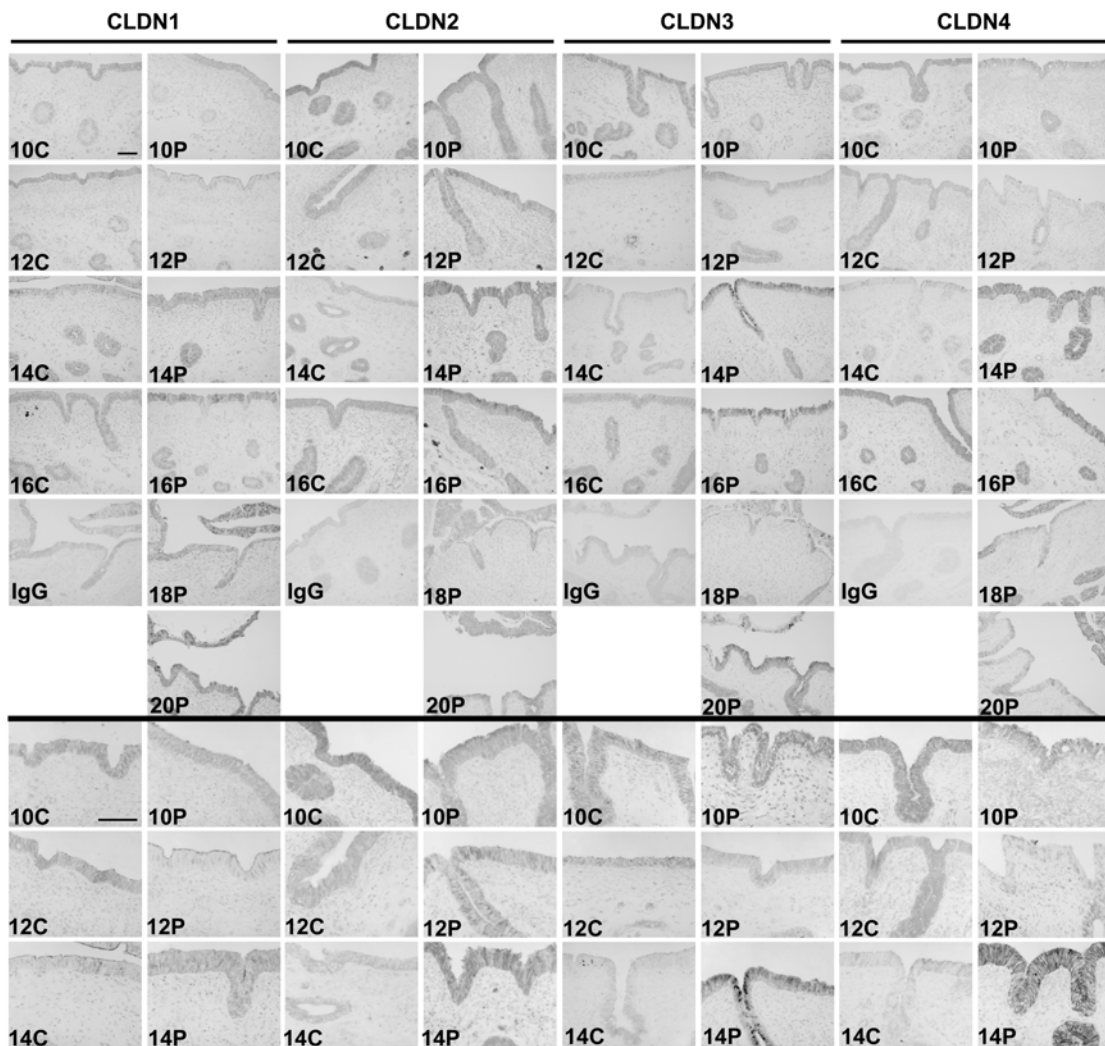


FIG. 4.2. Immunolocalization of CLDN1, CLDN2, CLDN3, and CLDN4 proteins in ovine endometria during the estrous cycle and pregnancy (Study One). Immunoreactive proteins were detected using specific rabbit polyclonal antibodies against the respective proteins. For the IgG control, normal rabbit IgG was substituted for the primary antibody. Sections were not counterstained. Sections in lower panels are from areas of upper panels shown at higher magnification. Legend: LE, luminal epithelium; GE, glandular epithelium; S, stroma; Tr, trophoblast. Bar = 10  $\mu$ m.

*CDHI and CTNNB1 mRNA (study 1)*

*CDHI* mRNA was localized to LE and GE of the ovine endometrium and was abundant on Day 10 of the estrous cycle and pregnancy (Figure 4.3). In both cyclic and pregnant ewes, *CDHI* mRNA declined between Days 10 and 14 and then increased between Days 14 and 16 of pregnancy. Abundant *CDHI* mRNA remained in endometrial LE and GE, as well as trophoctoderm of conceptuses on Days 18 and 20 of pregnancy.

Temporal and spatial changes in *CTNNB1* mRNA were also detected in ovine uteri throughout the estrous cycle and early pregnancy (Figure 4.3). *CTNNB1* mRNA was abundant in LE, GE, and stroma on Day 10 of the estrous cycle. A similar pattern was observed on Day 10 of pregnancy, but mRNA levels were lower in all cell types. By Day 12 of the cycle and pregnancy, less *CTNNB1* mRNA was detected in GE and stratum compactum stroma and, to a lesser extent, in LE. *CTNNB1* mRNAs then increased in LE and GE on Day 14 of both the cycle and pregnancy. In cyclic ewes, there was a marked decline in *CTNNB1* mRNA on Day 16 in both the LE and GE. In contrast, *CTNNB1* mRNA was abundant in uterine LE and GE of ewes between Days 16 and 20 of pregnancy, as well as in trophoctoderm of the conceptus on Days 18 and 20 of pregnancy. For all of the mRNAs and proteins analyzed in this study, there were no differences in their abundance in LE of intercaruncular or caruncular endometria.

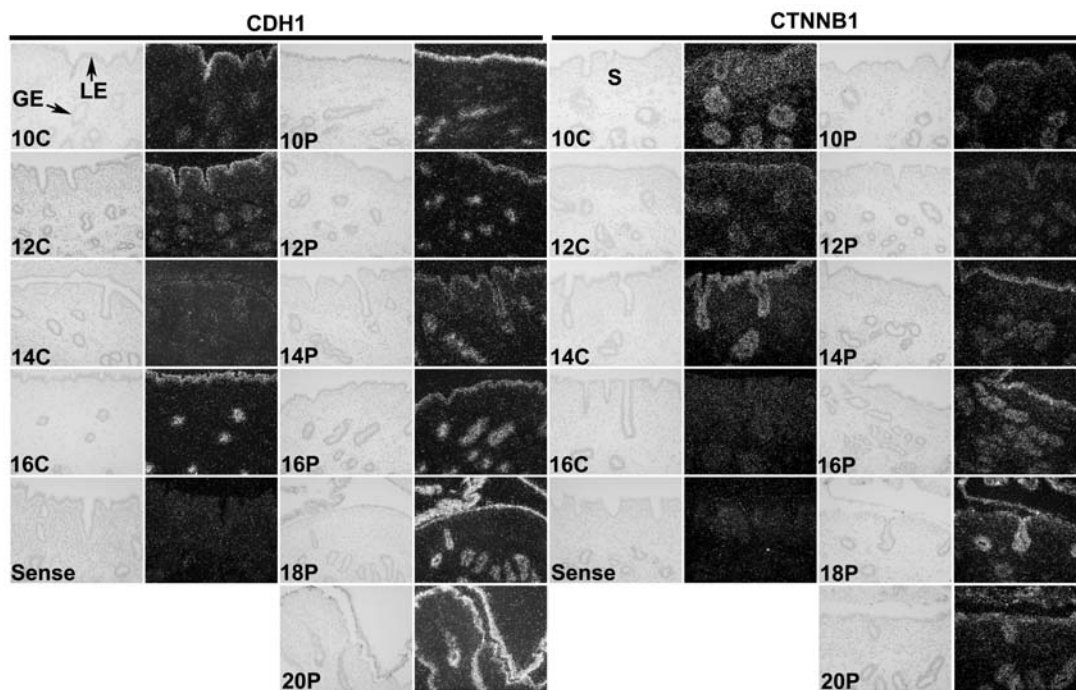


FIG. 4.3. *In situ* localization of *CDH1* and *CTNNB1* mRNAs in ovine endometria during the estrous cycle and pregnancy (Study One). Cross-sections of uteri were hybridized with radiolabeled antisense or sense ovine cRNA probes for each respective gene. The left panel is brightfield photomicrographs, and the right panel is darkfield photomicrographs. Legend: LE, luminal epithelium; GE, glandular epithelium; S, stroma; Tr, trophoctoderm. Bar = 10  $\mu$ m.

*CDH1 and CTNNB1 Protein (study 1)*

Overall, levels of CDH1 protein in the endometrium paralleled changes in *CDH1* mRNA in both cyclic and pregnant ewes (Figure 4.4). Immunoreactive CDH1 protein was particularly abundant in the endometrial GE of both cyclic and pregnant ewes and was located primarily at the apical region of epithelial cells. In cyclic ewes, CDH1 protein decreased in abundance from Days 10 to 14 and then increased to Day 16. Indeed, immunoreactive CDH1 protein was not detectable in the LE of Day 14 cyclic ewes. In pregnant ewes, CDH1 protein in the LE also decreased after Day 10, was almost undetectable on Days 14 and 16, and then increased on Day 18. Some LE in uteri of Days 18 and 20 pregnant ewes was not present due to assimilation of LE by trophoblast giant binucleate cells of the conceptus, which begin to differentiate on Days 14 to 15 of pregnancy.

Immunoreactive CTNNB1 protein was evaluated during the estrous cycle and early pregnancy by immunohistochemistry (Figure 4.4). On Day 10 of the cycle or pregnancy, CTNNB1 protein was localized predominantly in LE and GE. By Day 12 of both the cycle and pregnancy, there was a marked reduction in CTNNB1 protein, particularly in the LE and to a lesser extent in the GE. The pattern was maintained through Day 14; however, abundant CTNNB1 protein was detected in LE and GE on Day 16 of the estrous cycle. In uteri from Day 16 pregnant ewes, CTNNB1 protein increased in the GE and to a lesser and more varied extent in the LE. By Day 18 of pregnancy, more abundant levels of CTNNB1 protein were detected in endometrial GE and LE. Both mononuclear and binucleate cells of conceptus trophoderm contained high levels of CTNNB1 protein on Days 18 and 20 of pregnancy. In particular, nuclear CTNNB1 protein was present in trophoblast giant binucleate cells (data not shown). For all proteins analyzed in this study, no differences were observed in their abundance in the LE of intercaruncular and caruncular endometria.

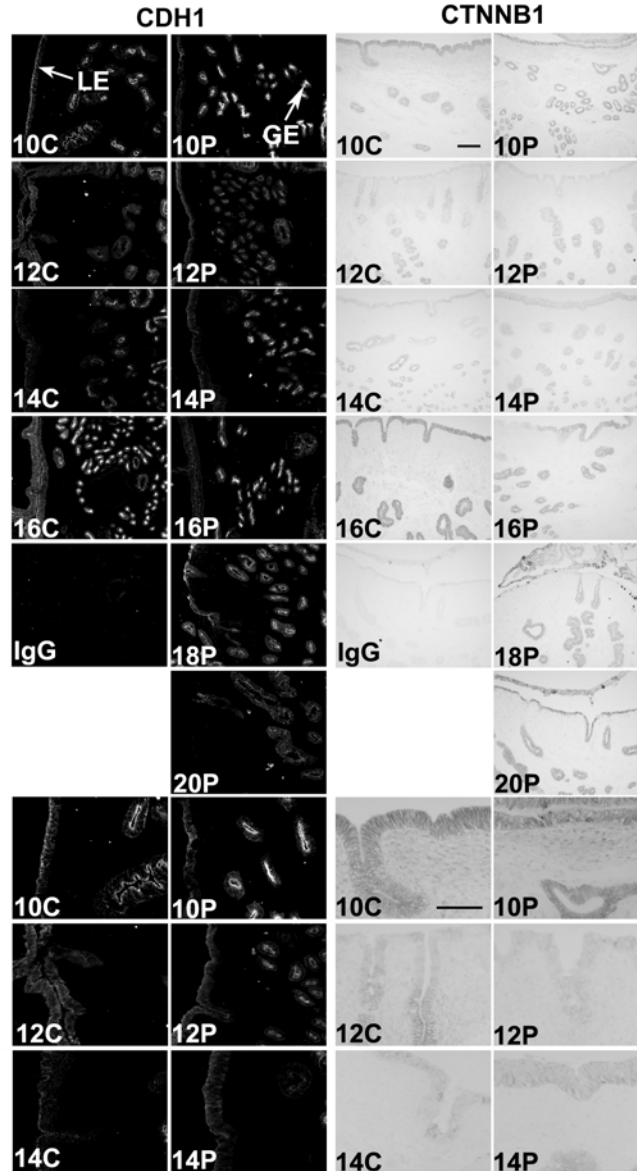


FIG. 4.4. Immunolocalization of CDH1 and CTNNB1 proteins in ovine endometria during the estrous cycle and pregnancy (Study One). Immunoreactive proteins were detected using specific rabbit polyclonal antibodies against the respective proteins. For the IgG control, normal rabbit IgG was substituted for the primary antibody. Sections were not counterstained. Legend: LE, luminal epithelium; GE, glandular epithelium; S, stroma; Tr, trophoctoderm. Bar = 10 μm.



*Progesterone Regulation of Tight-Junction-Associated Proteins (studies 2 and 3)*

To determine whether P4 regulates tight-junction-associated proteins, ewes were treated with either vehicle or P4 beginning on Day 1.5 postmating and then hysterectomized on either Day 9 or Day 12. This treatment regimen advances development of the conceptus and induces changes in gene expression in the uterine LE (Satterfield, Bazer et al. 2006). Immunoreactive TJP2 protein was most abundant in LE and GE of uteri from CO-treated ewes on Days 9 and 12 (Figure 4.5). Treatment with P4 decreased the abundance of TJP2 protein in LE compared with CO treatment, whereas treatment with P4 and RU486 (P4 + RU) increased TJP2 protein, particularly in the GE. Similar results were found for immunoreactive OCLN protein (Figure 5), although the P4-induced loss of OCLN protein was more pronounced in LE and GE on Day 12. Furthermore, treatment of ewes with P4 and RU486 increased OCLN protein in both LE and GE. Immunoreactive TJP2 and OCLN proteins were most abundant at the apical portions of the epithelial cells.

CLDN1 protein was detected at low levels in LE and GE on Day 9 in both CO- and P4-treated ewes (Figure 4.6). On Day 12, P4-treated ewes had less CLDN1 protein compared with CO controls, whereas administration of RU486 slightly increased CLDN1 protein in both LE and GE. CLDN2, CLDN3, and CLDN4 proteins were most abundant in uterine LE of CO-treated ewes on Days 9 and 12, decreased in LE of Day 12 ewes treated with P4 and were most abundant in GE of RU486-treated ewes on Day 12. For all of the proteins analyzed in this study, there were no differences in their abundance in LE of intercaruncular and caruncular endometria.

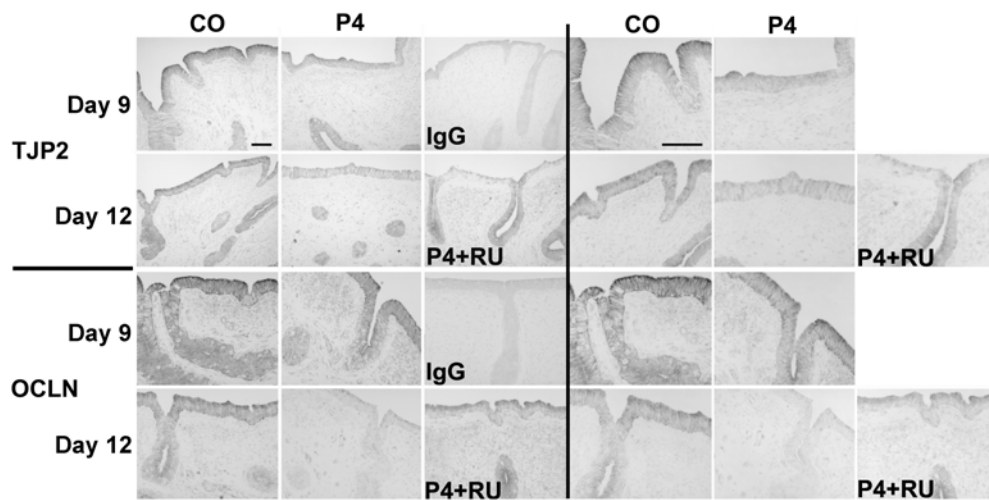


FIG. 4.5. Effects of corn oil (CO), progesterone (P4), or P4 and RU486 (P4+RU) on TJP2 and OCLN protein in endometria from Day 9 (Study Two) and Day 12 (Study Three) ewes. Immunoreactive OCLN and TJP2 proteins were detected using specific rabbit polyclonal antibodies. For the IgG control, normal rabbit IgG was substituted for the primary antibody. Sections were not counterstained. Legend: LE, luminal epithelium; GE, glandular epithelium; S, stroma. Bar = 10  $\mu$ m.

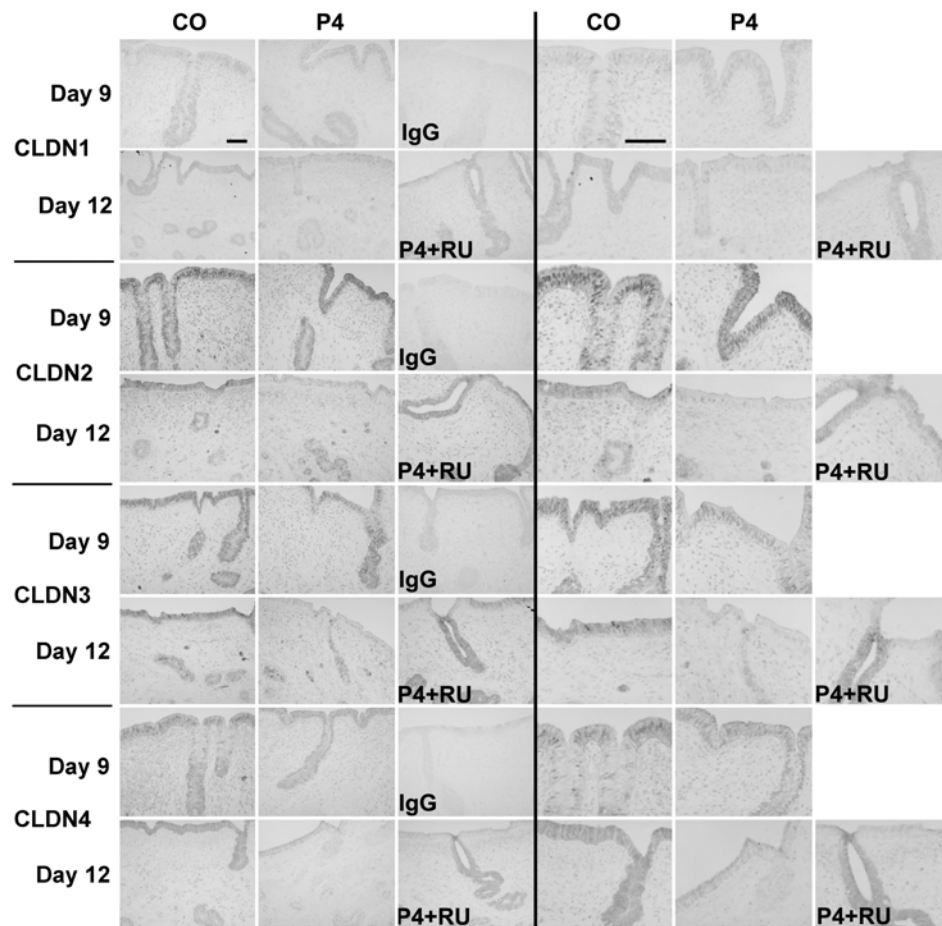


FIG. 4.6. Effects of corn oil (CO), progesterone (P4) or P4 and RU486 (P4+RU) on CLDN proteins in endometria from Day 9 (Study Two) and Day 12 (Study Three) ewes. Immunoreactive CLDNs 1, 2, 3, and 4 proteins were detected using specific rabbit polyclonal antibodies. For the IgG control, normal rabbit IgG was substituted for the primary antibody. Sections were not counterstained. Legend: LE, luminal epithelium; GE, glandular epithelium; S, stroma. Bar = 10  $\mu$ m.

### *CDH1 and CTNNB1 mRNA in Studies 2 and 3*

Steady-state levels of *CDH1* mRNA were not affected ( $P > 0.10$ ) by treatment on Day 9 or 12 (Figure 4.7A). On Day 9, endometrial *CTNNB1* mRNA was lower ( $P < 0.05$ ) in P4-treated compared with CO-treated ewes (Figure 4.7B). On Day 12, P4 treatment did not affect ( $P > 0.10$ ) *CTNNB1* mRNA in the endometrium; however, there was a 2-fold increase ( $P < 0.01$ ) in *CTNNB1* mRNA in endometria of RU486-treated ewes. *In situ* hybridization analysis identified *CTNNB1* mRNA in endometrial LE, GE and stroma of ewes (Figure 4.7C). The reduction in *CTNNB1* mRNA in endometria from Day 9 P4-treated ewes appeared to result from an overall decline in the LE, whereas RU486 treatment resulted in an increase in *CTNNB1* mRNA in Day 12 ewes in all endometrial cell types. For all of the mRNAs and proteins analyzed in this study, no differences were observed in their abundance in the LE of intercaruncular and caruncular endometria.

### *CDH1 and CTNNB1 Protein in Studies 2 and 3*

Immunoreactive CDH1 protein was less abundant in the endometrial LE of P4-treated ewes on Day 9 (Figure 4.8). However, CDH1 protein abundance in CO-treated compared with P4-treated ewes was not different on Day 12. Interestingly, the abundance of CDH1 protein decreased in LE of ewes treated with P4 and RU486 to Day 12 after onset of estrus.

Immunoreactive *CTNNB1* protein was localized predominantly in endometrial LE and GE of both CO- and P4-treated ewes on Day 9 (Figure 4.8). For CO-treated ewes, immunoreactive *CTNNB1* protein was observed in GE on Day 12, but was not detectable in LE. However, *CTNNB1* protein was very low to undetectable in GE of P4-treated ewes on Day 12. In contrast, *CTNNB1* protein was abundant in GE and detectable in LE of RU486-treated ewes on Day 12. For all of the proteins analyzed in this study, there were no differences in their abundance in LE of intercaruncular and caruncular endometria.

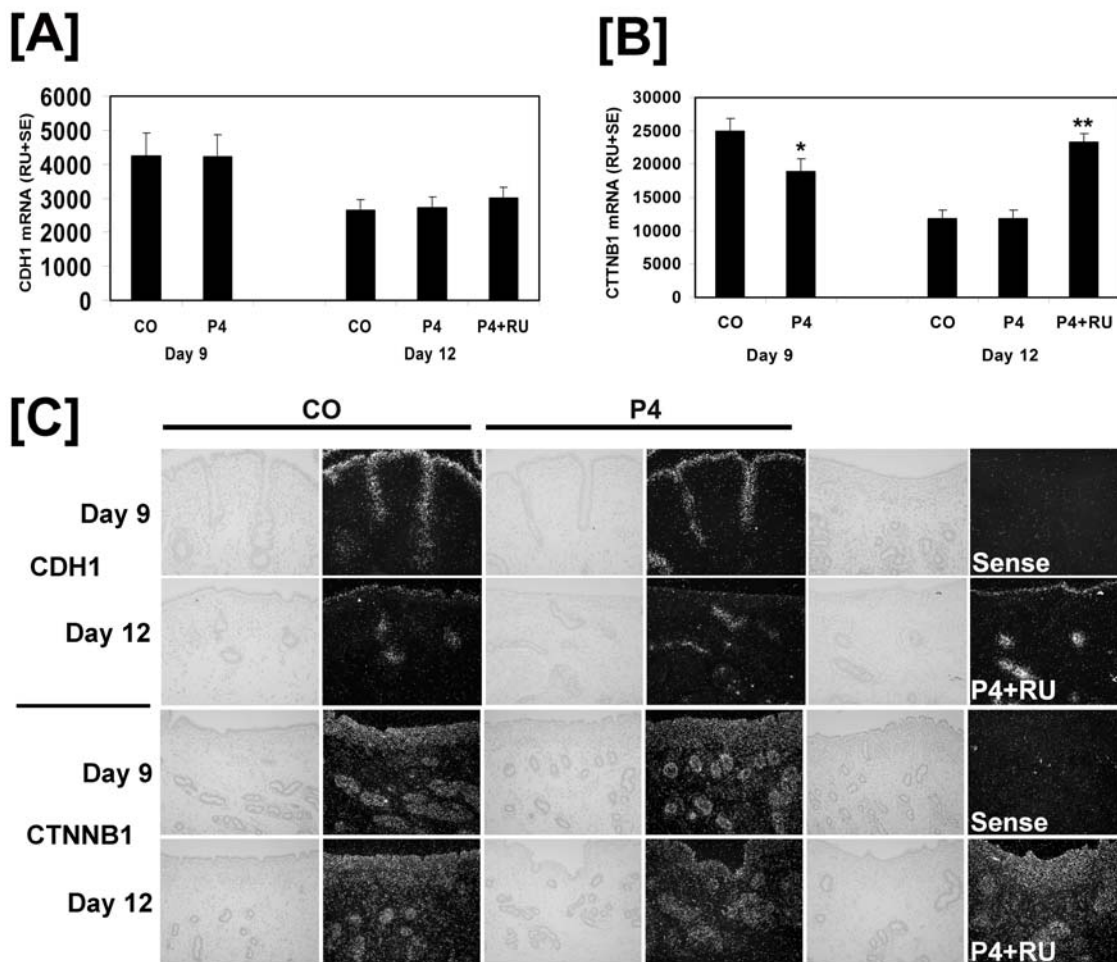


FIG. 4.7. Effects of corn oil (CO), progesterone (P4) or P4 and RU486 (P4+RU) on *CDH1* and *CTNNB1* mRNA in endometria from Day 9 (Study Two) and Day 12 (Study Three) ewes. (A & B) Steady-state levels of mRNAs were determined by slot blot hybridization analysis and are presented as relative units (RU) with standard error (SE). (C) Cross-sections of uteri were hybridized with radiolabeled antisense or sense ovine cRNA probes for each respective gene. Legend: LE, luminal epithelium; GE, glandular epithelium; S, stroma. Bar = 10  $\mu$ m.

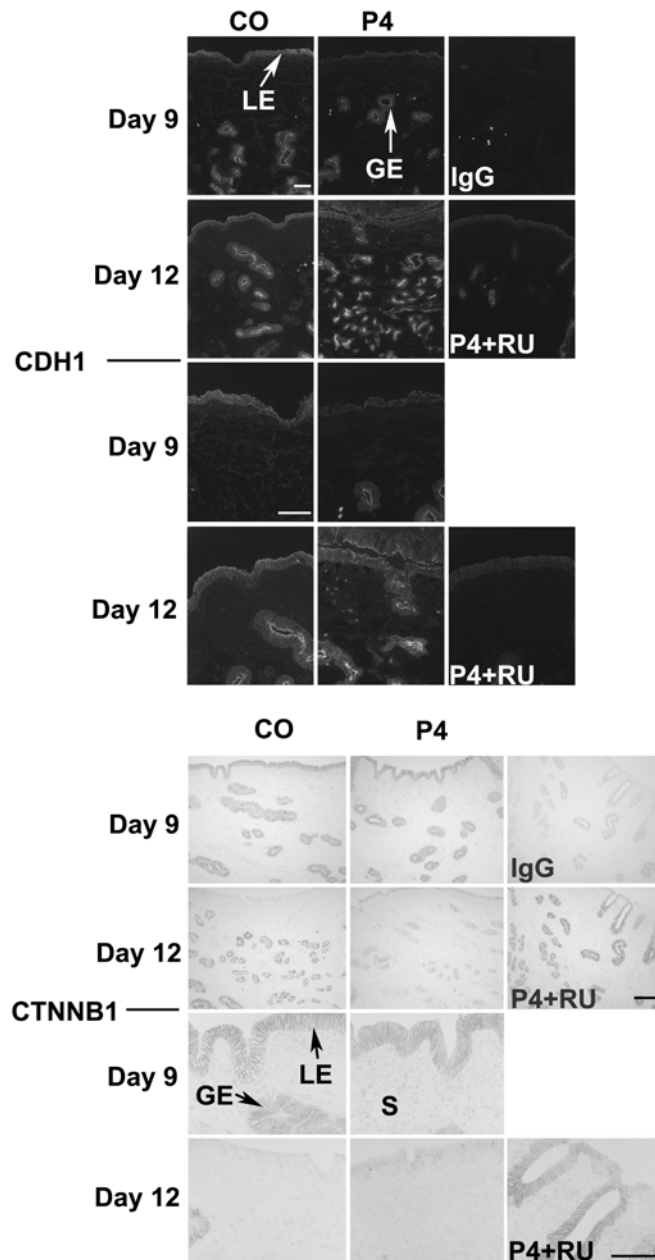


FIG. 4.8. Effects of corn oil (CO), progesterone (P4) or P4 and RU486 (P4+RU) on CDH1 and CTNNB1 protein in endometria from Day 9 (Study Two) and Day 12 (Study Three) ewes. Immunoreactive protein was detected using specific rabbit polyclonal antibodies. For the IgG control, normal rabbit IgG was substituted for the primary antibody. Sections were not counterstained. Legend: LE, luminal epithelium; GE, glandular epithelium; S, stroma. Bar = 10  $\mu$ m.

## ***Discussion***

Domestic animals are characterized as being nonmenstruating and having conceptuses that undergo a noninvasive type of implantation. These two distinguishing characteristics indicate the unique importance of and requirement for elasticity of function of uterine epithelia that must adapt to establish the appropriate microenvironment based on the physiological status of the animal. These adaptations are acutely monitored in response to appropriately timed cues from the ovary and paracrine effectors from the adjacent stroma and conceptus. In sheep, the spherical hatched blastocyst on Day 8 expands to Day 10 and then begins a morphological change to a tubular conceptus by Day 12 and subsequently undergoes rapid elongation into a filamentous conceptus by Day 14 (Rowson and Moor 1966a; Spencer et al. 2004a). The developing ovine conceptus undergoes elongation while maintaining superficial contact with the receptive LE before firm adhesion by Day 18 at which point the conceptus can no longer be easily flushed from the uterine lumen. Elongation of the conceptus trophoderm is required for the production of sufficient quantities of IFNT to elicit maternal recognition of pregnancy and inhibit development of the luteolytic mechanism (Farin et al. 1989; Gray et al. 2002; Guillomot et al. 1990; Spencer and Bazer 2004; Vallet et al. 1988). Beginning on Days 14 to 15, trophoblast giant binucleate cells begin differentiating from the mononuclear trophoderm cells in the conceptus and then migrate and fuse with the endometrial LE in both the caruncular and intercaruncular areas of the uterus and with each other to form multinucleated syncytia (Wooding 1984). These peri-implantation events begin synepitheliochorial placentation in ruminants.

Establishment and maintenance of the microenvironment within the uterine lumen to support pregnancy requires that epithelia serve as a selective transporter and/or barrier. Junctional complexes within the epithelia maintain this unique microenvironment by regulating the passage of water, ions, and other small molecules, regulating localization of various transporters within the epithelia to maintain appropriate molecular gradients, and changing cell shape and polarity to alter cell to cell communication and function. Results from the present studies on changes in endometrial

tight-junction and adherens-associated proteins provide insight into the functional requirements of the epithelia for the maintenance of pregnancy in sheep. All tight-junction- and adherens-associated proteins were moderately to abundantly present in the endometrial epithelia on Day 10 of the estrous cycle and pregnancy; however, by Day 12, the junctional proteins in the LE decreased to very low or undetectable levels, resulting in a potentially "leaky" paracellular space for transport of molecules from the uterine stroma and/or selective movement of serum transudate directly to the conceptus. This decline in tight junction and adherens junction-associated proteins would theoretically decrease tight junctions and increase the availability of stromal-derived factors, such as hepatocyte growth factor, fibroblast growth factor 7, and insulin-like growth factors (IGF1 and IGF2), as well as factors from serum, such as glucose, insulin, and essential amino acids, in the uterine lumen. Indeed, many of the proteins present in the uterine lumen are not directly synthesized by the endometrial epithelia or conceptus (Lee et al. 1998b). During early pregnancy, tubular blastocysts begin to elongate on Day 12 to form filamentous conceptuses, which involves proliferation and migration of trophoblast cells. Thus, accelerated blastocyst growth may result, in fact, from the influx of factors into the uterine lumen through the paracellular space as integrity of tight and adherens junctions decreases. Indeed, blastocyst elongation and formation of a filamentous conceptus has not been achieved *in vitro*.

The changes in tight junction and adherens junction-associated proteins observed in the present study are hypothesized to be promulgated by P4. In both cyclic and pregnant ewes, P4 receptors (PGR) decline to undetectable levels in endometrial LE and GE between Days 10 and 12 after onset of estrus (Spencer and Bazer 1995; Wathes and Hamon 1993; Wathes and Lamming 1995). This loss of PGR is induced by exposure of the endometrium to P4 for 8 to 10 days. Indeed, early P4 administration accelerates the loss of PGR from LE and GE, advances onset of expression of P4-stimulated genes such as galectin-15, and enhances blastocyst growth and development (Satterfield et al. 2006). Results from Studies 2 and 3 regarding analysis of tight-junction-associated proteins revealed that early P4 stimulated a decline in tight junction and adherens junction-



associated proteins in ovine uterine epithelia. This decline occurred in both cyclic and pregnant ewes because maternal recognition of pregnancy does not occur until Days 12 to 13. In fact, Guillomot *et al.* (Guillomot *et al.* 1986; Guillomot *et al.* 1981) observed that horseradish peroxidase injected into the uterine lumen of pregnant ewes and cows accumulated in the intracellular spaces beneath the basement membrane and in the stroma. This transport was mediated via transepithelial endocytotic activity (vesicles) as well as paracellular permeability and passage through intercellular spaces between tight junctions. These phenomena were especially marked when circulating concentrations of P4 were high during late diestrus when PGR are absent from endometrial LE and GE.

In addition to P4, the presence of the conceptus-affected tight-junction-associated proteins in the uterine LE. Consistently, tight-junction-associated proteins were much more abundant in the LE of pregnant ewes compared with cyclic ewes on Day 14, suggesting that a conceptus-derived factor stimulates tight-junction-associated proteins within the LE. During decidualization in mice, a stromal barrier surrounding the invading blastocyst uncharacteristically expresses tight-junction-associated proteins, which are believed to maintain an immunologically privileged environment assisting in the maintenance of pregnancy (Paria *et al.* 1999; Wang *et al.* 2004). Indeed, a similar mechanism may exist in sheep, whereby the developing conceptus signals its presence and produces a factor that stimulates tight junction formation that acts as a functional barrier to the maternal immune system. Simultaneously, reducing paracellular permeability of the LE may facilitate pregnancy recognition, allowing for sufficient quantities of IFNT to act on the LE and GE to inhibit luteolysis and induce genes encoding secreted proteins, such as galectin-15, cathepsin L, WNT7A, and cystatin C that may stimulate conceptus development and implantation. Furthermore, tight junction formation may maintain IFNT-induced or stimulated proteins at high levels in the uterine lumen. Expression of these tight-junction-associated proteins remains high to Day 20. The conceptus trophoctoderm also expresses high levels of tight-junction-associated proteins, which is not unexpected because a sophisticated cellular organization is

required to facilitate the rapid morphological and developmental changes during this period.

In the present studies, CDH1 and CTNNB1 mRNAs and proteins were abundant in the endometrial LE on Day 10 of the estrous cycle and pregnancy and then decreased in subsequent days corresponding with conceptus elongation. The abundance of *CDH1* and *CTNNB1* mRNAs increased on Days 16 and 14 of pregnancy, respectively. However, changes in levels of CDH1 protein within the LE did not parallel that of CDH1 mRNA suggesting posttranscriptional regulation of CDH1 levels. This phenomenon, based on discordant levels of mRNA and protein, began on Day 10 and continued on Day 12 when CDH1 protein was significantly lower than mRNA levels within LE, but not GE. The mechanism responsible for this decline in CDH1 protein is not known, but may be physiologically important for the initiation of the implantation process. Indeed, in rats, CDH1 is lost from the epithelium before invasion of the blastocyst into the endometrial stroma. These studies indicated a role for calcitonin in both *in vitro* and *in vivo* regulation of CDH1 expression during the period of implantation (Li et al. 2002). However in mice, the LE expresses abundant CDH1, which is highly localized to the apical domain at the implantation sites, whereas CDH1 is more diffusely localized at the inter-implantation sites (Jha et al. 2006). As the blastocyst begins to invade into the underlying stroma, these cells also uncharacteristically express CDH1 (Jha et al. 2006; Paria et al. 1999). Similarly, CTNNB1 protein declines from Days 10 to 14 within LE and to a lesser extent within GE, further supporting the decline in adherens junctions during the elongation process. In mice, inhibition of CTNNB1 signaling in both the developing blastocyst and uterine endometrium may have a role in synchronizing preimplantation embryonic development (Li et al. 2005). A similar mechanism may exist in sheep to synchronize events associated with the elongating conceptus and endometrial functions during this critical morphological transition. Results from this study indicate that two components of adherens junctions decline during the elongation process only to increase as the conceptus initiates implantation. The decline in adherens junction proteins also corresponds with loss of PGR from LE and subsequent loss of the

antiadhesive glyocalyx molecule, mucin 1 (Johnson et al. 2001a). These events may act in concert to allow the LE to become receptive to direct contact with conceptus trophoctoderm enabling the elongation process to be initiated. The presence of abundant quantities of CDH1 protein and moderate levels of CTNNB1 protein within the GE throughout the estrous cycle and pregnancy results in a polarized epithelium that can more efficiently secrete uterine histotroph. Previous research using the ovine uterine gland knockout ewe model established the requirement for secretions from the ovine uterine GE for conceptus survival beyond Day 12–14 (Gray et al. 2001c). Increases in CDH1 and CTNNB1 proteins in the LE from Days 16 to 20 of pregnancy in ewes may be in response to aggressive adhesion and attachment of the elongating ovine conceptus to restrict invasion of the trophoctoderm, thus maintaining appropriate contact for placental development and inhibiting adverse immunological reactions by the maternal endometrium.

Adherens junction proteins, in a manner similar to tight junctions, return on Day 16 of the cycle as the ewe returns to estrus. A highly polarized epithelium exhibiting a high degree of cell-to-cell contact may be necessary to provide a microenvironment suitable for capacitating sperm, as well as structural stability necessary for the high contractile rate of this tissue and the high degree of water imbibition occurring at estrus. Results of Studies 2 and 3 illustrate a subtle, but potentially physiologically relevant discord between *CDH1* mRNA and protein. Although *CDH1* mRNAs are not regulated by administration of P4 or blockade of its action, clearly P4 treatment resulted in a decline in CDH1 protein within the LE on Day 9 and a decline after RU486 administration on Day 12. *CTNNB1* mRNA and protein decreased in response to P4 administration to Day 9 and this may result in adherens junction breakdown and a decline in CDH1 protein as well. A decline in CDH1 and CTNNB1 within the LE on Day 9 could be hypothesized to be part of the cascade of epithelial alterations associated with loss of PGR from LE prior to implantation.

Collectively, results of the present study identify regulated changes within the epithelial architecture that modulate various functions of uterine epithelia. These

organizational changes are under the control of both steroid hormones and conceptus-derived factors. Future studies will aim to identify additional constituents of the uterine microenvironment derived from nonepithelial sources via epithelial paracellular transport to enhance conceptus growth and development. Identification of critical constituents of the uterine milieu of nonepithelial origin will give further insight into the role of the underlying stromal compartments in supporting growth and survival of the conceptus and ultimately decreasing periimplantation pregnancy losses.

**CHAPTER V**  
**PROGESTERONE REGULATION OF THE ENDOMETRIAL WNT SYSTEM**  
**IN THE OVINE UTERUS**

***Introduction***

In mammals, the period of uterine receptivity to the developing blastocyst is marked by specific histoarchitectural changes in the endometrial luminal (LE) and glandular epithelia (GE) (Burghardt et al. 2002; Carson et al. 2000; Satterfield et al. 2007; Spencer et al. 2004a; Tranguch 2005). Many of these changes occur coordinately with loss of progesterone receptors (PGR) from these epithelia, a phenomenon observed in all mammalian species studied to date (Brenner et al. 1991; Geisert et al. 1994; Lessey et al. 1988; Spencer and Bazer 1995; Spencer et al. 2004b; Tan et al. 1999). In sheep, 8 to 10 days of continuous progesterone (P4) exposure elicits loss of the PGR protein from the LE by Day 12 post-estrus followed by loss within the GE by Day 14 (Spencer and Bazer 1995) that is temporally associated with the onset of peri-implantation blastocyst elongation beginning on Day 12 to form a filamentous conceptus (Guillomot 1995). Indeed, blastocyst growth and development can be accelerated by prematurely increasing circulating levels of P4 in ruminants (Garrett et al. 1988b; Satterfield et al. 2006). In sheep, exogenous P4 administered from 36 h post-mating increased blastocyst diameter on Day 9 and accelerated elongation to a filamentous conceptus by Day 12 that was correlated with loss of PGR within the LE and GE and induction of several genes hypothesized to stimulate conceptus growth and development including *galectin 15 (LGALS15)* and *cathepsin L (CTSL)* (Satterfield et al. 2006). In addition to induction of genes encoding secreted proteins, components of adherens and tight junctions in the endometrial LE were reduced by P4 treatment (Satterfield et al. 2007), which likely results in altered intracellular signaling and cell to cell communication as well as increased histotroph in the uterine lumen (Li et al. 2002; Orchard and Murphy 2002; Paria et al. 1999; Wang et al. 2004). Recently, the WNT system has been implicated in the regulation of cell polarity and junctional complexes and their associated proteins

(Karner et al. 2006a; Karner et al. 2006b). Moreover, many components of the WNT system expressed in the ovine uterine endometrium may modulate trophectoderm migration and proliferation (Hayashi et al. 2007).

WNT genes are homologous to the *Drosophila* segment polarity gene wingless (*wg*). In humans and mice, the WNT family encodes a group of 19 highly conserved secreted glycoproteins that regulate cell and tissue growth and differentiation as well as components of junctional complexes (Davies 2002; Davies and Fisher 2002; Robinson et al. 2000). WNTs associate with their extracellular surface receptors, frizzled (FZD), in both an autocrine and paracrine manner, to mediate intracellular signal transduction pathways (Dale 1998). The ten FZDs are a family of seven transmembrane G protein-coupled receptors that possess an extracellular cysteine-rich domain for WNT binding (Liu et al. 1999; Wang et al. 1996). FZD then binds to low-density lipoprotein receptor-related proteins 5 and 6 (LRP5/6) which serve as coreceptors (Pinson et al. 2000). Another family of proteins, termed secreted FZD-related proteins (SFRPs) produced by six different genes, exerts inhibitory effects on WNT signaling by competing with WNT ligands for the FZD receptor and forming a nonfunctional complex with FZDs in a dominant-negative manner (Bafico et al. 1999; Kawano and Kypta 2003). WNT signaling is also antagonized by four Dickkopf (DKK) genes which encode secreted proteins that bind the LRP coreceptor (Nusse 2001). In the canonical WNT signaling pathway, FZD receptors inhibit glycogen synthase kinase 3 beta (GSK3B), resulting in stabilization of the transcriptional regulator beta-catenin (CTNNB1) (Logan and Nusse 2004; Pandur et al. 2002b). Nuclear CTNNB1 interacts with transcription factors, most notably members of the transcription factor 7 (T-cell specific, HMG-box) (TCF7/LEF) family, to regulate transcription of genes such as *JUN* (c-Jun) (Mann et al. 1999), *LEF1/TCF7* (Roose et al. 1999), *MET* (c-Met) (Boon et al. 2002), and *MSX2* (msh homeobox homolog 2) (Hussein et al. 2003; Willert et al. 2002). The noncanonical or planar cell polarity pathways, activated by *WNT5A* and *WNT11*, mediate cell polarity, cell movements during gastrulation, and other processes modifying the actin cytoskeleton via small GTPases of the Rho family, such as Rho, Rac, and Cdc42

(Huelsenken and Birchmeier 2001; Kuhl et al. 2000; Pandur et al. 2002a; Yamanaka et al. 2002). Rac activation stimulates c-Jun N-terminal kinase (JNK) activity (Boutros et al. 1998; Habas et al. 2003; Veeman et al. 2003). JNK activation plays essential roles in organogenesis by regulating cell survival, apoptosis, and proliferation (Nishina et al. 2004).

A recent encompassing study cataloged members of the WNT signaling family and a number of downstream target genes present in the ovine endometrium and identified roles for WNT signaling in regulation of trophoctoderm migration and proliferation (Hayashi et al. 2007). We hypothesize that P4 actions on the ovine uterus include a transient reduction in various components of the WNT signaling system that, in turn, reduces the expression of genes encoding tight junction associated proteins and adherens junctions, thereby increasing the availability of histotroph and stimulating blastocyst growth and development (Satterfield et al. 2007). Thus, this study evaluated the WNT system in an ovine model of advanced blastocyst growth and development reported by our laboratory (Satterfield et al. 2006).

## ***Material and Methods***

### ***Animals***

Mature Suffolk-type ewes (*Ovis aries*) were observed for estrus (designated as Day 0) in the presence of a vasectomized ram and used in experiments only after exhibiting at least two estrous cycles of normal duration (16-18 days). 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***

***Study One*** As described previously (Satterfield et al. 2006), ewes were mated at estrus to intact Suffolk rams and then assigned randomly to receive daily i.m. injections from Days 1.5 to 9 of either corn oil vehicle CO (n=6); or 25 mg progesterone (P4, n=6;

Sigma Chemical Co., St. Louis, MO). All ewes were hysterectomized on Day 9. At hysterectomy, sections (~0.5 cm) from the mid-portion 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 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 was physically dissected from myometrium, frozen in liquid nitrogen, and stored at -80°C for subsequent RNA or protein extraction. In monovulatory pregnant ewes, uterine tissue samples were marked as either contralateral or ipsilateral to the ovary bearing the CL. No tissues from the contralateral uterine horn were used for further analysis.

**Study Two** As described previously (Satterfield et al. 2006), ewes were mated at estrus to intact Suffolk rams and assigned randomly to receive daily intramuscular (i.m.) injections of either: (a) CO vehicle from Days 1.5 to 12 (n=8); (b) 25 mg P4 from Days 1.5 to 12 (n=7); or (c) 25 mg P4 from Days 1.5 to 12 and 75 mg RU486 (Sigma), a PGR antagonist, from Days 8 to 12 (P4+RU, n=5). All ewes were hysterectomized on Day 12, and the uteri were processed as described for Study One.

#### *RNA Isolation*

Total cellular RNA was isolated from frozen ipsilateral endometrium using Trizol reagent (Gibco-BRL, Bethesda, MD) according to manufacturer's recommendations. The quantity and quality of total RNA was determined by spectrometry and denaturing agarose gel electrophoresis, respectively.

#### *Slot Blot Hybridization Analysis*

Steady-state levels of *WNT2*, *WNT4*, *WNT5A*, *DKK1*, *LEF1*, *MSX1*, and *MSX2* mRNA in endometria were assessed by slot blot hybridization as described previously (Spencer et al. 1999c). Briefly, radiolabeled antisense ovine cRNA probes (Hayashi et al. 2007) were generated by *in vitro* transcription using linearized plasmid templates containing partial cDNAs, RNA polymerases, and [ $\alpha$ -<sup>32</sup>P]-UTP. Denatured total



endometrial RNA (20 µg) from each ewe in Studies One and Two was hybridized with radiolabeled cRNA probes. To correct for variation in total RNA loading, a duplicate RNA slot blot membrane was hybridized with radiolabeled antisense 18S cRNA (pT718S; Ambion, Austin, TX). Following washing, the blots were digested with ribonuclease A and radioactivity associated with slots was quantified using a Typhoon 8600 MultiImager (Molecular Dynamics, Piscataway, NJ). Data are expressed as relative units (RU) with overall standard errors (SE).

#### *In Situ Hybridization Analysis*

Cell-specific location of *WNT2*, *WNT2B*, *WNT4*, *WNT5A*, *WNT5B*, *WNT7A*, *WNT11*, *FZD6*, *DKK1*, *GSK3B*, *LEF1*, *MSX1*, and *MSX2* mRNAs in the ovine uteri was determined by radioactive *in situ* hybridization analysis as described previously (Spencer et al. 1999c). Radiolabeled antisense and sense ovine cRNA probes were generated by *in vitro* transcription using linearized partial plasmid cDNA templates (Hayashi et al. 2007), RNA polymerases, and [ $\alpha$ -<sup>35</sup>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 between 10 and 60 days determined by intensity of radioactive signal of slides placed on MR Kodak film for 16 h. All slides for each respective gene were exposed to photographic emulsion for the same period of time. Slides were developed in Kodak D-19 developer, counterstained with Gill's hematoxylin (Fisher Scientific, Fairlawn, NJ), and then dehydrated through a graded series of alcohol to xylene. Coverslips were then affixed with Permount (Fisher). Images of representative fields were recorded under brightfield or darkfield illumination using a Nikon Eclipse 1000 photomicroscope (Nikon Instruments Inc., Lewisville, TX) fitted with a Nikon DXM1200 digital camera.

### *Semi-Quantitative RT-PCR Analysis*

*WNT7A* and *WNT11* mRNA levels in ovine endometria were assessed using semi-quantitative RT-PCR as described previously (Stewart et al. 2000). Briefly, endometrial total RNA was treated with RQ1 RNase Free-DNase1 (Promega) and then ethanol-precipitated. The cDNA was synthesized from total RNA (5 µg) using random and oligo (dT) primers and SuperScript II Reverse Transcriptase (Life Technologies). Newly synthesized cDNA was acid-ethanol precipitated, resuspended in 20 µl sterile water, and stored at -20°C. The cDNAs were diluted (1:10) in sterile water before use in PCR. Primers for *WNT7A* and *WNT11* were derived from conserved sequences of the porcine and bovine gene, respectively using primer 3 (Rozen and Skaletsky 2000). Primer sequences for *WNT7A* were (Forward) 5'-CCTGGAGGAGAACATGAAGC-3' and (Reverse) 5'-CAGTAA TTGGGTGACTTCTCG-3' and for *WNT11* were (Forward) 5'-TTCCCGATGCTCCTATGAAGG-3' and (Reverse) 5'-ACAGCACATGAGG TCACAAGC-3' which amplified a 261 bp and 430 bp fragment, respectively. The PCR amplification was conducted as follows for *WNT7A* and *WNT11*: (1) 95°C for 5 min; (2) 95°C for 30s, 52°C for 40s (for *WNT7A*), 55°C for 40s (for *WNT11*), and 72°C for 1 min for 32 cycles; and (3) 72°C for 10 min. Housekeeping beta actin (*ACTB*) primers were forward (5'-ATGAA GATCCTCACGGAACG-3') and reverse (5'-GAAGGTGGTCTCGTGAATGC-3'), which amplified a 270 bp product. PCR amplification was conducted as follows for *ACTB*: (1) 95°C for 5 min; (2) 95°C for 30 s, 57°C for 30 s, and 72°C for 1 min for 25 cycles; and (3) 72 °C for 10 min. After PCR, equal amounts of reaction product were analyzed using a 1.5% agarose gel, and PCR products were visualized using ethidium bromide staining. The amount of DNA present was quantified by measuring the intensity of light emitted from correctly sized bands under u.v. light using a ChemiDoc EQ system and Quantity One software (Bio-Rad). Amplified PCR products of the predicted size were subcloned into the pCRII cloning vector using a T/A Cloning kit (Invitrogen, Carlsbad, CA) and sequenced in both directions using an ABI PRISM Dye Terminator Cycle Sequencing kit and ABI PRISM automated DNA sequencer (Applied Biosystems, Foster City, CA) to confirm identity.

### *Immunohistochemistry*

Immunocytochemical localization of immunoreactive JUN, phosphorylated JUN (p-JUN), CREB, p-CREB, and TCF7L2 protein in the ovine uterus was performed as described previously (Gray et al. 2004; Hayashi et al. 2007; Taylor et al. 2000). Proteins were localized using the following antibodies: rabbit Anti-c-JUN (06-225) at 1:2000, rabbit Anti-phospho-c-JUN (06-659) at 1:200, rabbit Anti-CREB (06-863) at 1:500, and rabbit Anti-phospho-CREB (06-519) at 1:1000 at 1:300 (Upstate Cell Signaling Solutions, Lake Placid, NY). Negative controls for these antibodies included substitution of the primary antibody with non-immune rabbit IgG (Sigma). Immunoreactive TCF7L2 protein was localized using a mouse Anti-TCF4 monoclonal antibody (05-511; Upstate Cell Signaling Solutions, Lake Placid, NY) at a 1:2000 final dilution. Negative controls for TFC7L2 included substitution of the primary antibody with mouse IgG (Sigma). A Vectastain ABC anti-rabbit or anti-mouse kit (Vector Laboratories, Burlingame, CA) was utilized for detection of all proteins following antigen retrieval with boiling citrate buffer as described previously (Taylor et al. 2000). Immunoreactive protein was visualized using diaminobenzidine tetrahydrochloride (Sigma) as the chromagen. Sections were dehydrated and coverslips affixed with Permount (Fisher).

### *Photomicroscopy*

Photomicrographs of *in situ* hybridization and immunocytochemistry slides were taken using a Nikon Eclipse E1000 photomicroscope (Nikon Instruments, Melville, NY). Digital images were captured using a Nikon DXM 1200 digital camera and assembled using Adobe Photoshop 7.0 (Adobe Systems, Seattle, WA).

### *Statistical Analyses*

Data from slot blot hybridization and RT-PCR analyses were subjected to least-squares analysis of variance using the General Linear Models procedures of the Statistical Analysis System (SAS Institute, Cary, NC). Slot blot hybridization data were

corrected for differences in sample loading by using the 18S rRNA data as a covariate. Data are presented as the least-squares means (LSM) with overall standard error (SE). Analysis of RT-PCR data incorporated the ACTB values as a covariate in the statistical model to correct for differences in the amounts of reverse transcriptase cDNA analyzed for each uterus. In all analyses, error terms used in tests of significance were identified according to the expectation of the mean squares for error.

## **Results**

### *Progesterone Regulation of Non-Canonical WNT mRNAs*

WNT5A, WNT5B, and WNT11 are members of the WNT family that signal via the non-canonical or planar cell polarity pathway signaling molecules (Logan and Nusse 2004) and are expressed in ovine uterine endometrium during early pregnancy (Hayashi et al. 2007). In Study One, steady-state levels of *WNT5A* mRNA in endometria were not different ( $P>0.10$ ) between CO- and P4-treated ewes (Figure 5.1A). *WNT5A* mRNA was detected in both LE and stromal cells of the endometrium with the preponderance of *WNT5A* mRNA in *stratum compactum* stromal cells underlying LE (Figure 5.1C). In Study Two, endometrial *WNT5A* mRNA levels were not different ( $P>0.10$ ) between CO- and P4-treated ewes on Day 12 (Figure 5.1A); however, *in situ* hybridization analyses revealed that *WNT5A* mRNA was clearly reduced in LE of both CO- and P4-treated ewes on Day 12 as compared to endometria of CO- and P4-treated ewes on Day 9. The endometrium of P4+RU-treated ewes had increased ( $P<0.03$ ) endometrial *WNT5A* mRNA levels on Day 12 (Figure 5.1A), and *WNT5A* mRNA was present in stromal cells and LE (Figure 5.1C). *WNT5B* mRNA was detected in both LE and stromal cells (data not shown). Endometrial *WNT5B* mRNA levels were not affected ( $P>0.10$ ) by treatment (data not shown).

Although steady-state levels of *WNT11* mRNA did not differ ( $P>0.10$ ) between endometria of CO- and P4-treated ewes on Day 9 (Figure 5.1B), *in situ* hybridization analyses revealed a clear reduction or absence of *WNT11* mRNA in LE of endometria from P4-treated ewes (Figure 5.1C). Endometrial *WNT11* mRNA levels were lower

( $P < 0.005$ ) in P4- as compared to CO-treated ewes on Day 9 and also in Day 12 as compared to Day 9 ewes regardless of treatment (Figure 5.1B). *In situ* hybridization revealed that *WNT11* mRNA was reduced in GE of both P4 and P4+RU ewes as compared to CO ewes on Day 12 (Figure 5.1C). Interestingly, *WNT11* mRNA was present in LE cells of uteri from P4+RU ewes, but not in LE of either CO- or P4-treated ewes on Day 12 (Figure 5.1C).

#### *Downstream Targets of Non-Canonical WNT Signaling*

JUN is a transcriptional regulator of non-canonical WNT signaling (Logan and Nusse 2004). Immunoreactive JUN was abundant in LE, GE and stromal cells of CO-treated ewes on Day 9 (Figure 5.2). An increased amount of JUN protein was observed in nuclei of LE and sGE in uteri of P4 as compared to CO ewes on Day 9. In Study Two, immunoreactive JUN was abundant in LE of CO-treated ewes on Day 12 and also detected in GE and stromal cells. A clear loss of JUN protein from LE and GE occurred in uteri of P4 as compared to CO ewes on Day 12.

Immunocytochemical analysis of activated JUN (p-JUN) in Study One revealed low levels of p-JUN in LE of CO-treated ewes and higher levels in the *stratum compactum* stromal cells (Figure 5.2). An increased abundance of p-JUN was noted in nuclei of LE from P4-treated ewes on Day 9. In Study Two, p-JUN was detected in LE, GE, and stromal cells of CO-treated ewes, but there were no differences in p-JUN in nuclei of LE or GE between CO- and P4-treated ewes. An increased abundance of p-JUN protein was detected in stromal cells of P4+RU ewes with little or no detectable p-JUN observed in LE (Figure 5.2).

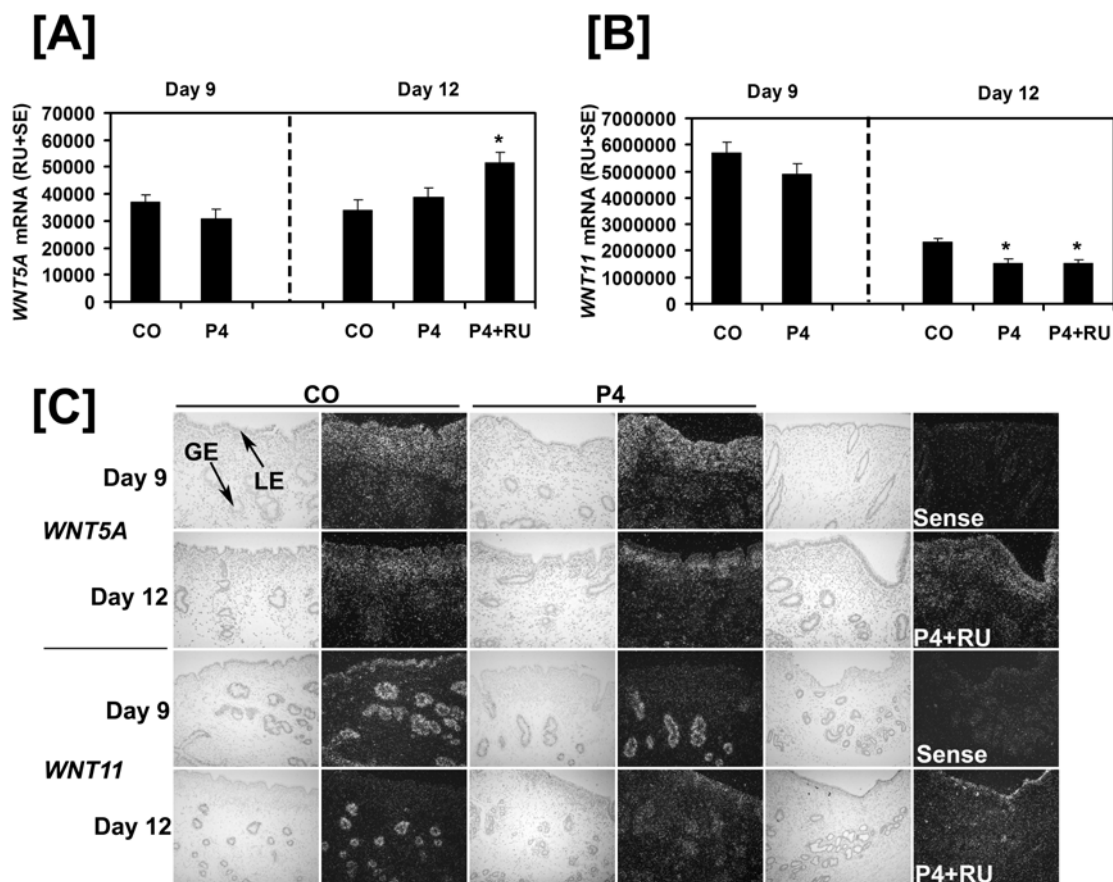


FIG. 5.1. Effects of treatments with corn oil (CO), progesterone (P4) or P4 and RU486 on *WNT5A* and *WNT11* mRNAs in ovine endometria. [A] Steady-state levels of *WNT5A* mRNA in endometria from Day 9 (Study One) and Day 12 (Study Two) ewes, as determined by slot blot analysis. *WNT5A* mRNA transcripts in P4+RU treated ewes were more abundant ( $p < 0.03$ ) than for CO- and P4-treated ewes on Day 12. [B] Steady-state levels of *WNT11* mRNA were determined by quantification of ethidium bromide intensity scanned on a phosphoimager. *ACTB* mRNA levels were utilized as a covariate for normalization of individual samples. *WNT11* mRNA decreased ( $p < 0.005$ ) with P4 and P4+RU treatment in Study Two. Data are presented as relative units with standard error. [C] *In situ* localization of *WNT5A* and *WNT11* mRNAs in endometria of Day 9 (Study One) and Day 12 ewes (Study Two) are presented. Cross-sections of uteri were hybridized with radiolabeled antisense or sense ovine cRNA probes for each respective gene. Legend: LE, luminal epithelium; S, stroma; GE, glandular epithelium.

*Progesterone Regulation of Canonical WNT mRNAs*

Several members of the canonical WNT signaling family including, *WNT2*, *WNT2B*, *WNT4*, and *WNT7A*, are expressed in the ovine uterus (Hayashi et al. 2007). In Study One, steady-state levels of endometrial *WNT2* mRNA were reduced ( $P < 0.05$ ) in P4 as compared to CO-treated ewes on Day 9. In Study Two, *WNT2* mRNA levels were not different ( $P > 0.10$ ) between CO- and P4-treated ewes on Day 12, but higher levels ( $P < 0.0002$ ) of *WNT2* mRNA were observed in stromal cells of P4+RU-treated ewes (Figure 5.3A and 5.3C). *WNT2B* and *WNT4* mRNAs were constitutively expressed at low levels in endometrial stromal cells and not affected by treatment in either study (data not shown).

Semi-quantitative RT-PCR analysis determined that *WNT7A* mRNA was lower ( $P < 0.03$ ) in endometria of P4 as compared to CO ewes on Day 9 (Figure 5.3B). As expected, *WNT7A* mRNA was observed only in endometrial LE and sGE (Figure 3C). *WNT7A* mRNA was very low to undetectable in endometria of both CO- and P4-treated ewes on Day 12 and lower ( $P < 0.01$ ) than in endometria from Day 9 ewes (Figure 5.3B); however, an increase ( $P < 0.002$ ) in *WNT7A* mRNA levels occurred in endometria of P4+RU as compared to P4 ewes on Day 12.

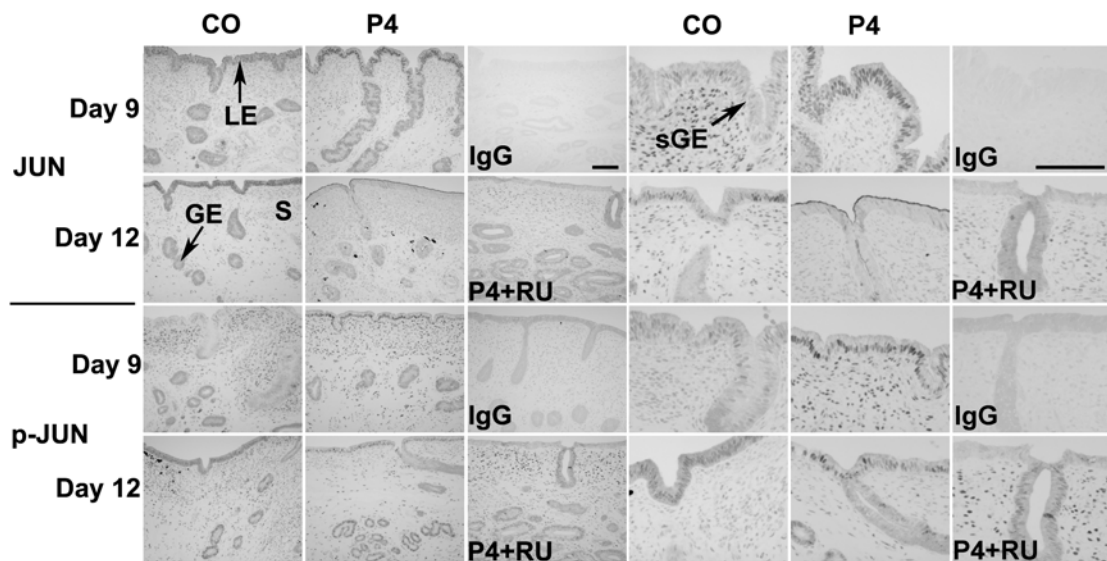


FIG. 5.2. Effects of corn oil (CO), progesterone (P4) or P4 and RU486 on localization of c-JUN and p-JUN proteins in endometria from Day 9 (Study One) and Day 12 (Study Two) ewes using specific antibodies against c-JUN and p-JUN proteins generated in rabbits. For the IgG control, normal rabbit IgG was substituted for the primary antibody. Legend: LE, luminal epithelium; S, stroma; sGE, superficial glandular epithelium; GE, glandular epithelium.



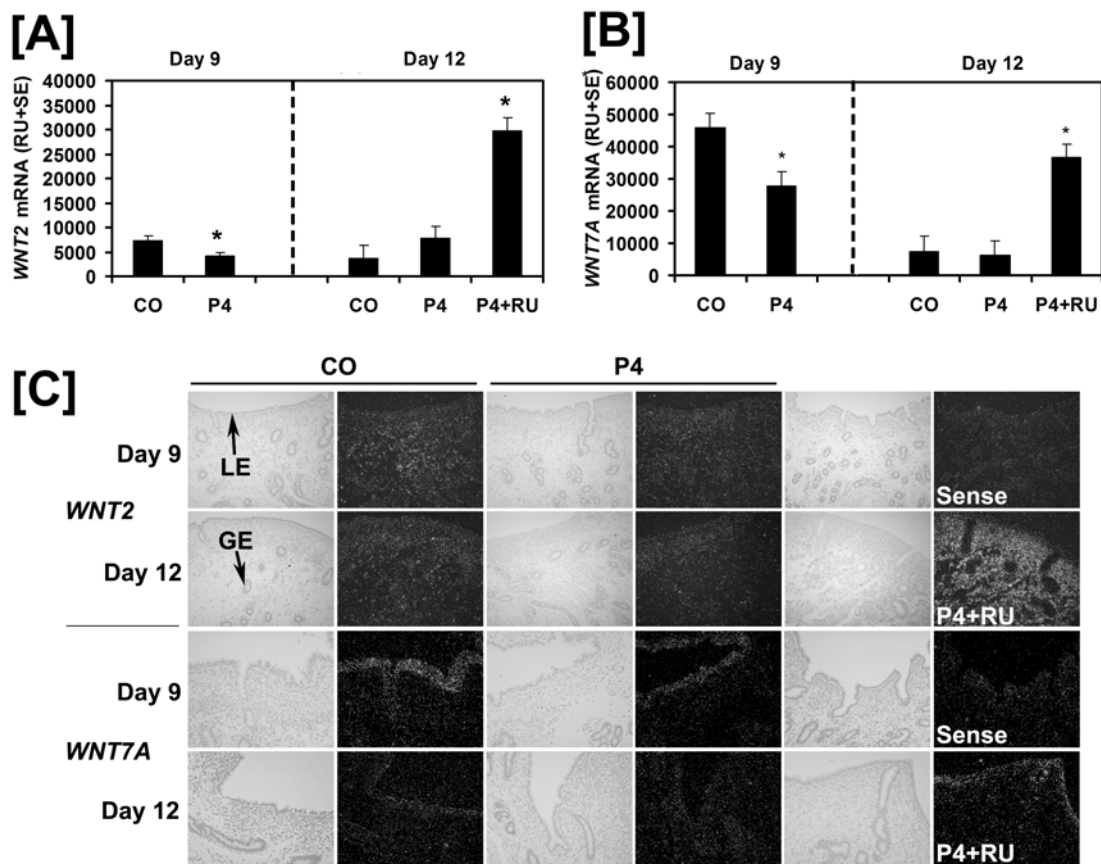


FIG. 5.3. Effects of treatments with corn oil (CO), progesterone (P4) or P4 and RU486 on endometrial *WNT2* and *WNT7A* mRNAs. [A] Steady-state levels of *WNT2* mRNA in endometria from Day 9 (Study One) and Day 12 (Study Two) ewes, as determined by slot blot analysis. *WNT2* mRNA transcripts were reduced by P4 treatment to Day 9 ( $p < 0.05$ ). In Study Two, RU486 markedly increased ( $p < 0.0002$ ) *WNT2* mRNA expression. [B] Steady-state levels of *WNT7A* mRNA were determined by quantification of ethidium bromide intensity scanned on a phosphorimager. *ACTB* mRNA levels were utilized as a covariate for normalization of individual samples. *WNT7A* mRNA decreased ( $p < 0.03$ ) with P4 treatment in Study One. In Study Two, administration of RU486 increased ( $p < 0.002$ ) endometrial *WNT7A* mRNA. Data are presented as relative units with standard error. [C] *In situ* localization of *WNT2* and *WNT7A* mRNAs in endometria from Day 9 (Study One) and Day 12 ewes (Study Two) are presented. Cross-sections of uteri were hybridized with radiolabeled antisense or sense ovine cRNA probes for each respective gene. Legend: LE, luminal epithelium; S, stroma; GE, glandular epithelium.

### *WNT Receptors and Signaling Components*

The most abundant receptor for WNT signaling in the ovine uterus is *FZD6* (Hayashi et al. 2007). Steady-state levels of *FZD6* mRNA in the endometrium were not affected ( $P>0.10$ ) by treatment in either study (data not shown). *In situ* hybridization analysis found that *FZD6* mRNA was abundant in LE and GE in both CO and P4-treated ewes on Day 9 (Figure 5.4B). On Day 12, *FZD6* mRNA abundance was lower in LE and GE in both CO and P4 ewes as compared to endometria from Day 9 ewes. *In situ* hybridization localized low levels of *FZD8* mRNA to LE and GE of Day 9 and Day 12 ewes, but expression was not affected ( $P>0.10$ ) by treatment in either study (data not shown). *GSK3B* mRNA was predominantly in LE and GE of uteri from all ewes on Days 9 and 12 and abundance was not affected ( $P>0.10$ ) by treatment (data not shown).

*DKK1* is a secreted inhibitor of WNT signaling. *DKK1* mRNA levels were higher ( $P<0.01$ ) in endometria of CO and P4 ewes on Day 12 as compared to Day 9, but not affected ( $P<0.01$ ) by P4 treatment in either study (Figure 5.4A). However, *DKK1* mRNA levels were reduced ( $P<0.02$ ) in endometria of P4+RU- as compared to P4-treated ewes on Day 12. *In situ* hybridization revealed that *DKK1* mRNA was present in the *stratum compactum* stromal cells of both CO- and P4-treated ewes, but not P4+RU-treated ewes on Day 12 (Figure 5.4B).

In Study One, *TCF7L2* was predominantly in nuclei of LE cells (Figure 5.5A) and was more abundant in LE and GE of uteri from P4-treated ewes on Day 9. *TCF7L2* was also abundant in the LE of CO ewes on Day 12, but was reduced in LE of uteri from P4-treated ewes. Interestingly, *TCF7L2* was abundant in LE and GE of uteri from P4+RU-treated ewes.

*LEF1* mRNA was not different ( $P>0.10$ ) in endometria of treated ewes on either Day 9 or Day 12 (Figure 5.5B). *In situ* hybridization analyses found that *LEF1* mRNA was present at low abundance in most endometrial cell types on Day 9, but was more abundant in LE/sGE of uteri from CO and P4 ewes on Day 12 with decreased expression in *stratum compactum* stromal cells (Figure 5.5C); however, this increase was not observed in LE/sGE of uteri from P4+RU treated ewes on Day 12 (Figure 5.5C).

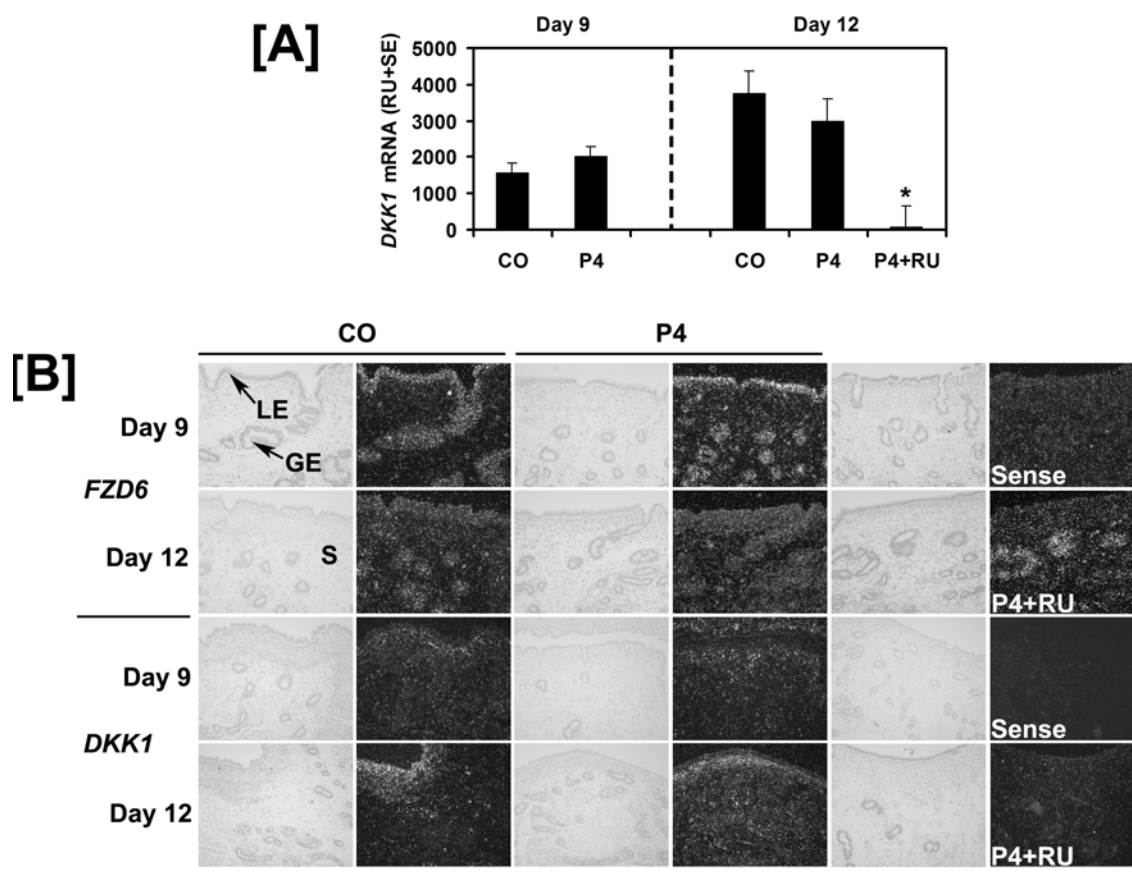


FIG. 5.4. Effects of treatments with corn oil (CO), progesterone (P4) or P4 and RU486 on endometrial *FZD6* and *DKK1* mRNAs. [A] Steady-state levels of *DKK1* mRNA in the endometria from Day 9 (Study One) and Day 12 (Study Two) ewes, as determined by slot blot analysis. *DKK1* mRNA transcripts were ablated ( $p < 0.02$ ) by administration of RU486 in Study Two. [B] *In situ* localization of *FZD6* and *DKK1* mRNAs in endometria of Day 9 (Study One) and Day 12 ewes (Study Two) are presented. Cross-sections of uteri were hybridized with radiolabeled antisense or sense ovine cRNA probes for each respective gene. Legend: LE, luminal epithelium; S, stroma; GE, glandular epithelium.

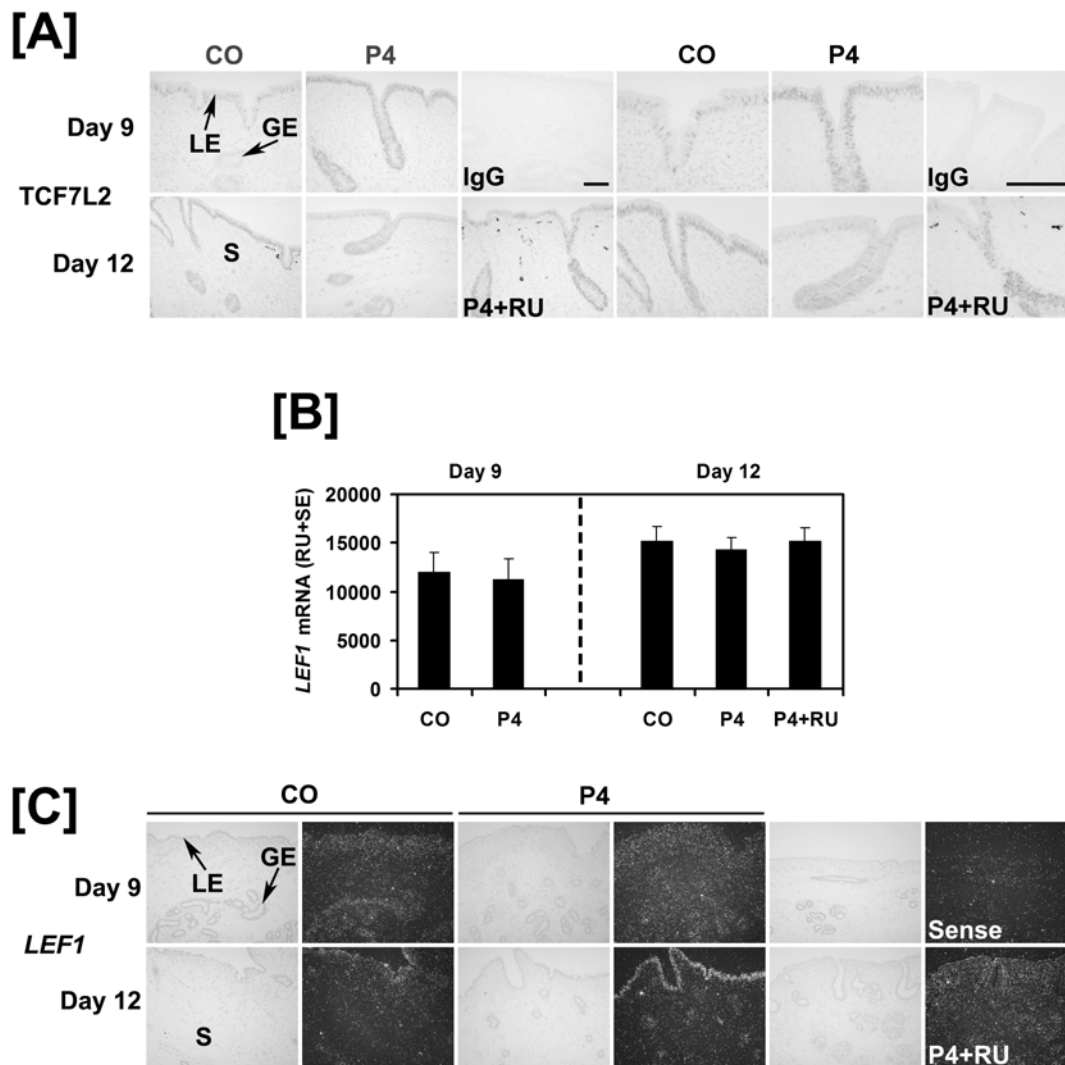


FIG. 5.5. Effects of corn oil (CO), progesterone (P4) or P4 and RU486 on localization of TCF7L2 protein and *LEF1* mRNAs in endometria from Day 9 (Study One) and Day 12 (Study Two) ewes. [A] Immunoreactive protein was detected using specific antibodies against TCF7L2 protein generated in mice. For the IgG control, normal mouse IgG was substituted for the primary antibody. [B] Steady-state levels of *LEF1* mRNA in endometria from Day 9 (Study One) and Day 12 (Study Two) ewes, as determined by slot blot analysis. Steady-state levels of *LEF1* mRNA did not differ by treatment in Studies One or Two. [C] *In situ* localization of *LEF1* mRNA in endometria of Day 9 (Study One) and Day 12 ewes (Study Two) are presented. Cross-sections of uteri were hybridized with radiolabeled antisense or sense ovine cRNA probes for each respective gene. Legend: LE, luminal epithelium; S, stroma; GE, glandular epithelium.

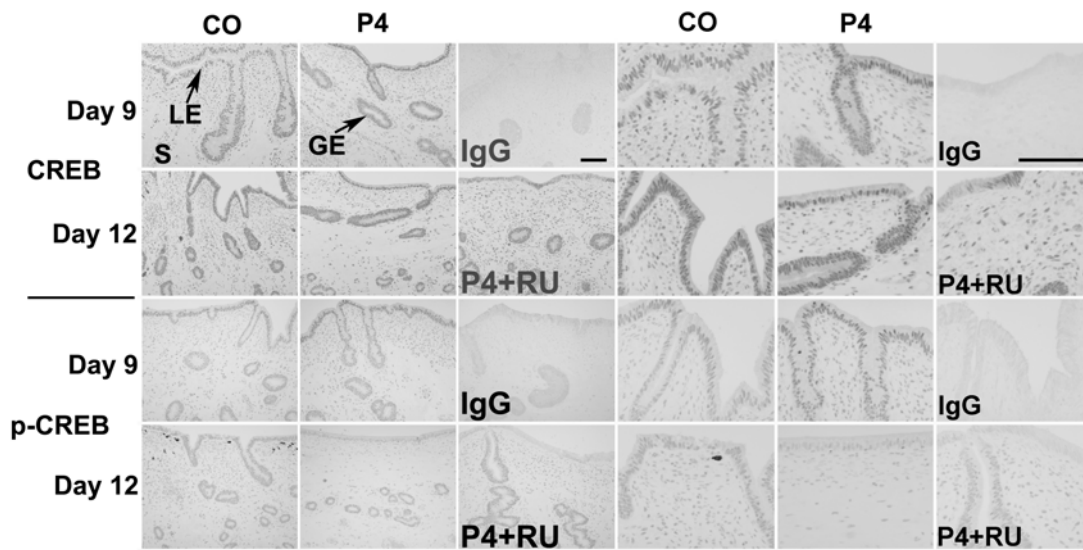


FIG. 5.6. Effects of corn oil (CO), progesterone (P4) or P4 and RU486 on localization of CREB and p-CREB proteins in endometria from Day 9 (Study One) and Day 12 (Study Two) ewes. Immunoreactive proteins were detected using specific antibodies against CREB, and p-CREB proteins generated in rabbits. For the IgG control, normal rabbit IgG was substituted for the primary antibody. Legend: LE, luminal epithelium; S, stroma; GE, glandular epithelium.

CREB, a target gene for WNT signaling (Chen et al. 2005), was detected in all cell types and treatment groups on Days 9 and 12, but was not affected by treatment in either study (Figure 5.6). Immunoreactive p-CREB was abundant in the nuclei of LE, GE, and stromal cells of CO ewes on Day 9 (Figure 5.6). The abundance of p-CREB increased in LE of P4-treated ewes on Day 9 compared to CO controls. On Day 12, a slight reduction in p-CREB was observed in LE of CO-treated ewes compared to Day 9, but this was not apparent in GE or stromal cells. Lower amounts of p-CREB were observed in LE, GE and stroma of P4- as compared to CO-treated ewes on Day 12, and p-CREB was less abundant in LE of uteri from P4+RU-treated ewes as compared to P4-treated ewes on Day 12.

#### *WNT Regulated Genes*

*MSX1* and *MSX2* are homeobox genes induced by WNTs via the canonical pathway. *MSX1* mRNA levels were reduced ( $P < 0.01$ ) by P4 in Study One (Figure 5.7A). *MSX1* mRNA was abundant in LE and GE of CO ewes on Day 9 and was reduced predominantly in GE of P4-treated ewes (Figure 5.7B). In Study Two, *MSX1* mRNA levels were not different ( $P > 0.10$ ) between CO- and P4-treated ewes on Day 12 and were localized to the deep GE; however, *MSX1* mRNA levels were higher ( $P < 0.001$ ) in endometria of P4+RU- as compared to P4-treated ewes, increasing in GE of deep, middle, and superficial uterine glands (Figure 5.7A and 5.7B). *MSX2* mRNA levels were not affected by P4 treatment in either study, but increased ( $P < 0.03$ ) in endometria of P4+RU ewes on Day 12 (Figure 5.7A), specifically in endometrial glands (Figure 5.7B).

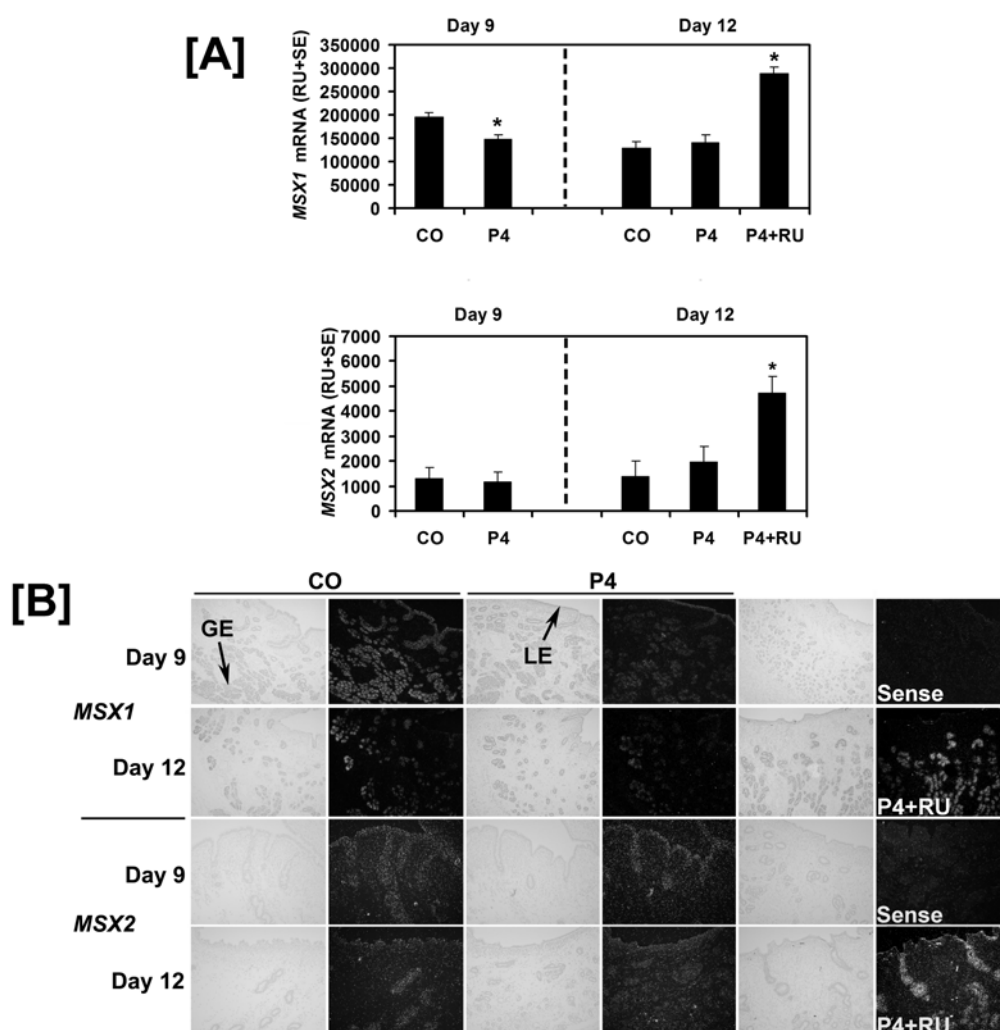


FIG. 5.7. Effects of corn oil (CO), progesterone (P4) or P4 and RU486 on localization of *MSX1* and *MSX2* mRNAs in endometria from Day 9 (Study One) and Day 12 (Study Two) ewes. [A] Steady-state levels of *MSX1* and *MSX2* mRNAs in endometria from Day 9 (Study One) and Day 12 (Study Two) ewes, as determined by slot blot analysis. *MSX1* mRNA levels were reduced ( $p < 0.01$ ) by P4 treatment in Study One. There was no difference in *MSX1* mRNA between CO- and P4-treated ewes on Day 12; however, administration of RU486 increased *MSX1* mRNAs levels above levels for control ewes ( $p < 0.02$ ) and tended ( $p < 0.09$ ) to decrease levels below those detected for ewes treated with P4 alone. *MSX2* mRNAs were expressed at low levels on Day 9, independent of treatment, and were not different between CO- and P4-treated ewes to Day 12, but P4+RU-treated ewes expressed more ( $p < 0.03$ ) *MSX2* mRNA. [B] *In situ* localization of *MSX1* and *MSX2* mRNAs in endometria of Day 9 (Study One) and Day 12 ewes (Study Two) are presented. Cross-sections of uteri were hybridized with radiolabeled antisense or sense ovine cRNA probes for each respective gene.

### ***Discussion***

These studies revealed complex effects of day of pregnancy, P4 and PGR on expression of WNTs and their inhibitors in ovine uterine endometria. A decrease in *WNT5A*, *WNT7A* and *WNT11* mRNAs was observed specifically in endometrial LE between Days 9 and 12, and *WNT7A* and *WNT11* mRNAs decreased in LE in response to early P4 treatment as assessed on Day 9. Those changes in epithelial WNT expression paralleled changes in epithelial PGR protein (Satterfield et al. 2006), suggesting direct regulation of those genes by PGR. Indeed, *WNT7A* expression is stimulated by progestins in human endometrial epithelial cells (Oehler et al. 2002). In contrast, estrogen stimulates expression of *WNT11* in breast cancer cells (Lin et al. 2007). Given the antagonistic actions between estrogen and progesterone, P4 may suppress *WNT11* gene expression. Indeed, the loss of PGR induced by P4 may be responsible for the decrease in *WNT5A*, *WNT7A* and *WNT11* expression in uterine LE during early pregnancy. In addition to those WNTs, results of the present study indicated that *DKK1* mRNA increased in endometrial stromal cells between Days 9 and 12 and decreased to undetectable levels in ewes treated with RU486, a PGR antagonist, in Study Two. Given that *DKK1* expression increases in response to P4 in human endometria (Tulac et al. 2006), it is likely that *DKK1* is also regulated by liganded PGR in ovine endometrial stromal cells. The collective loss of WNTs from uterine LE and the increase in *DKK1*, a WNT signaling inhibitor, suggests that canonical and non-canonical WNT signaling is reduced in uterine LE between Days 9 and 12 of pregnancy and this is hypothesized to be responsible for functional changes in the LE that support blastocyst growth and development.

*WNT5A* and *WNT11* signal via the non-canonical or planar cell polarity pathway that influences cell polarity and cell migration via activation of Rho kinase and JNK-JUN pathways (Karner et al. 2006b). Similarly, canonical WNT signaling through *CTNNB1* has numerous functions including regulation of cell differentiation and adhesion as well as changes in cell shape mediated via the actin microfilament cytoskeletal network (Widelitz 2005). Collectively, the temporal and/or P4-induced



loss of *WNT5A*, *WNT11* and *WNT7A* expression from LE and/or GE is closely correlated to changes in LE cell shape and histoarchitecture. Human cell culture models utilizing non-adhesive versus adhesive uterine epithelial cells have indicated that adhesive cells capable of trophoblast attachment exhibit, a lack of ZO-1 expression, tracer leakiness of the paracellular space, and atypical patterns in adherens junction formation (Thie et al. 1996). Further, the actin-based cytoskeleton lacked a polar organization in adhesive cells which also had fewer well formed desmosomes (Thie et al. 1996). Similarly, we reported distinct temporal and spatial alterations in occludin, tight junction protein 2, and claudin 1-4 proteins in endometria of cyclic and early pregnant ewes (Satterfield et al. 2007). In that study, dynamic changes in tight junction formation were characterized by an abundance of tight junction proteins on Day 10 of the estrous cycle and pregnancy that decreased significantly by Day 12. Early P4 administration that advanced blastocyst development on Day 9 and 12 was associated with loss of tight-junction-associated proteins. Further, cadherin 1 and CTNNB1, which form adherens junctions, were abundant in endometrial glands, but decreased after Day 10 of pregnancy in LE and then increased by Day 16 with the onset of implantation. Of particular note, regulation of tight junction associated proteins, principally claudins, by WNT signaling was reported for other cell types (Mankertz et al. 2004; Miwa et al. 2001). Therefore, the transient loss of WNTs in ovine uterine LE between Days 9 and 12 may be responsible for the decline in expression of genes encoding components of tight and adherence junctions. This loss is correlated to the onset of blastocyst growth and elongation into a filamentous conceptus. The physiological impact of a loss in epithelial tight junctions immediately preceding elongation may increase selective movement of serum or stromal derived molecules into the uterine lumen to stimulate the elongation of the blastocyst. Indeed, P4 modulates changes in HGF, FGF7, and several members of the IGF family as well as increases the abundance of glucose and essential amino acids including, arginine, leucine and glutamine in the uterine lumen (H. Gao, G. Wu, G. A. Johnson, T.E. Spencer and F.W. Bazer, unpublished results). Further, many of the proteins present in the uterine lumen are not directly synthesized by endometrial epithelia or the conceptus (Lee et al.

1998b). The return of tight junctions in endometrial cells in response to pregnancy associated signals from the conceptus is hypothesized to have multiple roles in supporting pregnancy, including concentrating IFNT in appreciable quantities to inhibit luteolysis, to regulate appropriate contact between the newly implanting conceptus and the maternal endometrium, and by providing an immunoprotective barrier to inhibit targeting of the conceptus by the maternal immune system.

In canonical WNT signaling, following stabilization and nuclear translocation, CTNNB1 protein complexes with TCF/LEF transcriptional activators to stimulate transcription of WNT target genes. In contrast, non-canonical WNT signaling occurs directly in response to receptor activation and results in activation of the Rho-ROCK and JNK-JUN pathways. Interestingly, available results indicate a similar trend in WNT signaling between the two pathways. Indeed, in the present study, P4 induced a transient rise followed by a rapid decline in p-JUN, TCF7L2, and p-CREB, a previously characterized WNT target gene (Chen et al. 2005). These results are expected based on the decline in their upstream regulators, *WNT5A*, *WNT11*, *WNT7A*, *FZD6*, and *CTNNB1* along with increasing abundance of the WNT inhibitor *DKK1*. Curiously, quantitative analysis of *LEF1* mRNA indicated relatively static expression, while *in situ* hybridization analyses revealed that cell-specific effects of P4 treatment to Day 12 to increase *LEF1* mRNA in the LE. The upregulation of *LEF1* in the LE may be critical for stimulating transcription of genes encoding paracrine factors which enhance conceptus development and morphogenesis during early pregnancy. This upregulation slightly precedes upregulation of *WNT7A* [reference] and may act cooperatively to induce transcription of *WNT7A* stimulated genes.

Knowledge gained from studies with our sheep model of early pregnancy, parallel that reported for mouse and human with respect to conserved mechanisms for maintaining appropriate conceptus/maternal synchrony and communication. Indeed, in mice, prior to Day 4, Wnt/Ctnnb1 signaling is not detected in uteri of non-pregnant mice; however, on the morning of Day 4 the blastocyst emits a signal that activates the Wnt/Ctnnb1 signaling pathway first in circular smooth muscle of the myometrium and

subsequently in uterine LE at the site of implantation (Mohamed et al. 2005). Inhibition of Wnt signaling using secreted frizzled related protein 2 resulted in a decline in the percentage of implanting blastocysts (Mohamed et al. 2005). In the sheep, *WNT7A* mRNA is lost in LE by Day 12 and is subsequently upregulated in pregnant, but not cyclic ewes, between Days 14 and 16 in response to conceptus derived IFNT (Kim et al. 2003). To date, the blastocyst derived factor responsible for the induction of the Wnt/Ctnnb1 signaling pathway in the mouse has not been identified.

Collectively, broad analysis of components of both the canonical and non-canonical WNT signaling pathways as well as downstream targets of these pathways reveal a general decline in WNT signaling following the essential loss of PGR from uterine epithelia prior to conceptus elongation. Interestingly, signals from the elongated conceptus reactivate the WNT signaling network via secretion of IFNT. The transient loss of WNT signaling may indeed have an essential role in synchronizing the developing conceptus and maternal endometrium prior to initiation of the implantation process as proposed for mice. Receptivity of the endometrium is characterized by changes in both form and function of the epithelia. In sheep, changes in both tight and adherens junctions reflect temporal changes in WNT signaling. Although limited, available results illustrate that expression of tight junction associated proteins is regulated by members of the WNT signaling family. These findings from studies of early pregnancy in sheep support the idea that alterations in junctional complexes prior to conceptus elongation are mediated by a loss in WNT signaling during this critical period and that these structural and functional changes may synchronize conceptus-maternal communications to initiate implantation.

**CHAPTER VI**  
**PROGESTERONE REGULATION OF CANDIDATE PROGESTAMEDINS AND**  
**THE IGF SYSTEM IN AN OVINE MODEL OF ACCELERATED CONCEPTUS**  
**DEVELOPMENT**

*Introduction*

Early conceptus growth and development requires the production of secretions which emanate from uterine luminal (LE) and glandular (GE) epithelia to form histotroph (for reviews see (Bazer 1975; Carson et al. 2000; Fazleabas et al. 1994; Kane et al. 1997; Roberts and Bazer 1988). In sheep, absence of uterine glands results in failure of the blastocyst to elongate and survive beyond Day 14 of pregnancy (Gray et al. 2002; Gray et al. 2001c). Indeed, an estimated 20% of blastocysts are lost between hatching of blastocysts from the zona pellucida and firm adhesion to the uterine endometrium signifying the end of implantation, which corresponds to the time of maternal recognition of pregnancy in domesticated livestock (Ayalon 1978; Diskin and Sreenan 1980; Maurer and Chenault 1983; Roche et al. 1981). Ovarian progesterone (P4) is the predominant steroid hormone responsible for the production and secretion of uterine factors during this critical period (Spencer et al. 2004b). However, detectable progesterone receptor (PGR) protein is lost first within the (LE) and then the (GE) by Day 13 of pregnancy (Spencer and Bazer 1995; Wathes and Hamon 1993). Loss of the PGR is thought to be required for initiation of epithelial gene expression required for uterine receptivity and blastocyst growth. Indeed, loss of epithelial PGR is a common phenomenon across all mammals studied to date and is likely critical for uterine receptivity (Geisert et al. 1994; Lessey et al. 1988; Press and Greene 1988; Spencer and Bazer 1995; Spencer and Bazer 2002; Tan et al. 1999). The endometrial stromal cells continue to localize PGR protein throughout pregnancy, thus it has been hypothesized that stromal derived factors regulated by P4 act on the adjacent epithelia as progestamedins to mediate P4-induced changes in epithelial gene expression. Our laboratory identified fibroblast growth factor 7 (FGF7), FGF10, and hepatocyte growth

factor (HGF) in the ovine endometrium produced by the PGR-positive uterine stroma that may act to modulate P4 actions via their respective receptors in epithelia and trophectoderm of the conceptus, i.e., fibroblast growth factor receptor 2 (FGFR2) and met proto-oncogene (MET) (Chen et al. 2000a; Chen et al. 2000b). FGF7 is an established paracrine growth factor of mesenchymal origin that stimulates epithelial cell proliferation and differentiation (Rubin et al. 1995) and mediates effects of P4 in primate endometria (Koji et al. 1994). Like FGF7, FGF10 is a specific mitogen for epithelial cells (Igarashi et al. 1998; Lu et al. 1999). HGF is a pleiotropic mesenchymal growth factor that has potent mitogenic, morphogenic, and motogenic activities on epithelial cells (Montesano et al. 1991; Nakamura et al. 1989; Stoker et al. 1987), angiogenic properties (Okada et al. 1999) and is an inhibitor of tumor growth and invasion (Shima et al. 1991). Interestingly, HGF regulates human endometrial epithelial cell proliferation and motility (Sugawara et al. 1997).

We have used an ovine model of accelerated blastocyst development achieved by the administration of exogenous P4 to elicit a premature increase in circulating concentrations of this hormone in blood of ewes (Satterfield et al. 2006). This treatment increased blastocyst growth on Day 9 and accelerated morphological transformation from a spherical blastocyst to a filamentous conceptus on Day 12. We hypothesized that the accelerated growth and development of the blastocyst was mediated by specific and accelerated changes in expression of endometrial genes. This hypothesis was supported by the observation that genes encoding secreted proteins expressed by the LE, such as *LGALS15* and *CTSL*, were advanced and increased by P4 treatment (Satterfield et al. 2006). We endeavored to determine the regulatory role of P4 on growth factor families which have been implicated in early conceptus development and/or uterine function.

The insulin-like growth factor (IGF) family consists of IGF1, IGF2, and their receptors IGF1R and IGF2R, as well as seven high affinity IGF binding proteins (IGFBPs 1-7) which modulate IGF activity and bioavailability (for review see (Wang and Chard 1999). IGF1 and IGF2 possess both mitogenic and differentiative properties and are implicated in early embryonic and placental development in a multitude of

species including humans, rodents, pigs, and domesticated ruminants (Baker et al. 1993; D'Ercole 1987; DeChiara et al. 1990; Gluckman et al. 1992; Wathes et al. 1998; Zhou and Bondy 1992). In humans, IGF1R has high affinity for IGF1 and moderate affinity for IGF2 and is the primary functional receptor expressed in the endometrium during pregnancy (Wang and Chard 1999). In sheep, IGF1R is localized to the LE, GE, and to a lesser extent, caruncular stromal cells and myometrium, as well as the pre-implantation blastocyst (Hayashi et al. 2005; Stevenson et al. 1994; Watson et al. 1999). IGFBPs can both enhance or retard IGF actions (Holly and Perks 2006). IGFBPs bind IGFs with high affinity and they can be cleaved by proteases yielding free IGF to act on adjacent cells expressing the IGF1R. IGFBP proteases include, matrix metalloproteinases (MMPs), kallikreins, cathepsins, pregnancy associated plasma protein A (PAPPA), calpain, and serine proteases (Bunn and Fowlkes 2003; Ghosh et al. 2005; Rajah et al. 1995). Collectively, the mechanisms regulating IGF actions involve, with equal importance, the availability of receptors, the IGFBPs, and the activity of available proteases to regulate local concentrations of free IGF. Gene mutagenesis studies in the mouse indicated critical roles for both *Igf1* and *Igf2* in embryonic, placental, and fetal growth; with *Igf1* null mice being infertile (Baker et al. 1993; DeChiara et al. 1990; Liu et al. 1993). Interestingly, uterine growth is not dependant upon *Igf1* production by the uterus, but it is supported by systemic *Igf1*. In pigs, the proteolytic cleavage of IGFBPs, to yield free IGF, has been associated with the elongation period of conceptus development (Lee et al. 1998a). Early studies in the cow revealed the presence of mRNAs encoding *IGFBPs 1-5* within the endometrium during early conceptus elongation (Keller et al. 1998). Interestingly, early embryonic and fetal development in the cow can be stimulated by components of the GH-IGF system (Moreira et al. 2002a; Moreira et al. 2002b).

Based on available information from the literature, we hypothesized that P4 regulates expression of the stromal growth factors, *FGF7*, *FGF10*, and *HGF* to act as progestamedins on the adjacent epithelium and conceptus trophectoderm, and members of the IGF system to enhance the morphological transformation of spherical hatched

blastocysts of less than 1 mm into elongated filamentous conceptuses of over 190 mm in just 2 days (Guillomot et al. 1993). To address this hypothesis, we utilized a previously characterized model of accelerated conceptus development induced by premature exposure of the uterus to P4 (Satterfield, Bazer et al. 2006). The objectives of the present study were to determine the hormonal regulation of candidate progesterone-induced genes, *FGF7*, *FGF10*, and *HGF* in the ovine endometrium and identify members of the IGF system that may augment blastocyst growth and development in response to early P4.

### ***Materials and Methods***

#### ***Animals***

Mature Suffolk-type ewes (*Ovis aries*) were observed for estrus (designated as Day 0) in the presence of a vasectomized ram and used in experiments only after exhibiting at least two estrous cycles of normal duration (16-18 Days). 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***

**Study One** As described previously (Satterfield et al. 2006), ewes were mated at estrus to intact Suffolk rams and then assigned randomly to receive daily i.m. injections from Days 1.5 to 9 of either corn oil vehicle (CO; n=6) or 25 mg progesterone (P4; n=6) (Sigma Chemical Co., St. Louis, MO). All ewes were hysterectomized on Day 9. At hysterectomy, sections (~0.5 cm) from the mid-portion 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 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 was physically dissected from myometrium, frozen in liquid nitrogen, and stored at -80°C for subsequent RNA or protein extraction. In monovulatory pregnant ewes, uterine tissue samples were marked as either contralateral

or ipsilateral to the ovary bearing the CL. No tissues from the contralateral uterine horn were used for further analysis.

**Study Two** As described previously (Satterfield et al. 2006), ewes were mated at estrus to intact Suffolk rams and assigned randomly to receive daily intramuscular (i.m.) injections of either: (a) CO vehicle from Days 1.5 to 12 (n=8); (b) 25 mg P4 from Days 1.5 to 12 (n=7); or (c) 25 mg P4 (Days 1.5 to 8, n=5) and 75 mg of RU486 (Sigma), a PGR antagonist, from Days 8 to 12 (P4+RU). All ewes were hysterectomized on Day 12, and the uteri were then processed as described for Study One.

#### *RNA Isolation*

Total cellular RNA was isolated from frozen ipsilateral endometrium (Studies One and Two) using Trizol reagent (Gibco-BRL, Bethesda, MD) according to manufacturer's instructions. The quantity and quality of total RNA was determined by spectrometry and denaturing agarose gel electrophoresis, respectively.

#### *Real-Time PCR Analysis*

The cDNA was synthesized from uterine total RNA (5 µg) using random primers (Invitrogen, Carlsbad, CA), oligo (deoxythymidine) primers, and SuperScript II Reverse Transcriptase (Invitrogen) as described previously (Stewart et al. 2000). Newly synthesized cDNA was acid-ethanol precipitated, resuspended in 20 µl water, and stored at -20°C. PCR analysis of *FGF7*, *FGF10*, and *HGF* mRNA expression was performed using an ABI PRISM 7700 (Applied Biosystems, Foster City, CA) with SYBR Green PCR Master Mix (Applied Biosystems) as the detector, according to manufacturer's recommendations, as described previously (Kim et al. 2003). Primers were designed to amplify cDNAs of less than 100 bp to maximize efficiency (Table 1). PCR cycle parameters were 95°C for 15 sec and 60°C for 1 min for 40 cycles. Data were analyzed by using GeneAmp 5700 SDS software (version 1.4, Applied Biosystems). The threshold line was set in the linear region of the plots above the baseline noise, and threshold cycle ( $C_T$ ) values were determined as the cycle number at which the threshold line crosses the



amplification curve. PCR without template or template substituted with total RNA was used as a negative control to verify experimental results. The results are expressed as raw  $C_T$  values for each target gene mRNA.

#### *In Situ Hybridization Analysis*

Location of *IGF1*, *IGF2*, *IGF1R*, *IGFBP1*, *IGFBP2*, *IGFBP3*, *IGFBP4*, *PAPP-A*, *IGFBP5*, *IGFBP6*, *IGFBP7*, *MET*, and *FGFR2* mRNAs in ovine uteri was determined by radioactive *in situ* hybridization analysis as described previously (Spencer et al. 1999c). Radiolabeled antisense and sense cRNA probes were generated by *in vitro* transcription using linearized partial plasmid cDNA templates (Chen et al. 2000a; Chen et al. 2000b; Hayashi et al. 2007), RNA polymerases, and [ $\alpha$ - $^{35}$ 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 between 5 and 30 days based on intensity of radioactive signal of slides placed on MR Kodak film for 16 h. All slides for each respective gene were exposed to photographic emulsion for the same period of time. Slides were developed in Kodak D-19 developer, counterstained with Gill's hematoxylin (Fisher Scientific, Fairlawn, NJ), and then dehydrated through a graded series of alcohol to xylene. Coverslips were then affixed with Permount (Fisher). Images of representative fields were recorded under brightfield or darkfield illumination using a Nikon Eclipse 1000 photomicroscope (Nikon Instruments Inc., Lewisville, TX) fitted with a Nikon DXM1200 digital camera.

#### *Slot Blot Hybridization Analysis*

Steady-state levels of *MET*, *FGFR2*, *IGF1*, *IGF2*, *IGFBP1*, *IGFBP2*, *IGFBP3*, *IGFBP4*, *PAPP-A*, *IGFBP5*, *IGFBP6*, and *IGFBP7* mRNAs in endometria were assessed by slot blot hybridization as described previously (Spencer et al. 1999c). Briefly, radiolabeled antisense cRNA probes were generated by *in vitro* transcription using

linearized plasmid templates containing partial cDNAs, RNA polymerases, and [ $\alpha$ - $^{32}$ P]-UTP. Denatured total endometrial RNA (20  $\mu$ g) from each ewe in Studies One and Two was hybridized with radiolabeled cRNA probes. To correct for variation in total RNA loading, a duplicate RNA slot blot membrane was hybridized with radiolabeled antisense 18S cRNA (pT718S; Ambion, Austin, TX). Following washing, the blots were digested with ribonuclease A and radioactivity associated with slots was quantified using a Typhoon 8600 MultiImager (Molecular Dynamics, Piscataway, NJ). Data are expressed as relative units (RU).

#### *Radioimmunoassay*

Total IGF1 protein was determined in uterine flushings using an IGF1 RIA kit (Mediagnost, Reutlingen, Germany) according to manufacturer's instructions. Briefly, samples of uterine flushings were prepared by acidification with acidification buffer at a rate of 10:1 uterine flush, to buffer. Total counts, standards, non-specific binding, and quality controls samples (duplicate) and unknown samples (triplicate) were then mixed with appropriate reagents in 12 x 75 mm polypropylene tubes. Samples remained at 4°C for 48 h and were centrifuged at 3500 g for 30 min following addition of appropriate kit components. All tubes except total counts were decanted gently and blotted to remove excess liquid. Tubes were then quick spun to ensure that all precipitates were located at the bottom of the tube and quantified using a gamma counter. Assay results were calculated using the AssayZap Version 3.1 program (Biosoft, Ferguson, CA).

#### *Photomicroscopy*

Photomicrographs were taken using a Nikon Eclipse E1000 photomicroscope (Nikon Instruments, Melville, NY). Digital images were captured using a Nikon DXM 1200 digital camera and assembled using Adobe Photoshop 7.0 (Adobe Systems, Seattle, WA).

### *Statistical Analyses*

Data from RIA, slot blot hybridization, and real-time PCR analyses were subjected to least-squares analysis of variance using the General Linear Models procedures of the Statistical Analysis System (SAS Institute, Cary, NC). Slot blot hybridization data were corrected for differences in sample loading by using the 18S rRNA data as a covariate. Data are presented as the least-squares means (LSM) with overall standard error (SE). Analysis of PCR data incorporated the cyclophilin values as a covariate in the statistical model to correct for differences in the amounts of reverse transcriptase cDNA analyzed for each uterus. In all analyses, error terms used in tests of significance were identified according to the expectation of the mean squares for error. Data are presented as LSM of untransformed values.

### ***Results***

We previously reported that premature exposure of the endometrium to P4 beginning on Day 1.5 post-estrus increased blastocyst diameter 2.2-fold on Day 9 and accelerated the morphological transformation of the spherical blastocyst into a filamentous conceptus by Day 12. Administration of the PGR antagonist, RU486, from Day 8 to Day 12, resulted in demise of conceptuses on Day 12. In addition, early P4 resulted in premature loss of PGR from LE and GE, although ewes receiving RU486 exhibited a return of PGR protein to GE on Day 12 (Satterfield et al. 2006).

### *FGF7, FGF10, and HGF and their Receptors*

*FGF7*, *FGF10*, and *HGF* expressed in stromal cells of ovine endometria (Chen et al. 2000a; Chen et al. 2000b) are hypothesized to act via their respective epithelial specific receptors to mediate P4 action and modulate epithelial gene expression and function (Chen et al. 2000a; Chen et al. 2000b). Real-time PCR was used to measure steady-state levels of *FGF*, *FGF10*, and *HGF* mRNA in endometria (Figure 6.1A and 6.1B). In Study One, *FGF7* mRNA levels were 13.9-fold higher ( $P < 0.02$ ) in endometria of P4 than CO ewes on Day 9; however, *FGF7* mRNA levels did not differ ( $P > 0.10$ ) in

between endometria of P4 and CO ewes on Day 12. In contrast, *FGF7* mRNA levels were 5.7 fold lower ( $P < 0.03$ ) in endometria of P4+RU treated ewes compared to P4-treated ewes on Day 12. *FGF10* mRNA levels were not different ( $P > 0.10$ ) between CO- and P4-treated ewes on either Day 9 or Day 12; however, *FGF10* mRNA was reduced 24.3-fold ( $P < 0.005$ ) in P4+RU- compared to P4-treated ewes on Day 12. In Study One, *HGF* mRNA levels were 6.3-fold higher ( $P < 0.001$ ) in endometria of P4 as compared to CO ewes on Day 9; however, *HGF* mRNA levels did not differ ( $P > 0.10$ ) between endometria of P4 and CO ewes on Day 12. In contrast, *HGF* mRNA levels were 6.8-fold lower ( $P < 0.04$ ) in endometria of P4+RU- compared to P4-treated ewes.

Endometrial *MET* mRNA levels tended to be higher ( $P < 0.07$ ) in endometria of P4-treated ewes on Day 9 (Figure 6.1C). *MET* mRNA levels were higher ( $P < 0.05$ ) in P4-treated as compared to CO ewes on Day 12, and endometria of P4+RU-treated ewes had lower ( $P < 0.04$ ) levels of *MET* mRNA compared to P4-treated ewes. *In situ* hybridization localized *MET* mRNA to endometrial LE and GE (Figure 6.1D). Endometrial *FGFR2* mRNA levels were not different ( $P > 0.10$ ) between CO and P4-treated ewes on Day 9 or Day 12, but P4+RU treated ewes had higher ( $P < 0.001$ ) levels of *FGFR2* mRNA compared to P4-treated ewes on Day 12 (Figure 6.1C). *FGFR2* mRNA was predominantly present in endometrial LE and GE and decreased within these cells types from Day 9 to Day 12 (Figure 1D).

#### *IGF1, IGF2 and the IGF1R*

Steady-state mRNA levels of *IGF1* were not different ( $P > 0.10$ ) in between endometria of CO and P4 treated ewes on Day 9 or Day 12, although *IGF1* mRNA was reduced ( $P = 0.0002$ ) in endometria of CO ewes on Day 12 compared to Day 9 (Figure 6.2A). *IGF1* mRNA was present primarily in caruncular and intercaruncular stromal cells (Figure 6.2B). *IGF2* mRNA levels were also not different ( $P > 0.10$ ) between CO and P4-treated ewes in Study One; however, *IGF2* mRNA levels tended ( $P < 0.06$ ) to be lower in P4+RU- compared to P4-treated ewes (Figure 6.2A). *IGF2* mRNA was present only in stromal cells of ewes receiving CO or P4 to Days 9 and 12. In contrast, *IGF2*

mRNA was most abundant in LE of uteri from P4+RU ewes (Figure 6.2B). Steady-state mRNA levels of *IGF1R* were not different between ewes on Day 9 or Day 12 (data not shown); however, *IGF1R* mRNA was localized predominantly in uterine LE and GE (Figure 6.2B).

#### *IGF1 Protein Levels within the Uterine Flush*

Results of the radioimmunoassay to detect IGF1 protein in uterine flushings following removal of IGFBPs by acidification indicated that total IGF1 was 2-fold lower ( $P=0.0007$ ) in the uterine lumen of P4-treated ewes on Day 9 (Figure 6.3), but not different ( $P>0.10$ ) in the uterine lumen on Day 12.

| <b>[A]</b> | <b>Day 9</b> | <b>CO</b>             | <b>P4</b>             |
|------------|--------------|-----------------------|-----------------------|
|            | <i>FGF7</i>  | 28.6±0.9 <sup>a</sup> | 24.8±0.9 <sup>b</sup> |
|            | <i>FGF10</i> | 25.6±1.5 <sup>a</sup> | 26.1±1.5 <sup>a</sup> |
|            | <i>HGF</i>   | 27.3±0.5 <sup>a</sup> | 24.6±0.5 <sup>b</sup> |

| <b>[B]</b> | <b>Day 12</b> | <b>CO</b>             | <b>P4</b>             | <b>P4+RU</b>          |
|------------|---------------|-----------------------|-----------------------|-----------------------|
|            | <i>FGF7</i>   | 27.5±0.7 <sup>a</sup> | 28.1±0.7 <sup>a</sup> | 30.7±0.8 <sup>b</sup> |
|            | <i>FGF10</i>  | 26.9±1.0 <sup>a</sup> | 27.3±1.0 <sup>a</sup> | 31.9±1.2 <sup>b</sup> |
|            | <i>HGF</i>    | 26.8±0.8 <sup>a</sup> | 25.6±0.9 <sup>a</sup> | 29.6±1.0 <sup>b</sup> |

FIG. 6.1. Effects of treatments with CO, P4, or P4+RU on endometrial *FGF7*, *FGF10*, and *HGF* mRNAs. [A-B] Steady-state levels of *FGF7*, *FGF10*, and *HGF* mRNAs in endometria from Day 9 (Study One) and Day 12 (Study Two) ewes were determined by real-time PCR. In Study One, P4 increased amounts of *FGF7* ( $P<0.02$ ) and *HGF* ( $P<0.001$ ) mRNAs 13.9- and 6.3 fold, respectively on Day 9, but not on Day 12. In contrast, P4+RU treated ewes reduced *FGF7* ( $P<0.03$ ), *FGF10* ( $P<0.005$ ), and *HGF* ( $P<0.04$ ) 5.7-, 24.3-, and 6.8-fold, respectively. Data are presented as raw CT values using cyclophilin as a covariate.

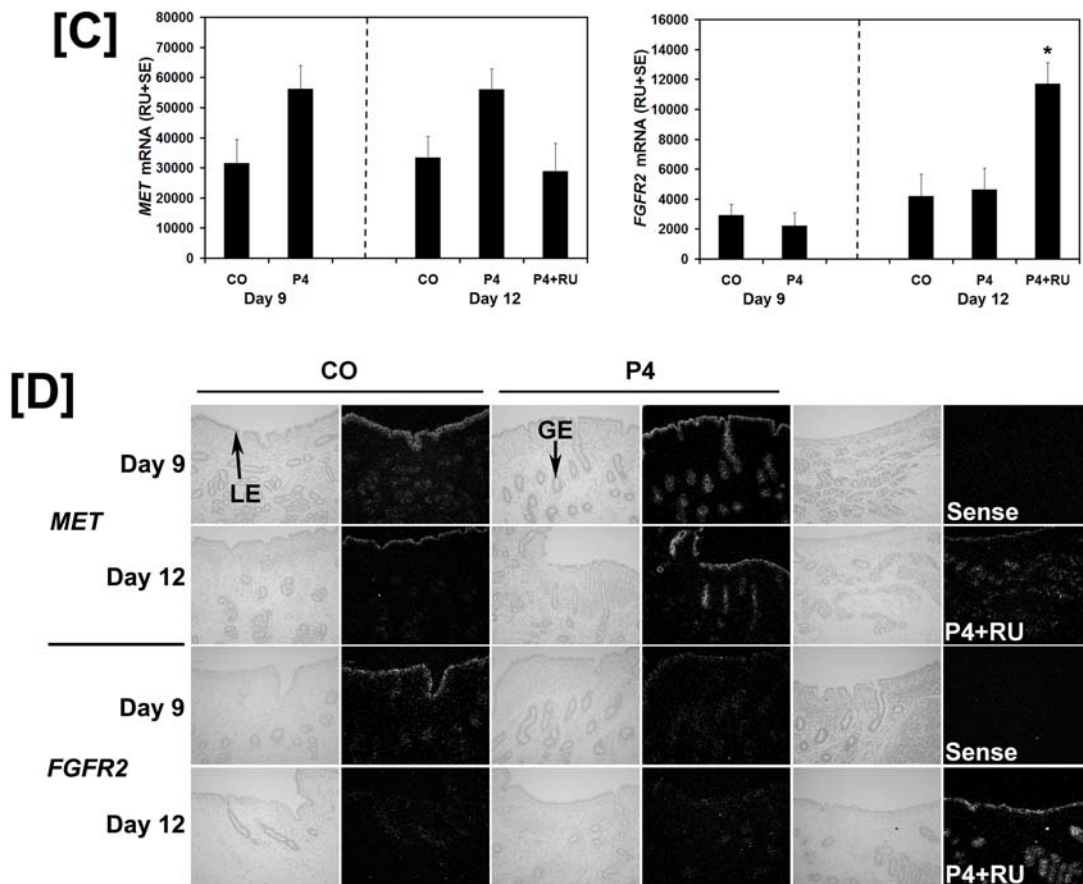


FIG. 6.1.cont. [C] Steady-state levels of *MET* and *FGFR2* mRNA in endometria from Day 9 (Study One) and Day 12 (Study Two) ewes. *MET* mRNA tended to increase ( $P < 0.07$ ) in P4-treated ewes on Day 9 and increased ( $P < 0.05$ ) in P4-treated ewes on Day 12. P4+RU-treated ewes had lower ( $P < 0.04$ ) quantities of *MET* mRNA compared to P4-treated ewes on Day 12. *FGFR2* mRNA was not different ( $P > 0.10$ ) between CO and P4-treated ewes on either Day 9 or Day 12; however, P4+RU treated ewes had higher ( $P < 0.001$ ) quantities of *FGFR2* mRNA compared to P4-treated ewes on Day 12. [D] *In situ* localization of epithelial specific *FGFR2* and *MET* which act as receptors for FGF7 and FGF10 and HGF, respectively. Cross-sections of uteri were hybridized with radiolabeled antisense or sense ovine cRNA probes for each respective gene. Legend: LE, luminal epithelium.

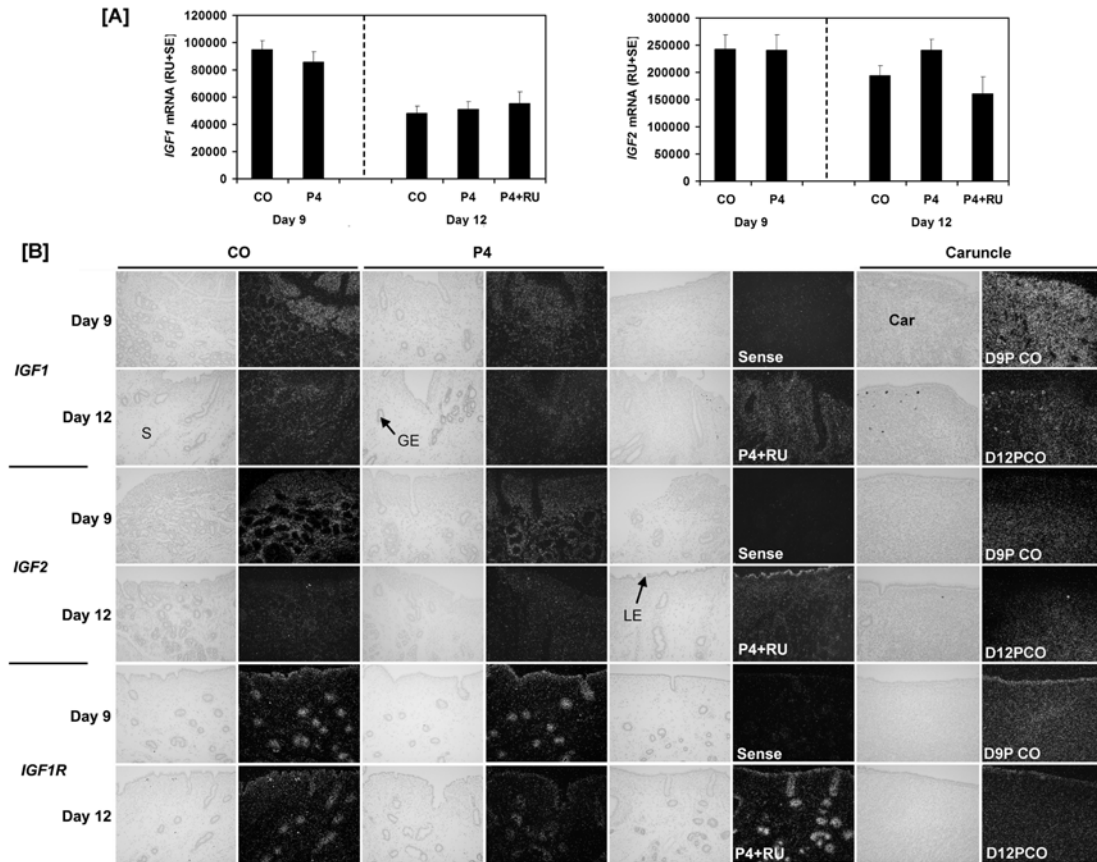


FIG. 6.2. Effects of treatments with CO, P4, or P4+RU on endometrial *IGF1*, *IGF2*, and *IGF1R* mRNAs. [A] Steady-state levels of *IGF1* and *IGF2* mRNAs in endometria from Day 9 (Study One) and Day 12 (Study Two) ewes, as determined by slot blot analysis. *IGF1* and *IGF2* mRNAs were not affected ( $P > 0.10$ ) by P4 treatment on Day 9 or Day 12. P4+RU-treated ewes had lower ( $P < 0.03$ ) quantities of *IGF2* mRNA than P4-treated ewes. [B] *In situ* localization of *IGF1*, *IGF2* and *IGF1R* mRNAs in endometria of Day 9 (Study One) and Day 12 ewes (Study Two) are presented. Cross-sections of uteri were hybridized with radiolabeled antisense or sense ovine cRNA probes for each respective gene. Legend: LE, luminal epithelium; S, stroma; GE, glandular epithelium.

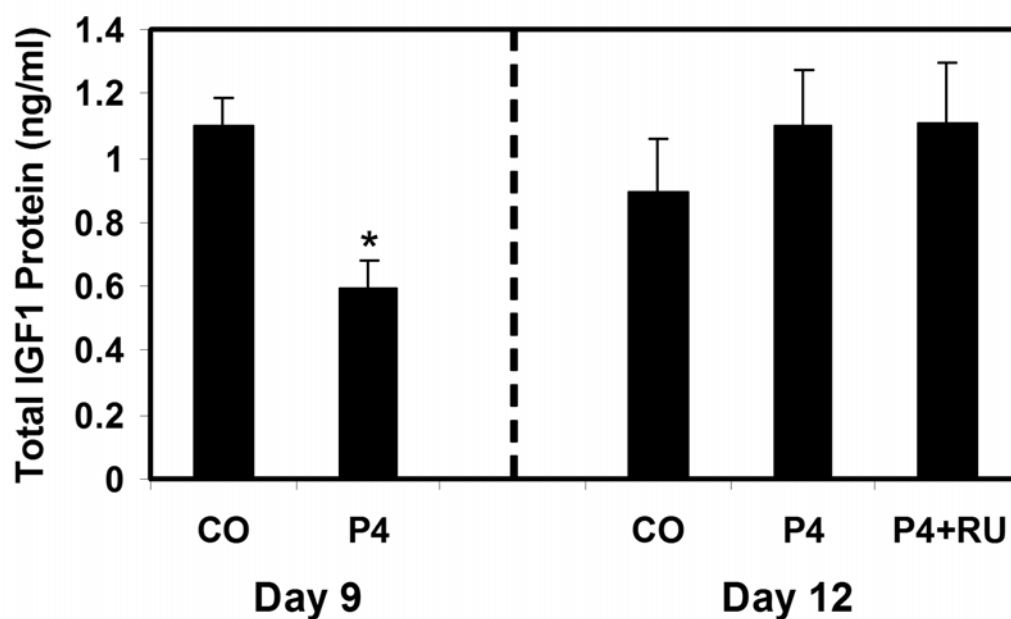


FIG. 6.3. Effects of CO, P4, or P4+RU on total IGF1 protein in uterine flushings. Total IGF1 protein in uterine flushings was determined by radioimmunoassay following acidification of samples to remove IGF1BPs. Total IGF1 protein was reduced ( $P=0.0007$ ) by P4 on Day 9, but there was no effect ( $P>0.10$ ) of treatment on Day 12.



### *Stromal IGFBPs*

Seven high affinity IGF binding proteins have been identified to date that modulate IGF bioavailability (Holly and Perks 2006; Tamura et al. 2007). Steady-state levels of endometrial *IGFBP* mRNAs are presented in Figure 4 and their localization by *in situ* hybridization in Figs. 6.5 and 6.6. *In situ* hybridization analysis revealed that *IGFBPs* 2, 4, 5, 6, and 7 were expressed predominantly in stromal cells of intercaruncular and caruncular endometria (Figure 6.5 and 6.6). *IGFBP5* was also expressed in LE on Day 9.

*IGFBP2* mRNA levels were not different ( $P>0.10$ ) between CO and P4-treated ewes on Days 9 and 12 or between CO ewes on Days 9 and 12; however, *IGFBP2* mRNA was 2-fold greater ( $P<0.02$ ) in P4+RU- compared to P4-treated ewes on Day 12 (Figure 6.4). *IGFBP4* and its known protease, *PAPPA*, were unaffected ( $P>0.10$ ) by P4 treatment on Days 9 and 12 and were not different ( $P>0.10$ ) between CO ewes on Days 9 and 12. *IGFBP5* mRNA was decreased ( $P<0.01$ ) in P4 ewes on Day 9. There was no difference ( $P>0.10$ ) in *IGFBP5* mRNA between CO and P4-treated ewes on Day 12 or between CO ewes on Days 9 and 12; however, *IGFBP5* mRNA levels were 3-fold greater ( $P<0.0001$ ) in P4+RU- compared to P4-treated ewes. *IGFBP6* mRNA was not different ( $P>0.10$ ) between CO and P4-treated ewes on Day 9, but *IGFBP6* mRNA was lower ( $P<0.03$ ) in P4-treated than CO- and P4+RU-treated ewes on Day 12. *IGFBP6* mRNA levels did not differ ( $P>0.10$ ) between CO ewes on Days 9 and 12. *IGFBP7* mRNA did not differ ( $P>0.10$ ) between CO and P4-treated ewes on Day 9 or Day 12 although, however *IGFBP7* mRNA levels were 2-fold greater ( $P<0.001$ ) in P4+RU-treated than P4-treated ewes on Day 12. *IGFBP7* mRNA levels tended to be lower ( $P<0.07$ ) in endometria of CO ewes on Day 12 as compared to Day 9.

*IGFBP5* mRNA decreased to undetectable levels in the LE between Day 9 and Day 12 (Figure 6.5). Interestingly, *IGFBP5* and *IGFBP7* mRNAs were also localized to stromal cells immediately surrounding the uterine vasculature (Figs. 6.5 & 6.6). *IGFBP5* mRNA was increased in stroma and myometrium of P4+RU ewes on Day 12. *IGFBP7* mRNA was also localized to the myometrium in CO ewes on Day 9 and was

reduced by P4 (data not shown). *IGFBPs 2, 4, 6 mRNAs* and *PAPPA* mRNA were undetectable in stromal cells specifically surrounding the uterine vasculature and in myometrium.

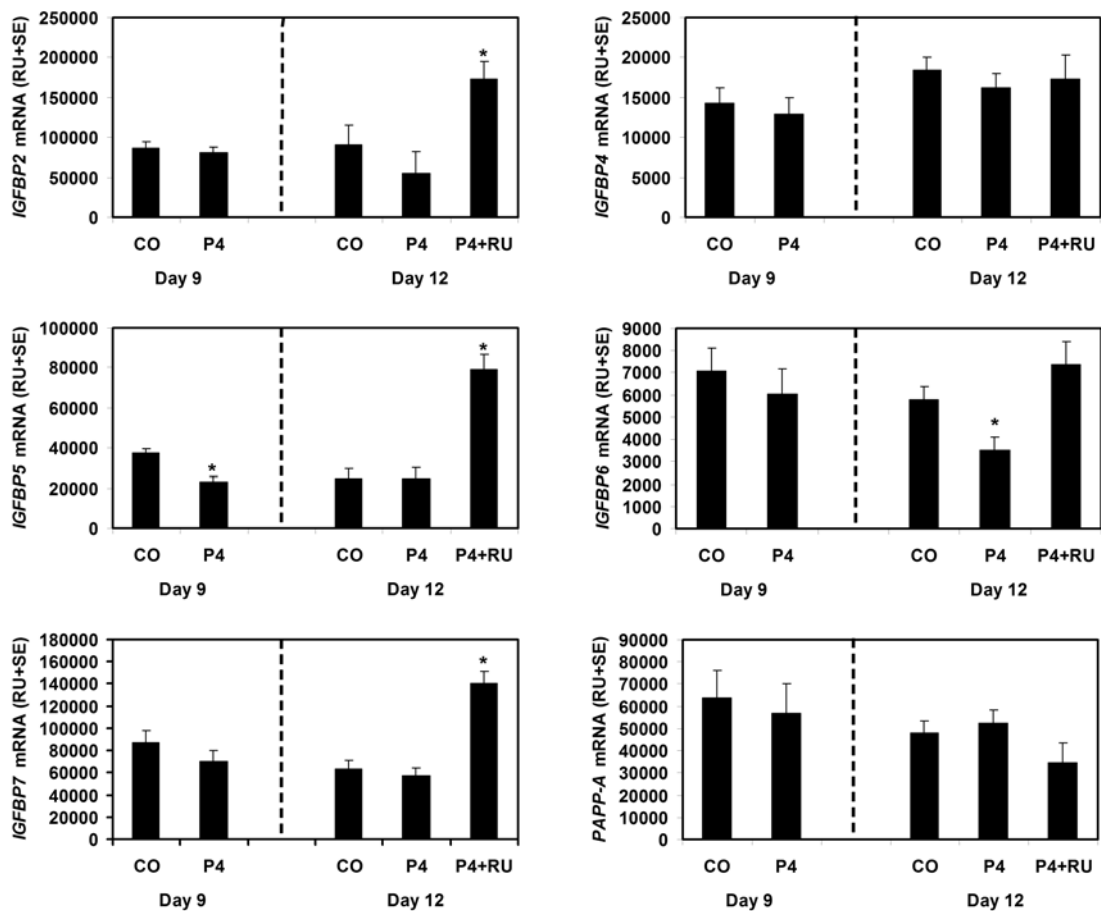


FIG. 6.4. Effects of CO, P4, or P4+RU on endometrial *IGFBP2*, *IGFBP4*, *IGFBP5*, *IGFBP6*, *IGFBP7*, and *PAPPA* mRNAs. [A] Steady-state levels of *IGFBP2*, *IGFBP4*, *IGFBP5*, *IGFBP6*, *IGFBP7*, and *PAPPA* mRNAs in endometria from Day 9 (Study One) and Day 12 (Study Two) ewes, as determined by slot blot analysis. In Study One, *IGFBP5* was reduced ( $P < 0.01$ ) by P4 on Day 9. In Study Two, *IGFBP2* ( $P < 0.04$ ), *IGFBP5* ( $P < 0.0001$ ), and *IGFBP7* ( $P < 0.0001$ ) increased in P4+RU treated ewes on Day 12. *IGFBP6* mRNA was decreased ( $P < 0.03$ ) in P4- compared to CO- and P4+RU- treated ewes on Day 12. There was no effect of treatment on *IGFBP4* or *PAPPA* on either Day 9 or Day 12.

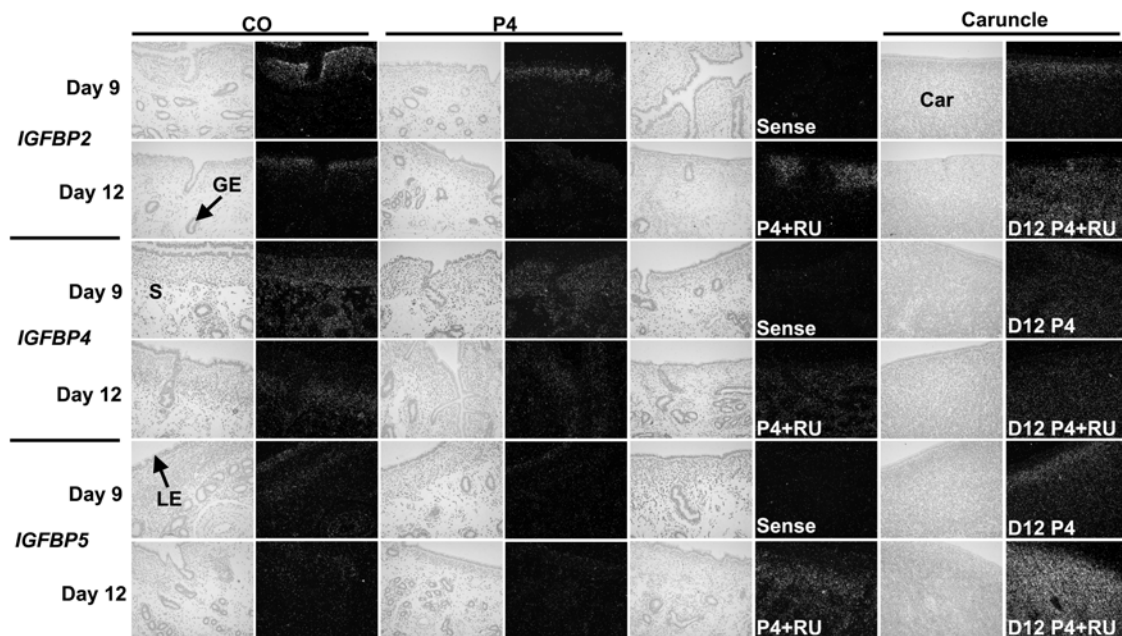


FIG. 6.5. Localization of stromal IGFbps in ovine endometria. *In situ* localization of *IGFBP2*, *IGFBP4*, and *IGFBP5* mRNAs in endometria of Day 9 (Study One) and Day 12 ewes (Study Two). Cross-sections of uteri were hybridized with radiolabeled antisense or sense ovine cRNA probes for each respective gene. Legend: LE, luminal epithelium; S, stroma; GE, glandular epithelium; CAR, caruncular stroma.

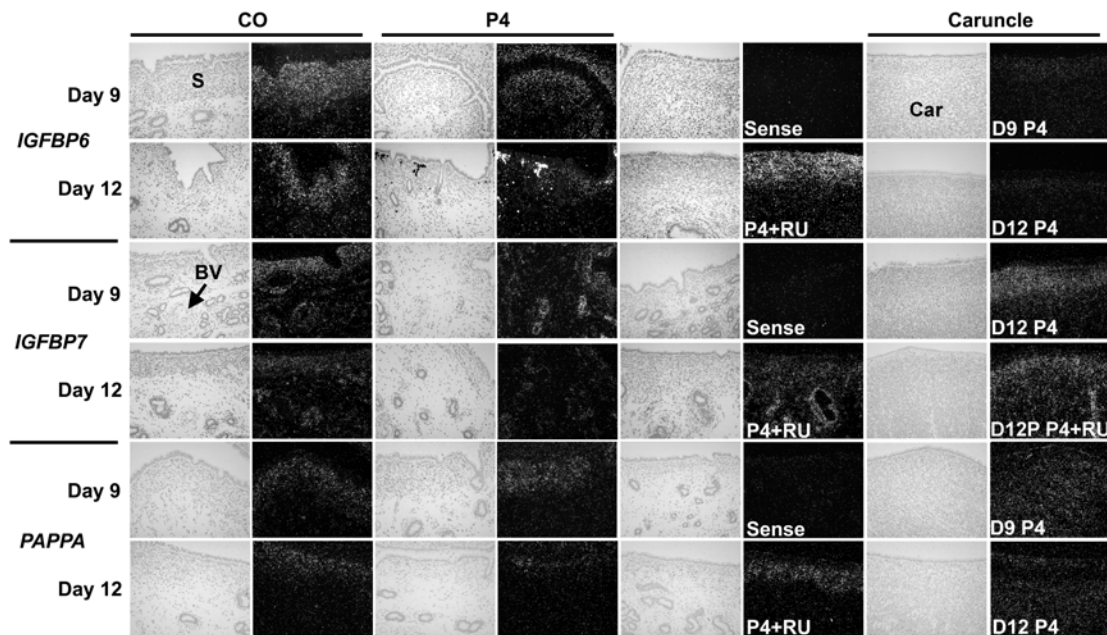


FIG. 6.6. Localization of stromal IGFbps in ovine endometria. *In situ* localization of *IGFBP6*, *IGFBP7*, and *PAPPa* mRNAs in endometria of Day 9 (Study One) and Day 12 ewes (Study Two). Cross-sections of uteri were hybridized with radiolabeled antisense or sense ovine cRNA probes for each respective gene. Legend: LE, luminal epithelium; S, stroma; CAR, caruncular stroma; and BV, blood vessel.

*IGFBPs 1 and 3 in the Uterine LE*

*In situ* hybridization analyses revealed that *IGFBP1* and *IGFBP3* mRNAs were present specifically in LE and sGE (Figure 6.7A). Endometrial *IGFBP1* mRNA was 9-fold higher ( $P<0.02$ ) in P4-treated ewes on Day 9 (Figure 6.7B). *IGFBP1* levels increased 28-fold from Day 9 to Day 12 in CO ewes, but there was no difference ( $P>0.10$ ) in *IGFBP1* mRNA between CO- and P4-treated ewes on Day 12. *IGFBP1* mRNA was 10-fold lower ( $P<0.0001$ ) in P4+RU- compared to P4-treated ewes on Day 12. *IGFBP3* mRNA was increased 3-fold ( $P<0.005$ ) in P4-treated ewes on Day 9 (Figure 6.7A). *IGFBP3* mRNA levels increased 6.4 fold ( $P<0.03$ ) in endometria of CO ewes on Day 12 as compared to Day 9. In Study Two, *IGFBP3* mRNA was reduced 2-fold ( $P<0.03$ ) in P4+RU- compared to P4-treated ewes. *In situ* hybridization detected mRNAs for *IGFBP1* and *IGFBP3* in LE and superficial GE of the ovine endometria (Figure 6.7B).

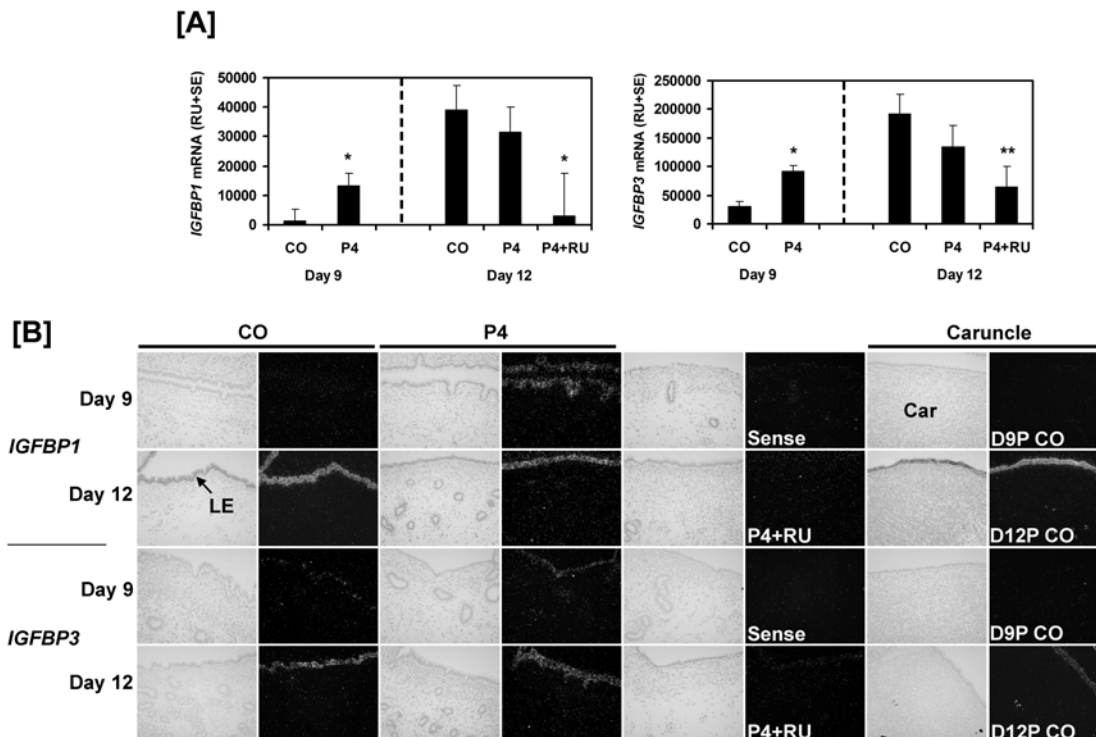


FIG. 6.7. Effects of treatments with CO, P4, or P4+RU on endometrial *IGFBP1* and *IGFBP3* mRNAs. [A] Steady-state levels of *IGFBP1* and *IGFBP3* mRNAs in endometria from Day 9 (Study One) and Day 12 (Study Two) ewes determined by slot blot analysis. *IGFBP1* ( $P > 0.02$ ) and *IGFBP3* ( $P > 0.005$ ) mRNAs were increased by P4 on Day 9, but not on Day 12. However, P4+RU-treated ewes expressed less *IGFBP1* ( $P < 0.0001$ ) and *IGFBP3* ( $P < 0.03$ ) mRNAs than CO- and P4-treated ewes. [B] *In situ* localization of *IGFBP1* and *IGFBP3* mRNAs in endometria of Day 9 (Study One) and Day 12 ewes (Study Two). Cross-sections of uteri were hybridized with radiolabeled antisense or sense ovine cRNA probes for each gene. Legend: LE, luminal epithelium.

### ***Discussion***

This study determined the role of P4 in regulating candidate stromal derived growth factors hypothesized to act on uterine epithelia as progestamedins and members of the IGF system. Results indicate that P4 differentially regulates *FGF7*, *FGF10*, *HGF*, *MET*, *FGFR2*, *IGF1*, *IGF2*, *IGFBP1*, *IGFBP2*, *IGFBP3*, *IGFBP5*, *IGFBP6*, and *IGFBP7* in a temporal and spatial manner which is hypothesized to result in a uterine environment conducive to blastocyst growth and development. Indeed, *FGF7* and *HGF* mRNAs were increased by P4 treatment on Day 9. Further, inhibition of P4 action with RU486, a PGR antagonist, decreased expression of all three candidate progestamedins (*FGF7*, *FGF10*, and *HGF*), supporting the hypothesis that these growth factors act as progestamedins to mediate P4 actions on uterine epithelia. Indeed, P4 upregulation of *FGF7* and *HGF* coincides with loss of PGR protein from the epithelia (Satterfield et al. 2006) suggesting that these molecules may mediate P4 effects on epithelial gene expression and function. Further, we have previously reported that genes encoding secreted proteins expressed in the LE such as *LGALS15* and *CTSL* were increased by P4 treatment (Satterfield et al. 2006). Indeed, expression of *LGALS15* and *CTSL* requires loss of PGR from uterine epithelia (Gray et al. 2006; Satterfield et al. 2006; Song et al. 2005), but it has yet to be determined if these genes are regulated by either FGF7 or HGF acting via FGFR2 or MET on LE. Roles for FGF7, FGF10, and HGF in mediating the actions of steroid hormones has also been reported for the primate uterus with FGF7 being produced in stromal cells in response to P4 (Koji et al. 1994); while HGF regulates human endometrial cell proliferation and motility (Sugawara et al. 1997) and mediates estrogen actions in the primate uterus (Brenner et al. 1997).

HGF regulates epithelial morphology and function (Kamalati et al. 1999). Alterations in epithelial junctional complexes occur in several forms of cancer in response to HGF actions via MET/beta catenin (CTNNB1) complexes (Davies et al. 2001; Hiscox and Jiang 1999; Murai et al. 2004). These alterations increase the invasive properties of cells. During pregnancy, we reported a transient reduction in CTNNB1 and CDH1 localized to the membranes of uterine epithelia following treatment of ewes with

P4 to Day 9 that upregulated expression of *HGF* (Satterfield et al. 2007). These alterations along with a reduction in tight junction complexes at the same time increase permeability of uterine epithelia to stromal and/or serum derived factors such as glucose, amino acids, ions and growth factors which may stimulate blastocyst growth and development. Receptors for these growth factors are also expressed by ovine conceptus trophoderm (Chen et al. 2000a; Chen et al. 2000b); therefore, FGF7, FGF10, and HGF may stimulate conceptus proliferation and differentiation directly. Indeed, HGF signaling in the trophoblast is aberrantly regulated during preeclampsia (Kauma et al. 1999; Nasu et al. 2000) and results in increased trophoblast apoptosis (Dash et al. 2005). The anti-apoptotic role of HGF was linked to glycogen synthase kinase-3beta and beta catenin which acted in concert to increase expression of inducible nitric oxide synthase.

The IGF system plays a critical role in many developmental processes and is a primary determinant of prenatal and postnatal fetal growth. Available results indicate that *IGF1* is not regulated by P4, although a reduction in steady-state mRNA levels was observed between Days 9 and 12 of pregnancy. In contrast, *IGF2* is regulated by P4 in a cell specific manner suggesting differential roles for *IGF2* in uterine function and conceptus development. The induction of *IGF2* mRNA in LE following P4 inhibition suggests that P4 suppresses *IGF2* expression in LE. In contrast, increased expression within the caruncular and *stratum compactum* stromal cells by P4 suggests a more classical epithelial/mesenchymal role for IGF2 in pregnancy, possibly increasing the secretory activity of the epithelia via its receptor in LE and GE. This idea is supported by evidence that, in human endometria, *IGF1* is preferentially expressed during the proliferative phase while *IGF2* is expressed in the mid to late secretory phase and in early pregnancy to regulate differentiated functions of the endometrium (Giudice et al. 1993).

IGF is hypothesized to act on the developing conceptus as well. Indeed, both ovine and bovine conceptuses express *IGFIR* (Watson et al. 1999). Previous research, primarily utilizing *in vitro* cultured embryos, elucidated both proliferative and anti-apoptotic roles for IGF1 (Block et al. 2003; Byrne et al. 2002; Sirisathien and Brackett



2003). The protective effects of IGF1 increased during periods of heat stress (Jousan and Hansen 2007). Extension of the roles of IGF1 to the elongation stage of conceptus development has not been achieved to date. Since blastocysts do not elongate in culture, it is not possible to monitor the effects of IGF1 on conceptus development during the morphological transformation from spherical to tubular and filamentous conceptuses.

Interestingly, although *IGF1* mRNA levels did not differ on Day 9, total IGF1 protein in uterine flushings was reduced by half in P4-treated ewes. Total IGF1 on Day 12 did not differ between treatment groups similar to the pattern of mRNA expression. It is possible that a very narrow window exists on Day 9 whereby the developing blastocyst has relatively unrestricted access to free IGF prior to upregulation of epithelial *IGFBPs 1* and *3*. If so, the reduction in total IGF1 protein may result from its rapid utilization by blastocysts. This access may be attenuated by rapid upregulation of LE specific IGFBPs to sustain a more constant level of total IGF protein. It is important to remember that only free IGF is available to the conceptus and that measures of total IGF1 protein based on our RIA is not a true measure of bioavailability, but only total quantity of IGF. Interestingly, early conceptus development in cows can be stimulated by components of the GH-IGF system (Moreira et al. 2002a; Moreira et al. 2002b). Administration of bovine somatotropin (bST) enhances pregnancy rates in lactating dairy cows, possibly via alterations in the IGF system (Moreira et al. 2001; Moreira et al. 2000), and also increased pregnancy rate, conceptus length, and IFNT secretion on Day 17 (Bilby et al. 2006a; Bilby et al. 2006b). These effects were accompanied by increased circulating concentrations of IGF1 in serum. In mice, systemic IGF1 rather than local IGF1 mediates estrogen-induced uterine growth and proliferation (Sato et al. 2002)

As modulators of IGF bioavailability, the IGFBPs present dynamic complexity to understanding IGF functions. Temporal and spatial alterations in expression of IGFBPs in P4-advanced uteri provide insight into potential regulatory functions of these molecules. *IGFBPs 1-6* have been identified and characterized in ovine endometria during early pregnancy (Gadd et al. 2000; Gadd et al. 2002; Osgerby et al. 1999;

Reynolds et al. 1997) results of the present study using *in situ* hybridization revealed that *IGFBPs* 2, 4, 5, 6, and 7 are expressed predominantly by stromal cells on Days 9 and 12. Interestingly, the majority of these IGFBPs were most abundant in ewes treated with RU486, a PGR antagonist. These findings suggest that IGFBPs localized to the stroma sequester IGF which inhibits their ability to effect actions on uterine epithelia. A reduction in availability of free IGF may, in part, account for the early demise of blastocysts in P4+RU-treated ewes.

*IGFBP1* and *IGFBP3* were specifically expressed within LE and superficial GE. Both *IGFBP1* and 3 were expressed at low levels on Day 9 in CO-treated ewes and strongly induced by P4 suggesting that these modulators of IGF action have a critical role in regulating early conceptus development and may act as mediators of cross-talk between the uterus and developing conceptus. This hypothesis is supported by the reduction of *IGFBP1* and *IGFBP3* mRNAs following inhibition of P4 actions with RU486. Limited results exist on the presence of IGFBPs in the uterine lumen; however, *IGFBP3* is the predominant IGFBP in the uterine lumen during early pregnancy in sheep (Peterson et al. 1998) and cattle (Keller et al. 1998). Interestingly, treatment of ewes with P4 for 10 days resulted in proteolysis of *IGFBP3* which would theoretically increase bioactive IGF available to the conceptus; however, treatment with P4 for 15 days subsequently decreased protease activity via apparent upregulation of an unidentified protease inhibitor. Thus, available studies support P4-induction of *IGFBP3* and actions of proteases to cleave *IGFBP3* to release free IGF for rapid utilization by developing blastocysts. Other IGFBPs were present in the uterine lumen at significantly lower abundances (Keller et al. 1998; Peterson et al. 1998), but further supporting the importance of IGFBPs from uterine epithelia. Interestingly, these studies did not identify *IGFBP1* in uterine flushings despite expression of *IGFBP1* within the adjacent epithelium and its regulation by P4.

The upregulation of *IGFBP1* during the peri-implantation period of pregnancy is not a unique event to sheep. Indeed, *IGFBP1* is a highly upregulated gene within the human secretory endometrium during the period of uterine receptivity to implantation

(Kao et al. 2002). Similarly, IGFBP1 is the primary secretory product of uterine decidual cells in the baboon (Fazleabas et al. 1993). IGFBP1 has actions independent of the IGF system (Gleeson et al. 2001; Irwin and Giudice 1998). IGFBP1 contains an RGD sequence and can act as a ligand for integrins such as  $\alpha 5\beta 1$ . IGFBP1 stimulated migration of human trophoblast cells was attenuated by mutation of the RGD sequence to WGD or pretreatment with an inhibitory peptide (Gleeson et al. 2001).

Curiously, in humans, overexpression of IGFBP1 has been detected during preeclampsia, a condition in pregnancy believed to result from superficial invasion of the placental trophoblast into the maternal endometrium resulting in fetal growth retardation and possibly pregnancy loss (Anim-Nyame et al. 2003; Giudice et al. 1997). These findings support a role for IGFBP1 as an integrin ligand responsible for maintaining appropriate fetal/maternal contact and regulating the invasion of the embryo into the endometrium, a process believed to be aberrantly regulated in the development of preeclampsia. At present, the mechanisms by which this may occur are unknown. Similar observations were made in mice following overexpression of IGFBP1 which resulted in abnormal trophoblast invasion and differentiation, increased placental mass, and intrauterine fetal growth retardation (Ben Lagha et al. 2006; Crossey et al. 2002). An increase in placental mass coincident with fetal intrauterine growth retardation indicates that IGFBP1 mediated invasion by developing blastocysts manifests long-term consequences resulting in placental insufficiency. Despite markedly different implantation schemes between primates, rodents, and ruminants, *IGFBP1* is a highly upregulated gene within endometria of all these mammals. The upregulation of this gene during implantation across species with differing implantation schemes suggests a critical conserved function for conceptus development and survival, although the mechanism remains elusive. In sheep, elongation of the spherical blastocyst is a uterine dependent event (Flechon et al. 1986) that correlates with loss of PGR and the anti-adhesive MUC1 glycoalex from the LE exposing LE integrins (Johnson et al. 2001a; Spencer and Bazer 1995) and induction of the integrin ligand IGFBP1. IGFBP1 may

play a central role in mediating blastocyst/uterine interactions required for elongation and implantation.

The present study is a first step in identifying progesterone regulated genes which are implicated in enhancing elongation of the blastocyst; a process required for the production of sufficient IFNT to block the luteolytic mechanism and maintain pregnancy. In this study, *IGFBPs 1* and *3* as well as *FGF7* and *HGF* were identified as potential critical regulators of endometrial function and/or conceptus development. Future studies will mechanistically dissect the role of each of these factors in sustaining pregnancy.

## CHAPTER VII

### SUMMARY AND CONCLUSIONS

#### *Summary*

Progesterone is unequivocally required for successful pregnancy and mediates peri-implantation conceptus growth and development via actions on the endometrium. This research utilized a model of accelerated conceptus development achieved via early administration of P4 (beginning 36 h after mating) to elucidate mechanisms of P4 actions including expression of P4-regulated genes important for blastocyst growth and development in sheep. Results of the present studies are the first to identify endometrial P4 regulated genes in the sheep utilizing a model of early exogenous P4 treatment.

The importance of an early rise circulating concentrations of P4 to promote conceptus growth and development and production of IFNT is well established (Mann et al. 2006; Mann and Lamming 2001). Further, administration of early exogenous progesterone illustrated that this treatment advances uterine functions and accelerates conceptus growth and development in sheep (Kleemann et al. 1994) and cattle (Garrett et al. 1988b). The acceleration of conceptus growth is believed to result from an increased or advanced secretory capacity of the endometrium as evidenced by alterations in secreted proteins from bovine endometrium on Day 5 following early P4 (Garrett et al. 1988b); however, no specific proteins were identified in that study. Data from UGKO ewes illustrated a requirement for secretions from LE and GE to support conceptus development beyond Day 14 (Gray et al. 2002; Gray et al. 2001c). Despite the importance of secreted factors from the endometrium, little is known about functions of components of uterine histotroph and temporal changes in expression of these molecules associated with different stages of implantation.

Identifying mechanisms whereby progesterone regulates conceptus growth and development utilizing a molecular biology approach is a first step in determining strategies to reduce the economic losses associated with pregnancy failure in domestic species of livestock. Development and characterization of an ovine model of accelerated

conceptus development during the peri-implantation period was undertaken (CHAPTER III). As expected, early administration of progesterone resulted in a premature and sustained rise in circulating concentrations of P4. Administration of P4 from Days 1.5 to Day 9 increased mean blastocyst diameter 2.2-fold over blastocysts from CO ewes. Administration of P4 from Days 1.5 to Day 12 advanced the morphological transformation from a spherical blastocyst to a filamentous conceptus. Administration of the PGR antagonist RU486 from Days 8 to Day 12 to block progesterone action resulted in demise of conceptuses. These observations demonstrate the ability of P4 to enhance blastocyst growth and development in sheep. The premature exposure of the endometrium to P4 accelerated loss of PGR from LE and GE which may initiate reprogramming of the epithelia to increase secretory activity due directly to loss of PGR or via upregulation of a stromal cell-derived factor responsive to P4. Western blot and protein slot blot analyses revealed that elongated filamentous conceptuses on Day 12 produced greater quantities of IFNT than spherical or tubular conceptuses from CO ewes, with no IFNT being detected from ewes receiving RU486. The increase in IFNT is important for multiple reasons. First, IFNT is the pregnancy recognition signal and higher levels of IFNT should improve the likelihood for successful maternal recognition of pregnancy. Second, IFNT induces or enhances the expression of a number of genes important for endometrial function and implantation. To determine if the increase in IFNT increased expression of known IFNT stimulated genes in the endometrium in a manner synchronous to the stage of the conceptus, slot blot and *in situ* hybridization techniques were employed. Indeed, *CTSL* and *RSAD2*, two previously characterized IFNT stimulated genes (Song et al. 2007; Song et al. 2005), were upregulated early in P4-treated ewes on Day 12 as compared to CO or P4+RU-treated ewes. Collectively, these findings establish and characterize a model system that can be explored to understand P4 actions on the uterine endometrium.

LGALS15, a recently characterized molecule in the ovine endometrium induced by P4 and further stimulated by IFNT, is hypothesized to enhance conceptus development and elongation via its adhesive properties (Farmer et al. 2007; Gray et al.

2006; Gray et al. 2004; Lewis et al. 2007). In support of this hypothesis, results of the present studies confirmed that LGALS15 was indeed induced by P4 and stimulated by IFNT (CHAPTER III). Further, the increase in LGALS15 protein expressed along the apical surface of the LE increased in uterine flushings in response to early P4 treatment. LGALS15 expression was ablated by administration of RU486. Collectively, available data from UGKO ewes (Gray et al. 2004) and present findings support a role for LGALS15 in early conceptus growth and development.

Existing information regarding uterine histotroph indicate the existence of factors of non-endometrial origin in the uterine lumen (Lee et al. 1998b). The specific identity and origin of many of these factors is unknown as is the mechanism by which these factors reach the uterine lumen. Interestingly, evidence obtained by injection of tracer compounds into the uterus suggested that molecules may enter the uterine luminal space via trafficking through the paracellular space between adjacent epithelial cells (Guillomot et al. 1986; Guillomot et al. 1981). The permeability of this space is regulated by abundance of specific tight junction associated proteins that are inversely proportional to permeability of the tissue (Gonzalez-Mariscal et al. 2000b).

We hypothesized that the uterine luminal microenvironment is altered by changes in endometrial epithelial junctional complexes (CHAPTER IV). Utilization of immunocytochemistry allowed for identification of a transient downregulation of tight junction associated proteins from Days 10 to 12 of the estrous cycle and pregnancy followed by an increase in tight junction proteins in uterine epithelia on Day 14 of pregnancy but not the cycle. Further, the decline in tight junction associated proteins was accelerated by administration of early exogenous P4. A similar decline in components of adherens junctions, specifically CDH1 and CTNNB1 occurred in endometrial LE during the period of conceptus elongation and in response to P4. These findings suggest the period of blastocyst elongation is initiated when there is a general decline in tight and adherens junction proteins in the epithelium, thus increasing the permeability of the epithelium to stromal and serum derived molecules such as HGF, FGF7, glucose, and/or essential amino acids. These factors may further regulate

morphological changes of spherical blastocysts into filamentous conceptuses. A return of junctional complexes to the epithelium in response to pregnancy on Day 14 has multiple implications such as reducing permeability to sequester IFNT to act first on adjacent epithelia to inhibit formation of the luteolytic mechanism prior to upregulating a number of IFNT stimulated genes in stromal cells. Further, an increase in epithelial integrity in response to more aggressive adhesion and attachment of the conceptus following elongation may limit and regulate the extent of superficial invasion of the conceptus. Indeed, decidual cells surrounding the invading embryo uncharacteristically express tight and adherens junctions proteins in mice (Paria et al. 1999; Wang et al. 2004). In addition, reducing the permeability of the epithelium during implantation may limit maternal immune responses to the conceptus and provide a somewhat immunoprivileged environment. Future studies combining proteomics and genomics will identify factors of non-endometrial and stromal origin that enhance blastocyst growth and development.

WNT genes are homologous to the *Drosophila* segment polarity gene wingless (*wg*). In humans and mice, the WNT family encodes a group of 19 highly conserved secreted glycoproteins that regulate cell and tissue growth and differentiation as well as regulate components of junctional complexes (Davies 2002; Davies and Fisher 2002; Robinson et al. 2000). Recently, the WNT system has been implicated in the regulation of cell polarity and junctional complexes and their associated proteins (Karner et al. 2006a; Karner et al. 2006b). Moreover, many components of the WNT system are expressed in the ovine uterine endometrium and modulate trophoctoderm migration and proliferation (Hayashi et al. 2007). These results coupled with our observations of decreased organization of both tight and adherens junctions in response to P4 support the hypothesis that components of the WNT signaling family may be involved in mediating changes in ovine epithelia in response to P4.

Initially, we endeavored to establish hormonal regulation of a number of WNTs and their downstream signaling and transcriptional partners previously identified in adult ovine endometria (Hayashi et al. 2007) (CHAPTER V). Analyses of WNT signaling



molecules in the model of P4-induced accelerated blastocyst development indicated that *WNT5A*, *WNT11*, *WNT2*, *WNT7A*, *FZD6*, *TCF7L2*, *JUN*, *p-JUN*, *p-CREB*, *MSX1*, and *MSX2* were downregulated in ovine endometrium between Days 9 and 12 in either a P4-dependent or P4-independent manner. *DKK1*, an inhibitor of WNT signaling, increased from Day 9 to Day 12. Further, inhibiting P4 action with RU486, increased endometrial *WNT5A*, *WNT11*, *WNT2*, *WNT7A*, *TCF7L2*, *p-CREB*, *MSX1*, and *MSX2* while ablating *DKK1* and *LEF1*, compared to ewes receiving P4 alone to Day 12. These findings confirm a correlation between the decline in tight and adherens junctions during this period of pregnancy and support the hypothesis that WNT signaling molecules may mediate changes in uterine epithelia to alter the uterine microenvironment.

In addition to potentially mediating changes in epithelial histoarchitecture and function, a decline in WNT signaling followed by a conceptus stimulated increase in the epithelium at the site of implantation occurred in ewes as has been observed in mice. This may be a novel mechanism whereby the blastocyst and uterine endometrium become developmentally synchronized to initiate appropriately timed conceptus/maternal communications required for successful implantation (Mohamed et al. 2005). Indeed, inhibition of WNT signaling via infusion of a WNT antagonist decreased the number of implanting blastocysts (Mohamed et al. 2005). Our observations, along with those previously described by Hayashi et al. (Hayashi et al. 2007), suggest that a similar mechanism exists in sheep, whereby the maternal endometrium is synchronized by signals from the conceptus following downregulation of all inherent endometrial WNT signals. Indeed, *WNT7A* is a novel IFNT stimulated gene expressed in endometrial LE on Day 14 of pregnancy, but not the cycle (Kim et al. 2003).

The mechanism(s) whereby P4 mediates its effects on the epithelium despite a loss of the PGR from both LE and GE is a conundrum hindering understanding of progesterone modulated events affecting uterine functions essential to implantation and successful establishment and maintenance of pregnancy. One mechanism involves activation of expression of stromal cell-derived growth factors by P4 act via PGR and actions of these “progestamedins” in a paracrine manner on adjacent epithelia to induce

functional differentiation and gene expression. Our laboratory identified *FGF7*, *FGF10*, and *HGF* as potential stromal mediators of progesterone action (i.e. progestamedins) (Chen et al. 2000a; Chen et al. 2000b). When analyzed in the early P4 model, there were increases in *FGF7* and *HGF* mRNA abundance on Day 9, but not on Day 12. Further, the receptor for *HGF*, *MET*, was increased in epithelia by P4 on both Days 9 and 12. The administration of the PGR antagonist, RU486, decreased the expression of *FGF7*, *FGF10*, and *HGF* mRNAs. These results are the first to identify hormonal regulation of these growth factors by P4 in the ovine endometrium and support the hypothesis of stromal derived signals mediating epithelial function in response to P4. In addition, the receptors for FGF7, FGF10, and HGF are present on conceptus trophectoderm (Chen et al. 2000a; Chen et al. 2000b) and may directly stimulate conceptus development. The existence of these stromal-derived growth factors in the uterine lumen and their potential trafficking through the epithelial paracellular space has not been determined.

The IGF system plays a critical role in many developmental processes and is a primary determinant of prenatal and postnatal fetal growth. Therefore we hypothesized that the enhanced growth and development observed in response to early exogenous P4 may in part be due to modulation of the IGF system by P4. Results of the present study indicate that P4 regulates multiple members of the IGF system including, IGF1, *IGF2*, *IGFBP1*, *IGFBP2*, *IGFBP3*, *IGFBP5*, *IGFBP6* and *IGFBP7*. Interestingly members of the IGF system were regulated in a temporal and spatial pattern that suggests differing roles for these family members during the peri-implantation period. IGF1 protein in the uterine lumen was reduced immediately prior to conceptus elongation and could represent a critical period wherein IGF1 is rapidly utilized by the developing blastocyst resulting in an overall decrease in IGF1 levels in uterine fluid. *IGFBP2*, *IGFBP5*, *IGFBP6*, and *IGFBP7* were expressed predominantly in endometrial stromal cells and were relatively low during the period of conceptus elongation. Interestingly, they were upregulated following blockade of P4 action with RU486. The demise of blastocysts observed in this treatment group may, in part, be due to sequestration of IGFs by these binding proteins in the stroma, thus limiting IGF bioavailability to blastocysts. In

contrast, *IGFBP1* and *IGFBP3* were induced by P4 in LE and increased from Day 9 to Day 12. This period coincides with loss of PGR and elongation of the conceptus indicating that *IGFBP1* and *IGFBP3* may indeed regulate blastocyst growth, development, and elongation. Indeed, IGFBP1 is a highly upregulated secretory product in human, baboon, and mouse during implantation and it is believed to attenuate the proliferative actions of IGF on the endometrium. Further, IGFBP1 has IGF independent actions as an integrin ligand. Therefore, in sheep, upregulation of IGFBP1 during the elongation period allows IGFBP1 to serve as an effective adhesion molecule for apposition of the blastocyst to LE, facilitating the process of expansion and elongation. *IGFBP1* and *IGFBP3* mRNAs were reduced in response to RU486 further implicating them as critical for conceptus growth and survival. Future studies will mechanistically dissect the role of these P4-regulated growth factors in blastocyst growth and development.

### ***Conclusions***

Results of the present studies establish and characterize a viable model for increasing our understanding of a number of factors important for implantation including: identification of P4-regulated genes, identification of novel mechanisms by which P4 mediates its action on different cell types, identification of molecules that act on the conceptus to stimulate conceptus elongation, and a mechanism by which factors secreted into the uterine lumen in response to P4 can be identified. Future studies will employ genomic and proteomics approaches to identify P4 regulated genes in ovine endometria and developing conceptuses. A proteomics approach using mass spectroscopy techniques will be used to identify components of uterine histotroph present during the period of conceptus elongation and further elucidate regulation of these molecules by P4. Melding the disciplines of genomics and proteomics will allow increased understanding of the origins of molecules present in the uterine lumen during the putative window of receptivity for implantation and provide insight into mechanisms by which these molecules are trafficked within the endometrium. Ultimately, results

from these and future studies will identify selection criteria and strategies for reducing early embryonic losses in economically important species of livestock.

## REFERENCES

- Aberle H, Butz S, Stappert J, Weissig H, Kemler R, Hoschuetzky H. 1994. Assembly of the cadherin-catenin complex in vitro with recombinant proteins. *J Cell Sci* 107 (Pt 12):3655-3663.
- Achache H, Revel A. 2006. Endometrial receptivity markers, the journey to successful embryo implantation. *Hum Reprod Update* 12(6):731-746.
- Afonso S, Romagnano L, Babiarz B. 1997. The expression and function of cystatin C and cathepsin B and cathepsin L during mouse embryo implantation and placentation. *Development* 124(17):3415-3425.
- Anim-Nyame N, Hills FA, Sooranna SR, Steer PJ, Johnson MJ. 2003. The relationship between insulin and insulin-like growth factor binding protein-1 is modified by pre-eclampsia. *Gynecol Endocrinol* 17(6):471-476.
- Aplin JD, Hey NA. 1995. MUC1, endometrium and embryo implantation. *Biochem Soc Trans* 23(4):826-831.
- Ashley RL, Clay CM, Farmerie TA, Niswender GD, Nett TM. 2006. Cloning and characterization of an ovine intracellular seven transmembrane receptor for progesterone that mediates calcium mobilization. *Endocrinology* 147(9):4151-4159.
- Ashworth CJ, Bazer FW. 1989b. Changes in ovine conceptus and endometrial function following asynchronous embryo transfer or administration of progesterone. *Biol Reprod* 40(2):425-433.
- Ashworth CJ, Bazer FW. 1989a. Interrelationships of proteins secreted by the ovine conceptus and endometrium during the periattachment period. *Animal Reproduction Science* 20:117-130.
- Ayalon N. 1978. A review of embryonic mortality in cattle. *J Reprod Fertil* 54(2):483-493.
- Bafico A, Gazit A, Pramila T, Finch PW, Yaniv A, Aaronson SA. 1999. Interaction of frizzled related protein (FRP) with Wnt ligands and the frizzled receptor suggests alternative mechanisms for FRP inhibition of Wnt signaling. *J Biol Chem* 274(23):16180-16187.
- Bagchi IC, Cheon YP, Li Q, Bagchi MK. 2003. Progesterone receptor-regulated gene networks in implantation. *Frontiers in Bioscience* 8:s852-861.

- Bain DL, Heneghan AF, Connaghan-Jones KD, Miura MT. 2007. Nuclear receptor structure: implications for function. *Annu Rev Physiol* 69:201-220.
- Baird DT. 2002. Medical abortion in the first trimester. *Best Pract Res Clin Obstet Gynaecol* 16(2):221-236.
- Baker J, Liu JP, Robertson EJ, Efstratiadis A. 1993. Role of insulin-like growth factors in embryonic and postnatal growth. *Cell* 75(1):73-82.
- Balda MS, Gonzalez-Mariscal L, Contreras RG, Macias-Silva M, Torres-Marquez ME, Garcia-Sainz JA, Cerejido M. 1991. Assembly and sealing of tight junctions: possible participation of G-proteins, phospholipase C, protein kinase C and calmodulin. *J Membr Biol* 122(3):193-202.
- Bartol FF, Johnson LL, Floyd JG, Wiley AA, Spencer TE, Buxton DF, Coleman DA. 1995. Neonatal exposure to progesterone and estradiol alters uterine and morphology and luminal protein content in adult beef heifers. *Theriogenology* 43:835-844.
- Battye KM, Evans G, O'Neill C. 1996. Ovine endometrium synthesizes and releases platelet-activating factor, which can cause the release of prostaglandin F2 alpha by the uterus in situ. *Biol Reprod* 54(2):355-363.
- Bazer FW. 1975. Uterine protein secretions: relationship to development of the conceptus. *J Anim Sci* 41(5):1376-1382.
- Bazer FW, Roberts RM, Basha SM, Zavy MT, Caton D, Barron DH. 1979a. Method for obtaining ovine uterine secretions from unilaterally pregnant ewes. *J Anim Sci* 49(6):1522-1527.
- Bazer FW, Roberts RM, Thatcher WW. 1979b. Actions of hormones on the uterus and effect on conceptus development. *J Anim Sci* 49(Suppl 2):35-45.
- Ben Lagha N, Seurin D, Le Bouc Y, Binoux M, Berdal A, Menuelle P, Babajko S. 2006. Insulin-like growth factor binding protein (IGFBP-1) involvement in intrauterine growth retardation: study on IGFBP-1 overexpressing transgenic mice. *Endocrinology* 147(10):4730-4737.
- Berendt FJ, Frohlich T, Schmidt SE, Reichenbach HD, Wolf E, Arnold GJ. 2005. Holistic differential analysis of embryo-induced alterations in the proteome of bovine endometrium in the preattachment period. *Proteomics* 5(10):2551-2560.
- Bilby TR, Guzeloglu A, MacLaren LA, Staples CR, Thatcher WW. 2006a. Pregnancy, bovine somatotropin, and dietary n-3 fatty acids in lactating dairy cows: II.

- endometrial gene expression related to maintenance of pregnancy. *J Dairy Sci* 89(9):3375-3385.
- Bilby TR, Sozzi A, Lopez MM, Silvestre FT, Ealy AD, Staples CR, Thatcher WW. 2006b. Pregnancy, bovine somatotropin, and dietary n-3 fatty acids in lactating dairy cows: I. ovarian, conceptus, and growth hormone-insulin-like growth factor system responses. *J Dairy Sci* 89(9):3360-3374.
- Bindon BM. 1971. Systematic study of preimplantation stages of pregnancy in the sheep. *Aust J Biol Sci* 24(1):131-147.
- Block J, Drost M, Monson RL, Rutledge JJ, Rivera RM, Paula-Lopes FF, Ocon OM, Krininger 3<sup>rd</sup>, CE, Liu J, Hansen PJ. 2003. Use of insulin-like growth factor-I during embryo culture and treatment of recipients with gonadotropin-releasing hormone to increase pregnancy rates following the transfer of in vitro-produced embryos to heat-stressed, lactating cows. *J Anim Sci* 81(6):1590-1602.
- Boon EM, van der Neut R, van de Wetering M, Clevers H, Pals ST. 2002. Wnt signaling regulates expression of the receptor tyrosine kinase met in colorectal cancer. *Cancer Res* 62(18):5126-5128.
- Boutros M, Paricio N, Strutt DI, Mlodzik M. 1998. Dishevelled activates JNK and discriminates between JNK pathways in planar polarity and wingless signaling. *Cell* 94(1):109-118.
- Brayman M, Thathiah A, Carson DD. 2004. MUC1: a multifunctional cell surface component of reproductive tissue epithelia. *Reprod Biol Endocrinol* 2(1):4.
- Brenner RM, McClellan MC, West NB, Novy MJ, Haluska GJ, Sternfeld MD. 1991. Estrogen and progestin receptors in the macaque endometrium. *Ann N Y Acad Sci* 622:149-166.
- Brenner RM, Slaydon OD, Koji T, Izumi M, Chedid M, Csaky KG, Rubin JS. 1997. Hormonal regulation of the paracrine growth factors HGF and KGF in the endometrium of the rhesus macaque. In: Stock G, Habenicht, U.F. editor. *The endometrium as a target for contraception* Verlag: Springer. pp 21-49.
- Bunn RC, Fowlkes JL. 2003. Insulin-like growth factor binding protein proteolysis. *Trends Endocrinol Metab* 14(4):176-181.
- Burghardt RC, Johnson GA, Jaeger LA, Ka H, Garlow JE, Spencer TE, Bazer FW. 2002. Integrins and extracellular matrix proteins at the maternal-fetal interface in domestic animals. *Cells Tissues Organs* 171:202-217.

- Burton GJ, Watson AL, Hempstock J, Skepper JN, Jauniaux E. 2002. Uterine glands provide histiotrophic nutrition for the human fetus during the first trimester of pregnancy. *Journal of Clinical Endocrinology & Metabolism* 87(6):2954-2959.
- Butler WT, Ridall AL, McKee MD. 1996. Osteopontin. In: Bilezikian JP, Raisz LG, Rodan GA, editors. *Principals of bone biology*: Academic Press, Inc., Orlando Florida pp 167-181.
- Byrne AT, Southgate J, Brison DR, Leese HJ. 2002. Regulation of apoptosis in the bovine blastocyst by insulin and the insulin-like growth factor (IGF) superfamily. *Mol Reprod Dev* 62(4):489-495.
- Cann CH, Fairclough RJ, Browne CA, Gow CB. 1998. Uterine luminal content of insulin-like growth factor (IGF)-I and endometrial expression of mRNA encoding IGF-binding proteins 1 and 2 during the oestrous cycle and early pregnancy in the ewe. *Reprod Fertil Dev* 10(2):155-163.
- Carson DD, Bagchi I, Dey SK, Enders AC, Fazleabas AT, Lessey BA, Yoshinaga K. 2000. Embryo implantation. *Dev Biol* 223(2):217-237.
- Casida LE, Warwick J. 1945. The necessity of the corpus luteum for maintenance of pregnancy in the ewe. *J Anim Sci*:34-39.
- Chang MC, Rowson LE. 1965. Fertilization and early development of Dorset Horn sheep in the spring and summer. *Anat Rec* 152(3):303-316.
- Chen AE, Ginty DD, Fan CM. 2005. Protein kinase A signalling via CREB controls myogenesis induced by Wnt proteins. *Nature* 433(7023):317-322.
- Chen C, Spencer TE, Bazer FW. 2000a. Expression of hepatocyte growth factor and its receptor c-met in the ovine uterus. *Biol Reprod* 62(6):1844-1850.
- Chen C, Spencer TE, Bazer FW. 2000b. Fibroblast growth factor-10: a stromal mediator of epithelial function in the ovine uterus. *Biol Reprod* 63(3):959-966.
- Choi Y, Johnson GA, Burghardt RC, Berghman LR, Joyce MM, Taylor KM, Stewart MD, Bazer FW, Spencer TE. 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.
- Conneely OM, Mulac-Jericevic B, DeMayo F, Lydon JP, O'Malley BW. 2002. Reproductive functions of progesterone receptors. *Recent Prog Horm Res* 57:339-355.



- Cooper DN. 2002. Galectinomics: finding themes in complexity. *Biochim Biophys Acta* 1572(2-3):209-231.
- Cowin P, Rowlands TM, Hatsell SJ. 2005. Cadherins and catenins in breast cancer. *Curr Opin Cell Biol* 17(5):499-508.
- Crossey PA, Pillai CC, Miell JP. 2002. Altered placental development and intrauterine growth restriction in IGF binding protein-1 transgenic mice. *J Clin Invest* 110(3):411-418.
- D'Ercole AJ. 1987. Somatomedins/insulin-like growth factors and fetal growth. *J Dev Physiol* 9(6):481-495.
- Dale TC. 1998. Signal transduction by the Wnt family of ligands. *Biochem J* 329 (Pt 2):209-223.
- Das SK, Chakraborty I, Paria BC, Wang XN, Plowman G, Dey SK. 1995. Amphiregulin is an implantation-specific and progesterone-regulated gene in the mouse uterus. *Mol Endocrinol* 9(6):691-705.
- Dash PR, Whitley GS, Ayling LJ, Johnstone AP, Cartwright JE. 2005. Trophoblast apoptosis is inhibited by hepatocyte growth factor through the Akt and beta-catenin mediated up-regulation of inducible nitric oxide synthase. *Cell Signal* 17(5):571-580.
- Davies G, Jiang WG, Mason MD. 2001. Matrilysin mediates extracellular cleavage of E-cadherin from prostate cancer cells: a key mechanism in hepatocyte growth factor/scatter factor-induced cell-cell dissociation and in vitro invasion. *Clin Cancer Res* 7(10):3289-3297.
- Davies JA. 2002. Do different branching epithelia use a conserved developmental mechanism? *Bioessays* 24(10):937-948.
- Davies JA, Fisher CE. 2002. Genes and proteins in renal development. *Exp Nephrol* 10(2):102-113.
- DeChiara TM, Efstratiadis A, Robertson EJ. 1990. A growth-deficiency phenotype in heterozygous mice carrying an insulin-like growth factor II gene disrupted by targeting. *Nature* 345(6270):78-80.
- Devreker F, Hardy K, Van den Bergh M, Winston J, Biramane J, Englert Y. 2000. Noninvasive assessment of glucose and pyruvate uptake by human embryos after intracytoplasmic sperm injection and during the formation of pronuclei. *Fertil Steril* 73(5):947-954.

- Dey SK, Lim H, Das SK, Reese J, Paria BC, Daikoku T, Wang H. 2004. Molecular cues to implantation. *Endocr Rev* 25(3):341-373.
- Diskin MG, Niswender GD. 1989. Effect of progesterone supplementation on pregnancy and embryo survival in ewes. *J Anim Sci* 67(6):1559-1563.
- Diskin MG, Sreenan JM. 1980. Fertilization and embryonic mortality rates in beef heifers after artificial insemination. *J Reprod Fertil* 59(2):463-468.
- Dore JJ, Jr., Wilkinson JE, Godkin JD. 1996. Ovine endometrial expression of transforming growth factor beta isoforms during the peri-implantation period. *Biol Reprod* 54(5):1080-1087.
- Dunphy JL, Balic A, Barcham GJ, Horvath AJ, Nash AD, Meeusen EN. 2000. Isolation and characterization of a novel inducible mammalian galectin. *J Biol Chem* 275(41):32106-32113.
- Farin CE, Imakawa K, Hansen TR, McDonnell JJ, Murphy CN, Farin PW, Roberts RM. 1990. Expression of trophoblastic interferon genes in sheep and cattle. *Biol Reprod* 43(2):210-218.
- Farin CE, Imakawa K, Roberts RM. 1989. In situ localization of mRNA for the interferon, ovine trophoblast protein-1, during early embryonic development of the sheep. *Mol Endocrinol* 3(7):1099-1107.
- Farmer JL, Burghardt RC, Jousan FD, Hansen PJ, Bazer FW, Spencer TE. 2007. Galectin 15 (LGALS15) functions in trophectoderm migration and attachment. *FASEB J* 22(2):548:60.
- Fazleabas AT, Donnelly KM, Mavrogianis PA, Verhage HG. 1993. Secretory and morphological changes in the baboon (*Papio anubis*) uterus and placenta during early pregnancy. *Biol Reprod* 49(4):695-704.
- Fazleabas AT, Hild-Petito S, Verhage HG. 1994. Secretory proteins and growth factors of the baboon (*Papio anubis*) uterus: potential roles in pregnancy. *Cell Biol Int* 18(12):1145-1153.
- Fazleabas AT, Kim JJ, Srinivasan S, Donnelly KM, Brudney A, Jaffe RC. 1999. Implantation in the baboon: endometrial responses. *Semin Reprod Endocrinol* 17(3):257-265.

- Fazleabas AT, Miller JB, Verhage HG. 1988. Synthesis and release of estrogen- and progesterone-dependent proteins by the baboon (*Papio anubis*) uterine endometrium. *Biol Reprod* 39(3):729-736.
- Flechon JE, Guillomot M, Charlier M, Flechon B, Martal J. 1986. Experimental studies on the elongation of the ewe blastocyst. *Reprod Nutr Dev* 26(4):1017-1024.
- Fleming JA, Choi Y, Johnson GA, Spencer TE, Bazer FW. 2001. Cloning of the ovine estrogen receptor-alpha promoter and functional regulation by ovine interferon-tau. *Endocrinology* 142(7):2879-2887.
- Fleming JG, Spencer TE, Safe SH, Bazer FW. 2006. Estrogen regulates transcription of the ovine oxytocin receptor gene through GC-rich SP1 promoter elements. *Endocrinology* 147(2):899-911.
- Gadd TS, Osgerby JC, Wathes DC. 2000. Regulation and localization of insulin-like growth factor binding protein-5 gene expression in the uterus and placenta of the cyclic and early pregnant ewe. *Biol Reprod* 62(5):1415-1421.
- Gadd TS, Osgerby JC, Wathes DC. 2002. Regulation of insulin-like growth factor binding protein-6 expression in the reproductive tract throughout the estrous cycle and during the development of the placenta in the ewe. *Biol Reprod* 67(6):1756-1762.
- Garrett JE, Geisert RD, Zavy MT, Gries LK, Wettemann RP, Buchanan DS. 1988a. Effect of exogenous progesterone on prostaglandin F2 alpha release and the interestrus interval in the bovine. *Prostaglandins* 36(1):85-96.
- Garrett JE, Geisert RD, Zavy MT, Morgan GL. 1988b. Evidence for maternal regulation of early conceptus growth and development in beef cattle. *J Reprod Fertil* 84(2):437-446.
- Geisert RD, Pratt TN, Bazer FW, Mayes JS, Watson GH. 1994. Immunocytochemical localization and changes in endometrial progesterin receptor protein during the porcine oestrous cycle and early pregnancy. *Reprod Fertil Dev* 6(6):749-760.
- Geisert RD, Renegar RH, Thatcher WW, Roberts RM, Bazer FW. 1982. Establishment of pregnancy in the pig: I. interrelationships between preimplantation development of the pig blastocyst and uterine endometrial secretions. *Biol Reprod* 27(4):925-939.
- Genbacev OD, Prakobphol A, Foulk RA, Krtolica AR, Ilic D, Singer MS, Yang ZQ, Kiessling LL, Rosen SD, Fisher SJ. 2003. Trophoblast L-selectin-mediated adhesion at the maternal-fetal interface. *Science* 299(5605):405-408.

- Ghosh M, Shanker S, Siwanowicz I, Mann K, Machleidt W, Holak TA. 2005. Proteolysis of insulin-like growth factor binding proteins (IGFBPs) by calpain. *Biol Chem* 386(1):85-93.
- Giancotti FG, Ruoslahti E. 1999. Integrin signaling. *Science* 285(5430):1028-1032.
- Giblin PA, Hwang ST, Katsumoto TR, Rosen SD. 1997. Ligation of L-selectin on T lymphocytes activates beta1 integrins and promotes adhesion to fibronectin. *J Immunol* 159(7):3498-3507.
- Giraud A, Parker L, Taupin D, Hardy K, Shulkes A. 1993. Mammalian bombesin as a hormone in ovine pregnancy: ontogeny, origin, and molecular forms. *Am J Physiol* 265(6 Pt 1):E866-873.
- Giudice LC, Dsupin BA, Jin IH, Vu TH, Hoffman AR. 1993. Differential expression of messenger ribonucleic acids encoding insulin-like growth factors and their receptors in human uterine endometrium and decidua. *J Clin Endocrinol Metab* 76(5):1115-1122.
- Giudice LC, Martina NA, Crystal RA, Tazuke S, Druzin M. 1997. Insulin-like growth factor binding protein-1 at the maternal-fetal interface and insulin-like growth factor-I, insulin-like growth factor-II, and insulin-like growth factor binding protein-1 in the circulation of women with severe preeclampsia. *Am J Obstet Gynecol* 176(4):751-757; discussion 757-758.
- Gleeson LM, Chakraborty C, McKinnon T, Lala PK. 2001. Insulin-like growth factor-binding protein 1 stimulates human trophoblast migration by signaling through alpha 5 beta 1 integrin via mitogen-activated protein Kinase pathway. *J Clin Endocrinol Metab* 86(6):2484-2493.
- Gluckman PD, Morel PC, Ambler GR, Breier BH, Blair HT, McCutcheon SN. 1992. Elevating maternal insulin-like growth factor-I in mice and rats alters the pattern of fetal growth by removing maternal constraint. *J Endocrinol* 134(1):R1-3.
- Gonzalez-Mariscal L, Betanzos A, Avila-Flores A. 2000a. MAGUK proteins: structure and role in the tight junction. *Semin Cell Dev Biol* 11(4):315-324.
- Gonzalez-Mariscal L, Betanzos A, Nava P, Jaramillo BE. 2003. Tight junction proteins. *Prog Biophys Mol Biol* 81(1):1-44.
- Gonzalez-Mariscal L, Chavez de Ramirez B, Cereijido M. 1985. Tight junction formation in cultured epithelial cells (MDCK). *J Membr Biol* 86(2):113-125.

- Gonzalez-Mariscal L, Contreras RG, Bolivar JJ, Ponce A, Chavez De Ramirez B, Cerejido M. 1990. Role of calcium in tight junction formation between epithelial cells. *Am J Physiol* 259(6 Pt 1):C978-986.
- Gonzalez-Mariscal L, Namorado MC, Martin D, Luna J, Alarcon L, Islas S, Valencia L, Muriel P, Ponce L, Reyes JL. 2000b. Tight junction proteins ZO-1, ZO-2, and occludin along isolated renal tubules. *Kidney Int* 57(6):2386-2402.
- Gray CA, Abbey CA, Beremand PD, Choi Y, Farmer JL, Adelson DL, Thomas TL, Bazer FW, Spencer TE. 2006. Identification of endometrial genes regulated by early pregnancy, progesterone, and interferon tau in the ovine uterus. *Biol Reprod* 74(2):383-394.
- Gray CA, Adelson DL, Bazer FW, Burghardt RC, Meeusen EN, Spencer TE. 2004. Discovery and characterization of an epithelial-specific galectin in the endometrium that forms crystals in the trophoctoderm. *Proc Natl Acad Sci U S A* 101(21):7982-7987.
- Gray CA, Bartol FF, Tarleton BJ, Wiley AA, Johnson GA, Bazer FW, Spencer TE. 2001a. Developmental biology of uterine glands. *Biol Reprod* 65(5):1311-1323.
- Gray CA, Bartol FF, Taylor KM, Wiley AA, Ramsey WS, Ott TL, Bazer FW, Spencer TE. 2000a. Ovine uterine gland knock-out model: effects of gland ablation on the estrous cycle. *Biol Reprod* 62(2):448-456.
- Gray CA, Bazer FW, Spencer TE. 2001b. Effects of neonatal progestin exposure on female reproductive tract structure and function in the adult ewe. *Biol Reprod* 64(3):797-804.
- Gray CA, Burghardt RC, Johnson GA, Bazer FW, Spencer TE. 2002. Evidence that absence of endometrial gland secretions in uterine gland knockout ewes compromises conceptus survival and elongation. *Reproduction* 124(2):289-300.
- Gray CA, Dunlap KA, Burghardt RC, Spencer TE. 2005. Galectin-15 in ovine uteroplacental tissues. *Reproduction* 130(2):231-240.
- Gray CA, Taylor KM, Bazer FW, Spencer TE. 2000b. Mechanisms regulating norgestomet inhibition of endometrial gland morphogenesis in the neonatal ovine uterus. *Mol Reprod Dev* 57(1):67-78.
- Gray CA, Taylor KM, Ramsey WS, Hill JR, Bazer FW, Bartol FF, Spencer TE. 2001c. Endometrial glands are required for preimplantation conceptus elongation and survival. *Biol Reprod* 64(6):1608-1613.

- Guillomot M. 1995. Cellular interactions during implantation in domestic ruminants. *J Reprod Fertil Suppl* 49:39-51.
- Guillomot M, Betteridge KJ, Harvey D, Goff AK. 1986. Endocytotic activity in the endometrium during conceptus attachment in the cow. *J Reprod Fertil* 78(1):27-36.
- Guillomot M, Flechon JE, Leroy F. 1993. Blastocyst development and implantation. In: Thibault C, Levasseur MC, Hunter RHF, editors. *Reproduction in mammals and man*. Paris: Ellipses. pp 387-411.
- Guillomot M, Flechon JE, Wintenberger-Torres S. 1981. Conceptus attachment in the ewe: an ultrastructural study. *Placenta* 2(2):169-182.
- Guillomot M, Michel C, Gaye P, Charlier N, Trojan J, Martal J. 1990. Cellular localization of an embryonic interferon, ovine trophoblastin and its mRNA in sheep embryos during early pregnancy. *Biol Cell* 68(3):205-211.
- Habas R, Dawid IB, He X. 2003. Coactivation of Rac and Rho by Wnt/Frizzled signaling is required for vertebrate gastrulation. *Genes Dev* 17(2):295-309.
- Hampton AL, Butt AR, Riley SC, Salamonsen LA. 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.
- Hanlon DW, Davidson PJ, Hittmann AR, Joe AK. 2005. Supplementing previously treated anestrous dairy cows with progesterone does not increase first-service conception rate. *Theriogenology* 63(1):239-245.
- Hansen TR, Austin KJ, Perry DJ, Pru JK, Teixeira MG, Johnson GA. 1999. Mechanism of action of interferon-tau in the uterus during early pregnancy. *J Reprod Fertil* 54(Suppl):329-339.
- Hayashi K, Burghardt RC, Bazer FW, Spencer TE. 2007. WNTs in the ovine uterus: potential regulation of periimplantation ovine conceptus development. *Endocrinology* 148(7):3496-3506.
- Hayashi K, Carpenter KD, Welsh TH, Jr., Burghardt RC, Spicer LJ, Spencer TE. 2005. The IGF system in the neonatal ovine uterus. *Reproduction* 129(3):337-347.
- Heyman Y, Camous S, Fevre J, Meziou W, Martal J. 1984. Maintenance of the corpus luteum after uterine transfer of trophoblastic vesicles to cyclic cows and ewes. *J Reprod Fertil* 70(2):533-540.

- Hiscox S, Jiang WG. 1999. Hepatocyte growth factor/scatter factor disrupts epithelial tumour cell-cell adhesion: involvement of beta-catenin. *Anticancer Res* 19(1A):509-517.
- Holly J, Perks C. 2006. The role of insulin-like growth factor binding proteins. *Neuroendocrinology* 83(3-4):154-160.
- Hommeida A, Nakao T, Kubota H. 2004. Luteal function and conception in lactating cows and some factors influencing luteal function after first insemination. *Theriogenology* 62(1-2):217-225.
- Huelsken J, Birchmeier W. 2001. New aspects of Wnt signaling pathways in higher vertebrates. *Curr Opin Genet Dev* 11(5):547-553.
- Hussein SM, Duff EK, Sirard C. 2003. Smad4 and beta-catenin co-activators functionally interact with lymphoid-enhancing factor to regulate graded expression of Msx2. *J Biol Chem* 278(49):48805-48814.
- Hwang ST, Singer MS, Giblin PA, Yednock TA, Bacon KB, Simon SI, Rosen SD. 1996. GlyCAM-1, a physiologic ligand for L-selectin, activates beta 2 integrins on naive peripheral lymphocytes. *J Exp Med* 184(4):1343-1348.
- Hynes RO. 1996. Targeted mutations in cell adhesion genes: what have we learned from them? *Dev Biol* 180(2):402-412.
- Igarashi M, Finch PW, Aaronson SA. 1998. Characterization of recombinant human fibroblast growth factor (FGF)-10 reveals functional similarities with keratinocyte growth factor (FGF-7). *J Biol Chem* 273(21):13230-13235.
- Imakawa K, Helmer SD, Nephew KP, Meka CS, Christenson RK. 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 SR, Chang KT, Echterkamp SE, Christenson RK. 2006. Regulation of conceptus adhesion by endometrial CXC chemokines during the implantation period in sheep. *Mol Reprod Dev* 73(7):850-858.
- Ing NH, Francis H, McDonnell JJ, Amann JF, Roberts RM. 1989. Progesterone induction of the uterine milk proteins: major secretory proteins of sheep endometrium. *Biol Reprod* 41(4):643-654.
- Irwin JC, Giudice LC. 1998. Insulin-like growth factor binding protein-1 binds to placental cytotrophoblast alpha5beta1 integrin and inhibits cytotrophoblast

- invasion into decidualized endometrial stromal cultures. *Growth Horm IGF Res* 8(1):21-31.
- Jha RK, Titus S, Saxena D, Kumar PG, Laloraya M. 2006. Profiling of E-cadherin, beta-catenin and Ca(2+) in embryo-uterine interactions at implantation. *FEBS Lett* 580(24):5653-5660.
- Johnson GA, Bazer FW, Jaeger LA, Ka H, Garlow JE, Pfarrer C, Spencer TE, Burghardt RC. 2001a. Muc-1, integrin, and osteopontin expression during the implantation cascade in sheep. *Biol Reprod* 65(3):820-828.
- Johnson GA, Burghardt RC, Bazer FW, Spencer TE. 2003. Osteopontin: roles in implantation and placentation. *Biol Reprod* 69(5):1458-1471.
- Johnson GA, Burghardt RC, Spencer TE, Newton GR, Ott TL, Bazer FW. 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 GA, Spencer TE, Burghardt RC, Bazer FW. 1999b. Ovine osteopontin: I. Cloning and expression of messenger ribonucleic acid in the uterus during the periimplantation period. *Biol Reprod* 61(4):884-891.
- Johnson GA, Spencer TE, Burghardt RC, Taylor KM, Gray CA, Bazer FW. 2000. Progesterone modulation of osteopontin gene expression in the ovine uterus. *Biol Reprod* 62(5):1315-1321.
- Johnson GA, Spencer TE, Hansen TR, Austin KJ, Burghardt RC, Bazer FW. 1999c. Expression of the interferon tau inducible ubiquitin cross-reactive protein in the ovine uterus. *Biol Reprod* 61(1):312-318.
- Johnson GA, Stewart MD, Gray CA, Choi Y, Burghardt RC, Yu-Lee LY, Bazer FW, Spencer TE. 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 KR, Ross, RH, Fourt, DL 1958. Effect of progesterone administration on reproductive efficiency. *Journal of Animal Science*:386-390.
- Jousan FD, Hansen PJ. 2007. Insulin-like growth factor-I promotes resistance of bovine preimplantation embryos to heat shock through actions independent of its anti-apoptotic actions requiring PI3K signaling. *Mol Reprod Dev* 74(2):189-196.



- Kamalati T, Niranjana B, Yant J, Buluwela L. 1999. HGF/SF in mammary epithelial growth and morphogenesis: in vitro and in vivo models. *J Mammary Gland Biol Neoplasia* 4(1):69-77.
- Kane MT, Morgan PM, Coonan C. 1997. Peptide growth factors and preimplantation development. *Hum Reprod Update* 3(2):137-157.
- Kao LC, Tulac S, Lobo S, Imani B, Yang JP, Germeyer A, Osteen K, Taylor RN, Lessey BA, Giudice LC. 2002. Global gene profiling in human endometrium during the window of implantation. *Endocrinology* 143(6):2119-2138.
- Karner C, Wharton KA, Carroll TJ. 2006a. Apical-basal polarity, Wnt signaling and vertebrate organogenesis. *Semin Cell Dev Biol* 17(2):214-222.
- Karner C, Wharton KA, Jr., Carroll TJ. 2006b. Planar cell polarity and vertebrate organogenesis. *Semin Cell Dev Biol* 17(2):194-203.
- Karteris E, Zervou S, Pang Y, Dong J, Hillhouse EW, Rande HS, Thomas P. 2006. Progesterone signaling in human myometrium through two novel membrane G protein-coupled receptors: potential role in functional progesterone withdrawal at term. *Mol Endocrinol* 20(7):1519-1534.
- Kastner P, Krust A, Turcotte B, Stropp U, Tora L, Gronemeyer H, Chambon P. 1990. Two distinct estrogen-regulated promoters generate transcripts encoding the two functionally different human progesterone receptor forms A and B. *EMBO J* 9(5):1603-1614.
- Kauma SW, Bae-Jump V, Walsh SW. 1999. Hepatocyte growth factor stimulates trophoblast invasion: a potential mechanism for abnormal placentation in preeclampsia. *J Clin Endocrinol Metab* 84(11):4092-4096.
- Kawano Y, Kypta R. 2003. Secreted antagonists of the Wnt signalling pathway. *J Cell Sci* 116(Pt 13):2627-2634.
- Kayser JP, Kim JG, Cerny RL, Vallet JL. 2006. Global characterization of porcine intrauterine proteins during early pregnancy. *Reproduction* 131(2):379-388.
- Kazemi M, Amann JF, Keisler DH, Ing NH, Roberts RM, Morgan G, Wooding FB. 1990. A progesterone-modulated, low-molecular-weight protein from the uterus of the sheep is associated with crystalline inclusion bodies in uterine epithelium and embryonic trophectoderm. *Biol Reprod* 43(1):80-96.

- Keller ML, Roberts AJ, Seidel GE, Jr. 1998. Characterization of insulin-like growth factor-binding proteins in the uterus and conceptus during early conceptus elongation in cattle. *Biol Reprod* 59(3):632-642.
- Kenyon PR, Morris ST, West DM. 2005. The effect of progesterone supplementation post mating and shearing of ewes in early pregnancy on the reproductive performance of ewes and birthweight of lambs. *N Z Vet J* 53(5):321-325.
- Kim S, Choi Y, Bazer FW, Spencer TE. 2003. Identification of genes in the ovine endometrium regulated by interferon tau independent of signal transducer and activator of transcription 1. *Endocrinology* 144(12):5203-5214.
- Kirby DR, Billington WD, Bradbury S, Goldstein DJ. 1964. Antigen barrier of the mouse placenta. *Nature* 204:548-549.
- Kleemann DO, Walker SK, Hartwich KM, Fong L, Seamark RF, Robinson JS, Owens JA. 2001. Fetoplacental growth in sheep administered progesterone during the first three days of pregnancy. *Placenta* 22(1):14-23.
- Kleemann DO, Walker SK, Seamark RF. 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 CY, Ott TL, Davis MA, Simmen RC, Bazer FW, Simmen FA. 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.
- Koji T, Chedid M, Rubin JS, Slayden OD, Csaky KG, Aaronson SA, Brenner RM. 1994. Progesterone-dependent expression of keratinocyte growth factor mRNA in stromal cells of the primate endometrium: keratinocyte growth factor as a progestomedin. *J Cell Biol* 125(2):393-401.
- Kuhl M, Sheldahl LC, Park M, Miller JR, Moon RT. 2000. The Wnt/Ca<sup>2+</sup> pathway: a new vertebrate Wnt signaling pathway takes shape. *Trends Genet* 16(7):279-283.
- Kwon H, Spencer TE, Bazer FW, Wu G. 2003a. Developmental changes of amino acids in ovine fetal fluids. *Biol Reprod* 68(5):1813-1820.
- Kwon H, Wu G, Bazer FW, Spencer TE. 2003b. Developmental changes in polyamine levels and synthesis in the ovine conceptus. *Biol Reprod* 69(5):1626-1634.

- Larson SF, Butler WR, Currie WB. 1997. Reduced fertility associated with low progesterone postbreeding and increased milk urea nitrogen in lactating cows. *J Dairy Sci* 80(7):1288-1295.
- Larson SF, Butler WR, Currie WB. 2007. Pregnancy rates in lactating dairy cattle following supplementation of progesterone after artificial insemination. *Anim Reprod Sci* 102(1-2):172-179.
- Lasky LA, Singer MS, Dowbenko D, Imai Y, Henzel WJ, Grimley C, Fennie C, Gillett N, Watson SR, Rosen SD. 1992. An endothelial ligand for L-selectin is a novel mucin-like molecule. *Cell* 69(6):927-938.
- Lawson RA, Cahill LP. 1983. Modification of the embryo-maternal relationship in ewes by progesterone treatment early in the oestrous cycle. *J Reprod Fertil* 67(2):473-475.
- Lawson RA, Parr RA, Cahill LP. 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.
- Lee CY, Green ML, Simmen RC, Simmen FA. 1998a. Proteolysis of insulin-like growth factor-binding proteins (IGFBPs) within the pig uterine lumen associated with peri-implantation conceptus development. *J Reprod Fertil* 112(2):369-377.
- Lee RS, Wheeler TT, Peterson AJ. 1998b. Large-format, two-dimensional polyacrylamide gel electrophoresis of ovine periimplantation uterine luminal fluid proteins: identification of aldose reductase, cytoplasmic actin, and transferrin as conceptus-synthesized proteins. *Biol Reprod* 59(4):743-752.
- Leese HJ. 1995. Metabolic control during preimplantation mammalian development. *Hum Reprod Update* 1(1):63-72.
- Lessey BA. 1998. Endometrial integrins and the establishment of uterine receptivity. *Hum Reprod* 13 Suppl 3:247-258; discussion 259-261.
- Lessey BA. 2002. Adhesion molecules and implantation. *J Reprod Immunol* 55(1-2):101-112.
- Lessey BA, Killam AP, Metzger DA, Haney AF, Greene GL, McCarty KS, Jr. 1988. Immunohistochemical analysis of human uterine estrogen and progesterone receptors throughout the menstrual cycle. *J Clin Endocrinol Metab* 67(2):334-340.

- Lewis SK, Farmer JL, Burghardt RC, Newton GR, Johnson GA, Adelson DL, Bazer FW, Spencer TE. 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.
- Leymarie P, Martal J. 1993. The corpus luteum from cycle to gestation. In: Thibault C, Levasseur MC, Hunter RHF, editors. *Reproduction in mammals and man*. pp 413-434.
- Li J, Zhang JV, Cao YJ, Zhou JX, Liu WM, Fan XJ, Duan EK. 2005. Inhibition of the beta-catenin signaling pathway in blastocyst and uterus during the window of implantation in mice. *Biol Reprod* 72(3):700-706.
- Li Q, Wang J, Armant DR, Bagchi MK, Bagchi IC. 2002. Calcitonin down-regulates E-cadherin expression in rodent uterine epithelium during implantation. *J Biol Chem* 277(48):46447-46455.
- Lilien J, Balsamo J. 2005. The regulation of cadherin-mediated adhesion by tyrosine phosphorylation/dephosphorylation of beta-catenin. *Curr Opin Cell Biol* 17(5):459-465.
- Lin Z, Reierstad S, Huang CC, Bulun SE. 2007. Novel estrogen receptor-alpha binding sites and estradiol target genes identified by chromatin immunoprecipitation cloning in breast cancer. *Cancer Res* 67(10):5017-5024.
- Liu JP, Baker J, Perkins AS, Robertson EJ, Efstratiadis A. 1993. Mice carrying null mutations of the genes encoding insulin-like growth factor I (Igf-1) and type 1 IGF receptor (Igf1r). *Cell* 75(1):59-72.
- Liu T, Liu X, Wang H, Moon RT, Malbon CC. 1999. Activation of rat frizzled-1 promotes Wnt signaling and differentiation of mouse F9 teratocarcinoma cells via pathways that require Galpha(q) and Galpha(o) function. *J Biol Chem* 274(47):33539-33544.
- Logan CY, Nusse R. 2004. The Wnt signaling pathway in development and disease. *Annu Rev Cell Dev Biol* 20:781-810.
- Lu W, Luo Y, Kan M, McKeehan WL. 1999. Fibroblast growth factor-10. A second candidate stromal to epithelial cell andromedin in prostate. *J Biol Chem* 274(18):12827-12834.
- Lucy MC. 2001. Reproductive loss in high-producing dairy cattle: where will it end? *J Dairy Sci* 84(6):1277-1293.

- Lydon JP, DeMayo FJ, Funk CR, Mani SK, Hughes AR, Montgomery CA, Jr., Shyamala G, Conneely OM, O'Malley BW. 1995. Mice lacking progesterone receptor exhibit pleiotropic reproductive abnormalities. *Genes Dev* 9(18):2266-2278.
- MacLaughlin DT, Santoro NF, Bauer HH, Lawrence D, Richardson GS. 1986. Two-dimensional gel electrophoresis of endometrial protein in human uterine fluids: qualitative and quantitative analysis. *Biol Reprod* 34(3):579-585.
- Mankertz J, Hillenbrand B, Tavalali S, Huber O, Fromm M, Schulzke JD. 2004. Functional crosstalk between Wnt signaling and Cdx-related transcriptional activation in the regulation of the claudin-2 promoter activity. *Biochem Biophys Res Commun* 314(4):1001-1007.
- Mann B, Gelos M, Siedow A, Hanski ML, Gratchev A, Ilyas M, Bodmer WF, Moyer MP, Riecken EO, Buhr HJ, Hanski C. 1999. Target genes of beta-catenin-T cell-factor/lymphoid-enhancer-factor signaling in human colorectal carcinomas. *Proc Natl Acad Sci U S A* 96(4):1603-1608.
- Mann GE, Fray MD, Lamming GE. 2006. Effects of time of progesterone supplementation on embryo development and interferon-tau production in the cow. *Vet J* 171(3):500-503.
- Mann GE, Lamming GE. 2001. Relationship between maternal endocrine environment, early embryo development and inhibition of the luteolytic mechanism in cows. *Reproduction* 121(1):175-180.
- Mann GE, Lamming, G.E. 1999. The influence of progesterone during early pregnancy in cattle. *Reprod Domest Anim* 34:269-274.
- Mann GE, Merson P, Fray MD, Lamming GE. 2001. Conception rate following progesterone supplementation after second insemination in dairy cows. *Vet J* 162(2):161-162.
- Martin PM, Sutherland AE. 2001. Exogenous amino acids regulate trophectoderm differentiation in the mouse blastocyst through an mTOR-dependent pathway. *Dev Biol* 240(1):182-193.
- Martin PM, Sutherland AE, Van Winkle LJ. 2003. Amino acid transport regulates blastocyst implantation. *Biol Reprod* 69(4):1101-1108.
- Maurer RR, Chenault JR. 1983. Fertilization failure and embryonic mortality in parous and nonparous beef cattle. *J Anim Sci* 56(5):1186-1189.

- McCrea PD, Gumbiner BM. 1991. Purification of a 92-kDa cytoplasmic protein tightly associated with the cell-cell adhesion molecule E-cadherin (uvomorulin). Characterization and extractability of the protein complex from the cell cytostructure. *J Biol Chem* 266(7):4514-4520.
- McCrea PD, Turck CW, Gumbiner B. 1991. A homolog of the armadillo protein in *Drosophila* (plakoglobin) associated with E-cadherin. *Science* 254(5036):1359-1361.
- McDonnell DP, Shahbaz MM, Vegeto E, Goldman ME. 1994. The human progesterone receptor A-form functions as a transcriptional modulator of mineralocorticoid receptor transcriptional activity. *J Steroid Biochem Mol Biol* 48(5-6):425-432.
- McMaster MT, Teng CT, Dey SK, Andrews GK. 1992. Lactoferrin in the mouse uterus: analyses of the preimplantation period and regulation by ovarian steroids. *Mol Endocrinol* 6(1):101-111.
- Michael DD, Alvarez IM, Ocon OM, Powell AM, Talbot NC, Johnson SE, Ealy AD. 2006. Fibroblast growth factor-2 is expressed by the bovine uterus and stimulates interferon-tau production in bovine trophectoderm. *Endocrinology* 147(7):3571-3579.
- Mirando MA, Short EC, Jr., Geisert RD, Vallet JL, Bazer FW. 1991. Stimulation of 2',5'-oligoadenylate synthetase activity in sheep endometrium during pregnancy, by intrauterine infusion of ovine trophoblast protein-1, and by intramuscular administration of recombinant bovine interferon-alpha 11. *J Reprod Fertil* 93(2):599-607.
- Miwa N, Furuse M, Tsukita S, Niikawa N, Nakamura Y, Furukawa Y. 2001. Involvement of claudin-1 in the beta-catenin/Tcf signaling pathway and its frequent upregulation in human colorectal cancers. *Oncol Res* 12(11-12):469-476.
- Moffatt J, Bazer FW, Hansen PJ, Chun PW, Roberts RM. 1987a. Purification, secretion and immunocytochemical localization of the uterine milk proteins, major progesterone-induced proteins in uterine secretions of the sheep. *Biol Reprod* 36(2):419-430.
- Moffatt RJ, Bazer FW, Roberts RM, Thatcher WW. 1987b. Secretory function of the ovine uterus: effects of gestation and steroid replacement therapy. *J Anim Sci* 65(5):1400-1410.
- Mohamed OA, Jonnaert M, Labelle-Dumais C, Kuroda K, Clarke HJ, Dufort D. 2005. Uterine Wnt/beta-catenin signaling is required for implantation. *Proc Natl Acad Sci U S A* 102(24):8579-8584.

- Montesano R, Matsumoto K, Nakamura T, Orci L. 1991. Identification of a fibroblast-derived epithelial morphogen as hepatocyte growth factor. *Cell* 67(5):901-908.
- Moreira F, Badinga L, Burnley C, Thatcher WW. 2002a. Bovine somatotropin increases embryonic development in superovulated cows and improves post-transfer pregnancy rates when given to lactating recipient cows. *Theriogenology* 57(4):1371-1387.
- Moreira F, Orlandi C, Risco CA, Mattos R, Lopes F, Thatcher WW. 2001. Effects of presynchronization and bovine somatotropin on pregnancy rates to a timed artificial insemination protocol in lactating dairy cows. *J Dairy Sci* 84(7):1646-1659.
- Moreira F, Paula-Lopes FF, Hansen PJ, Badinga L, Thatcher WW. 2002b. Effects of growth hormone and insulin-like growth factor-I on development of in vitro derived bovine embryos. *Theriogenology* 57(2):895-907.
- Moreira F, Risco CA, Pires MF, Ambrose JD, Drost M, Thatcher WW. 2000. Use of bovine somatotropin in lactating dairy cows receiving timed artificial insemination. *J Dairy Sci* 83(6):1237-1247.
- Muggleton-Harris AL, Johnson MH. 1976. The nature and distribution of serologically detectable alloantigens on the preimplantation mouse embryo. *J Embryol Exp Morphol* 35(1):59-72.
- Mulac-Jericevic B, Mullinax RA, DeMayo FJ, Lydon JP, Conneely OM. 2000. Subgroup of reproductive functions of progesterone mediated by progesterone receptor-B isoform. *Science* 289(5485):1751-1754.
- Murai M, Shen X, Huang L, Carpenter WM, Lin CS, Silverman S, Regezi J, Kramer RH. 2004. Overexpression of c-met in oral SCC promotes hepatocyte growth factor-induced disruption of cadherin junctions and invasion. *Int J Oncol* 25(4):831-840.
- Nagaoka K, Sakai A, Nojima H, Suda Y, Yokomizo Y, Imakawa K, Sakai S, Christenson RK. 2003. A chemokine, interferon (IFN)-gamma-inducible protein 10 kDa, is stimulated by IFN-tau and recruits immune cells in the ovine endometrium. *Biol Reprod* 68(4):1413-1421.
- Nakamura T, Nishizawa T, Hagiya M, Seki T, Shimonishi M, Sugimura A, Tashiro K, Shimizu S. 1989. Molecular cloning and expression of human hepatocyte growth factor. *Nature* 342(6248):440-443.

- Nasu K, Zhou Y, McMaster MT, Fisher SJ. 2000. Upregulation of human cytotrophoblast invasion by hepatocyte growth factor. *J Reprod Fertil Suppl* 55:73-80.
- Nephew KP, McClure KE, Ott TL, Dubois DH, Bazer FW, Pope WF. 1991. Relationship between variation in conceptus development and differences in estrous cycle duration in ewes. *Biol Reprod* 44(3):536-539.
- Nguyen DA, Parlow AF, Neville MC. 2001. Hormonal regulation of tight junction closure in the mouse mammary epithelium during the transition from pregnancy to lactation. *J Endocrinol* 170(2):347-356.
- Nishina H, Wada T, Katada T. 2004. Physiological roles of SAPK/JNK signaling pathway. *J Biochem (Tokyo)* 136(2):123-126.
- Nusse R. 2001. Developmental biology. Making head or tail of Dickkopf. *Nature* 411(6835):255-256.
- Oehler MK, MacKenzie IZ, Wallwiener D, Bicknell R, Rees MC. 2002. Wnt-7a is upregulated by norethisterone in human endometrial epithelial cells: a possible mechanism by which progestogens reduce the risk of estrogen-induced endometrial neoplasia. *Cancer Lett* 186(1):75-81.
- Okada M, Matsumori A, Ono K, Miyamoto T, Takahashi M, Sasayama S. 1999. Hepatocyte growth factor is a major mediator in heparin-induced angiogenesis. *Biochem Biophys Res Commun* 255(1):80-87.
- Okegawa T, Li Y, Pong RC, Hsieh JT. 2002. Cell adhesion proteins as tumor suppressors. *J Urol* 167(4):1836-1843.
- Orchard MD, Murphy CR. 2002. Alterations in tight junction molecules of uterine epithelial cells during early pregnancy in the rat. *Acta Histochem* 104(2):149-155.
- Oskerby JC, Gadd TS, Wathes DC. 1999. Expression of insulin-like growth factor binding protein-1 (IGFBP-1) mRNA in the ovine uterus throughout the oestrous cycle and early pregnancy. *J Endocrinol* 162(2):279-287.
- Pandur P, Lasche M, Eisenberg LM, Kuhl M. 2002a. Wnt-11 activation of a non-canonical Wnt signalling pathway is required for cardiogenesis. *Nature* 418(6898):636-641.
- Pandur P, Maurus D, Kuhl M. 2002b. Increasingly complex: new players enter the Wnt signaling network. *Bioessays* 24(10):881-884.



- Paria BC, Das N, Das SK, Zhao X, Dileepan KN, Dey SK. 1998. Histidine decarboxylase gene in the mouse uterus is regulated by progesterone and correlates with uterine differentiation for blastocyst implantation. *Endocrinology* 139(9):3958-3966.
- Paria BC, Lim H, Das SK, Reese J, Dey SK. 2000. Molecular signaling in uterine receptivity for implantation. *Semin Cell Dev Biol* 11(2):67-76.
- Paria BC, Zhao X, Das SK, Dey SK, Yoshinaga K. 1999. Zonula occludens-1 and E-cadherin are coordinately expressed in the mouse uterus with the initiation of implantation and decidualization. *Dev Biol* 208(2):488-501.
- Parr RA, Davis IF, Fairclough RJ, Miles MA. 1987. Overfeeding during early pregnancy reduces peripheral progesterone concentration and pregnancy rate in sheep. *J Reprod Fertil* 80(1):317-320.
- Parr RA, Davis IF, Miles MA, Squires TJ. 1993a. Feed intake affects metabolic clearance rate of progesterone in sheep. *Res Vet Sci* 55(3):306-310.
- Parr RA, Davis IF, Miles MA, Squires TJ. 1993b. Liver blood flow and metabolic clearance rate of progesterone in sheep. *Res Vet Sci* 55(3):311-316.
- Peterson AJ, Ledgard AM, Hodgkinson SC. 1998. The proteolysis of insulin-like growth factor binding proteins in ovine uterine luminal fluid. *Reprod Fertil Dev* 10(4):309-314.
- Pinson KI, Brennan J, Monkley S, Avery BJ, Skarnes WC. 2000. An LDL-receptor-related protein mediates Wnt signalling in mice. *Nature* 407(6803):535-538.
- Press MF, Greene GL. 1988. Localization of progesterone receptor with monoclonal antibodies to the human progestin receptor. *Endocrinology* 122(3):1165-1175.
- Rajah R, Katz L, Nunn S, Solberg P, Beers T, Cohen P. 1995. Insulin-like growth factor binding protein (IGFBP) proteases: functional regulators of cell growth. *Prog Growth Factor Res* 6(2-4):273-284.
- Reynolds TS, Stevenson KR, Wathes DC. 1997. Pregnancy-specific alterations in the expression of the insulin-like growth factor system during early placental development in the ewe. *Endocrinology* 138(3):886-897.
- Richer JK, Jacobsen BM, Manning NG, Abel MG, Wolf DM, Horwitz KB. 2002. Differential gene regulation by the two progesterone receptor isoforms in human breast cancer cells. *J Biol Chem* 277(7):5209-5218.

- Ricketts AP, Flint AP. 1980. Onset of synthesis of progesterone by ovine placenta. *J Endocrinol* 86(2):337-347.
- Riley SC, Butt AR, Doughton BW, Li SX, Zheng SH, Findlay JK, Salamonsen LA. 1994. Endothelin in the ovine uterus during the oestrous cycle and early pregnancy. *J Reprod Fertil* 100(2):451-459.
- Rimm DL, Koslov ER, Kebriaei P, Cianci CD, Morrow JS. 1995. Alpha 1(E)-catenin is an actin-binding and -bundling protein mediating the attachment of F-actin to the membrane adhesion complex. *Proc Natl Acad Sci U S A* 92(19):8813-8817.
- Roberts GP, Parker JM, Symonds HW. 1976. Macromolecular components of genital tract fluids from the sheep. *J Reprod Fertil* 48(1):99-107.
- Roberts RM, Bazer FW. 1988. The functions of uterine secretions. *J Reprod Fertil* 82(2):875-892.
- Roberts RM, Ealy AD, Alexenko AP, Han CS, Ezashi T. 1999. Trophoblast interferons. *Placenta* 20(4):259-264.
- Robinson GW, Hennighausen L, Johnson PF. 2000. Side-branching in the mammary gland: the progesterone-Wnt connection. *Genes Dev* 14(8):889-894.
- Robinson NA, Leslie KE, Walton JS. 1989. Effect of treatment with progesterone on pregnancy rate and plasma concentrations of progesterone in Holstein cows. *J Dairy Sci* 72(1):202-207.
- Robinson RS, Fray MD, Wathes DC, Lamming GE, Mann GE. 2006. In vivo expression of interferon tau mRNA by the embryonic trophoblast and uterine concentrations of interferon tau protein during early pregnancy in the cow. *Mol Reprod Dev* 73(4):470-474.
- Robinson RS, Mann GE, Lamming GE, Wathes DC. 2001. Expression of oxytocin, oestrogen and progesterone receptors in uterine biopsy samples throughout the oestrous cycle and early pregnancy in cows. *Reproduction* 122(6):965-979.
- Roblero LS, Croxatto HB. 1991. Effect of RU486 on development and implantation of rat embryos. *Mol Reprod Dev* 29(4):342-346.
- Roche JF, Bolandl MP, McGeady TA. 1981. Reproductive wastage following artificial insemination of heifers. *Vet Rec* 109(18):401-404.

- Roose J, Huls G, van Beest M, Moerer P, van der Horn K, Goldschmeding R, Logtenberg T, Clevers H. 1999. Synergy between tumor suppressor APC and the beta-catenin-Tcf4 target Tcf1. *Science* 285(5435):1923-1926.
- Rosen SD. 1993. Ligands for L-selectin: where and how many? *Res Immunol* 144(9):699-703; discussion 754-662.
- Rowson LE, Moor RM. 1966a. Development of the sheep conceptus during the first fourteen days. *J Anat* 100(4):777-785.
- Rowson LE, Moor RM. 1966b. Embryo transfer in the sheep: the significance of synchronizing oestrus in the donor and recipient animal. *J Reprod Fertil* 11(2):207-212.
- Rozen S, Skaletsky H. 2000. Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* 132:365-386.
- Rubin JS, Bottaro DP, Chedid M, Miki T, Ron D, Cheon G, Taylor WG, Fortney E, Sakata H, Finch PW, et al. 1995. Keratinocyte growth factor. *Cell Biol Int* 19(5):399-411.
- Sato T, Wang G, Hardy MP, Kurita T, Cunha GR, Cooke PS. 2002. Role of systemic and local IGF-I in the effects of estrogen on growth and epithelial proliferation of mouse uterus. *Endocrinology* 143(7):2673-2679.
- Satterfield MC, Bazer FW, Spencer TE. 2006. Progesterone regulation of preimplantation conceptus growth and galectin 15 (LGALS15) in the ovine uterus. *Biol Reprod* 75(2):289-296.
- Satterfield MC, Dunlap KA, Hayashi K, Burghardt RC, Spencer TE, Bazer FW. 2007. Tight and adherens junctions in the ovine uterus: differential regulation by pregnancy and progesterone. *Endocrinology* 148(8):3922-3931.
- Schaiff WT, Barak Y, Sadovsky Y. 2006. The pleiotropic function of PPAR gamma in the placenta. *Mol Cell Endocrinol* 249(1-2):10-15.
- Schaiff WT, Knapp FF, Jr., Barak Y, Biron-Shental T, Nelson DM, Sadovsky Y. 2007. Ligand-activated peroxisome proliferator activated receptor gamma alters placental morphology and placental fatty acid uptake in mice. *Endocrinology* 148(8):3625-3634.
- Sekine K, Ohuchi H, Fujiwara M, Yamasaki M, Yoshizawa T, Sato T, Yagishita N, Matsui D, Koga Y, Itoh N, Kato S. 1999. Fgf10 is essential for limb and lung formation. *Nat Genet* 21(1):138-141.

- Shima N, Nagao M, Ogaki F, Tsuda E, Murakami A, Higashio K. 1991. Tumor cytotoxic factor/hepatocyte growth factor from human fibroblasts: cloning of its cDNA, purification and characterization of recombinant protein. *Biochem Biophys Res Commun* 180(2):1151-1158.
- Sirisathien S, Brackett BG. 2003. TUNEL analyses of bovine blastocysts after culture with EGF and IGF-I. *Mol Reprod Dev* 65(1):51-56.
- Song G, Bazer FW, Spencer TE. 2007. Pregnancy and interferon tau regulate RSAD2 and IFIH1 expression in the ovine uterus. *Reproduction* 133(1):285-295.
- Song G, Bazer FW, Wagner GF, Spencer TE. 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, Spencer TE, Bazer FW. 2005. Cathepsins in the ovine uterus: regulation by pregnancy, progesterone, and interferon tau. *Endocrinology* 146(11):4825-4833.
- Song G, Spencer TE, Bazer FW. 2006b. Progesterone and interferon-tau regulate cystatin C in the endometrium. *Endocrinology* 147(7):3478-3483.
- Spencer TE, Bartol FF, Bazer FW, Johnson GA, Joyce MM. 1999a. Identification and characterization of glycosylation-dependent cell adhesion molecule 1-like protein expression in the ovine uterus. *Biol Reprod* 60(2):241-250.
- Spencer TE, Bazer FW. 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 TE, Bazer FW. 2002. Biology of progesterone action during pregnancy recognition and maintenance of pregnancy. *Front Biosci* 7:d1879-1898.
- Spencer TE, Bazer FW. 2004. Conceptus signals for establishment and maintenance of pregnancy. *Reprod Biol Endocrinol* 2:49.
- Spencer TE, Becker WC, George P, Mirando MA, Ogle TF, Bazer FW. 1995a. Ovine interferon-tau inhibits estrogen receptor up-regulation and estrogen-induced luteolysis in cyclic ewes. *Endocrinology* 136(11):4932-4944.
- Spencer TE, Gray A, Johnson GA, Taylor KM, Gertler A, Gootwine E, Ott TL, Bazer FW. 1999b. Effects of recombinant ovine interferon tau, placental lactogen, and growth hormone on the ovine uterus. *Biol Reprod* 61(6):1409-1418.

- Spencer TE, Ing NH, Ott TL, Mayes JS, Becker WC, Watson GH, Mirando MA, Brazer FW. 1995b. Intrauterine injection of ovine interferon-tau alters oestrogen receptor and oxytocin receptor expression in the endometrium of cyclic ewes. *J Mol Endocrinol* 15(2):203-220.
- Spencer TE, Johnson GA, Bazer FW, Burghardt RC. 2004a. Implantation mechanisms: insights from the sheep. *Reproduction* 128:657-668.
- Spencer TE, Johnson GA, Bazer FW, Burghardt RC. 2007a. Fetal-maternal interactions during the establishment of pregnancy in ruminants. *Soc Reprod Fertil Suppl* 64:379-396.
- Spencer TE, Johnson GA, Bazer FW, Burghardt RC, Palmarini M. 2007b. Pregnancy recognition and conceptus implantation in domestic ruminants: roles of progesterone, interferons and endogenous retroviruses. *Reprod Fertil Dev* 19(1):65-78.
- Spencer TE, Johnson GA, Burghardt RC, Bazer FW. 2004b. Progesterone and placental hormone actions on the uterus: insights from domestic animals. *Biol Reprod* 71(1):2-10.
- Spencer TE, Ott TL, Bazer FW. 1996. tau-Interferon: pregnancy recognition signal in ruminants. *Proc Soc Exp Biol Med* 213(3):215-229.
- Spencer TE, Stagg AG, Joyce MM, Jenster G, Wood CG, Bazer FW, Wiley AA, Bartol FF. 1999c. Discovery and characterization of endometrial epithelial messenger ribonucleic acids using the ovine uterine gland knockout model. *Endocrinology* 140(9):4070-4080.
- Sreenan JM, Diskin MG. 1983. Early embryonic mortality in the cow: its relationship with progesterone concentration. *Vet Rec* 112(22):517-521.
- Starbuck GR, Darwash, A.O., Lamming, G.E. 1999. The importance of progesterone during early pregnancy in the dairy cow. *Cattle Practice* 7:397-399.
- Stevenson KR, Gilmour RS, Wathes DC. 1994. Localization of insulin-like growth factor-I (IGF-I) and -II messenger ribonucleic acid and type 1 IGF receptors in the ovine uterus during the estrous cycle and early pregnancy. *Endocrinology* 134(4):1655-1664.
- Stewart CL, Kaspar P, Brunet LJ, Bhatt H, Gadi I, Kontgen F, Abbondanzo SJ. 1992. Blastocyst implantation depends on maternal expression of leukaemia inhibitory factor. *Nature* 359(6390):76-79.

- Stewart MD, Johnson GA, Gray CA, Burghardt RC, Schuler LA, Joyce MM, Bazer FW, Spencer TE. 2000. Prolactin receptor and uterine milk protein expression in the ovine endometrium during the estrous cycle and pregnancy. *Biol Reprod* 62(6):1779-1789.
- Stoker M, Gherardi E, Perryman M, Gray J. 1987. Scatter factor is a fibroblast-derived modulator of epithelial cell mobility. *Nature* 327(6119):239-242.
- Stronge AJ, Sreenan JM, Diskin MG, Mee JF, Kenny DA, Morris DG. 2005. Post-insemination milk progesterone concentration and embryo survival in dairy cows. *Theriogenology* 64(5):1212-1224.
- Sugawara J, Fukaya T, Murakami T, Yoshida H, Yajima A. 1997. Hepatocyte growth factor stimulated proliferation, migration, and lumen formation of human endometrial epithelial cells in vitro. *Biol Reprod* 57(4):936-942.
- Tamura K, Matsushita M, Endo A, Kutsukake M, Kogo H. 2007. Effect of insulin-like growth factor-binding protein 7 on steroidogenesis in granulosa cells derived from equine chorionic gonadotropin-primed immature rat ovaries. *Biol Reprod* 77(3):485-491.
- Tan J, Paria BC, Dey SK, Das SK. 1999. Differential uterine expression of estrogen and progesterone receptors correlates with uterine preparation for implantation and decidualization in the mouse. *Endocrinology* 140(11):5310-5321.
- Taylor KM, Gray CA, Joyce MM, Stewart MD, Bazer FW, Spencer TE. 2000. Neonatal ovine uterine development involves alterations in expression of receptors for estrogen, progesterone, and prolactin. *Biol Reprod* 63(4):1192-1204.
- Teixeira MG, Austin KJ, Perry DJ, Dooley VD, Johnson GA, Francis BR, Hansen TR. 1997. Bovine granulocyte chemotactic protein-2 is secreted by the endometrium in response to interferon-tau (IFN-tau). *Endocrine* 6(1):31-37.
- Thatcher WW, Hansen PJ, Gross TS, Helmer SD, Plante C, Bazer FW. 1989. Antiluteolytic effects of bovine trophoblast protein-1. *J Reprod Fertil Suppl* 37:91-99.
- Thie M, Fuchs P, Butz S, Sieckmann F, Hoschutzky H, Kemler R, Denker HW. 1996. Adhesiveness of the apical surface of uterine epithelial cells: the role of junctional complex integrity. *Eur J Cell Biol* 70(3):221-232.
- Toyokawa K, Carling SJ, Ott TL. 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.
- Tranguch S, Daikoku, T., Wang, G.H., Dey S.K. 2005. Molecular complexity in establishing uterine receptivity and implantation. *Cellular and Molecular Life Sciences* 62:1964-1973.
- Tsukita S, Furuse M, Itoh M. 2001. Multifunctional strands in tight junctions. *Nat Rev Mol Cell Biol* 2(4):285-293.
- Tulac S, Overgaard MT, Hamilton AE, Jumbe NL, Suchanek E, Giudice LC. 2006. Dickkopf-1, an inhibitor of Wnt signaling, is regulated by progesterone in human endometrial stromal cells. *J Clin Endocrinol Metab* 91(4):1453-1461.
- Ulbrich SE, Kettler A, Einspanier R. 2003. Expression and localization of estrogen receptor alpha, estrogen receptor beta and progesterone receptor in the bovine oviduct in vivo and in vitro. *J Steroid Biochem Mol Biol* 84(2-3):279-289.
- Vallet JL, Barker PJ, Lamming GE, Skinner N, Huskisson NS. 1991. A low molecular weight endometrial secretory protein which is increased by ovine trophoblast protein-1 is a beta 2-microglobulin-like protein. *J Endocrinol* 130(2):R1-4.
- Vallet JL, Bazer FW, Fliss MF, Thatcher WW. 1988. Effect of ovine conceptus secretory proteins and purified ovine trophoblast protein-1 on interoestrous interval and plasma concentrations of prostaglandins F-2 alpha and E and of 13,14-dihydro- 15-keto prostaglandin F-2 alpha in cyclic ewes. *J Reprod Fertil* 84(2):493-504.
- Van Cleeff J, Drost M, Thatcher WW. 1991. Effects of postinsemination progesterone supplementation on fertility and subsequent estrous responses of dairy heifers. *Theriogenology* 36(5):795-807.
- Van Cleeff J, Macmillan KL, Drost M, Lucy MC, Thatcher WW. 1996. Effects of administering progesterone at selected intervals after insemination of synchronized heifers on pregnancy rates and resynchronization of returns to service. *Theriogenology* 46(7):1117-1130.
- Van Heeke G, Ott TL, Strauss A, Ammaturo D, Bazer FW. 1996. High yield expression and secretion of the ovine pregnancy recognition hormone interferon-tau by *Pichia pastoris*. *J Interferon Cytokine Res* 16(2):119-126.
- Veeman MT, Axelrod JD, Moon RT. 2003. A second canon. Functions and mechanisms of beta-catenin-independent Wnt signaling. *Dev Cell* 5(3):367-377.

- Villa-Godoy A, Hughes TL, Emery RS, Chapin LT, Fogwell RL. 1988. Association between energy balance and luteal function in lactating dairy cows. *J Dairy Sci* 71(4):1063-1072.
- Villaruel A, Martino A, BonDurant RH, Deletang F, Sisco WM. 2004. Effect of post-insemination supplementation with PRID on pregnancy in repeat-breeder Holstein cows. *Theriogenology* 61(7-8):1513-1520.
- Vogiagis D, Salamonsen LA, Sandeman RM, Squires TJ, Butt AR, Fry RC. 1997. Effect of immunisation against leukaemia inhibitory factor on the establishment of pregnancy in sheep. *Reprod Nutr Dev* 37(4):459-468.
- Wang HS, Chard T. 1999. IGFs and IGF-binding proteins in the regulation of human ovarian and endometrial function. *J Endocrinol* 161(1):1-13.
- Wang X, Matsumoto H, Zhao X, Das SK, Paria BC. 2004. Embryonic signals direct the formation of tight junctional permeability barrier in the decidualizing stroma during embryo implantation. *J Cell Sci* 117(Pt 1):53-62.
- Wang Y, Macke JP, Abella BS, Andreasson K, Worley P, Gilbert DJ, Copeland NG, Jenkins NA, Nathans J. 1996. A large family of putative transmembrane receptors homologous to the product of the *Drosophila* tissue polarity gene *frizzled*. *J Biol Chem* 271(8):4468-4476.
- Wathes DC, 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.
- Wathes DC, Lamming GE. 1995. The oxytocin receptor, luteolysis and the maintenance of pregnancy. *J Reprod Fertil Suppl* 49:53-67.
- Wathes DC, Reynolds TS, Robinson RS, Stevenson KR. 1998. Role of the insulin-like growth factor system in uterine function and placental development in ruminants. *J Dairy Sci* 81(6):1778-1789.
- Watson AJ, Westhusin ME, Winger QA. 1999. IGF paracrine and autocrine interactions between conceptus and oviduct. *J Reprod Fertil Suppl* 54:303-315.
- Whitley JC, Shulkes A, Salamonsen LA, Voggiagis D, Familiar M, Giraud AS. 1998. Temporal expression and cellular localization of a gastrin-releasing peptide-related gene in ovine uterus during the oestrous cycle and pregnancy. *J Endocrinol* 157(1):139-148.



- Widelitz R. 2005. Wnt signaling through canonical and non-canonical pathways: recent progress. *Growth Factors* 23(2):111-116.
- Willert J, Epping M, Pollack JR, Brown PO, Nusse R. 2002. A transcriptional response to Wnt protein in human embryonic carcinoma cells. *BMC Dev Biol* 2:8.
- Wiltbank JN, Hawk, H.W., Kidder, H.E., Black, W.G., Ulberg, L.C., Casida, L.E. 1956. Effect of progesterone therapy on embryo survival in cows of lowered fertility. *Journal of Dairy Science* 39:456-461.
- Wittchen ES, Haskins J, Stevenson BR. 1999. Protein interactions at the tight junction. Actin has multiple binding partners, and ZO-1 forms independent complexes with ZO-2 and ZO-3. *J Biol Chem* 274(49):35179-35185.
- Wooding FB. 1984. Role of binucleate cells in fetomaternal cell fusion at implantation in the sheep. *Am J Anat* 170(2):233-250.
- Wooding FB. 1992. Current topic: the synepitheliochorial placenta of ruminants: binucleate cell fusions and hormone production. *Placenta* 13(2):101-113.
- Yamanaka H, Moriguchi T, Masuyama N, Kusakabe M, Hanafusa H, Takada R, Takada S, Nishida E. 2002. JNK functions in the non-canonical Wnt pathway to regulate convergent extension movements in vertebrates. *EMBO Rep* 3(1):69-75.
- Yang RY, Liu FT. 2003. Galectins in cell growth and apoptosis. *Cell Mol Life Sci* 60(2):267-276.
- Zhou J, Bondy C. 1992. Insulin-like growth factor-II and its binding proteins in placental development. *Endocrinology* 131(3):1230-1240.
- Zhu LJ, Bagchi MK, Bagchi IC. 1998. Attenuation of calcitonin gene expression in pregnant rat uterus leads to a block in embryonic implantation. *Endocrinology* 139(1):330-339.
- Zhu Y, Bond J, Thomas P. 2003a. Identification, classification, and partial characterization of genes in humans and other vertebrates homologous to a fish membrane progesterin receptor. *Proc Natl Acad Sci U S A* 100(5):2237-2242.
- Zhu Y, Rice CD, Pang Y, Pace M, Thomas P. 2003b. Cloning, expression, and characterization of a membrane progesterin receptor and evidence it is an intermediary in meiotic maturation of fish oocytes. *Proc Natl Acad Sci U S A* 100(5):2231-2236.

Ziomek CA, Johnson MH. 1980. Cell surface interaction induces polarization of mouse 8-cell blastomeres at compaction. *Cell* 21(3):935-942.

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