INSULIN-LIKE GROWTH FACTOR BINDING PROTEINS-1 AND -3, AND HYDROXYSTEROID (11-BETA) DEHYDROGENASE ONE: POTENTIAL ROLES IN RUMINANT CONCEPTUS DEVELOPMENT AND ENDOMETRIAL FUNCTION

A Thesis

by

REBECCA MARIE SIMMONS

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

MASTER OF SCIENCE

December 2009

Major Subject: Physiology of Reproduction

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Approved by:

Chair of Committee:	Thomas E. Spencer
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ABSTRACT

Insulin-Like Growth Factor Binding Proteins-1 and -3, and Hydroxysteroid (11-Beta) Dehydrogenase One: Potential Roles in Ruminant Conceptus Development and Endometrial Function. (December 2009)

Rebecca Marie Simmons, B.S., Oregon State University

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Maternal contributions from the uterine endometrial luminal (LE) and glandular (GE) epithelia are unequivocally required to support ruminant conceptus growth and development, elongation and implantation. Therefore, studies were conducted to examine expression of endometrial genes hypothesized to regulate conceptus development.

The first study investigated two genes specifically expressed in the LE and superficial GE of the ovine uterus. Insulin-like growth factor binding protein (*IGFBP1)* and (*IGFBP3)* expression was coordinate with ovine conceptus elongation. Treatment with P4 induced and IFNT stimulated *IGFBP1*, but not *IGFBP3*; however, IFNT only moderately stimulated *IGFBP1*, indicating that another conceptus-derived factor stimulates endometrial *IGFBP1* expression. IGFBP1 did not affect proliferation of ovine trophectoderm (oTr) cells in *vitro*, but stimulated their migration and attachment. Results indicated that IGFBP1, but not IGFBP3 is a marker of conceptus elongation in ruminants and stimulates cell migration and attachment.

The second study evaluated the effects of pregnancy, P4 and IFNT on expression of hydroxysteroid (11-beta) dehydrogenases (HSD11B1 and HSD11B2), nuclear receptor subfamily 3, group C, member 1 (NR3C1), and prostaglandin-endoperoxide synthase 2 (PTGS2) in the ovine uterus. Expression of *HSD11B1* mRNA and PTGS2 protein in endometrial LE and sGE were coordinate with conceptus elongation, while *HSD11B2* mRNA was expressed primarily in the conceptus. Further, P4 induced, but

IFNT only moderately stimulated *HSD11B1*. Thus, *HSD11B1* expression may be regulated by prostaglandins (PGs) during early pregnancy. The presence of *NR3C1* in the ovine uterus implicates cortisol, the main product of *HSD11B1*, in peri-implantation period events that include elongation of the ovine conceptus.

The third study determined *in vivo* effects of PGs on ovine conceptus elongation and endometrial gene expression. Compared to control ewes, intrauterine infusions of a PTGS2 inhibitor, meloxicam, retarded elongation and decreased expression of elongation-related genes including *IGFBP1*, *IGFBP3*, *HSD11B1*, galectin 15 (*LGALS15*), solute carrier family 2, member 1 (*SLC2A1*), gastrin-releasing peptide (*GRP*), cystatin C (*CST3*), radical S-adenosyl methionine domain containing 2 (*RSAD2*), and ISG15 ubiquitin-like modifer (*ISG15*).

Collectively, these studies assessed the effects of pregnancy, P4, IFNT, and PGs on endometrial genes implicated in conceptus growth. These results indicate that *IGFBP1* is a marker of conceptus elongation in ruminants and provide novel roles for both cortisol and PGs in endometrial gene expression and conceptus elongation.

DEDICATION

This thesis is dedicated to my Dad, who taught me the value of hard work and humor. Dad, you have always encouraged me to follow my dreams and find happiness in whatever career path I've wandered down. And no matter how many times I've changed directions over the years, you've been my number one supporter and I can't thank you enough.

This thesis is also dedicated to my Mom, for understanding me better than anyone else. Mom, you know the perfect remedy for a rough day, and have always been there if I needed advice, someone to listen to, or just a big hug. I see so much of you in me the older I get, and it makes me so proud to be your daughter and friend.

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Lastly, I would like to express my immense gratitude for my parents and big sister Carly. I would not be where I am today without their love, support, and infallible belief in my abilities. I will always remember and be thankful for the endless sacrifices you have made over the years. And to my godmother Nancy, you've been like a mother, cool aunt, and best friend, all wrapped into one. Thanks for always being there to love and encourage me through everything, and for always making me feel special.

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TABLE OF CONTENTS

CHAPTER Page

LIST OF FIGURES

FIGURE Page

LIST OF TABLES

CHAPTER I
INTRODUCTION

In all species, early pregnancy is a critical period of time for conceptus (embryo/fetus and associated extraembryonic membranes) survival. Extensive prenatal mortality has been observed in all mammals studied, although significant differences exist among species in the extent and timing of embryonic death (Wilmut *et al.* 1986). In particular, ruminants experience relatively high levels of pregnancy loss during the peri-implantation period. Cattle, for example, have an estimated fertilization rate of 90% with an average calving rate of about 55%, and thus an approximate 35% embryonic/fetal mortality. Further, embryonic loss is higher between Days 8 and 16 after insemination which results in 70-80% of pregnancy failures (Diskin *et al.* 2006). In mated ewes, as much as 20 to 40% of ova ovulated do not result in live births (Edey 1979). While fertilization failure accounts for the loss of 5-10% of ova in sheep, the greatest proportion of the remaining loss occurs during the first 3 weeks of gestation. Prenatal death contributes to economic loss, resulting in reduced litter size in pigs and prolific sheep. Moreover, in animals with only one ovulation at each estrus, such as cattle and some breeds of sheep, early embryonic death leads to an increased interval between births (Wilmut *et al.* 1986).

To establish and maintain pregnancy, the conceptus must signal its presence to the mother, and, in turn, the uterus must respond accordingly. Various hormones, such as progesterone (P4) secreted from the corpus luteum (CL) and interferon tau (IFNT) released from the conceptus, regulate the expression of numerous endometrial genes that ensure an optimal environment for implantation and placentation. Genes in the endo-

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This thesis follows the style of *Reproduction*.

metrium are both positively and negatively regulated by P4 (Hansen *et al.* 1999). Both P4 and IFNT co-regulate numerous genes important during early pregnancy (Spencer & Bazer 2002, Spencer *et al.* 2008). Inadequate levels of either P4 (Savio *et al.* 1993, Wehrman *et al.* 1993, Mann & Lamming 2001) and IFNT (Nephew *et al.* 1991, Mann & Lamming 2001) have been implicated in early embryonic loss.

Prostaglandins (PGs) from the uterus and/or conceptus may also interact with other factors to regulate gene expression during the peri-implantation period. In many species, PGs regulate numerous physiologically important events and are key players in reproduction. PGs can stimulate gene expression, and, in turn, increase the activity of enzymes such as hydroxysteroid (11-beta) dehydrogenase 1 (HSD11B1) to produce active cortisol. Cortisol, a ligand for both the mineralocorticoid (MR) and glucocorticoid (NR3C1) receptors, can stimulate either water and ion transport or regulate gene transcription (Marver 1984, Michael *et al.* 2003).

Although the exact mechanisms behind pregnancy success are poorly understood, it is believed that pre- and post-ovulatory conditions as well as proper concentrations of hormones all play a role in establishment and maintenance of pregnancy (Inskeep 2004). It is important to define causes of such loss due to the substantial economic loss that follows prenatal death in farm animals. Understanding the temporal and spatial (cell-specific) expression of implantation-related genes and their secreted products provides important insight into conceptus survival and growth. In addition, gene regulation by established hormones of pregnancy, such as P4 and IFNT, as well as novel regulatory hormones, offers a new area of exploration into pregnancy success. Results from this research will facilitate the development of markers for conceptus elongation in ruminants, as well as contribute to a greater working knowledge of the uterine environment and its effects on pregnancy and conceptus elongation.

CHAPTER II
LITERATURE REVIEW

Uterine Development and Structure in Ruminants

In many species including livestock (cattle, swine, and sheep), development of the uterus occurs during both fetal and neonatal life. Uterine adenogenesis is completed during the postnatal period in mammals with long gestation periods (Marion & Gier 1971, Wiley *et al.* 1987, Bartol *et al.* 1993). In all mammals, the mature uterine wall consists of two functional units, the endometrium and myometrium (Fig. 2.1). The endometrium or the inner lining of the uterus is comprised of two epithelial cell types, luminal epithelium (LE) and glandular epithelium (GE), two stratified stromal compartments, a dense zone of fibroblasts (stratum compactum) and a more loosely organized zone in the deeper endometrium (stratum spongiosum), blood vessels and immune cells. The myometrium includes an inner circular layer and outer longitudinal layer of smooth muscle (Wiley *et al.* 1987, Bartol *et al.* 1988, Bartol *et al.* 1993).

The uteri of sheep are dichotomous in nature because the endometrium consists of numerous raised aglandular caruncles and glandular intercaruncular areas (Wimsatt 1950, Atkinson *et al.* 1984). Caruncles serve as the site of superficial implantation and placentation and eventually fuse with placental cotyledons to form placentomes. These placentomes are the major sites of fetal-maternal gas exchange and provide fetal access to micronutrients such as glucose and amino acids (Wimsatt 1950, Mossman 1987). Intercaruncular areas are filled with branched, coiled uterine glands which synthesize and secrete a complex mixture of substances termed histotroph that is absorbed by placental areolae (Bazer 1975).

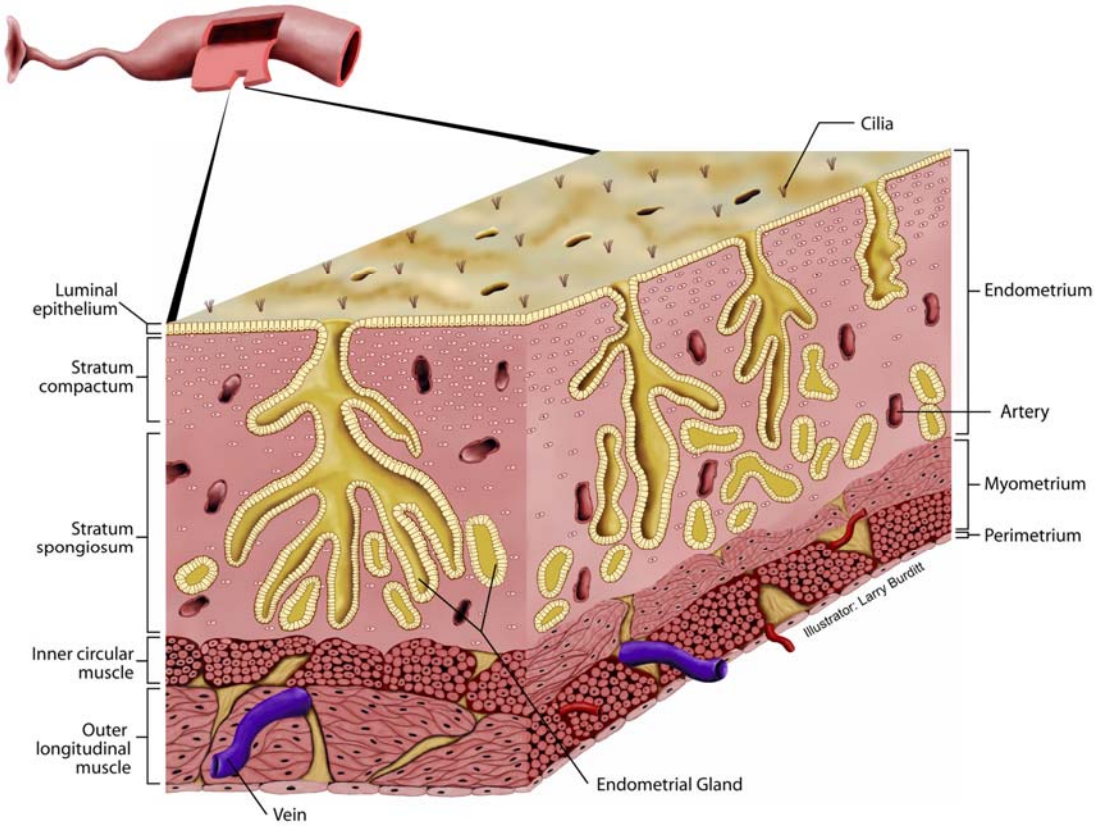

Fig. 2.1. Schematic illustration of uterine histoarchitecture. The uterus is divided into endometrial and myometrial compartments. The myometrium consists of an inner circular and outer longitudinal smooth muscle layers. The endometrium consists of luminal epithelial cells lining the lumen of the uterus, glandular epithelial cells lining the coiling, branching endometrial glands, and stromal cells subdivided into dense, stratum compactum and loose, stratum spongiosum. The endometrium additionally contains a large population of immune cells and vasculature. (Graphic courtesy of Rodney Geisert and Larry Burdett, Oklahoma State University, Stillwater, USA). Originally published by (Spencer *et al.* 2005).

Early Pregnancy Events in Ruminants

Establishment and maintenance of pregnancy in ruminants (sheep, cow, and goat) involves synchronization of key events. It is imperative for the conceptus to signal its presence to the mother, and for the mother, in turn, to create an optimal environment for conceptus elongation, implantation and placentation (Geisert *et al.* 1992, Burghardt *et al.* 2002, Spencer & Bazer 2002, Spencer & Bazer 2004). In sheep and cows, ovulation occurs approximately 30 hours after onset of estrus and the ovum enters the ampulla of the oviduct (Fig. 2.2). Fertilization takes place at the ampullary-isthmic junction of the oviduct where the zygote then undergoes consecutive rounds of cleavage. By Day 4 (Day 0=estrus/mating), the embryo has reached the morula stage and enters the uterus where it forms a blastocyst by Day 6. Blastocysts contain a blastocoele, or fluid-filled cavity, surrounded by a monolayer of trophectoderm and extra-embryonic endoderm (Guillomot 1995, Spencer *et al.* 2004, Spencer *et al.* 2007b). The expanded blastocysts hatch from the zona pellucida on Days 8 or 9 (sheep) or Days 9 to 10 (cattle) and then become ovoid to tubular by Day 11 (sheep) or Day 13 (cattle) and are then termed conceptuses. On Day 12 (sheep) and Day 14 (cattle), the conceptus begins to elongate to a filamentous form that can reach 25 cm or more in length within a few days (Wales & Cuneo 1989). The entire uterine horn ipsilateral to the CL is occupied by Days 16 to 18 in sheep and Days 19 to 21 in cattle.

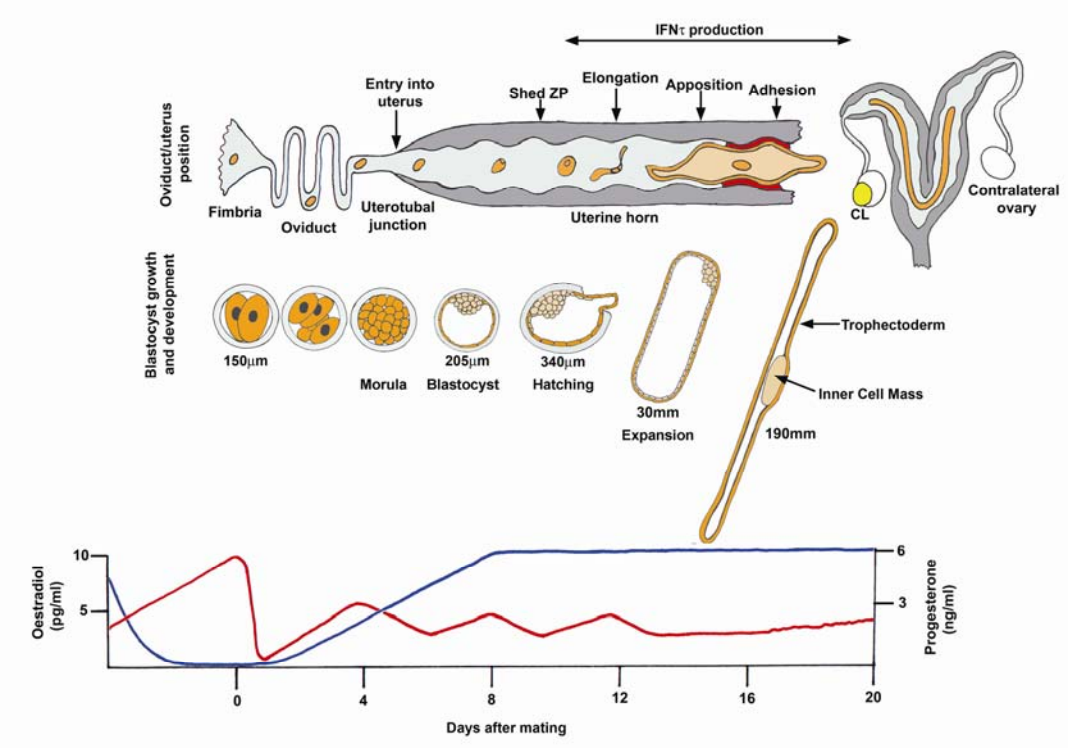

Fig. 2.2. Schematic of events occurring during early pregnancy in sheep. Position of the conceptus in the female reproductive tract, stages of blastocyst development and circulating levels of estradiol and progesterone are illustrated. Following ovulation, the oöcyte enters the oviduct, is fertilized at the ampullary-isthmic junction and the embryo enters into the uterus on Day 4 post-mating. The zona pellucida (ZP) is shed on Days 8 to 9, after which the conceptus expands to a tubular form, followed by elongation to a filamentous conceptus by Day 15. Conceptus elongation coincides with the onset of implantation. (Graphic courtesy of Dr. Greg Johnson, Texas A&M University, College Station, USA). From (Spencer *et al*. 2004).

Conceptus Development and Elongation

Maternal recognition of pregnancy was first defined in 1969 by Roger Short as the physiological process by which the conceptus signals its presence to the maternal system and extends the lifespan of the CL (Short 1969). In ruminants, this recognition process requires conceptus elongation from a spherical to a tubular and then filamentous form after hatching from the ZP (Spencer & Bazer 2004). It is hypothesized that elongation occurs as cells proliferate and redistribute towards the ends of tubular blastocysts, resulting in the characteristic increase in length and decrease in diameter. Studies in the pig by Geisert and coworkers (Geisert *et al.* 1982) first documented the mechanisms involved in elongation. Measurement of DNA and mitotic index indicated no significant changes during the transition from spherical to filamentous forms, supporting the notion that elongation is accomplished through cellular remodeling as well as cellular hyperplasia. This idea of migration was further supported by scanning and transmission electron microscopy (Geisert *et al.* 1982). Blastocyst elongation appears to result from surface and ultrastructural changes involving both the trophectoderm and endoderm. Not only did the trophectoderm flatten, but there was also an increase in microvilli on the cell surface and the formation of large intercellular spaces between trophectoderm cells, possibly to allow for remodeling. A dense band of cells composed of both migrating endoderm and trophectoderm cells comprised a region known as the elongation zone. Filapodia formed between the endoderm and the basement membrane of trophectoderm, possibly to allow endodermal cells to migrate freely and rapidly across the trophectodermal surface.

A major event in conceptus elongation includes the differentiation of the extraembryonic membranes (Wooding & Flint 1994). On Day 13 the embryonic disc is raised above the surface of the elongated trophoblast, and by Day 15 it has grown in size and is more elongated. Both the neural groove and the beginning of the yolk sac are also evident. By Day 17, the embryo has increased in length and the allantois is clearly discernible at the caudal end. The embryo takes on a characteristic rounded shape by Day 19 when many more structural features can be identified (Wales & Cuneo 1989).

Conceptus elongation in sheep involves an exponential increase in length and weight of the trophectoderm (Wales & Cuneo 1989). While the conceptus almost doubles in length every two days, increases in width remain minimal between Days 13 and 19 of pregnancy (Wales & Cuneo 1989). The conceptus can reach 190 mm or more in length by Day 15, but the width remains at approximately 1 to 1.5 mm throughout elongation, resulting in a filamentous appearance (Chang & Rowson 1965, Bindon 1971, Wintenberger-Torres & Flechon 1974). In addition, growth of the conceptus is reflected in its dry weight which increases 4- to 5-fold every two days (Wales & Cuneo 1989). The dry weights of both the trophoblast and yolk sac double between Days 17 and 19, while the allantois, just forming on Day 17, undergoes rapid growth to reach 2 mg by Day 19 (Wales & Cuneo 1989).

The onset of trophoblast giant binucleate cell (BNC) formation is also characteristic of elongation (Wooding & Flint 1994). The BNC first appear on Day 14 in the sheep (Wooding 1984) and are thought to arise from mononuclear trophectoderm cells by successive nuclear divisions without cytokinesis (Wooding 1992), also known as mitotic polyploidy. By Day 16, BNC comprise 15-20% of trophectoderm cells and begin to migrate to the microvillar junction of the LE. They fuse with individual uterine LE cells to produce trinucleate fetomaternal hybrid cells (Wooding 1984), eventually giving rise to multinucleated syncytial plaques that cover the caruncles. The two main functions for BNC include: (1) formation of syncytia necessary for implantation and cotyledonary growth in the placentome; and (2) synthesis and secretion of protein and steroid hormones important during pregnancy (Wooding 1992).

Elongation of conceptus trophectoderm is required for the production of IFNT, which is the pregnancy recognition signal that inhibits development of the endometrial luteolytic mechanism (Spencer *et al.* 1996, Roberts *et al.* 1999, Spencer & Bazer 2002). IFNT is an exclusive product of the mononuclear cells of the conceptus trophectoderm and is synthesized and secreted from Days 10 to 21 of pregnancy, with maximal production on Days 14 to 16 (Bazer 1992, Roberts *et al.* 1999). During 24 hours in

culture, a single Day 16 conceptus produces approximately 1 x 10^8 antiviral units of IFNT (Ashworth & Bazer 1989).

Implantation

Conceptus elongation coincides with the onset of the highly coordinated events of implantation. Implantation is common to all mammals, but the timing of implantation differs among species and can last anywhere from a few hours in rodents to days in humans and domestic animals (Guillomot *et al.* 1993). In general, implantation involves shedding of the zona pellucida, precontact and blastocyst orientation, apposition, adhesion, and endometrial invasion (Guillomot *et al.* 1981, Guillomot *et al.* 1993, Guillomot 1995). Endometrial invasion by the conceptus does not occur in domestic ruminants. After hatching from the ZP, the blastocyst is positioned and immobilized within the uterus from Days 9 to 14 while elongation commences (Fig. 2.3). By Day 14 the trophectoderm is closely associated with the endometrial LE and will weakly adhere to the uterine epithelia. In most species, apposition results in the reduction of apical microvilli covering the trophectoderm from Days 13 to 15 in sheep (Guillomot *et al.* 1981, Guillomot *et al.* 1993). While loss of apical microvilli does not occur on the uterine LE in sheep, apposition of the blastocyst is ensured by interdigitation of cytoplasmic projections of the trophectoderm cells and uterine epithelial microvilli. Furthermore, uterine glands also serve as sites of apposition in ruminants (Guillomot *et al.* 1981, Guillomot & Guay 1982, Guillomot *et al.* 1993). On Day 16, firm adhesion of trophoblast to the LE begins and is completed around Day 22.

In sheep, it is proposed that elongation from a spherical blastocyst to a filamentous form requires transitory attachment and adhesion of conceptus trophectoderm to LE. The LE may initially be nonadhesive, in part, due to the apical expression of mucin glycoprotein one (MUC1); however, as MUC1 expression decreases between Days 9 and 17 of early pregnancy, the process of implantation can begin (Johnson *et al.* 2001). Loss of MUC1 is common to sheep (Johnson *et al.* 2001), cattle (Davies *et al.* 2008), rodents (McNeer *et al.* 1998), and pigs (Bowen *et al.* 1996),

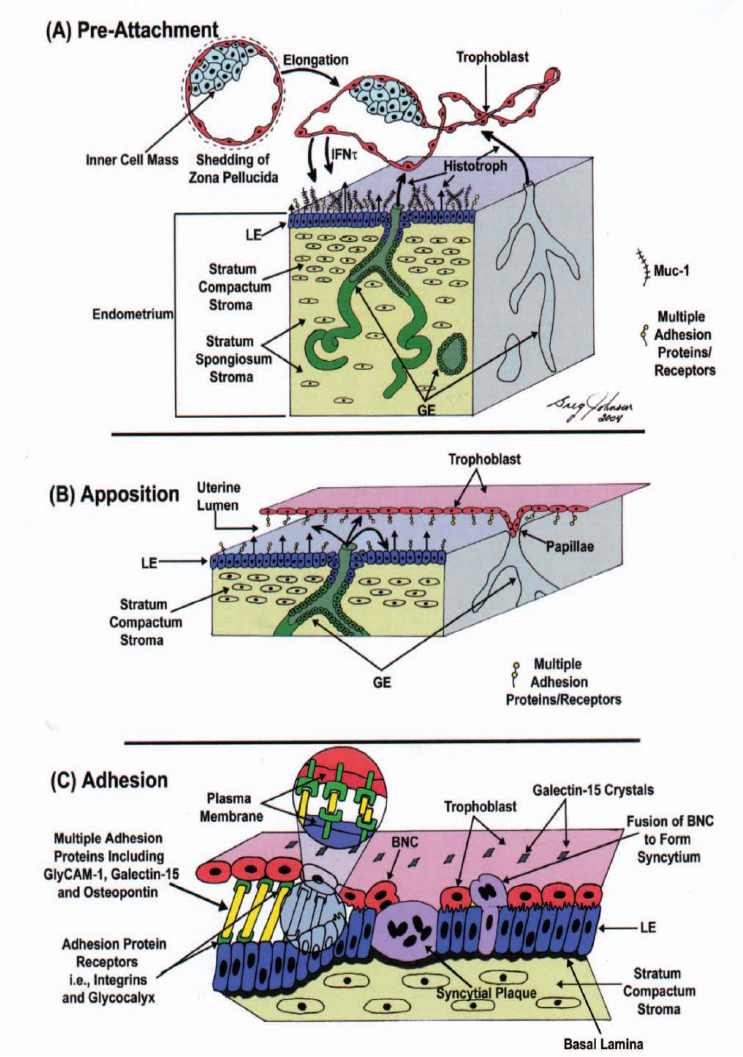

Fig. 2.3. Schematic illustration of the pre-attachment, apposition, and adhesion stages of superficial implantation in sheep. Pre-attachment (A) involves shedding of the zona pellucida, followed by conceptus expansion, as well as precontact and blastocyst orientation. Mucin 1, cell surface associated (MUC1) expression prevents contact with integrin subunits and/or carbohydrate receptors. Apposition (B) involves a decrease in MUC1 expression by the LE and infiltration of the necks of the uterine glands by trophoblast papillae to aid in elongation. Adhesion (C) between the apical surfaces of the conceptus trophectoderm and LE is mediated by uterine secretory proteins, such as secreted phosphoprotein 1 (SPP1) and glycosylation-dependent cell adhesion molecule-1 **(**Gly-CAM-1), binding to receptors. Trophoblast binucleate cells fuse with the luminal epithelial (LE) cells to form syncytial plaques, but are not invasive beyond this single cell layer. (Graphic courtesy of Dr. Greg Johnson, Texas A&M University, College Station, USA). From (Spencer *et al*. 2004).

but in rabbits and humans MUC1 increases overall but is locally reduced at points of conceptus attachment (Hoffman *et al.* 1998, Aplin 1999). As MUC1 levels decline, various integrin subunits expressed by both the LE and conceptus trophectoderm can bind bridging molecules such as secreted phosphoprotein one (SPP1) and other extracellular matrix (ECM) ligands (Yoshinaga 1989, Burghardt *et al.* 1997, Johnson *et al.* 2001).

Uterine Histotroph

Growth and development of the blastocyst into an elongated conceptus requires histotroph, transported or secreted predominantly by the endometrial LE and GE (Bazer 1975, Bazer *et al.* 1979, Roberts RM 1988, Gray 2001a). All mammalian uteri contain endometrial epithelia that synthesize and secrete or transport a rather undefined, complex mixture of serum proteins, transport, and adhesion proteins, protease inhibitors, cytokines, growth factors, hormones, amino acids, sugars, and ions termed 'histotroph' (Wimsatt 1950, Amoroso 1952, Bazer 1975). Experiments by Flechon and coworkers (Flechon *et al.* 1986) demonstrated that Day 12 ovine blastocysts were unable to elongate *in vitro* until surgically transferred into the uterine horn ipsilateral to the CL of ewes on Day 12 of the estrous cycle. When Day 4 ovine conceptuses were transferred into Day 7 recipient ewes, all conceptuses survived but were unable to elongate (Lawson *et al.* 1983); furthermore, these conceptuses stopped growing after Day 11. Results of these experiments support the idea that the maternal uterine environment regulates conceptus survival and development. In addition, uterine gland knockout (UGKO) ewes, which lack endometrial GE and have much reduced LE, experience recurrent early pregnancy loss between Days 12 and 14 post-mating (Gray 2001, Gray 2002). Collectively, these studies indicate that uterine-derived histotroph is required for conceptus survival and elongation in ruminants.

Research has provided insight into both the components of histotroph and patterns of gene expression during pregnancy in ruminants (Spencer *et al*. 2007b, Spencer *et al.* 2008). Histotroph is the product of the selective transport of serum-

derived substances by the endometrium (amino acids, glucose) as well as synthesis and secretion of factors by the endometrium (cytokines, growth factors, adhesion proteins). A comprehensive study by Gao et al. (Gao *et al.* 2009b) identified the following nutrients in uterine histotroph of sheep: glucose, arginine, glutamine, leucine, aspartic acid, glutamic acid, asparagine, histidine, beta-alanine, tyrosine, tryptophan, methionine, valine, phenylalanine, isoleucine, lysine, cysteine, proline, glutathione, calcium, and sodium. While quantities of glucose, amino acids, glutathione, calcium, sodium, and potassium increase during early pregnancy, particular nutrients are hypothesized to play a more direct role in conceptus elongation. Further, specific genes expressed by the endometrium function in transport of histotroph and encode for secreted proteins that govern conceptus elongation; however, these genes are dependent upon ovarian P4 and conceptus-derived IFNT for adequate expression.

Hormonal Regulation of Endometrial Epithelial Functions

Progesterone

Often termed the hormone of pregnancy, P4 has long been known to act on the uterus and stimulate uterine functions required for histotroph production, and thus conceptus survival, growth, and development (Bazer 1975, Spencer *et al.* 2004). Bred ewes treated with mifepristone (RU486), a *PGR* and *NR3C1* antagonist (Baulieu 1989), from Days 8 to 12 post-mating lacked conceptuses on Day 12 (Satterfield *et al.* 2006). Hormone replacement studies in sheep confirmed that P4 induces endometrial LE/sGE gene expression of *lectin, galactoside-binding, soluble, 15* (*LGALS15*), *cathepsin L1* (*CTSL1*), *cystatin C* (*CST3*), *gastrin-releasing peptide* (*GRP*), *insulin-like growth factor binding protein 1* (*IGFBP1*), *insulin-like growth factor binding protein 3* (*IGFBP3*), *hydroxysteroid (11-beta) dehydrogenase 1* (*HSD11B1*), *prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)* (*PTGS2*), *solute carrier family 2 (facilitated glucose transporter), member 1* (*SLC2A1*), *solute carrier family 5 (sodium/glucose cotransporter), member 1* (*SLC5A1*), *solute carrier family 7 (cationic*

amino acid transporter, y+ system), member 2 (*SLC7A2*), and GE gene expression of *solute carrier family 5 (sodium/glucose cotransporter), member 11* (*SLC5A11*), and *GRP*, and *SPP1* (see (Spencer *et al*. 2007a, Spencer *et al.* 2008) for reviews). Interestingly, the onset of expression of many genes by uterine LE/sGE that are associated with conceptus elongation is associated with loss of *PGR* between Days 10 and 11 in the LE and between Days 12 and 13 in the GE post-mating (Wathes & Hamon 1993, Spencer & Bazer 1995). However, *PGR* expression does not decrease in the stroma or myometrium after 8 to 10 days of P4 exposure (Spencer & Bazer 2002). Immediately prior to implantation, loss of *PGR* in uterine epithelia is a common feature across mammals and allows for expression of key genes associated with implantation (Carson *et al.* 2000).

Interferons and Interferon Tau

Interferons (IFNs) are cytokines that have potent antiviral and antiproliferative effects, and are important modulators of the immune system (Platanias 2005). The IFN family includes numerous Type I IFNs, all of which share considerable structural homology, but only one Type II IFN, IFN gamma (IFNG) (Pestka *et al.* 1987, Pestka *et al.* 2004). Type I IFNs include interferons alpha (IFNA), beta (IFNB), delta (IFND), tau (IFNT), and omega (IFNW1). Some IFNs are species specific; for example, IFNT is unique to ruminants, while IFND is specific to pigs (Roberts *et al.* 1999, Roberts *et al.* 2003, Pestka *et al.* 2004, Krause & Pestka 2005) and horses (Tayade C 2008; Abstract 83, Cochet *et al.* 2009). Although IFNT in ruminants is the only known IFN to be involved in pregnancy signaling, IFNs likely affect uterine receptivity, decidualization and placental growth and development in primates, ruminants, pigs and rodents (Austin *et al.* 2003, Pestka *et al.* 2004, Popovici *et al.* 2006, Hess *et al.* 2007, Spencer *et al.* 2007a).

IFNT Signaling

All Type I IFNs bind a common receptor known as the Type I IFN receptor (Fig. 2.4). It consists of two subunits, IFNAR1 and IFNAR2, which are associated with the Janus-activated kinases (JAKs) tyrosine kinase 2 (TYK2) and JAK1, respectively (Darnell *et al.* 1994, Stark *et al.* 1998, Platanias 2005). Type I IFNs induce homodimerization of signal transducer and activator of transcription (STAT1), which can then translocate to the nucleus and bind gamma-activation site elements (Bridges *et al.* 2008) in the promoter region of certain ISGs to initiate transcription (Leanza *et al.* 2007). IFN regulatory factor one (IRF1) is a GAS-regulated gene that intensifies the effects of IFNs by binding and activating interferon-response elements (ISREs) of many ISGs. Type I IFNs act predominately through interferon-stimulated gene factor 3 (ISGF3), which is a STAT1-STAT2-IRF9 (IFN-regulatory factor 9) complex, as opposed to exerting effects through the gamma activation factor (GAF) (Platanias 2005, Bazer *et al.* 2009).

Type II IFNG signaling involves activation of the JAK family due to a constitutive association between JAK1 and JAK2 with IFNGR1 and IFNGR2 subunits of Type II IFNR, respectively (Chard 1989, Bazer & Johnson 1991, Krause & Pestka 2005). Activation of the JAKs by IFNG results in their tyrosine phosphorylation and activation, leading to tyrosine phosphorylation and homo-dimerization of STAT1. STAT1 can then translocate to the nucleus and bind GAS elements in promoter regions of IFNG-regulated genes to initiate transcription (Leanza *et al.* 2007, Bazer *et al.* 2009).

Action of IFNT

As the conceptus elongates, IFNT, the maternal recognition signal in ruminants, is synthesized and secreted by the mononuclear trophectoderm cells (Bazer 1975, Bazer *et al.* 1979, Spencer & Bazer 2004). IFNT inhibits transcription of the *estrogen receptor alpha* (*ESR1*) gene and, therefore, estrogen-induced expression of the *oxytocin receptor* (*OXTR*) in the endometrial LE/sGE, which is required for the generation of oxytocin-induced luteolytic pulses of PGF2α in cyclic or non-pregnant animals. Moreover, IFNT

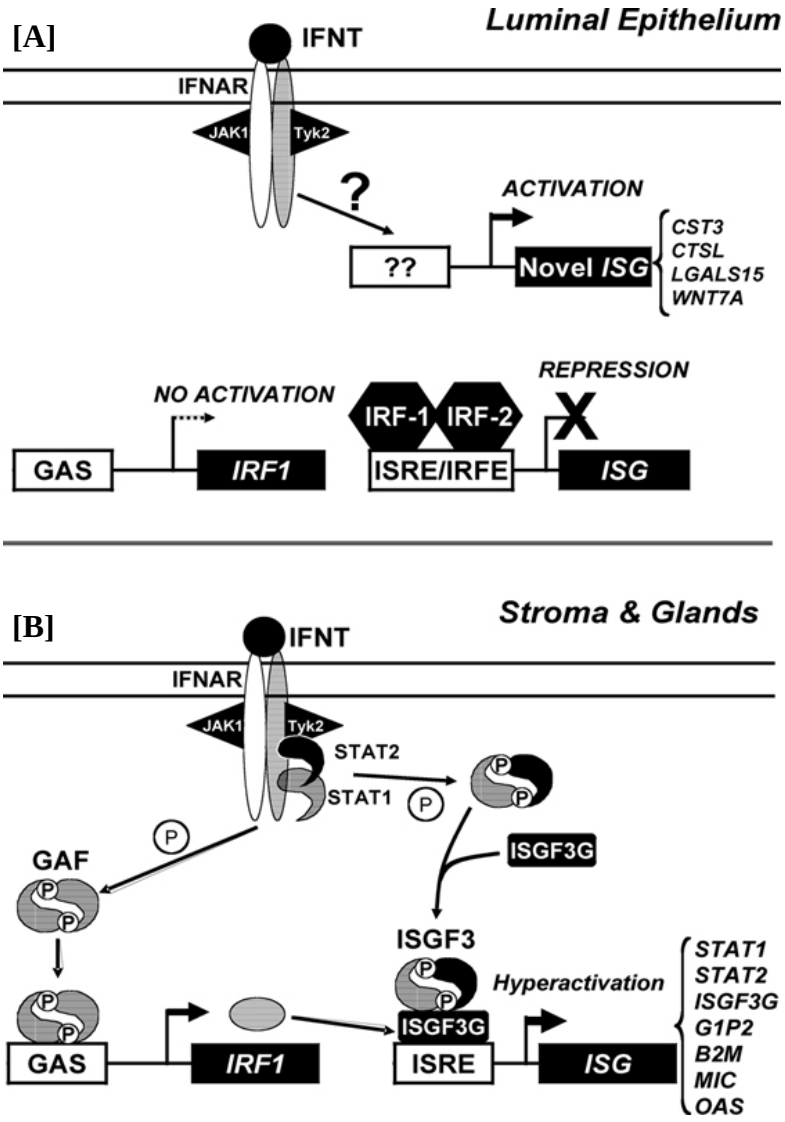

Fig. 2.4. Interferon tau (IFNT) signaling in the ovine endometrium. [A] In luminal and superficial glandular epithelia (LE/sGE), IRF-2, a transcriptional repressor, prevents IFNT induction of expression of IFN-stimulate genes (ISGs), including STAT1, STAT2, and interferon-stimulated gene factor 3 (ISGF3G). The LE/sGE also lack progesterone receptors; however, progesterone induces and IFNT stimulates novel cell signaling pathways to induce expression of *WNT7A, CST3, CTSL, HIF2A, GLUT1, CAT2,* and *LGALS15* genes only in LE/sGE. [B] In stromal cells and GE, IFNT activates the classical Janus-activated kinase-signal transducer and activator of transcription 1-interferon regulatory factor 1 (JAK-STAT1-IRF-1) cell signaling pathway for formation of ISGF3 and gamma-activation factor (GAF) transcription factor complexes to induce ISG expression. Additional transcriptional regulators are likely involved in IFNT actions on stromal cells and GE (Bazer *et al.* 2009).

inhibition of *ESR1* expression prevents *PGR* reappearance in endometrial epithelia (Spencer *et al.* 1996). The maintained CL produces an increasing amount of P4 that, along with IFNT, induces and/or stimulates a myriad of genes in the endometrium implicated in the events of early pregnancy (Spencer *et al.* 2008). IFNT acts on the endometrium to induce or enhance expression of many genes believed to regulate conceptus development, known collectively as IFN-stimulated genes or ISGs. Early embryonic losses and pregnancy failure are the result of inadequate reaction of the endometrium to progesterone and IFNT or insufficient secretion of IFNT by the conceptus (Hernandez-Ledezma *et al.* 1993). The combined effects of P4 and IFNT have been attributed to stimulating a myriad of endometrial implantation-related genes important for conceptus survival and growth.

Combinatorial Effects of P4 and IFNT

Uterine receptivity to implantation is P4 dependent; however, implantation events, endometrial gene expression, and selective transport of molecules into the uterine lumen that support conceptus development, all require loss of expression of *PGR* after 8-10 days of exposure of the uterus to P4 (Bazer *et al.* 2009). Loss of *PGR* by endometrial epithelia, but not by stromal or myometrial cells, is hypothesized to be required for uterine receptivity as well as blastocyst survival and growth, and is common among all studied mammals prior to implantation (Lessey *et al.* 1988, Press & Greene 1988, Geisert *et al.* 1994, Spencer & Bazer 1995, Tan *et al.* 1999, Spencer & Bazer 2002). In addition, reduced expression of anti-adhesive genes such as *MUC1* from uterine LE appears to be a prerequisite for uterine receptivity to implantation (Spencer & Bazer 2002, Spencer *et al.* 2007b). Thus, P4 likely acts on *PGR*-positive stromal cells in a classical manner to induce expression of progestamedins, including *fibroblast growth factor -7* (*FGF7*) and *-10* (*FGF10*) and *hepatocyte growth factor* (*HGF*) (Fig. 2.5). In the ovine uterus, *FGF10* and *HGF* are expressed in the endometrial stroma, and *FGF7* is expressed in the tunica media of blood vessels within the endometrium (Chen *et al.* 2000a, Chen *et al.* 2000b). These progestamedins exert paracrine effects on uterine epithelia and conceptus trophectoderm that express receptors for FGF7 and FGF10

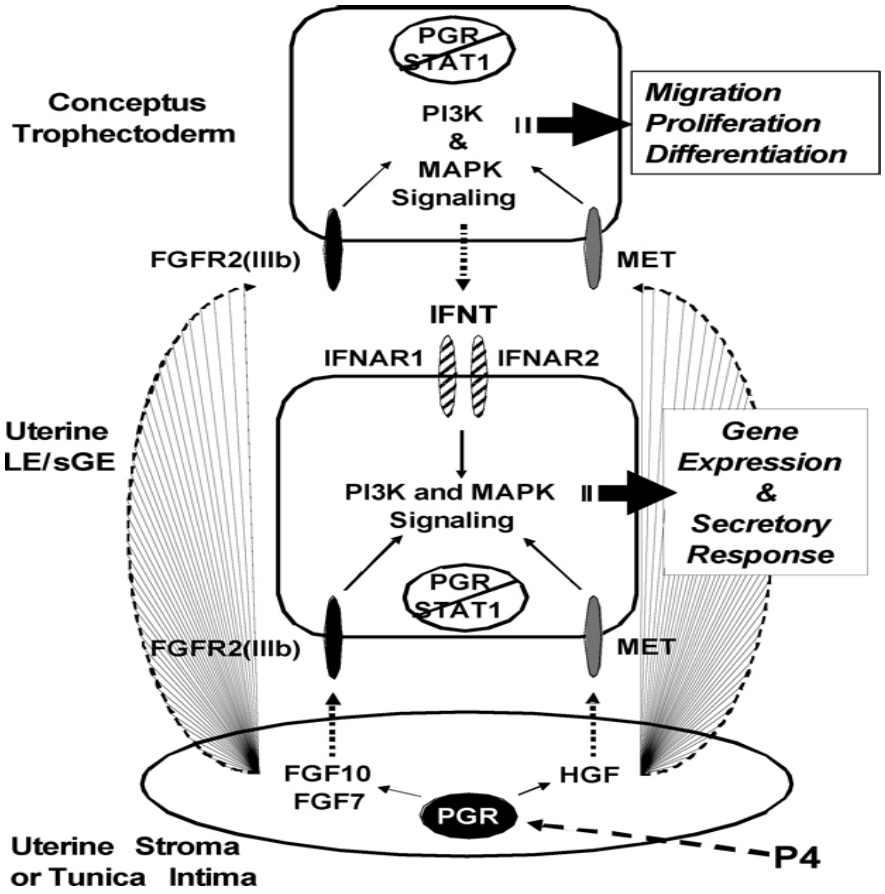

Fig. 2.5. Hypothesis on the roles of progesterone, progestamedins, and interferon tau on gene expression and secretory function by ovine uterine luminal and superficial glandular epithelia that lack both progesterone receptor and signal transducer and activator of transcription 1 (see Bazer *et al*. 2009). Ovine uterine LE/sGE lack detectable PGR and STAT1, indicating that P4 and IFNT use nonclassical signaling pathways to regulate expression of P4-induced and IFNT-stimulated genes. The stroma remains PGR positive. Results from preliminary studies indicate that P4 increases production of stromal-derived progestamedins in the ovine uterus that act on uterine LE/sGE and conceptus trophectoderm cells that express FGFR2(IIIb) and MET receptors for FGF7/FGF10 and HGF, respectively, to activate MAPK and PI3K cell signaling. Progestamedins and type I interferons (IFNs) activate the PI3K and AKT1 signaling pathways in other cell types (Platanias 2005). Unpublished results (J. Kim, G. Wu, G.A. Johnson, and F.W. Bazer) indicate that IFNT activates p38 MAPK and PI3K-AKT1-FRAP1 (mTOR) signaling pathways and that promoter/enhancer regions of novel P4-induced and IFNT-stimulated genes expressed in uterine LE/sGE have binding sites for transcription factors activated by MAPK and PI3K signaling pathways.

(*FGFR2IIIb*) and HGF (*MET*; *proto-oncogene MET*) (Chen *et al.* 2000a, Chen *et al.* 2000b, Spencer & Bazer 2002, Spencer *et al.* 2007b). The primary progestamedin responsive to P4 in ewes appears to be FGF10, which presumably mediates P4 actions on *PGR*-negative uterine epithelia that express their receptors (Satterfield *et al*. 2008).

Because uterine LE/sGE of sheep express *interferon regulatory factor 2* (*IRF2*), a potent inhibitor of gene transcription that silences expression of genes such as *ESR1* and *STAT1*, classical ISGs are limited to uterine GE and stromal cells (Choi *et al.* 2001). Lack of *PGR*, *STAT1*, *STAT2*, and *IRF*s in the ovine uterine LE/sGE renders IFNT unable to affect gene transcription through the canonical JAK-STAT cell signaling pathways. It has been proposed that both P4-induced progestamedins and IFNT are working through nonclassical cell signaling pathways independent of *PGR* and *STAT1*, such as mitogen-activated protein kinases (MAPKs) and phosphoinositide-3 kinase (PI3K), to stimulate uterine gene expression (Platanias 2005, Spencer *et al.* 2007a, Bazer *et al.* 2009). Progestamedins and Type I IFNs have been shown to activate to PI3K and AKT1 signaling pathways in other cells types, such as endothelial cells in the uterine artery (Bird *et al.* 2003). Recent evidence suggests that P4 and IFNT work together to induce and/or enhance endometrial genes, many of which transfer nutrients or encode for secreted proteins that contribute to uterine histotroph and conceptus elongation.

Transporters

Glucose serves as the primary energy source for ovine conceptuses during implantation and can be metabolized into glycogen, nucleic acids, proteins, and lipids (Wales & Waugh 1993). Glucose levels increase in the uterine lumen during early pregnancy (Gao *et al.* 2009b) and, in turn, utilization of glucose progressively increases between Days 13 and 19 of pregnancy (Wales & Waugh 1993) to regulate trophoblast cell growth and proliferation through the glutamine fructose-6-phosphate amidotransferase (GFAT)-mediated FKBP12-rapamycin complex-associated protein 1 (FRAP1) signaling pathway (Wen *et al.* 2005). Because neither fetal nor maternal tissues can perform gluconeogenesis, transport of glucose from maternal circulation into

the uterine lumen requires glucose transporters (Leese & Barton 1984, Pantaleon & Kaye 1998).

Two distinct families of glucose transporters are responsible for transfer of glucose across the plasma membrane: facilitative transporters (solute carriers SLC2A) and sodium-dependent transporters (sodium/glucose cotransporters SLC5A) (Wood & Trayhurn 2003). *SLC2A1*, *SLC5A1* and *SLC5A11* are three transporters that have been identified in the ovine endometrium as well as the conceptus trophectoderm and endoderm; *SLC5A1* and *SLC2A1* are specific to the LE and sGE, while *SLC5A11* is predominately found in the endometrial GE, and *SLC2A3* is expressed by trophectoderm cells (Gao *et al.* 2009). During early pregnancy, P4 induces expression of *SLC2A1*, responsive to P4 in ewes appears to be FGF10, which presumably mediates P4 actions on *PGR*-negative uterine epithelia that express their receptors (Satterfield *et al.* 2008). *SLC5A1*, and *SLC5A11*, and IFNT further stimulates *SLC2A1* and *SLC5A11* (Gao *et al.* 2009). Thus, P4 and/or IFNT induce expression of transporters that move glucose across the endometrium and into the uterine lumen to support conceptus growth and development.

Cathepsins

Cathepsins are a family of lysosomal proteinases responsible for degradation of extracellular matrix molecules and influencing catabolism of intracellular proteins and pro-hormone processing (Kirschke H 1998). Two members of the family, CST3 and CTSL1, are necessary for normal embryonic development and uterine decidualization in mice (Afonso *et al.* 1997). Recent studies by Song and coworkers (Song *et al.* 2005) identified both cathepsins in the ovine endometrium, with *CTSL* expression abundant in the endometrial epithelia and conceptus trophectoderm during early pregnancy. *CST3* is expressed coordinately in the same tissues and is thought to control cathepsin actions during the peri-implantation period (Afonso *et al.* 1997). In addition, both *CTSL* and *CST3* are induced by P4 and stimulated by IFNT in the endometrial LE and sGE (Song *et al.* 2005, Song *et al.* 2006). Because both cathepsins and their inhibitor *CTSB* are

primarily in intercaruncular endometrial glands and intercotyledonary placenta during later pregnancy, they are hypothesized to be involved in endometrial remodeling and placentation in sheep (Song *et al.* 2007).

Radical s-Adenosyl Methionine Domain Containing 2 (RSAD2)

RSAD2 is a cytoplasmic antiviral protein induced by Type I IFNs with the ability to prevent cells from being infected with human cytomegalovirus (Chin & Cresswell 2001). Produced in response to IFNs during viral infections, RSAD2 limits viral replication and modifies subsequent adaptive immunity (Katze *et al.* 2002, Helbig *et al.* 2005). During early pregnancy an antiviral state in the endometrium may be advantageous by blocking sexually transmitted viruses as well as modulating local immune cells to ensure acceptance of the allogenic conceptus and support conceptus survival and growth by stimulating production of cytokines (Hansen 1995, Tekin & Hansen 2002, Croy *et al.* 2003). Endometrial *RSAD2* in sheep increases between days 12 and 16 of pregnancy, but not of the estrous cycle, with primary expression being in endometrial glands, stroma, and immune cells (Song *et al.* 2007b).

ISG15 Ubiquitin-Like Modifier (ISG15)

This ubiquitin homolog is one of many classical interferon stimulated genes expressed in the uteri of ruminants in response to conceptus-derived IFNT, and it can also be found in decidual cells of pregnant humans and baboons (Bebington *et al.* 1999), the decidualized stroma and surrounding GE of pregnant mice (Austin *et al.* 2003), and in the endometrial stroma of pigs during the peri-implantation period (Joyce *et al.* 2003). In sheep, steady state levels of *ISG15* mRNA decrease after Day 25 of pregnancy; however, ISG15 persists in the endometrium through Day 120, but is restricted to the maternal-placental interface (Joyce *et al.* 2005). In several different mammalian species, ISG15 activity increases in the uterine endometria during pregnancy; therefore, ISG15 may be part of a critical temporal and spatial response of the stroma as it prepares for embryo implantation and placentation (Joyce *et al.* 2005). Bovine ISG15 has also been

shown to conjugate to intracellular proteins during pregnancy, suggesting its involvement in protein regulation critical for establishment and maintenance of pregnancy (Johnson *et al.* 1998).

Gastrin-Releasing Peptide (GRP)

GRP, another secreted protein, is widely expressed in various mammalian organs such as the lung, pancreas, hypothalamus, anterior pituitary, and gastrointestinal tract (Patel *et al.* 2006), as well as the uteri of pregnant sheep (Fraser *et al.* 1994, Giraud *et al.* 1994), cattle (Budipitoj *et al.* 2001, Budipitojo *et al.* 2003), and humans (Whitley *et al.* 1996, Xiao *et al.* 1996). Previous studies have identified *GRP* in the ovine endometrial glands and have implicated GRP as a potential regulator of fetal kidney growth and development (Whitley *et al.* 1996, Fleischmann *et al.* 2005). While the major known role of GRP is to elicit the release of gastrin, GRP also can stimulate various lines of cancer cells to induce carcinogenesis both *in vitro* and *in vivo* (Patel *et al.* 2006). Biological roles of GRP include effects on cell morphogenesis, migration, adhesion, and angiogenesis (Martinez *et al.* 2005, Patel *et al.* 2006).

Galectin 15 (LGALS15)

Galectins are proteins with a conserved carbohydrate recognition domain (CRD), allowing them to bind glycoproteins and glycolipid receptors on the surface of cells and initiate biological responses (Cooper 2002, Yang & Liu 2003, Liu & Rabinovich 2005). Galectins play a functional role in cell adhesion, chemoattraction, migration, growth, differentiation, and apoptosis (Hughes 2001, Wang *et al.* 2004), all biological roles necessary for early blastocyst growth and differentiation (Guillomot 1995, Spencer *et al.* 2004). In sheep, *LGALS15* mRNA is present in the endometrial LE and sGE after Day 10 of pregnancy and is a P4 induced, IFNT stimulated gene (Gray *et al.* 2004, Satterfield *et al.* 2006). The ovine LGALS15 protein contains predicted CRD, leu-asp-val (LDV), and arg-gly-asp (RGD) recognition sequences, which can bind and activate integrins

(Ruoslahti 1996), leading to stimulation of trophectoderm cell migration and attachment (Farmer *et al.* 2008).

Insulin-Like Growth Factors

Background

The IGF system is comprised of a complex network of ligands (IGF1 and IGF2), receptors (Type 1 IGFR and Type 2 IGFR), high affinity insulin-like growth factor binding proteins (IGFBPs), and specific proteases that regulate their biological activities (Jones & Clemmons 1995, Rajaram *et al.* 1997). The IGFs share significant structural homology with insulin; however, unlike insulin, IGFs circulate in plasma complexed to a family of binding proteins that influence IGF actions (Rajaram *et al.* 1997). IGFs control many important biological functions, such as placental, embryonic, and fetal growth (Osgerby *et al.* 1999). Both IGF1 and IGF2 are involved in early embryonic development (Schultz & Heyner 1993, Giudice & Saleh 1995), and *in vitro* studies have demonstrated that IGF1 can enhance glucose uptake, protein synthesis, cell proliferation, and conceptus development (Harvey & Kaye 1992, Pantaleon & Kaye 1996, Herrler *et al.* 1998). Additionally, IGF1 acts as a survival factor by decreasing apoptosis in numerous cell types (Giudice *et al.* 1998, Herrler *et al.* 1998).

Actions of both IGF1 and IGF2 are primarily mediated by the Type 1 IGF receptor (IGF1R), which is structurally similar to the insulin receptor. In contrast, the Type 2 IGF receptor (IGF2R) is structurally distinct, binds primarily IGF2, and serves as a receptor for other mannose-6-phosphate containing ligands (Nissley & Lopaczynski 1991). While binding of IGFs to IGF1R activates a tyrosine kinase cell signaling pathway (Jones & Clemmons 1995), IGF2R lacks a signaling function but, removes excess IGF2 from the circulation (Kornfeld 1992) which can interfere with normal development (Filson *et al.* 1993). While the role of IGF2R in mediating IGF actions is less clear, IGF2 can stimulate extravillous trophoblast cell migration *in vitro* by signaling through IGF2R (McKinnon *et al.* 2001).

IGFs in the Ovine Uterus

In sheep, components of the IGF system are located throughout the reproductive tract and the developing conceptus. Caruncular and intercaruncular stromal cells express IGF1, while the LE and GE predominately express *IGF1R* (Satterfield *et al.* 2008). Lower levels of IGF1R are also present in the caruncular stroma and myometrium of the uterus and conceptus trophectoderm (Stevenson *et al.* 1994, Kim *et al.* 2008). *IGF2* mRNA is present in the endometrium during the preimplantation period, specifically in the caruncles and, to a lesser extent, the surrounding endometrial stroma. IGF2 levels are unaffected by day of pregnancy and or P4 treatment (Satterfield *et al.* 2008). During the first half of gestation, *IGF2* expression decreases after fetal interdigitation, but low levels of expression continue in the maternal caruncles. Interestingly, expression is higher at the base of the maternal caruncle and lower near the fetal membranes, suggesting that fetal tissue may inhibit maternal expression of *IGF2* in sheep (Reynolds *et al.* 1997). Intense expression of *IGF2* mRNA is also present in mesodermal tissue of the allantochorion, particularly at the tips of the invading villi, and may be playing a role in stimulating proliferation within the developing placentome (Reynolds *et al.* 1997). It has been suggested that these high levels of IGF2 might be responsible for the downregulation of IGF1R in caruncles at the fetal-maternal interface (Papa *et al.* 1991) and shift localization to the deep uterine glands, which could influence secretion of histotroph into the uterine lumen (Wathes *et al.* 1998).

IGF2 has also been identified in uterine flushes and, along with IGF1, may regulate blastocyst growth and development (Ko *et al.* 1991, Kim *et al.* 2008). IGF2 levels are elevated on Days 12 to 14 of pregnancy (Ko *et al.* 1991). Studies by Kim and coworkers (Kim *et al.* 2008) demonstrated that IGF2 stimulated activation of ERK 1/2 and MAPK p38 phosphorylation in ovine trophectoderm cells, two pathways important in embryonic and placental development in other species (Wang *et al.* 2004, Daoud *et al.* 2005). Further, IGF2 increased migration of trophectoderm cells (Kim *et al.* 2008) and a combination of IGF1 and IGF2 promoted IFNT secretion by ovine conceptuses *in vitro* (Ko *et al.* 1991). *IGF1*, *IGF2*, and *IGF1R* mRNAs are present in ovine preimplantation

embryos (Watson *et al.* 1994), perhaps to produce IGFs and respond to IGFs released by the oviduct or uterus.

IGFs in the Bovine Uterus

The temporal and cell-specific patterns of expression of IGF mRNAs are similar in uteri of cattle and sheep, i.e., *IGF1* is abundant in subepithelial stroma of intercaruncular and caruncular endometria, and *IGF2* and *IGF1R* are present in deep endometrial stroma, caruncular stroma and myometrium (Robinson *et al.* 2000, Llewellyn *et al.* 2008). *IGF1*, *IGF2* and the *IGF1R* and *IGF2R* mRNAs have also been identified at all stages of bovine preimplantation embryos from mature oocyte to blastocyst (Watson *et al.* 1992), and *IGF1R* is also present in Day 15 elongated bovine conceptuses (Sawai *et al.* 2007).

IGF1 is one of the most studied regulatory molecules for the bovine pre-implantation embryo. In cattle, IGF1 is secreted by the oviduct (Pushpakumara *et al.* 2002), uterine endometrium (Geisert *et al.* 1991), and embryo (Lonergan *et al.* 2000), and has receptors in the ovary (Perks *et al.* 1999), oviduct (Pushpakumara *et al.* 2002), uterus (Robinson *et al.* 2000), preimplantation embryo (Watson *et al.* 1992), and elongated conceptus (Bertolini *et al.* 2002). Numerous *in vitro* studies have demonstrated positive effects of IGF1 on bovine pre-implantation embryonic development through modification of cellular physiology and increased cell survival (Matsui *et al.* 1997, Palma *et al.* 1997, Prelle *et al.* 2001). Interestingly, pre-implantation bovine embryos cultured with IGF1 are protected from stress, as IGF1 blocks the induction of apoptosis and inhibition of development caused by elevated temperature (Jousan & Hansen 2004, Jousan & Hansen 2007). IGF1 can also stimulate P4 production, which is important for maintaining production of IFNT by the conceptus (Einspanier *et al.* 1990, Sauerwein *et al.* 1992).

While IGF2 is also present in uterine flushes, its maximal values are from Days 0 to 5 of the cycle (Ko *et al.* 1991). IGF2 protein is also present in bovine conceptus trophectoderm, but is less abundant in the inner cell mass (Wang *et al.* 2009).

Supplementation of maturation medium for bovine oocytes with 20 ng/mL IGF2 or development medium with 100 ng/mL IGF2 benefited bovine preimplantation embryo development; however, excess IGF2 decreased the number of embryos developing to blastocysts *in vitro* (Wang *et al.* 2009).

IGFs in the Porcine Uterus

Maintenance of pregnancy in pigs is dependent upon rapid expansion and elongation of conceptuses throughout the two uterine horns; however, unlike ruminants, pig conceptuses release estrogen (E2) as the maternal recognition signal of pregnancy on Days 11-12 (Bazer & Thatcher 1977, Geisert & Yelich 1997). IGF1 synthesis and release are tightly correlated with the time of conceptus elongation (Simmen *et al.* 1992, Persson *et al.* 1997) and are thought to increase E2 synthesis by stimulating aromatase gene expression in the conceptus (Green *et al.* 1995). Levels of endometrial *IGF1* mRNA peak on Day 12, but are undetectable by midgestation. Interestingly, *IGF1* levels in the myometrium are greater during the preimplantation period when compared to the endometrium, and myometrial *IGF1* remains detectable in later pregnancy (Simmen *et al.* 1992). In addition, conceptus tissues constitutively express low levels of *IGF1* during early pregnancy (Dominguez *et al.* 2003).

Endometrial *IGF2* and *IGF1R* remain low and relatively invariant during the estrous cycle and early pregnancy of pigs (Simmen *et al.* 1992). These low levels of *IGF2* mRNA expression are characteristic of the uterine endometrium of pigs during implantation as both E2 and/or P4 can induce the accumulation of *IGF1* mRNA in the pig uterus without affecting *IGF2* levels (Simmen *et al.* 1990). *IGF2* increases with stage of pregnancy, however, and placental *IGF2* levels are greater than either endometrial or myometrial *IGF2* (Simmen *et al.* 1992).

Differential expression patterns of endometrial *IGF1* and *IGF2* in the pig suggest a preferential role for IGF1 during the preimplantation period and for IGF2 during postimplantation stages of conceptus development. The pregnant uterus is the major site of IGF1 biosynthesis in pigs (Tavakkol *et al.* 1988) and high levels are also produced by

the oviduct (Wiseman *et al.* 1992). Only at the time of implantation is there a notable induction of *IGF2* mRNA in the endometria of pigs. Therefore, IGF1 may function more in endometrial remodeling during the estrous cycle and implantation, while IGF2 is perhaps regulating growth and differentiation of the endometrium and placenta during fetal development (Simmen *et al.* 1992).

IGFs in the Primate Uterus

IGF1 mRNA is expressed in mid- to late-proliferative and early-secretory phase endometria in humans (Giudice *et al.* 1993, Zhou *et al.* 1994). *IGF2* mRNA is highest in mid- to late-secretory phase human endometria and is suggested to be a progestamedin (Zhou *et al.* 1994, Giudice & Irwin 1999), and additional studies have implicated P4 in *IGF2* regulation (Rutanen *et al.* 1997). Both *IGF1R* and *IGF2R* are highly expressed in endometrial epithelia and to a lesser degree in the stroma, but do not change during the cycle (Zhou *et al.* 1994). Thus, IGF actions in the endometrium are more likely regulated by their binding proteins, and not by their receptors.

IGFs are important mediators of metabolic function because they influence nutrient transfer across the placenta. In cultured human trophoblasts, IGF1 can stimulate transport of both glucose and amino acids (Kniss *et al.* 1994). In the human placenta, all mRNAs of the IGF system except *IGF2* are expressed in a similar spatial pattern and relative abundance throughout gestation. Both *IGF1* and *IGF2* show comparable tissue distribution, but *IGF2* is more abundant than *IGF1* at all gestational stages (Han *et al.* 1996). Of note, *IGF2* is found in the villous cytotrophoblasts only during the first trimester of pregnancy, and the greatest concentrations are in invading cytotrophoblasts that must gain access to maternal blood vessels (Han *et al.* 1996).

IGFs in the Rodent Uterus

In rodents, *Igf1* mRNA is intensely expressed in the mesometrial decidua on Day 10 of pregnancy (Correia-da-Silva *et al.* 1999) and may participate in endometrial stromal cell decidualization. While *Igf1* mRNA increases during the estrous cycle in

rodents (Murphy & Ghahary 1990), low levels of *Igf2* are expressed in the uterus with no distinctive changes (Norstedt *et al.* 1989). In placentae of rodents, *Igf2* mRNA is expressed in all cellular elements of the labyrinth (Correia-da-Silva *et al.* 1999), trophoblast invading the decidua (Zhou & Bondy 1992), endothelial cells of fetal capillaries, mesenchymal cores of trabeculae (Correia-da-Silva *et al.* 1999), and fetal vessels of the placenta (Zhou & Bondy 1992). Thus, *IGF2* may be associated with both trophoblast invasion and growth of the fetal vasculature.

In mice, *IGF1* mRNA increases during the estrous cycle (Murphy & Ghahary 1990) and is expressed diffusely throughout decidua during implantation, with highest expression in the primary decidual zone (Kapur *et al.* 1992). *IGF2* is found in primitive endoderm and extra-embryonic mesoderm (Lee *et al.* 1990). On Day 9.5 and 12.5, the spongiotrophoblast, which is homologous to the cytotrophoblast columns and shell in early human pregnancy, strongly expresses *IGF2* (Redline *et al.* 1993). *IGF1R* mRNA is present in endometrial epithelial cells of mice (Henemyre & Markoff 1999).

The preimplantation mouse embryo also expresses IGFs and their receptors. *IGF2* and *IGF2R* mRNA are present in 2-cell mouse embryos, and the *IGF1R* in 8-cell embryos (Rappolee *et al.* 1992). *IGF1* mRNA is not expressed in preimplantation mouse blastocysts; however, maternally derived IGF1 within reproductive tract fluids may compensate for lack of this receptor (Smith *et al.* 1993). This idea is supported by *in vitro* experiments in which IGF1 stimulated growth of mouse preimplantation embryos (Harvey & Kaye 1992) as well as proliferation and migration of ectoplacental cone cells (Kanai-Azuma *et al.* 1993).

Various gene knockout studies in mice have demonstrated the importance of IGFs and their receptors. *Igf1* (-/-) null mutant mice experience embryonic and postnatal growth restriction, with pups exhibiting a 40% reduction in birth weight (Baker *et al.* 1993). *Igf2* (p-) null (paternally derived heterozygous) mutants are growth restricted at birth, but grow postnatally at a normal rate (Baker *et al.* 1993). Mutagenesis of the *Igf1R* (-/-) results in more severely growth restricted fetuses because of loss of both IGF1 and IGF2 signaling (Liu *et al.* 1993). Placental growth is impaired in *Igf2* (p-)

mutant mice, but not until Day 13.5, suggesting its significance in placental growth (DeChiara *et al.* 1990, Baker *et al.* 1993). Further, mice lacking *Igf2R*, which contributes to IGF2 turnover, but is not essential for IGF2 signal transduction, exhibit a 25% increase in placental mass (DeChiara *et al.* 1990).

The Insulin-Like Growth Factor Binding Protein Family

Background

The IGFBP family consists of seven members that can modulate actions of IGFs, facilitate storage of IGFs in extracellular matrices, and/or exert IGF-independent effects (Rosenfeld *et al.* 1990, Shimasaki & Ling 1991, Rechler 1993) (Table 2.1). The majority of circulating IGF1 and IGF2 are complexed to IGFBPs due to a higher affinity for these binding proteins than the *IGF1R*, thus preventing receptor association (Clemmons 1997). Although IGFBPs limit IGF interactions with their receptors, IGFBPs can enhance IGF effects by slowly releasing IGFs for receptor interactions (Conover & Powell 1991) or targeting IGFs to specific tissues (Bar *et al.* 1990). Post-translational modifications of the IGFBPs can alter their affinity for IGFs and help maintain a large, stable reservoir of IGFs in a more favorable equilibrium with their receptors (Clemmons *et al.* 1995, Jones & Clemmons 1995). For example, IGFBP phosphorylation increases its affinity for the IGFs, while proteolysis and glycosylation of IGFBPs decreases IGFBP binding to IGFs (Clemmons 1997).

The nomenclature used to distinguish the IGFBPs was developed from the historical order in which their sequences were identified. IGFBP1 was the first to be purified and sequenced because of its abundance in amniotic fluid (Drop *et al.* 1984, Povoa *et al.* 1984), followed by the cloning and sequencing of rat IGFBP2 (Brown *et al.* 1989). IGFBP3 was next to be cloned and sequenced, and purified to homogeneity from human serum (Martin & Baxter 1986), with Shimasaki and coworkers (Shimasaki & Ling 1991) identifying the sequences of IGFBP4, -5 and -6. Recently a new member of the family, IGFBP7, was identified and is under investigation for its roles in early pregnancy (Oh *et al.* 1996). While all cells that have been examined synthesize at least

one form of IGFBP, patterns and combinations of synthesis vary among tissues and cell types. Expression of IGFBPs changes significantly in many tissues during the transition from fetal life to adulthood (Clemmons 1997).

Binding proteins are components of uterine histotroph in ruminants (Bazer 1975), pigs (Bazer *et al.* 1991), and humans (Giudice *et al.* 1991). *IGFBPs 1-6* have previously been reported during early pregnancy in the ovine uterus (Stevenson *et al.* 1994, Reynolds *et al.* 1997, Wathes *et al.* 1998, Osgerby *et al.* 1999, Gadd *et al.* 2000, Gadd *et al.* 2002). In sheep, Satterfield and coworkers (Satterfield *et al.* 2008) found mRNAs for *IGFBPs 2*, *4*, *5*, *6*, and *7* in uterine stroma, most of which increased in ewes receiving RU486, a *PGR* antagonist, suggesting that P4 decreases expression of those stromal IGFBPs. In contrast, *IGFBP1* and *IGFBP3* mRNA were upregulated in the ovine endometrial LE/sGE in the presence of P4, coincident with loss of *PGR* in the uterine LE/sGE (Spencer & Bazer 1995, Satterfield *et al.* 2008). These results suggest that, unlike other binding proteins, epithelial-derived IGFBP1 and IGFBP3 have a biological role in the ovine uterus during the peri-implantation period of pregnancy and may be important for the events of conceptus elongation.

Table 2.1. General characteristics of the insulin-like growth factor binding proteins and their biological roles in mammals [a]

	Special features and Post-Translational Modifications	IGF affinity	Modulation of IGF action	Biological Roles in Mammals[b]
IGFBP1	RGD sequence[c], specifically binds α5β1 integrin Phosphorylation	I = II	Inhibition and/or potentiation	Stimulates cell migration, limits trophoblast invasion
IGFBP2	RGD sequence Heparin binding if IGFs present, proteolysis	II > I	Inhibition and/or potentiation	Pituitary development and transfer of IGFII to CSF, stimulates glucose transport
IGFBP3	Complexed to ALS[c] subunit to retain IGFs in serum N glycosylation, heparin binding, proteolysis	I = II	Inhibition and/or potentiation	Prolongs half-life of IGFs, alters their interaction with cell surface receptors IGF independent actions
IGFBP4	2 extra cysteine residues N glycosylation, proteolysis	I = II	Inhibition	Inhibits IGF-stimulated cell proliferation, Inhibitory effect relieved by proteolysis
IGFBP5	Protected from proteolysis when bound to IGFs O glycosylation, heparin binding	II > I	Inhibition and/or potentiation	Localizes IGFI and IGFII in ECM, stimulates mitogenesis, inhibits cell growth in ovary and kidney
IGFBP6	2 fewer cysteine residues O glycosylation	II > I	Inhibition	Regulates cell proliferation, differentiation, and transformation
IGFBP7[d]	Low affinity for IGFs	I = II	Inhibition	Regulates cell proliferation, adhesion, angiogenesis

[a]Table adapted from Rajaram *et al*. 1997 and Clemmons *et al.* 1997
[b]Biological roles of IGFBPs 1-6 from Kelley *et al*. 1996
[c]RGD, Arg-Gly-Asp; ALS, acid-labile α-subunit [d]
IGFBP7 data obtained from Kim *et al*. 1997 and Tamura *et al*. 2007

Insulin-Like Growth Factor Binding Protein One

Background

IGFBP1 is present in many biological fluids, including amniotic and seminal fluid, milk, urine, and serum, and also in many tissues including placentae (Kelley *et al.* 1996). It is synthesized most abundantly in the liver, but has been detected in uteri of cows, cats, rats, primates, and humans (Lee *et al.* 1997, Osgerby *et al.* 1999). In serum, IGFBP1 is often phosphorylated (Fowler *et al.* 2000), which increases its affinity for IGF1 six-fold (Jones *et al.* 1991); thus, IGFBP1 can influence the ability of IGFs to bind IGF1R and either inhibit or potentiate IGF actions. In experiments designed to mimic the normal physiological ratio of IGF1 to IGFBP1, IGFBP1 potentiates actions of IGF1. For example, when non-phosphorylated forms are added with a molar excess of IGF1, DNA synthesis is enhanced (Koistinen *et al.* 1990) as is wound healing (Jyung *et al.* 1994, Tsuboi *et al.* 1995). Conversely, addition of exogenous IGFBP1 decreases cell growth and/or inhibits differentiation of cells (Jones & Clemmons 1995).

Interactions of IGFBP1 with Integrins

Unlike other IGFBPs, IGFBP1 contains an RGD sequence in the C-terminus that binds the $\alpha5\beta1$ integrin heterodimer (Irwin & Giudice 1998, Gleeson *et al.* 2001). Integrins are dominant glycoproteins composed of non-covalently linked α and β subunits that bind to various ECM components and cell adhesion molecules (Giancotti & Ruoslahti 1999). They play a central role in mediating adhesion, stabilizing adhesion through cytoskeletal reorganization, and transducing cellular signals through various signaling intermediates during implantation (Yoshinaga 1989, Burghardt *et al.* 1997, Giancotti & Ruoslahti 1999, Johnson *et al.* 2001). Because integrins lack endogenous activity, they must interact with other catalytic molecules to initiate signaling to the cell interior (Juliano & Haskill 1993, Clark & Brugge 1995). Binding of integrins to ligands leads to formation of focal adhesions *in vitro* (Kornberg *et al.* 1992) and to activation of members of the MAPK pathway, especially extracellular-regulated protein kinase (ERK) (Morino *et al.* 1995), as well as focal adhesion kinase (FAK) (Guan & Shalloway 1992).

The binding of IGFBP1 to the α5β1 integrin stimulates trophoblast cell migration in a RGD-dependent manner that is independent of effects of IGF1 (Irving & Lala 1995, Gleeson *et al.* 2001). Various studies have demonstrated that cell migration is abrogated by mutation of the RGD domain in IGFBP1, as well as inhibition of the FAK and MAPK pathways (Jones *et al.* 1993, Gleeson *et al.* 2001).

Integrins in Sheep

Both conceptus trophectoderm and endometrial LE in sheep express integrins (Johnson *et al.* 2001), which are essential for conceptus development and implantation (Burghardt *et al.* 2002). Recent studies in sheep identified the presence of α_v, α_4, α_5, β_1, β_3, and β_5 mRNAs and proteins in endometria of both cyclic and pregnant ewes, and conceptus trophectoderm (Johnson *et al.* 2001). These integrins are constitutively expressed on the apical portion of the LE, GE and conceptus trophectoderm, and do not increase during the peri-implantation period. Unlike primates, in which integrins frame the window of implantation (Lessey *et al.* 1994), sheep may be more dependent on integrin access through downregulation of *MUC1* (Johnson *et al.* 2001). After *MUC1* declines, exposed integrins on the LE are hypothesized to bind RGD and non-RGD amino-acid sequences of proteins to participate in cytoskeletal reorganization events of conceptus elongation and migration (Burghardt *et al.* 2002).

IGFBP1 Regulation

The *IGFBP1* gene contains specialized regions that allow for regulation of IGFBP1 production, including a binding site for hepatic nuclear factor 1 (HNF1), glucocorticoid response elements (GREs) 1 and 2, a 3'-5'-cyclic adenosine monophosphate (cAMP) response element (CRE), and a steroid hormone response element (HRE) (Lee *et al.* 1997). The effects of insulin, which can both inhibit and stimulate IGFBP1 secretion, are mediated through an insulin response element (Suwanichkul *et al.* 1994). In the presence of insulin, *IGFBP1* mRNA and protein production in human endometrial stromal cells is downregulated in a dose-dependent

manner (Powell *et al.* 1991). Further, both insulin and IGF1 block a relaxin-mediated increase in decidual *IGFBP1*, possibly via a negative feedback mechanism to control cellular growth (Thraikill *et al.* 1990). Insulin is also inhibitory in Hep G2 cultures but can stimulate the release of IGFBP1 in myocytes (McCusker & Clemmons 1988, Conover *et al.* 1989), suggesting cell type-specific regulation of IGFBP1 secretion. Conversely, *IGFBP1* is stimulated by glucocorticoids (GCs) (Lee *et al.* 1993) and hypoxia (Tazuke *et al.* 1998) in hepatic cells, and by human chorionic gonadotropin (hCG) (Tang & Gurpide 1993) and interleukin 1 beta (IL1B) (Strakova *et al.* 2000) in endometrial stromal cells. *In vitro*, IGFBP1 secretion is stimulated by IGF1 in liver explants (Binoux *et al.* 1984), myocytes (McCusker & Clemmons 1988), and human fibroblasts (Hill *et al.* 1989).

IGFBP1 in the Ovine Uterus

IGFBP1 is abundant in the ovine uterus during both the estrous cycle and pregnancy (Wathes *et al.* 1998) and is localized to endometrial LE/sGE (Waites *et al.* 1990) during the period of conceptus elongation. *IGFBP1* is highest during early pregnancy (Waites *et al.* 1990, Osgerby *et al.* 1999) and expression increases considerably between Days 9 and 12 of pregnancy. In sheep, early P4 treatment beginning on Day 1.5 of pregnancy increases endometrial expression of *IGFBP1* and blastocyst growth on Day 9, and advances the transformation from a spherical blastocyst to an elongated filamentous conceptus on Day 12 (Satterfield *et al.* 2008). However, RU486 blocks these effects of P4 (Satterfield *et al.* 2008) and blastocysts do not survive (Satterfield *et al.* 2006). Thus, *IGFBP1* may mediate effects of P4 on blastocyst growth and development (Reynolds *et al.* 1997, Satterfield *et al.* 2008). Interestingly, *IGFBP1* mRNA levels are higher in the gravid horn of unilaterally pregnant ewes (Osgerby *et al.* 1999), suggesting a local effect of the conceptus and P4 on gene regulation.

IGFBP1 in the Bovine Uterus

Similar to sheep, *IGFBP1* is expressed exclusively in the endometrial LE/sGE of cattle (Wathes *et al.* 1998, Robinson *et al.* 2000). Expression increases between Days 8 to 12 and peaks on Day 14, consistent with the early period of conceptus elongation (Robinson *et al.* 2000). Increased expression of bovine endometrial *IGFBP1* occurs when P4 levels are high. Since the human *IGFBP1* gene contains a P4 response element and ovine endometrial *IGFBP1* is stimulated by P4, it is hypothesized that P4 increases *IGFBP1* expression in the bovine endometrium (Lee *et al.* 1997, Tseng *et al.* 1997). However, low levels of *IGFBP1* are also detectable during estrus when P4 is minimal (Robinson *et al.* 2000), suggesting that P4 is not the sole regulator of endometrial *IGFBP1* expression.

IGFBP1 in the Porcine Uterus

While *IGFBP 2-6* are expressed in the pig endometrium, *IGFBP1* is undetectable (Song *et al.* 1996, Robinson *et al.* 2000). *IGFBP2* is the most abundant binding protein in porcine endometria (Robinson *et al.* 2000) and, along with *IGF2*, increases with advancing stages of pregnancy (Simmen *et al.* 1992). This variation suggests differences in either importance or functions of IGFBP1 among species.

IGFBP1 in the Primate Uterus

Unlike ruminants, successful implantation, placental development and subsequent fetal growth in primates and rodents requires adequate invasion of the intermediate trophoblasts into the maternal decidua to gain access to maternal blood vessels (Nayak & Giudice 2003). Differentiation of the uterine endometrial stromal cells to decidual cells occurs after establishment of pregnancy. These decidual cells are imperative for proper implantation and nutritional support for the embryo, as they produce factors that promote trophoblast invasion and modulate the maternal immune system to prevent rejection of the conceptus allograft (Bell 1983, Hunt *et al.* 2000).

IGFBP1 is present in human decidua, placenta, and fetal membranes (Hill *et al.* 1993), as well as in uterine epithelia and a small population of stromal cells during the late secretory phase (Zhou *et al.* 1994). During implantation in primates, IGFBP1 is the most prevalent IGF binding protein in the uterus and is thought to facilitate trophoblast penetration at the maternal interface (Tarantino *et al.* 1992, Han *et al.* 1996). IGFBP1 is secreted primarily by decidualized endometrial cells (Koistinen *et al.* 1986) and its concentration increases in maternal circulation during pregnancy (Drop *et al.* 1984b). High levels of IGFBP1 are also present in amniotic fluid (Drop *et al.* 1984a), and fetal plasma (Drop *et al.* 1984b). In non-human primates, endogenous E2 and P4 act synergistically to increase concentrations of IGFBP1 in blood and results of *in vitro* studies demonstrated that P4 is required for endometrial stromal cells to synthesize and secrete IGFBP1 (Richards *et al.* 1995).

Human implantation is a complex process that is similar to tumor invasion, as it involves proteolysis of the ECM, cellular attachment and migration, as well as inhibition of these processes (Stetler-Stevenson *et al.* 1993). IGFBP1 is proposed to mediate the events of implantation because it has both pro- and anti-invasive roles in terms of human cytotrophoblast invasion. IGFBP1 may act independently of IGFs via binding of its RGD sequence to the $\alpha5\beta1$ integrin heterodimer, which is uniquely expressed by the invading trophoblast (Fisher & Damsky 1993). It is hypothesized that when IGFBP1 binds this specific integrin heterodimers it inhibits fibronectin (FN), another RGD ligand in the placenta that favors the $\alpha5\beta1$ integrin. Inhibition of FN leads to improved cell detachment and potentiates invasion (Irwin & Giudice 1998). In addition, binding of the RGD sequence to the $\alpha5\beta1$ integrin stimulates human trophoblast cell migration (Jones *et al.* 1993, Irving & Lala 1995) via the MAPK pathway (Gleeson *et al.* 2001).

IGFBP1 binding to the $\alpha5\beta1$ integrin heterodimer is conducive to trophoblast invasion in humans. However, maternal IGFBP1 can also inhibit cytotrophoblast invasion. Metalloproteinases (MMPs), which digest the ECM of maternal tissue and favor invasiveness of tumor cells, are expressed by human cytotrophoblasts *in vivo* (Polette *et al.* 1994) and *in vitro* (Fisher *et al.* 1989). In contrast, tissue inhibitor of

metalloproteinases (TIMPs) inhibit the invasiveness of cytotrophoblast cells (Librach *et al.* 1991). IGFBP1 can stimulate TIMP1 secretion by cytotrophoblasts (Bischof *et al.* 1998), thus providing a maternal restraint on invasion. IGFBP1 may also restrict trophoblast migration and invasion by preventing IGF interactions with cell surface receptors (Lee *et al.* 1997). Cytotrophoblast invasion is limited when co-cultured with human endometrial stromal cells, which produce inordinate amounts of IGFBP1 when decidualized *in vitro* (Irwin & Giudice 1998). Moreover, excessive concentrations of IGFBP1 in human maternal and fetal serum have been implicated in shallow cytotrophoblast invasion, resulting in preeclampsia and underweight offspring (Giudice *et al.* 1997).

IGFBP1 in the Rodent Uterus

The preimplantation rat uterus expresses *Igfbp1* in the deep GE on Day 3.5, but only minimal expression in LE and sGE (Cerro & Pintar 1997). By Day 7 the LE and GE express *Igfbp1*, and as decidualization proceeds, *Igfbp1* mRNA expression remains high in uterine epithelia of inter-implantation sites, but is undetectable in epithelia surrounding the decidua (Cerro & Pintar 1997). Decidualization, however, is a progressive process that begins at the LE and moves to the myometrium (Krehbiel 1937). Since *Igfbp1* follows an opposite pattern of expression, it may be more involved in pre-decidual transformation of the rat uterus (Cerro & Pintar 1997).

In the mouse uterus, Damario and coworkers (Damario *et al.* 1998) first detected IGFBP1 on Day 1.5 postcoitum in LE, GE and decidual stroma, with greater staining in GE compared to LE (Damario *et al.* 1998). By Day 7.5 to 8.5, moderate staining was present in decidualized stromal cells at implantation sites, with lighter staining throughout the non-implantation decidua. Intense decidual stromal staining was localized immediately adjacent to the conceptus and implantation site, and by Day 9.5 to 10.5 a dense band of IGFBP1 comprised of maternal placenta and the adjacent decidua was evident (Damario *et al.* 1998). Presence of an embryo increased IGFBP1 protein in murine uterine epithelia (Damario *et al.* 1998), as well as *IGFBP1* mRNA expression in

endometrial stromal cells on Days 3, 5, and 8 of culture (Soong *et al.* 1998). Thus IGFBP1 is likely playing a role in decidualization and regulation of implantation in mice and is stimulated by factors from the conceptus (Soong *et al.* 1998).

Transgenic mice overexpressing *Igfbp1* have reduced brain size, somatic growth retardation, alterations in glucose homeostasis, and decreased reproductive performance including impaired fertility, implantation, and interrupted or prolonged pregnancies that result in fetal and neonatal death (Gay *et al.* 1997). Interestingly, *Igfbp1 -/-* mice are phenotypically indistinguishable from wild-type or heterozygous littermates, with no embryonic lethality or significant developmental defects. Adult mice of both sexes are fertile, produce a normal litter size, and females can lactate (Leu *et al.* 2003a). However, *Igfbp1* knockout mice suffer from massive hepatic apoptosis, indicating that Igfbp1 may function as a critical survival factor in the liver via its regulation of integrin-mediated signaling (Leu *et al.* 2003b).

Insulin-Like Growth Factor Binding Protein Three

Background

IGFBP3, the most abundant binding protein in human serum, exists in various glycosylated forms between 40 and 44 kDa (Butt & Williams 2001). Circulating IGFBP3 originates mainly from hepatic nonparenchymal cells, with insulin being responsible for increased gene transcription (Kelley *et al.* 1996). Concentrations of IGFBP3 in blood are regulated by age, nutrition, and various hormones, primarily growth hormone (Rechler 1993). Approximately 75% of the IGFs in blood are bound to IGFBP3 in a high molecular weight ternary complex that includes an acid-labile subunit (ALS) responsible for prolonging the half-lives of IGFs (Baxter & Martin 1989). While free IGFBP3 is cleared from serum, the ALS/IGFBP3/IGF complex is present at high levels in serum (Clemmons 1997).

IGFBP3 binds both IGF1 and IGF2 with high affinity (Cubbage *et al.* 1990) and must be degraded by serine and/or metal dependent enzymes, such as plasmin, cathepsin D (CTSD), matrix metalloproteinases (MMPs), and prostate specific antigen, to increase

IGF availability (Giudice 1995, Maile *et al.* 1998). Recent studies have identified an interaction between IGFBP3 and numerous molecules that may bind IGFBP3, thus regulating its action on cells. These include transferrin (Weinzimer *et al.* 1999), type 1 alpha collagen (Liu *et al.* 1999), heparin (Yang *et al.* 1996b), and the latent TGFB binding protein (LTBP1) (Xu & Murphy 1998).

IGFBP3 Action

IGFBP3, which contains a nuclear localization sequence, can translocate to the nucleus and either directly control gene expression or exert IGF-independent actions (Jaques *et al.* 1997, Schedlich *et al.* 1998). In addition, IGFBP3 has the ability to both potentiate and inhibit IGF actions. Demello and Baxter (De Mellow & Baxter 1988) first reported that IGFBP3 associated with cell surfaces could stimulate DNA synthesis in human fibroblasts; however, pre-incubation of the cultures with IGFBP3 prior to addition of IGF1 was required. Thus, it was hypothesized that IGFBP3 induced modifications in *IGF1R* activity since *IGF1R* downregulation was prevented by pre-incubation with IGFBP3 (Conover 1992), which is a possible mechanism whereby IGFBP3 enhances the effects of IGF1 (Clemmons 1997). Further, co-incubation of IGF1 and IGFBP3 potentiates the effect of IGF1 on alpha-aminoisobutyric acid (AIB) uptake in rat osteoblasts (Ernst & Rodan 1990) and increases DNA synthesis by 90% in baby hamster kidney (BHK) fibroblast cells (Blum *et al.* 1989). IGFBP3 and IGF1 also accelerate wound healing in rats (Sommer *et al.* 1991) and stimulate growth and an increase in bone mineral density in ovariectomized rats (Bagi *et al.* 1994).

IGFBP3 can also inhibit growth and induce apoptosis through its ability to bind IGFs. When bound to IGF1, IGFBP3 modulates the amount of IGF1 available for receptor binding (McCusker *et al.* 1991). The addition of excess IGFBP3 inhibits IGF1-stimulated DNA synthesis in cultured fibroblasts (Blat *et al.* 1989, Villaudy *et al.* 1991), as well as IGF1-stimulated steroidogenesis and cAMP generation in rat granulosa cells (Bicsak *et al.* 1990). Studies by Lee and Cohen (Lee & Cohen 2002) demonstrated that IGFBP3 forms a complex with the retinoid X-receptor (RXR) homodimer that induced

gene transcription, leading to apoptosis in cancer cells. In the prostate carcinoma cell line PC-3, Rajah et al. (Rajah *et al.* 1997) verified both a dose-dependent induction of apoptosis in response to the addition of recombinant IGFBP3 that was independent of effects of IGFs.

While IGFBP3 affects cell growth and DNA synthesis in the presence of IGF1, IGFBP3 also has direct effects in the absence of IGF1. IGFBP3 cDNA inhibited DNA synthesis independent of IGF1 after it was transfected into Balb/c3T3 cells (Cohen *et al.* 1993). Effects of FSH on granulosa cell functions were attenuated by IGFBP3 directly, thus negating a response of mouse embryo fibroblasts to either serum or fibroblast growth factor (Blat *et al.* 1989, Liu *et al.* 1992). Further, the inhibitory effects of TGFB1 on breast carcinoma cell division is mediated through induction of IGFBP3 (Oh *et al.* 1993a), and these actions may be controlled by a cell-surface receptor specific for IGFBP3 (Oh *et al.* 1993b).

The local microenvironment can also affect actions of IGFBP3. In a neutral environment, IGFBP3 blocks IGF1-stimulated [^{3}H]-aminobutyric acid uptake, but in an acidic environment IGFBP3 stimulates effects of IGF1 (Conover *et al.* 1996). Because cell membranes of conceptuses are acidic due to high levels of sialic acid (Denker & Gerdes 1979, Oriol *et al.* 1993), IGFBP3 may attract maternal IGF1 to the conceptus and potentiate its actions. Studies in the horse and rabbit identified IGFBP3 in close proximity to the extraembryonic matrices where it may accumulate maternal IGFs to promote development of the conceptus by controlled release of IGFs (Herrler *et al.* 1997, Herrler & Beier 2000, Herrler *et al.* 2002). IGFBP3 may also contribute to early embryonic development, since human embryos co-cultured with the high levels of IGFBP3 and Vero cells (derived from kidney epithelial cells of the African Green Monkey) were the most developed (Menezo *et al.* 1990, Lai *et al.* 1996).

Proteolysis of IGFBP3

A large number of IGFBP3 fragments have been found in close association with the conceptus in ovine uterine luminal fluid. Studies in sheep determined this

fragmentation to result from actions of maternal IGFBP proteases (Peterson *et al.* 1998b) that release IGFs into the environment. Two types of fragments have been identified; one has a weak affinity for IGF1 and is thus a weak antagonist of IGF action, and the other lacks affinity for IGFs but inhibits IGF-stimulated mitogenesis through an IGF-independent mechanism (Lalou *et al.* 1996). Others have demonstrated that IGFBP3 fragments, but not intact IGFBP3, bind and block insulin action (Yamanaka *et al.* 1997, Vorwerk *et al.* 1998). While IGFBP3 has many well-established roles in IGF regulation, potential functions in conceptus elongation require further study.

IGFBP3 in the Ovine Uterus

IGFBP3 is expressed in endometria of sheep (Reynolds *et al.* 1997, Peterson *et al.* 1998a) and is the predominant IGFBP in the uterine lumen (Peterson *et al.* 1998b). While high levels of *IGFBP3* mRNA are found in specific regions of the LE during the preimplantation period and increase in the caruncular stroma following fetal villi penetration, the majority of expression is in the wall of maternal blood vessels (Reynolds *et al.* 1997). Further work by Satterfield and colleagues (Satterfield *et al.* 2008) localized *IGFBP3* mRNA to ovine uterine LE and sGE and found that expression increased substantially between Days 9 and 12 of pregnancy, and further on Day 9 in response to early P4 beginning on Day 1.5 post-mating; however, *IGFBP3* decreased on Day 12 in ewes receiving a combination of P4 and RU486. Further, early P4 treatment increased blastocyst growth on Day 9 and advanced the transformation from a spherical blastocyst to an elongated filamentous conceptus on Day 12 (Satterfield *et al.* 2008), implicating *IGFBP3* in stimulation of conceptus elongation.

Similar to IGFBP1, ovine IGFBP3 may mediate effects of P4 on blastocyst growth and development (Reynolds *et al.* 1997, Satterfield *et al.* 2008). Within the uterine lumen, IGFBP3 exists primarily in a fragmented form between Days 12 and 15 of pregnancy, perhaps to increase availability of IGFs for actions on the elongating conceptus. Interestingly, ovariectomized ewes treated with P4 for 10 days exhibited increased proteolytic activity; however, by Day 15 of P4 treatment, proteolytic activity

decreased in uterine luminal fluid, suggesting upregulation of an unidentified inhibitor (Peterson *et al.* 1998b). Treatment of ewes with estradiol down-regulated *IGFBP3* expression in the endometrium; therefore, low levels of IGFBP3 in the uterine lumen early in the estrous cycle may be due to high levels of estradiol in plasma during this period (Peterson *et al.* 1998a).

IGFBP3 in the Bovine Uterus

Robinson and coworkers (Robinson *et al.* 2000) evaluated *IGFBP3* mRNA expression during the bovine estrous cycle and pregnancy. Expression was detected specifically in the sub-epithelial stroma, LE and GE (Robinson *et al.* 2000). There was no significant change in endometrial *IGFBP3* mRNA during the estrous cycle, indicating that ovarian steroid hormones do not regulate expression of *IGFBP3* (Robinson *et al.* 2000). *IGFBP3* mRNA was also moderately expressed in the caruncular stroma on Day 16 of pregnancy and may modulate effects of IGF2 on caruncular development (Robinson *et al.* 2000). Similar to results of Keller and coworkers (Keller *et al.* 1998), *IGFBP3* mRNA was lower on Day 16 of pregnancy and not different from values for inseminated non-pregnant cows (Robinson *et al.* 2000). Lower *IGFBP3* expression in pregnant heifers led to the hypothesis that a factor(s) from bovine conceptuses may reduce *IGFBP3* expression, thus increasing mitogenic action of IGFs on the endometrial stroma (Cerro & Pintar 1997). *IGFBP3* has also been identified in vascular endothelial cells (Keller *et al.* 1998), myometrium and oviduct (Kirby *et al.* 1996), as well as the pre-implantation bovine conceptus (Winger *et al.* 1997), and is the most abundant IGFBP in the uterine lumen during early pregnancy (Keller *et al.* 1998).

IGFBP3 in the Porcine Uterus

In pigs, IGFBP3 is the most prevalent IGF binding protein within the uterine lumen during the estrous cycle and pregnancy in pigs (Geisert *et al.* 2001). IGFBPs are first detected on Day 10, but are very low to undetectable by Day 12, coinciding with the morphological transition of conceptuses from spherical to filamentous forms (Lee *et al.*

1998). Further, proteolytic activity is detected in the uterine luminal fluid of filamentous, but not spherical conceptuses, indicating the importance of IGFs during elongation of porcine conceptuses (Lee *et al.* 1998). While IGFBP3 is undetectable by Day 12, *IGFBP3* mRNA remains constitutive in the uterine endometrium, indicating that loss of IGFBP3 in uterine luminal fluid is most likely due to IGFBP-specific proteolysis or activity of estrogens secreted by conceptuses (Lee *et al.* 1998).

IGFBP3 in the Primate Uterus

IGFBP3 is found in endometria of humans (Giudice *et al.* 1991) in a small population of decidual cells, as well as in certain intermediate trophoblast cells of the basal plate, anchoring villi of placenta, mesodermal core, amnion and chorion (Han *et al.* 1996). In humans, IGFBP3 levels decrease as gestation progresses and by eight weeks of gestation it is barely detectable (Giudice *et al.* 1990). Because IGF distribution does not change during human pregnancy but IGFBP3 levels decrease, it is thought that altered IGFBPs carry IGFs, but with a lower affinity, thus allowing the developing conceptus access to IGFs (Davies *et al.* 1991a).

Proteolytic activity of IGFBP3 was first identified in plasma and serum of pregnant women (Giudice *et al.* 1990, Hossenlopp *et al.* 1990), and in patients in various catabolic states including post-operative recovery after major surgery, severe illness, growth hormone-resistant and -deficient states, malignancies and malnutrition (Davies *et al.* 1991b, Davenport *et al.* 1992a, Fielder *et al.* 1992, Muller *et al.* 1993). In humans, IGFBP3 proteolysis is evident at six weeks of gestation and persists through pregnancy, but is undetectable by Day 5 postpartum, consistent with the notion that proteolysis requires the presence of trophoblast (Giudice *et al.* 1990). The placenta is hypothesized to regulate proteolysis (Wiesli *et al.* 2006) since isolated human placental trophoblasts produce a neutral IGFBP3 protease with characteristics similar to activity in pregnancy serum (Irwin *et al.* 2000). Both syncytiotrophoblast and extravillous trophoblast may be sources of IGFBP3 protease, as both are in direct contact with maternal blood and secrete products into the maternal circulation. Further, IGFBP3 protease activity

increases with both gestational age and fetal number, indicating a direct correlation between placental tissue mass and protease activity in maternal serum (Irwin *et al.* 2000). However, IGFBP3 protease activity is absent from fetal serum (Bang *et al.* 1994, Giudice *et al.* 1995).

IGFBP3 in the Rodent Uterus

In the rat uterus, *Igfbp3* is detected on Day 3.5 in scattered stromal cells, but as decidualization proceeds, expression shifts to the LE (Cerro & Pintar 1997). After implantation, *Igfbp3* is found in non-decidualized stromal cells adjacent to the LE with very strong expression in sub-luminal stromal cells near the decidua. By Day 7 an intense ring is detectable throughout non-decidualized stromal cells surrounding the anti-mesometrial decidua; expression extends mesometrially by Day 11.5, possibly to buffer effects of IGF on LE and decidua (Cerro & Pintar 1997).

As pregnancy proceeds and the decidua cells proliferate, the uterine vasculature undergoes remodeling. *Igfbp3* mRNA is abundant in the capillary plexus, which surrounds the primary decidual zone. By Day 11.5, expression is strong in cells that line sinusoids in decidua (Cerro & Pintar 1997), indicating that *Igfbp3* is associated with development of the uterine vasculature. IGFBP3 is the most abundant binding protein in the placenta and uterus on Day 12 of pregnancy, but is undetectable by Day 18 (Davenport *et al.* 1992). Proteolytic activity has been detected in rat serum between Days 12 and 15 of pregnancy, corresponding with the dramatic decrease in serum IGFBP3 (Davenport *et al.* 1990).

In mice, *Igfbp3* mRNA expression is strong in the LE, weaker and more diffuse in the stroma, but greater on the anti-mesometrial side where the blastocyst attaches. This expression is similar but less intense in pseudopregnant animals (Sherwin *et al.* 2004). After implantation, *Igfbp3* mRNA is strong in the primary zone of decidualization immediately surrounding the implanting blastocyst and in the LE, and is possibly mediated by local embryo-derived factors (Sherwin *et al.* 2004).

Igfbp3 expression decreases in response to both E2 and P4, but increases in response to leukemia-inhibitory factor (LIF), a cytokine important for murine implantation (Sherwin *et al.* 2004). *Igfbp3* mRNA is first observed on Day 3 of pregnancy before endogenous LIF on Day 4, which may represent actions of *nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor)* (*NR3C1*) (Cheon *et al.* 2003). Thus, the intense expression of *Igfbp3* on Day 4 in the LE may require a combination of LIF and P4 (Sherwin *et al.* 2004).

While Igfbp3 protein is not detected on Day 3.5 postcoitum in mice, by Day 7.5-8.5 moderate staining is present in decidualized stromal cells at the implantation site (Damario *et al.* 1998). By Day 9.5-10.5, a thick band of immunostaining is evident in the maternal surface of the placenta and adjacent decidua (Damario *et al.* 1998). As in other primates, proteolytic activity has been detected in the mouse uterus (Fielder *et al.* 1990). Igfbp3 levels gradually decrease during gestation and are undetectable in serum by Day 10 (Fielder *et al.* 1990). Inactivation of *Igfbp3* in mice does not result in a phenotype different from control mice (Schneider *et al.* 2000), but ALS null mice experience a post-natal growth defect after three weeks with a 62 to 88% reduction in Igfbp3 concentrations in plasma (Ueki *et al.* 2000).

Hydroxysteroid (11-Beta) Dehydrogenases -1 and -2

Background

HSD11B1 is a member of the short-chain dehydrogenase/reductase (SDR) family of oxidoreductases (Oppermann *et al.* 2001) that generally catalyze NADPH-dependent reactions with substrates including polyols, retinoids, steroids, and xenobiotics (Bray *et al.* 2008). The presence of one or more amino-terminal transmembrane domains makes the HSD11Bs unique from other HSD family members (Tomlinson *et al.* 2004). Both HSD11B1 and HSD11B2 are responsible for the interconversion of active cortisol (humans) or corticosterone (mice and rats) and inactive cortisone (Stewart & Krozowski 1999). HSD11B1 activity is bidirectional; however, it has a higher affinity for cortisone

than for cortisol, causing it to act primarily as a reductase to generate active cortisol (Alfaidy *et al.* 2003).

The expression of *HSD11B1* is widespread throughout the body, with the liver being the major site of enzyme production (Agarwal *et al.* 1989, Yang *et al.* 1992). *HSD11B2*, an exclusive oxidase, is localized to the placenta to protect the fetus from excess glucocorticoids (GCs) (Sun *et al.* 1999). HSD11B2 activity is thought to regulate both the transplacental passage of maternal GCs to the fetus (Yang 1997) and access of GCs to their intracellular receptors (Burton & Waddell 1999), since both excessive and inadequate GC exposure *in utero* are detrimental to fetal development (Reinisch *et al.* 1978, Mosier *et al.* 1982).

While HSD11B2 serves as a protective enzyme to regulate corisol levels, the significance of HSD11B1 and cortisol production in early pregnancy is a more recent area of interest. Cortisol regulates many of the processes required for successful implantation of blastocysts, as well as subsequent growth and development of the conceptus; however, actions of GCs are balanced between positive effects promote pregnancy versus adverse effects that compromise the pregnancy (see (Michael & Papageorghiou 2008) for review). In later pregnancy, prostaglandins (PGs) increase the activity of HSD11B1 and subsequent cortisol production (Challis *et al.* 1997). As a result, the relationships between *HSD11B1*, cortisol, and PGs in the induction of *HSD11B1* expression during early pregnancy are being evaluated in primates and livestock (Klemcke *et al.* 2003, Tomlinson *et al.* 2004, Draper & Stewart 2005).

HSD11B1 and HSD11B2 in the Ovine Uterus

In sheep, *HSD11B1* is expressed in the endometrium during both the estrous cycle and pregnancy (Yang *et al.* 1996), as well as in the endometrium and, to a lesser extent, in myometrium of pregnant sheep at term (Yang *et al.* 1995). *HSD11B1* expression is localized exclusively to the LE throughout pregnancy (Yang *et al.* 1996a) and may regulate GCs which, in turn, could impact local PG and peptide production as well as fetal growth and maturation (Liggins 1976).

In late pregnancy, fetal cortisol acts at levels of intrauterine tissues to alter steroidogenesis and induce upregulation of *prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)* (*PTGS2*) expression within trophoblast cells, leading to increased PG production (Challis *et al.* 1997) (Fig. 2.6). PGs can then stimulate HSD11B1-induced cortisol production which leads to a decline in P4 and an increase in E2. Higher E2 levels initiate specific contraction-associated proteins (CAPs) within the myometrium that permit myometrial contractions in response to PG and oxytocin (Lye 1994), allowing for induction of labor. Progesterone is hypothesized to regulate *HSD11B1* mRNA expression throughout gestation and after parturition. *HSD11B1* mRNA in the endometrium is undetectable in cycling ewes until Day 10 of the late luteal phase, when circulating levels of P4 are elevated. Further, *HSD11B1* mRNA is undetectable in either myometrial or endometrial tissue at three months postpartum, possibly due to low P4 levels (Challis *et al.* 1997). Induction of *HSD11B1* when P4 levels increase supports the idea of *HSD11B1* persisting through pregnancy.

HSD11B1 and HSD11B2 in the Bovine Uterus

HSD11B1 and *HSD11B2* mRNAs have also been identified during early pregnancy in endometria of cattle (Lee *et al.* 2007, Komiyama *et al.* 2008), which suggests a role for GCs in endometrial and conceptus development. Endometrial *HSD11B1* mRNA is low during the estrous cycle and luteal phase, but increases during the follicular phase; however, *HSD11B2* mRNA is lowest during the follicular phase (Lee *et al.* 2007).

Increased levels of *HSD11B1* mRNA and bioactivity are temporally coincident with an increase in basal release of PGF2α, but not PGE2, during the estrous cycle (Miyamoto *et al.* 2000, Murakami *et al.* 2001). Using cultured bovine endometrial cells, Lee and coworkers (Lee *et al.* 2008) found that PGF2α stimulated HSD11B1 bioactivity and protein expression in a dose-dependent manner. When cultured with indomethacin, a non-specific inhibitor of PTGS1 and PTGS2, HSD11B1 bioactivity and PGF2α levels

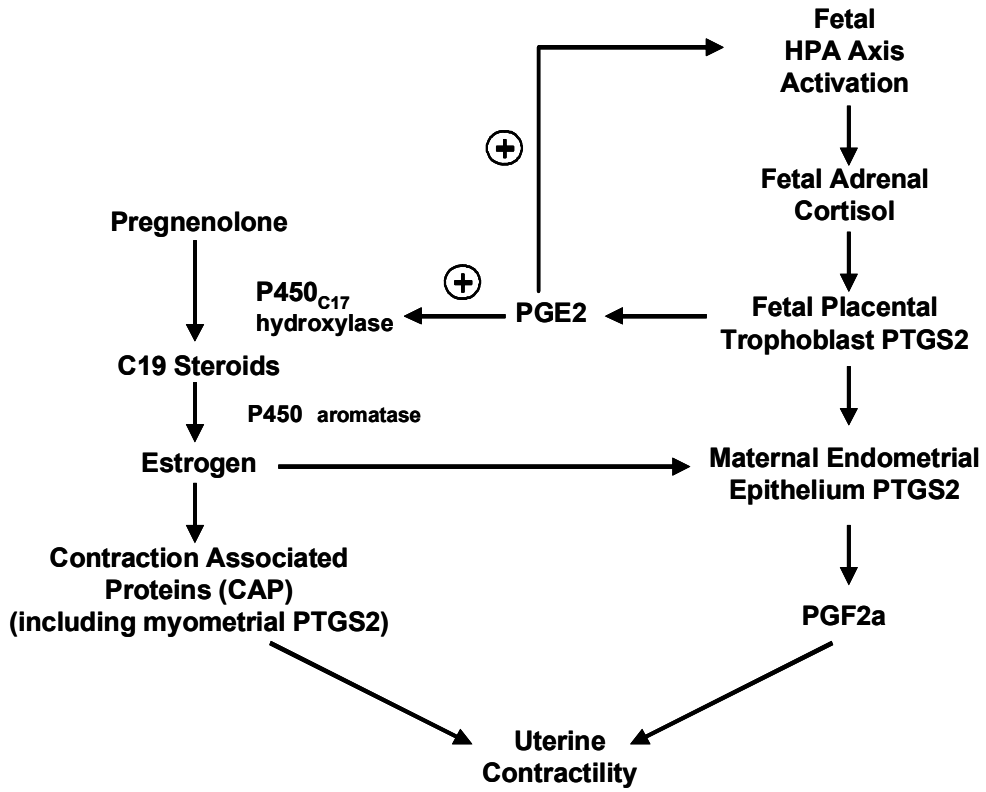

Fig. 2.6. Endocrine events at the onset of ovine parturition. Fetal cortisol increases placental trophoblast expression and activity of PTGS2 leading to PGE2 production. Placental PGE2 in turn mediates an autocrine/paracrine increase in placental P450C17 expression/activity resulting in a surge in placental estrogen production that is superimposed on a gestation-dependent increase in estrogen output. Placental PGE2 is also secreted into the fetal compartment where it acts to sustain fetal HPA axis activation. Estrogen upregulates the expression of maternal endometrial PTGS2, leading to increased PGF2α output and also induces the expression of CAPs. Consequently, myometrial contractility is stimulated and labor and delivery of the fetus ensues (Whittle *et al.* 2001).

decreased. HSD11B1 bioactivity was restored, however, when co-cultured with PGF2α, indicating a suppressive effect of indomethacin on HSD11B1 activity was mediated by the decrease in endogenous PG production (Lee *et al.* 2008). Thus, PGF2α may be exerting direct stimulatory effects on HSD11B1 bioactivity.

Because high levels of PGF2α are believed to cause premature luteolysis and early embryonic loss (Adams *et al.* 1987), the authors suggest that activation of HSD11B1 by PGF2α and subsequent cortisol production may be a protective mechanism against excessive PGF2α production (Lee *et al.* 2008). In the non-pregnant bovine endometrium, cortisol suppresses basal PGF2α production and tumor necrosis factor-stimulates both PGE2 and PGF2α production by endometrial stromal cells, thus initiating a negative feedback loop between local PG production and cortisol synthesis (Lee *et al.* 2007). The inhibitory effect of cortisol on PGF2α production in bovine endometrial stromal cells, as well as the stimulatory effect of GCs during pregnancy, suggests that reproductive status alters the interaction of GCs and PGs in the uterus (Alfaidy *et al.* 2001, Lee *et al.* 2008).

HSD11B1 and HSD11B2 in the Porcine Uterus

At Day 75 of gestation, Klemcke and coworkers (Klemcke 2000) first identified both HSD11B oxoreductase and dehydrogenase activities, with dehydrogenase activity 5-fold greater than oxoreductase activity. They also noted a significant positive linear association between net dehydrogenase activity and fetal/placental size. *HSD11B1* and *HSD11B2* mRNAs were also identified during early pregnancy in porcine placentae (Klemcke *et al.* 2003). While *HSD11B2* mRNA and enzymatic activity were present in porcine placentae as early as Day 24 of gestation, placental *HSD11B1* mRNA was absent on Day 24. Both isoforms increased during gestation, as did dehydrogenase activity (Klemcke & Christenson 1996, Klemcke *et al.* 2003).

Since HSD11B2 is proposed to act as a placental barrier, levels of fetal cortisol were analyzed and, as expected, the percentage of fetal cortisol contributed by the maternal compartment decreased from 22.8% at Day 50 of gestation to 5.9% at Day 100

in piglets (Klemcke 1995). HSD11B2 activity was greater in crowded than in a roomy uterine environment, and placental activity from male fetuses was sometimes greater than in female fetuses, indicating a fetal influence on HSD11B2 activity (Klemcke & Christenson 1996). Cortisol was also detected in the porcine embryo as early as Day 25, and levels increased 8-fold by Day 35 (Klemcke *et al.* 1999).

HSD11B1 and HSD11B2 in the Primate Uterus

HSD11B1 mRNA is expressed at low, invariable levels between the proliferative and secretory phases, but is significantly higher in endometrium during the menstrual period (McDonald *et al.* 2006). *HSD11B1* is also present in the human decidua (Albiston *et al.* 1995) and levels increase in the first trimester of pregnancy. Greater expression is also seen in uterine natural killer cells, a group of phenotypically unique cells that increase in number prior to pregnancy (McDonald *et al.* 2006). *HSD11B2* mRNA is expressed in normal endometria throughout the menstrual cycle at higher levels than *HSD11B1*, but there was no increase in *HSD11B2* mRNA in the endometrium during the menstrual phase or in uterine NK cells (McDonald *et al.* 2006).

HSD11B1 and *HSD11B2* mRNA are also present in human placenta, vascular endothelium, and more abundantly in trophoblast cells of the chorion, amnion, and decidua (Sun *et al.* 1997). The placental syncytiotrophoblast of both humans and baboons also express *HSD11B1* (Pepe *et al.* 1999). However, HSD11B1 protein is not detectable by immunohistochemistry (Sun *et al.* 1997) or western blot analyses (Ricketts *et al.* 1998) in humans. Therefore, unlike the baboon, HSD11B2 is the primary dehydrogenase in the human placentae (Ricketts *et al.* 1998).

Additional research with human placentae supports evidence for *HSD11B1* and PGs working together to induce labor as demonstrated in sheep at parturition. In this model, locally produced PGs stimulate HSD11B1 reductase activity in fetal membranes to increase local production of cortisol (Alfaidy *et al.* 2001). Accumulation of cortisol stimulates PG synthesis and decreases metabolism of PGs by 15-hydroxyprostaglandin dehydrogenase (HPGD), allowing PGs to further stimulate HSD11B1 activity.

Increased PGs, cortisol, and HSD11B1 create and then accelerate a feed-forward loop within trophoblast cells of the chorion responsible for human labor (Alfaidy *et al.* 2003).

HSD11B1 and HSD11B2 in the Rodent Uterus

In the rat, *HSD11B1* mRNA is evident in trophoblast cells within the basal zone on Day 16, and is undetectable by Day 22. However, expression increases in trophoblast cells in the labyrinth zone as gestation progresses (Waddell *et al.* 1998), which may be the result of decreasing levels of P4 at parturition (Mark *et al.* 2009). Conversely, *HSD11B2* mRNA is highest in trophoblast cells within the labyrinth zone on Day 16 (Waddell *et al.* 1998). In rodents, as for humans, there is no detectable HSD11B1 bioactivity, but HSD11B2 is detectable in both placental zones and varies with changes in the abundance of mRNA (Waddell *et al.* 1998).

In the mouse endometrium, *HSD11B1* mRNA is localized to the LE, while *HSD11B2* is only expressed in the stroma and inner circular layer of the myometrium from Days 12.5 to 18.5 (Thompson *et al.* 2002). *HSD11B1* mRNA is highly expressed in the decidualized stroma above and below the compact decidua from Days 12.5 to 18.5. Expression of *HSD11B2* mirrors that of *HSD11B1*, but expression is only present below the compact decidua. Both isoforms are undetectable in the compact decidualized region (Thompson *et al.* 2002).

In mice, the labyrinthine zone serves as the major site of maternal and fetal exchange of nutrients (Cross 2000). *HSD11B2* mRNA is found in both the labyrinthine zone of the placenta and extra-embryonic membranes on Day 15.5, but is undetectable in both the labyrinth trophoblast (Brown *et al.* 1996) and the yolk sac (Thompson *et al.* 2002) by Day 16.5. The onset of *HSD11B1* expression in the placenta coincides with silencing of *HSD11B2* and the corticosterone surge during late gestation, which peaks on Day 16.5 of gestation (Barlow *et al.* 1974). This switch may allow for increased fetal exposure to maternal GCs, which are essential for final GC-dependent maturation of fetal organ systems (Venihaki *et al.* 2000).

Hsd11b1 null mice are fertile, have unaltered birth weight and litter size, and appear generally normal; however, they lack reductase activity (Holmes *et al.* 2001). A null mutation of *Hsd11b2* results in a 50% increase in mortality of pups within 48 hours after birth, and death is preceded by motor weakness and reduced suckling. However, the remaining mice develop normally to adulthood (Holmes *et al.* 2001).

Corticosteroid Hormones and Receptors

Background

Mineralocorticoids (MCs) and GCs are the two major classes of corticosteroid hormones that bind intracellular receptors, specifically the glucocorticoid and mineralocorticoid receptors (NR3C1 and MR), and thereby stimulate or silence gene transcription (Tomlinson *et al.* 2004). NR3C1 and MR are members of the steroid/thyroid hormone receptor superfamily of ligand-inducible transcription factors (Pippal & Fuller 2008) and are homologous both in structure and function. In humans, for example, NR3C1 and MR are approximately 56% identical in the steroid-binding domain (Arriza *et al.* 1987). Despite acting through closely related receptors and a common DNA response element, GCs and MCs induce considerably different physiological responses in a tissue-specific manner (Pippal & Fuller 2008).

Mineralocorticoids

The synthesis of MCs occurs in the zona glomerulosa of the adrenal cortex, where there is no 17-hydroxylase (Baulieu & Kelly 1990). Progesterone is hydroxylated at 21 and 11β, producing corticosterone. A hydroxylation at 18 (18-hydroxycorticosterone) occurs, followed by an oxidation of the alcohol function to aldehyde, leading to aldosterone production (Baulieu & Kelly 1990). MCs are imperative for maintaining sodium homeostasis and primarily stimulate sodium transport by epithelial cells when bound to the MR (Marver 1984). MR is a physiologically important receptor for two classes of hormones, the MCs, aldosterone and deoxycorticosterone (Penning *et al.* 1997) and the GCs, cortisol (in humans) and

corticosterone (in rodents), making it unique among the steroid receptors (Pippal & Fuller 2008). As with all nuclear receptors, the activated *MR* binds to response elements that enhance the promoter activity of target genes. Although the *MR* predominantly acts as a transcription factor, it may also mediate non-nuclear activation of second messenger pathways (Fuller & Young 2005). Furthermore, some actions of aldosterone may involve a receptor other than the *MR* that mediate rapid, nongenomic responses that have been reported for other steroid hormones such as E2 and P4 (Luconi *et al.* 2004).

Glucocorticoids

Synthesis of GCs occurs primarily in cells of the zona fasciculata of the adrenal cortex where pregnenolone is oxidized at C3, resulting in a ketone function (Baulieu & Kelly 1990). Isomerization of the double bond occurs to produce progesterone, which then undergoes a series of hydroxylations at C17 (17-hydroxyprogesterone), at C21 (11-deoxycortisol), and then at C11β, terminating in cortisol (Baulieu & Kelly 1990). GCs, such as cortisol and corticosterone, are important for their physiological roles in the regulation of carbohydrate and amino acid metabolism, maintenance of blood pressure, and modulation of stress and inflammatory responses (Michael *et al.* 2003). In most tissues, GCs exert anti-inflammatory effects by decreasing the expression and/or activity of phospholipase A2, group IB (PLA2G1B), thus limiting synthesis of prostaglandins (PGs) and thromboxanes (TBXs) (Bailey 1991, Flower & Rothwell 1994, Barnes 1998); however, in the placenta and fetal membranes, GCs paradoxically increase PG synthesis (Sun & Myatt 2003, Zhang *et al.* 2006), possibly to facilitate parturition. GCs can also directly alter uterine physiology (Brann & Mahesh 1991) by altering decidual gene expression in humans (Gellersen *et al.* 1994), modifying uterine blood flow and inducing labor in sheep (Monheit & Resnik 1981, Gupta *et al.* 2003), regulating prostaglandin biosynthesis in cows (Lee *et al.* 2007), and inhibiting fetal growth in pigs (Klemcke & Christenson 1997). While GCs have been evaluated in late pregnancy and at parturition, the significance of GCs during early pregnancy in livestock remains relatively unknown.

GCs have been studied extensively in humans during early pregnancy; however, the majority of the women evaluated were undergoing assisted reproduction (Quenby *et al.* 2005, Boomsma *et al.* 2007). During the peri-implantation period, secretion of hCG from the trophoblast of early human blastocysts is pivotal in maintaining P4 secretion from the CL and may act locally to promote implantation and differentiation of the conceptus (Fazleabas *et al.* 1999, Islami *et al.* 2001, Licht *et al.* 2001). Treatment with synthetic GCs can stimulate the secretion of hCG *in vitro* from human term trophoblast cells or from first trimester cytotrophoblast cells (Guller *et al.* 1994). GCs can also suppress the expression of trophoblast integrins and modulate blastocyst attachment (Ryu *et al.* 1999). During implantation, GCs may have a role in improving the intra-uterine environment and facilitate implantation of blastocysts, possibly by correcting imbalances in growth factors, cytokines, and uterine NK cells (Quenby *et al.* 2005). Interestingly, GCs can either suppress fibronectin expression in term human cytotrophoblast cells and amnion, or act in synergy with TGFB to upregulate fibronectin in chorion and placental mesenchymal cells (Guller *et al.* 1995).

While GCs are involved in many facets of reproduction, it is well documented in humans that overexposure of the fetus to GCs in mid- to late pregnancy may result in adverse outcomes, such as intra-uterine growth restriction (Reinisch *et al.* 1978), increased risk of preterm labor (Shams *et al.* 1998), programming of post-natal hypertension (Tangalakis *et al.* 1992), programming of increased activity in the hypothalamo-pituitary-adrenal axis (Uno *et al.* 1994), or effects on fetal brain development (Muneoka *et al.* 1997). Thus, tight regulation of GC production and degradation throughout gestation is critical for successful growth of the conceptus, as well as postnatal development of the neonate (Michael & Papageorghiou 2008).

Mechanism of Glucocorticoid Signaling

While cortisol and corticosterone can activate both *NR3C1* and *MR*, they primarily serve as ligands for *NR3C1*. Two isoforms of *NR3C1* have been identified, both of which originate from the same gene by alternative splicing of the *NR3C1*

primary transcript and are thus termed *NR3C1α* and *NR3C1β* (Hollenberg *et al.* 1985, Giguere *et al.* 1986, Funder 1993). The ligand-dependent *NR3C1α* stimulates gene transcription in target tissues, while *NR3C1β* is unable to bind GCs due to an altered ligand-binding domain (Lu & Cidlowski 2004); therefore, *NR3C1α* is thought to be the active receptor (Hollenberg *et al.* 1985).

Residing primarily in the cytoplasm of cells, *NR3C1* interacts with a chaperone complex that holds the receptor in an inactive state ready to bind its ligand. This complex consists of various heat-shock proteins (hsp), including hsp40, hsp70, hsp90, in addition to p23 and p60 (Morishima *et al.* 2000). When no ligand is present, the receptor is phosphorylated and becomes hyper-phosphorylated upon cortisol binding (Bodwell *et al.* 1998). This induces the translocation of *NR3C1* to the nucleus, were it can act as a ligand-dependent transcription factor. Phosphorylated NR3C1 dimers can then recruit either co-activator proteins to increase target gene expression, or co-repressor proteins to decrease gene expression. NR3C1 dimers can modulate gene expression by controlling histone acetylation (Li *et al.* 2003, Hayashi *et al.* 2004) and permanently alter cellular functions (Michael & Papageorghiou 2008).

Once in the nucleus, NR3C1 can alter the level of gene expression by interacting with the regulatory regions of responsive genes. Four types of binding sites have been identified: GC response elements (Prasit *et al.* 1999) and GC response element half-sites (GRE1/2s) which activate gene expression; negative GC response elements (nGREs) that suppress gene activity; and tethering GC response elements, which can both inhibit and stimulate gene expression (Schoneveld *et al.* 2004). In many genes, the GC response depends both on NR3C1 binding to a GRE, but also requires the binding of other transcription factors to binding sites in close proximity. Since these response elements are both spatially and functionally clustered, they are referred to as GC response units (GRUs) (Schoneveld *et al.* 2004).

Alternatively, NR3C1 can associate with other transcription factors and form protein-protein interactions (McKay & Cidlowski 1999), a mode referred to as crosstalk. This process involves the association of the NR3C1 complex with the AP-1 transcription

factor family; interactions can be direct through protein-protein interaction, indirect through bridging proteins, or through competition for a limiting cofactor (Gottlicher *et al.* 1998, McKay & Cidlowski 1999). Crosstalk results in either transcription factor synergism or negative interference of gene expression, and is dependent on specific cell conditions (Whittle *et al.* 2001). Thus, GCs can exert a wide array of effects on gene transcription independent of a GRE site (Bamberger *et al.* 1996, Gottlicher *et al.* 1998).

MR and NR3C1 in the Ovine Uterus

Neither *MR* nor *NR3C1* have been reported for the ovine uterus during early pregnancy, but NR3C1 protein has been identified in intrauterine tissues (Gupta *et al.* 2003). NR3C1 is present in trophoblast cells, but not maternal tissue of the placentome. NR3C1α is specific to the nuclei of trophoblast cells, while NR3C1β resides in the cytosol (Gupta *et al.* 2003). Both isoforms are also present in epithelial cells of the amnion and chorionic epithelium, as well as the maternal endometrial epithelium. NR3C1α protein is also present in mesenchymal cells of the amnion and uterine GE. While NR3C1α levels are elevated during labor, NR3C1β levels remain unchanged (Gupta *et al.* 2003). In sheep, *NR3C1* is also expressed on Days 80 and 140 in placentomes, and increase throughout gestation (Gnanalingham *et al.* 2007). In moderately nutrient restricted ewes, however, the increase in NR3C1 is much lower by mid-gestation when compared to adequately fed ewes (Gnanalingham *et al.* 2007). *NR3C1* has also been identified in the uninucleate trophoblast cells of the placentome, but not in maternal stroma or BNC, and can be stimulated by infusion of cortisol into the fetus (Whittle *et al.* 2006).

MR and NR3C1 in the Bovine Uterus

NR3C1 mRNA is present in endometria of non-pregnant cattle, as well as in the bovine corpus luteum during the luteal stage (Lee *et al.* 2007, Komiyama *et al.* 2008). Endometrial *NR3C1* mRNA was greater in the mid-luteal stage compared to other stages of the estrous cycle (Lee *et al.* 2007), and levels were inversely correlated with PGF2α

output from the bovine endometrium (Miyamoto *et al.* 2000). Because concentrations of cortisol in plasma were low during the luteal phase, differential expression of *NR3C1* during the estrous cycle may allow GCs to control endometrial PG production (Lee *et al.* 2007). Schäubli and coworkers (Schaubli *et al.* 2008) evaluated NR3C1 in cows at 8 and 9 months of pregnancy and in cows undergoing a premature Caesarean section with or without induction of birth with a PGF2α agonist, or receiving a Caesarean section due to severe dystocia. They noted a cell type- and location-specific pattern of NR3C1, with greater NR3C1 staining in endometrial stromal cells in cows undergoing premature Caesarean section after induction of birth compared to cows that were slaughtered during month 8 or 9 of pregnancy or cows receiving Caesarean section following dystocia (Schaubli *et al.* 2008). During late pregnancy and at term NR3C1 protein was detectable in nuclei of LE (Schaubli *et al.* 2008), as in ewes (Gupta *et al.* 2003). The LE express enzymes necessary for endometrial PG synthesis during pregnancy; thus, GCs may be directly stimulating PGF2α synthesis and secretion leading to luteolysis and labor or even premature birth (Schaubli *et al.* 2008).

MR and NR3C1 in the Porcine Uterus

While *MR* have not been identified in pig reproductive tissues, *NR3C1* mRNA is present during early pregnancy in porcine placentae, with expression first noted on Day 24 of gestation. Both *NR3C1* mRNA and dehydrogenase activity increased by Day 40 of gestation (Klemcke *et al.* 2003). Klemcke and coworkers (Klemcke *et al.* 2006) also evaluated effects of maternal cortisol on porcine conceptuses following treatment with metyrapone, a cortisol and aldosterone synthesis inhibitor, or metyrapone plus cortisol. *NR3C1* mRNA was detectable in porcine conceptuses, embryonic tissue, and allantoic tissue with no treatment effects on *NR3C1* mRNA expression. But in trophectoderm, *NR3C1* mRNA expression was reduced in metyrapone, and in metyrapone plus cortisol injected pigs when compared to controls. These results suggest that *NR3C1* mRNA in porcine conceptuses during early gestation may be capable of responding to cortisol in

the uterine environment. Tissue aldosterone may also be regulating *NR3C1* expression (Klemcke *et al.* 2006).

MR and NR3C1 in the Primate Uterus

McDonald and coworkers (McDonald *et al.* 2006) extensively studied both the *MR* and *NR3C1* in the cyclic and early pregnant human endometrium. They identified *MR* expression in non-pregnant endometria throughout the menstrual cycle, in first-trimester decidua, and in an isolated population of uterine NK cells. *MR* was higher in mid-secretory phase endometria compared to proliferative phase endometria, and *MR* expression decreased significantly in the late secretory phase when P4 levels decreased; thus, *MR* is likely regulated by circulating P4 (McDonald *et al.* 2006). *NR3C1* mRNA was present at low levels in cyclic endometria, but was more abundant in endometria during the menstrual period and in uterine NK cells (Henderson *et al.* 2003, McDonald *et al.* 2006). NR3C1 protein was present in stromal cells and was minimal in uterine LE during the proliferative and early secretory phases, but NR3C1 protein increased in the late secretory phase (McDonald *et al.* 2006).

In pregnant tissues, MR protein was highly expressed in first trimester decidua and increased coordinately, but not significantly, with increases in circulating levels of P4. Both *NR3C1* and *HSD11B1* mRNA were upregulated in first trimester decidua and may be involved in eliciting an anti-inflammatory response to tissue remodeling (McDonald *et al.* 2006). *NR3C1* mRNA is abundant in decidua, chorion, amnion, stromal fibroblasts, vascular smooth muscle cells and endothelial cells from term human placental villi, and is moderately expressed in term cytotrophoblasts with negligible expression in term syncytiotrophoblast (Kossmann *et al.* 1982, Lopez Bernal *et al.* 1984, Sun *et al.* 1996, Weisbart & Huntley 1997, Driver *et al.* 2001, Chan *et al.* 2007, Yang *et al.* 2007).

MR and NR3C1 in the Rodent Uterus

Both *Mr* and *Nr3c1* mRNAs have been identified in the rat ovary (Schreiber *et al.* 1982) and uterus (Panko *et al.* 1981), but *Mr* expression in these tissues is very low to undetectable. In the rat uterus, NR3C1 protein is present in all uterine cell populations except for GE (Korgun *et al.* 2003). Major sites of expression are LE and decidual cells in the stroma, similar to Nr3c1 distribution in the human uterus. The subcellular localization of Nr3c1 protein differs in fetal and maternal tissues; in embryonic tissues it is in the cytoplasm, while in maternal tissues it is in the nucleus (Korgun *et al.* 2003).

Ho and coworkers (Ho *et al.* 1999) investigated effects of sex steroids on *Nr3c1* mRNA using a rat model. *Nr3c1* was downregulated in rats treated with equine chorionic gonadotrophin (eCG), but gradually recovered after treatment with human gonadotrophin (hCG) injection. This downregulation may result from increases in E2 in blood after eCG injection that either directly or indirectly suppresses *Nr3c1* gene transcription. Further, at 24 hours after hCG injection, both *Hsd11b1* and *Nr3c1* mRNAs were abundant (Ho *et al.* 1999). Perhaps *Hsd11b1* supplies more cortisol than its receptor can accommodate, thus preparing the uterus for implantation.

Similar to *Hsd11b1*, *Nr3c1* is abundant in endometrial epithelia but not stromal cells of murine uteri. While *Nr3c1* mRNA is readily detectable in myometrium throughout late gestation, Nr3c1 protein is relatively unchanged in the myometrium between Days 12.5 to 18.5 of gestation (Thompson *et al.* 2002). In uteri of mice, *Nr3c1* mRNA is absent from the compact decidua between Days 12.5 and 16.5, but is localized to the decidualized stroma both above and below the compact decidua (Thompson *et al.* 2002). More *Nr3c1* mRNA is detected in the labyrinth layer and yolk sac of placentae, and is abundant in spongiotrophoblast and giant cells (Thompson *et al.* 2002). Because *Hsd11b1* and *Hsd11b2* mRNA are co-expressed with Nr3c1 protein, they may regulate placental growth and function in an autocrine fashion by influencing synthesis of glucose transporters (Shin *et al.* 1997), growth factors, and their binding proteins (Han & Carter 2000).

Null mutations of *Mr* and *Nr3c1* in mice are lethal. In *Mr* knockout mice, pups undergo normal prenatal development, but by week 1 the neonates begin to show signs of pseudohypoaldosteronism (Wintermantel *et al.* 2004). By Day 8, pups exhibit high levels of circulating potassium, decreased levels of sodium, and a significant increase in rennin, angiotensin II, and aldosterone plasma concentrations. Pups lose weight and suffer from impaired renal sodium reabsorption and water loss, leading to death in the second week of life (Wintermantel *et al.* 2004).

Nr3c1 knockout mice die shortly after birth from atelectasis of the lungs. The induction of gluconeogenic enzymes is impaired and T-cell apoptosis is abolished. Lack of *Nr3c1* also impairs the ability of red blood progenitor cells to proliferate and renew erythrocytes (Tronche *et al.* 1998). Further, negative feedback mediated by *Nr3c1* is absent. Although the hypothalamic-pituitary-adrenal axis is not fully functional at birth, GCs and ACTH levels are greater compared to wild type mice (Tronche *et al.* 1998) due to an increase in transcription of the corticotropin-releasing hormone (CRH) and proopiomelanocortin (POMC) genes (Reichardt & Schutz 1996).

Prostaglandins

Background

PGs mediate responses to pain and have been implicated in pathological conditions such as hypertension, cancer, and inflammation; thus, their production has been a target for pharmacological therapy with non steroidal anti-inflammatory drugs (NSAIDs) for more than a century (Fortier *et al.* 2008). Normal functions of the female reproductive system also require presence of PGs to regulate numerous reproductive events including ovulation, implantation, parturition, luteolysis, and they may play a secondary role in pregnancy recognition (Poyser 1995, Dubois *et al.* 1998). Apart from sex steroids, PGs are probably the most critical regulators of female reproductive function and associated pathologies (Lindstrom & Bennett 2004).

Synthesis and Receptors

PGs are ubiquitously expressed and exert their effects in a paracrine or autocrine fashion (Kudo & Murakami 2002). The primary precursor of PGs is arachidonic acid, an essential fatty acid stored in membrane phospholipids (Bergstroem *et al.* 1964, Smith & Lands 1972) (Fig. 2.7). Arachidonic acid is converted into prostaglandin H_2 (PGH2) via the cyclooxygenase PTGS1 and PTGS2 pathway, making PTGS the rate-limiting enzyme in PG production (Smith *et al.* 2000, Parent *et al.* 2003). Cell-specific isomerases and synthases then convert PGH2 into various PGs, including PGE2, PGF2α, PGD2, prostacyclin (PGI2), and thromboxane A2 (TXA2) (Smith *et al.* 1996).

PGs exert a wide array of effects governed by specific receptors, some of which exist in multiple isoforms to bind a single PG (Sales & Jabbour 2003b, Bos *et al.* 2004). Each class of PG acts through its cognitive G-protein coupled receptor, a family of rhodopsin-like seven transmembrane spanning receptors. The prostanoid receptor subfamily consists of eight members (PTGER1–4, PTGDR, PTGFR, PTGIR and TBXA2R), each named for the prostanoid ligand for which individual receptors have the greatest affinity (Breyer *et al.* 2001). PGF2α, for example, acts through PTGFR receptors coupled to Gq, PLC and calcium release, whereas PGE2 acts through four subtypes of receptors, PTGER1 coupled to Gi and calcium channels, PTGER2 and PTGER4 coupled to Gs and cAMP generation, and PTGER3 coupled primarily to the inhibitory Gi system (Hata & Breyer 2004). PTGDR, PTGIR and TBXA2R serve as receptors for PGD2, PGI2, and TBXA2, respectively (Kennedy *et al.* 2007).

Peroxisome Proliferators-Activated Receptors (PPARs)

Though not part of the traditional family of PG receptors, peroxisome proliferators-activated receptors (PPAR A,G,D) have been linked to reproductive events in numerous animal models (Berger & Moller 2002). PPARs are a family of nuclear transcription factors thought to act as receptors for polyunsaturated fatty acids and to reduce production of PGs responsible for swelling and inflammation, also referred to as series 2 PGs (Desvergne & Wahli 1999). Activation of PPAR receptors may be ligand

dependent or independent, and cross-talk often occurs with other nuclear receptors, their response elements, as well as several transcription factors (Nunez *et al.* 1997).

While infamous for their roles in lipid metabolism, PPARs are also involved in reproduction (Berger & Moller 2002). *PPAR*s have been proposed as nuclear receptors for PGD2 and PGI2 (Desvergne & Wahli 1999), and, more recently, for PGE2 after the discovery of functional *PTGER3* and *PTGER4* in nuclear membranes, as well as in primary and transfected cells of human embryonic kidney (HEK) 293 cells, porcine microvascular endothelial cells, brain of newborn piglets and liver of adult rats (Bhattacharya *et al.* 1999).

Prostaglandin Production in Ruminants

Studies in the 1970s and 1980s found that both conceptuses and endometria of sheep and cattle synthesize numerous PGs during early pregnancy (Lewis 1989). PGI2, PGE2, PGF2α, TBXA2, and PGD2 are all synthesized and secreted by ovine conceptuses, and Day 16 conceptuses produce a larger amount of these PGs than Day 14 conceptuses (Lewis & Waterman 1985). Similarly, bovine conceptuses synthesize and secrete PGE2 and PGF2α, and Day 19 conceptuses produce more of those PGs than Day 16 conceptuses (Lewis *et al.* 1982). The Day 14 ovine conceptus produces 251 ng PGE2 per day and 409 ng PGF2α per day, while the endometrium produces 50 ng and 281 ng of PGE2 and PGF2α per day, respectively (Lewis & Waterman 1985). Levels of PGE2 and PGF2α are thus higher in the lumen of pregnant compared to cyclic ewes (Days 14 and 16) and cattle (Days 16 and 19), despite the fact that IFNT from the elongating conceptus inhibits expression of oxytocin receptors by endometrial cells and, therefore, oxytocin-induced pulses of PGF2α.

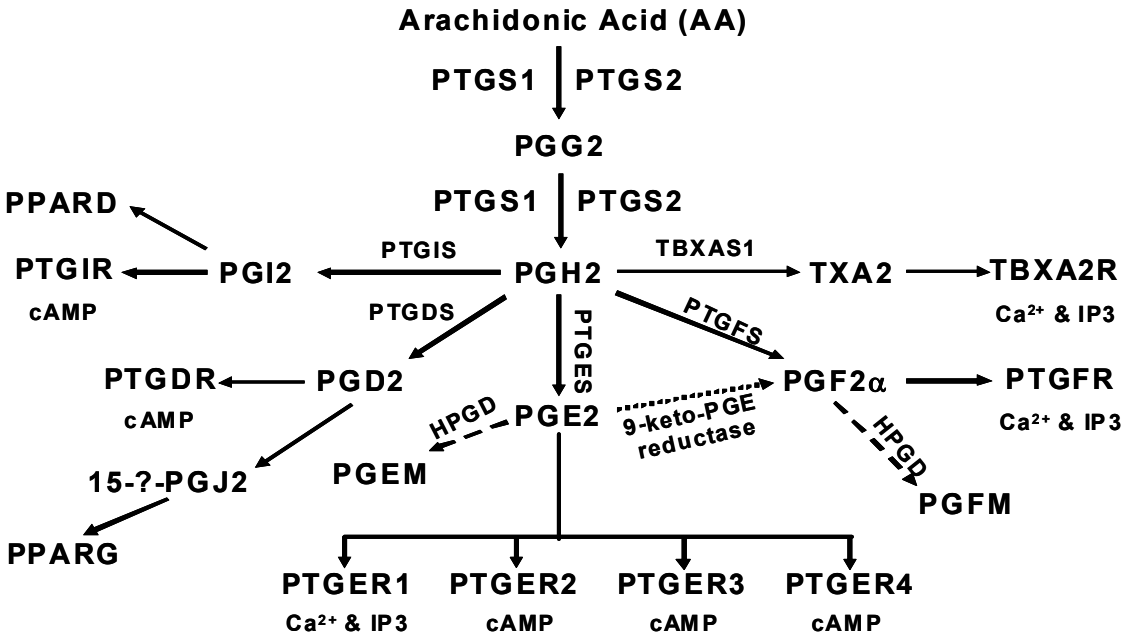

Fig. 2.7. Schematic representation of prostaglandin biosynthesis. Arachidonic acid is liberated from the cell membrane by PLA2 and converted into PGH2 by the rate-limiting enzymes, cyclooxygenases (PTGS1 and PTGS2). PGH2 is then converted into primary PGs, including PGE2, PGF2α, PGD2, PGI2, and thromboxane (TXA2), by cell-specific isomerases and synthases that exert their effects through G protein-coupled receptors, designated prostaglandin E receptors (PTGERs), PTGFR, PTGDR, PTGIR, and thromboxane A2 receptor (TBXA2R), respectively. PTGER1 is coupled to phospholipase C (PLC), generating two second messengers, inositol trisphosphate (IP3), which liberates intracellular calcium (Ca^{2+}), and diacylglycerol (DAG). PTGER2 and PTGER4 are coupled to adenylate cyclase (AC) and generate cAMP. The signaling of PTGER3 is more complex with four subtypes, A, B, C, and D, having a wide range of actions from inhibition of cAMP production to increases in Ca^{2++} and IP3. Nuclear peroxisome proliferator-activating receptors (PPARs) PPARD and PPARG are also activated by PGI2 and a product of PGD2, 15-deoxy-Δ12,14-PGJ2, respectively.

Biological Roles of Prostaglandins

The two PGs most pertinent to reproduction, PGF2α and PGE2, have been evaluated for their roles in early pregnancy in many species. It is well established that oxytocin-induced pulsatile release of PGF2α from the endometrium is responsible for luteolysis and subsequent return to estrus in ruminants (McCracken *et al.* 1999). Actions of PGE2, while more controversial, are believed to favor establishment of pregnancy through luteotrophic or antiluteolytic mechanisms (Pratt *et al.* 1977, Magness *et al.* 1981). Less studied PGs such as PGI2 may also have supportive roles in implantation and pregnancy.

PGE2 and PGF2α

In the reproductive system, PGF2α and PGE2 work in opposition to one another (Vincent & Inskeep 1986). In the presence of oxytocin, endometrial release of PGF2α initiates luteolysis in animals and ovarian PGF2α in primates contributes to the luteolytic process (Nagle *et al.* 2005). When a viable conceptus is present, however, an antiluteolytic or luteotrophic signal or a combination of both counteracts the default luteolytic mechanism to allow maintenance of a functional CL for continued production of P4. IFNT from the conceptus inhibits the expression of uterine *ESR1* and *OXTR* via paracrine mechanisms, blocks oxytocin-induced pulsatile secretion of PGF2α, and inhibits luteolysis (Spencer *et al.* 1999). PGF2α has been suggested to constrict the myometrial and uterine blood vessels (Olson 2005), inhibiting CL function, leading to a decline in P4 production and an eventual return to estrus.

PGE2, on the other hand, is a vasodilator that exerts a strong luteotrophic action in humans (Hagstrom *et al.* 1996). In species such as sheep and pigs, uterine PGE2 production increases in early pregnancy, and intrauterine infusions of PGE2 can transiently protect the CL from the luteolytic effects of PGF2α, but non-physiological amounts of PGE2 are required (McCracken *et al.* 1999). Moreover, PGE2 has been reported to counteract the effects of PGF2α when given simultaneously to cycling ewes via the uterine artery (Henderson *et al.* 1977) or into the uterine lumen (Magness *et al.*

1978). In addition, secreted factors from the conceptus such as estrogen in pigs and IFNT in ruminants are thought to increase the endometrial PGE2:PGF2α production ratio (Asselin *et al.* 1997, Ziecik 2002). PGE2 synthesis may therefore be important during maternal recognition of pregnancy.

In terms of early pregnancy, PGs are hypothesized to modulate the uterine environment to prepare for pregnancy. PGE2 and PGI2 are critical regulators of blastocyst implantation, decidualization, and uterine angiogenesis during pregnancy in mice, rats, and likely humans (Dey *et al.* 2004, Wang & Dey 2006, Kennedy *et al.* 2007). PGs also prevent rejection of the conceptus allograft by modulating the immune system to suppress activity of maternal natural killer cells (Lala 1990). In primates, PGE2 binds to its specific receptor (*PTGER2* or *PTGER4*) and activates adenyl cyclase, resulting in increased intracellular levels of cAMP that upregulate *IGFBP1* gene transcription in stromal fibroblasts (Fazleabas *et al.* 1999). Thus, PGs facilitate the events of early pregnancy by protecting the developing blastocyst and upregulating genes that are potentially important for blastocyst growth and development.

Prostacyclin

Along with PGE2 and PGF2α, PGI2 is produced by the ruminant endometrium and trophoblast (Charpigny *et al.* 1997a, Charpigny *et al.* 1999). PGI2, generated in the presence of its specific isomerase PTGIS, acts through the *PTGIR* to stimulate various G proteins, leading to an increase in cAMP generation and phosphatidylinositol responses (Narumiya *et al.* 1999).

Cammas and colleagues (Cammas *et al.* 2006) identified strong expression of *PTGIS* in the ovine endometrium before Day 12. Expression decreased thereafter, coincident with the rise in circulating estradiol that occurs after Day 12 (Cox *et al.* 1973). Conversely, *PTGIS* was nearly undetectable before Day 12 in the trophoblast and expression was upregulated thereafter. At the time of implantation, PTGIS protein was 10 times more abundant in trophoblast than in endometrium. Thus, PGI2 is constantly present during conceptus elongation, first as a product of the endometrium from Days 7

to 9, then the conceptus from Day 12 and beyond (Cammas *et al.* 2006). *PTGIR* was also present in the endometrium and conceptus (Cammas *et al.* 2006), further implicating PGI2 in the events of early pregnancy.

PGI2 is hypothesized to activate the PPARD/RXRA heterodimer and control implantation in mice (Lim *et al.* 1999) and trophoblast invasion in humans (Tarrade *et al.* 2001). Similarly, transcripts for all *PPAR* and their *RXR* heterodimerization partners are expressed in the ovine endometrium, with PPARD and PPARG proteins present in the LE after Day 12 (Cammas *et al.* 2006). In addition, *PPARD*, *PPARG*, *RXRA*, and *RXRG* were simultaneously upregulated in the trophoblast after Day 12 (Cammas *et al.* 2006), indicating that ovine PGI2 signaling in sheep involves both the uterus and the trophoblast. More recently, PGI2 was found to improve the developmental competency of embryos via regulation of the cAMP and PTGS2-signaling pathways in cattle (Song *et al.* 2009). Thus, PGI2, along with PGE2 and PGF2α, may mediate the establishment of pregnancy and the initiation of implantation in ruminants as in other species.

Prostaglandin Receptor Knockout Models

Deletions of various PG receptors have demonstrated physiological functions of PGs in the murine model. All four *PTGER*s are expressed in uteri of nice during the peri-implantation period (Yang *et al.* 1997); however, knockout models have shown that *Ptger1*- and *Ptger3*-deficient female mice are fertile, suggesting that these receptors are nonessential for ovulation, fertilization, and implantation (Ushikubi *et al.* 1998). In contrast, *Ptger4* deficiency results in embryonic perinatal lethality (Nguyen *et al.* 1997), while *Ptger2* null mutants develop normally but produce small litters and exhibit ovulation and peri-implantation defects (Kennedy *et al.* 1999, Tilley *et al.* 1999). *Ptgfr* null mice develop normally but are unable to successfully deliver pups (Sugimoto *et al.* 1997). While these mice exhibit normal estrous cycles, ovulation, fertilization, and implantation, they fail to respond to exogenous oxytocin (Sugimoto *et al.* 1997). No reproductive effects have been demonstrated in *Ptgdr* null mice, yet they suffer from ataxia, abnormal astrocyte morphology, and premature death (Matsuoka *et al.* 2000,

Mohri *et al.* 2006). *Ptgir* null mice exhibit normal implantation potential, but have fewer hatched blastocysts compared to wild type mice, approximately half of which fail to reach the expanded blastocyst stage within 48 hours after hatching from the zona pellucida (Huang *et al.* 2007).

Null mutations of the PPARs further implicate PGs in normal reproductive functions. *Ppara* null mice experience a 20% increase in abortion rate, increased neonatal mortality, and downregulation of *interleukin 4* (*Il4*) mRNA (Yessoufou *et al.* 2006). These mice experience greater weight gain, higher adiposity and lipidemia, as well as a reduced capacity to metabolize long-chain fatty acids (Kroetz *et al.* 1998). Homozygous *Ppard*-null mice exhibit placental defects and reduced birth weight (Peters *et al.* 2000), implicating this nuclear receptor in murine placental function and fetal development. *Pparg* deficiency results in death by Day 10 of gestation (Barak *et al.* 1999) due to various placental defects. Placentae of *Pparg* mutant mice fail to form the vascular labyrinth, and the labyrinthine trophoblast fails to undergo typical morphological and cellular changes, such as compaction and syncytium formation (Barak *et al.* 1999).

Prostaglandin-Endoperoxide Synthases

Background

PGs are synthesized in the presence of one or more isoforms of the rate-limiting enzyme PTGS (PTGS1, PTGS2, and PTGS3) (Chandrasekharan *et al.* 2002, Morita 2002). *PTGS1* is constitutively expressed in a variety of cell types (Kim *et al.* 2003) and is generally necessary for normal physiological functions (Sales & Jabbour 2003), but only responds to levels of AA above 10 μM (Parent *et al.* 2003). Conversely, *PTGS2* can be rapidly induced by growth factors, oncogenes, carcinogens and tumor-promoting esters, and has been implicated in rheumatic disease, inflammation and tumorigenesis (Morita 2002). In addition, *PTGS2* supports sustained production of PGs from very low levels of AA, i.e., 2.5 μM or lower (Parent *et al.* 2003). PTGS2 has been linked to reproductive events such as ovulation, implantation of blastocysts, and parturition

(Parent *et al.* 2003). *PTGS3* mRNA, a third variant transcript of the *PTGS1* gene, retains intron 1 in its mRNA and shares all the structural and catalytic features of *PTGS1* and *PTGS2*, but it is sensitive to drugs that that have low anti-inflammatory properties (Sales & Jabbour 2003). PTGS3 has been identified primarily in the cerebral cortex of dogs and humans, as well as human heart, but has no known function in reproduction (Chandrasekharan *et al.* 2002).

PTGS1 and PTGS2 in the Ovine Uterus

Charpigny and coworkers (Charpigny *et al.* 1997b) identified both PTGS1 and PTGS2 proteins in the ovine endometrium, and PTGS2 protein in the ovine conceptus (Charpigny *et al.* 1997a). While PTGS1 is present at steady state levels during both the estrous cycle and pregnancy, PTGS2 was abundant and transiently expressed in uterine endometria between Days 12 and 15 of the estrous cycle and Days 12 and 17 of pregnancy (Charpigny *et al.* 1997b). Further studies localized *PTGS2* mRNA and protein in endometrial LE and sGE of cyclic and pregnant ewes. Similar to findings by Charpigny, *PTGS2* mRNA was also found to increase between Days 10 and 12 post-mating and then decline in another study (Kim *et al.* 2003).

In the ovine conceptus, PTGS2 protein is abundant from Days 8 to 17 of pregnancy, while PTGS1 is undetectable during that time (Charpigny *et al.* 1997a). In addition, PTGS2 levels are maximal between Days 14 and 16 and are specific to trophoblast cells, but not the inner cell mass (Charpigny *et al.* 1997a). Furthermore, in the second half of pregnancy, *PTGS2* mRNA is abundant in placentae of ewes while *PTGS1* is constitutively expressed throughout pregnancy and at parturition (Wimsatt *et al.* 1993, Rice *et al.* 1995, Zhang *et al.* 1996).

Similar to sheep, PTGS2 is the predominant enzyme expressed in the bovine uterus (Arosh *et al.* 2002) and uterine LE and sGE, and is required for production of luteolytic pulses of PGF2α. Endometrial *PTGS2* mRNA is transiently induced during late diestrus around the expected time of luteolysis, but *PTGS1* expression is undetectable in cows throughout the estrous cycle (Arosh *et al.* 2004a, Emond *et al.*

2004). *PTGS2* mRNA and protein are low from Days 1 to 12 of the estrous cycle, and higher on Days 13 to 21 (Arosh *et al.* 2002). PTGS2 has also been identified during the early stages of bovine embryonic development (Gurevich & Shemesh 1994, Emond *et al.* 2004), suggesting an important physiological role for PG production prior to implantation.

The effects of IFNT on PG production remain controversial. Using an *in vitro* model with bovine endometrial cells, IFNT was found to decrease *PTGS2* mRNA and protein (Xiao *et al.* 1999, Binelli *et al.* 2000). Conversely, Krishnaswamy and colleagues (Krishnaswamy *et al.* 2009) demonstrated that IFNT increased PTGS2 and PG production in bovine endometrial stromal cells in a dose-dependent manner, and that inhibition of the p38 MAPK pathway abolished IFNT action on PG production. Moreover, PTGS2 is upregulated by IFNT in endometria of early pregnant cattle (Arosh *et al.* 2004b, Emond *et al.* 2004). IFNT can also affect PGE2 synthesis directly via stimulation of PGES and even increase PGE2 receptors, particularly *PTGER2*, in endometria and myometria of cattle (Arosh *et al.* 2004b).

PTGS1 and 2 Knockout Models

Knockout studies in mice have demonstrated the importance of PTGS1 and PTGS2 in reproduction. Compared to their wild-type counterparts, mice deficient in *Ptgs1* exhibit prolonged gestation periods, protracted parturition and deliver fewer live young. However, conception and conceptus development are unaffected in *Ptgs1* null mice, indicating that *Ptgs1* is not critical for ovulation, fertilization, or implantation, but is required for induction of labor (Langenbach *et al.* 1995, Gross *et al.* 1998). However, *Ptgs2* knockout mice exhibit multiple reproductive failures, including ovulation, fertilization, implantation and decidualization defects (Dinchuk *et al.* 1995, Lim *et al.* 1997, Langenbach *et al.* 1999a, Langenbach *et al.* 1999b).

These findings suggest that while PTGS enzymes catalyze the same reaction, they differ in their tissue-specific distribution in the reproductive tract and, therefore, serve very different functions. In addition, *Ptgs2* expressed in *Ptgs1* knockout mice

partially compensates for the PG deficiency (Reese *et al.* 1999). *Ptgs1* and *Ptgs2* double knockouts result in death of pups soon after birth, indicating that PGs play a more significant role in survival than previously anticipated (Reese *et al.* 2000, Loftin *et al.* 2001).

Inhibition of PG Synthesis

Various inhibitors of both PTGS1 and PTGS2 have been tested in animal models. As early as 1982, LaCroix and Kann reported that pregnant ewes receiving either indomethacin (300 mg subcutaneous daily) or acetylsalicylic acid (aspirin) (1 g intravenous daily) from Days 7 to 22 experienced normal pregnancies (Lacroix & Kann 1982). While their experiments resulted in a sharp decline in concentrations of PGs in endometria and conceptuses on Day 23, an apparently normal conceptus was present in the uterus. Thus, they surmised that PGs were not involved during early pregnancy in sheep (Lacroix & Kann 1982). The dose of indomethacin and aspirin, both non-selective PTGS inhibitors, should have resulted in circulating levels that exceeded the half maximal inhibitory concentration (IC_{50}) for each compound; however, it was not determined if those compounds gained entry into the uterine lumen or if treatment inhibited PTGS2 in the conceptus.

These results highlight the importance of choosing a proper PTGS inhibitor and dosage, but more importantly, an effective mode of entry. The inhibitors used by LaCroix and Kann (Lacroix & Kann 1982) were likely administered in insufficient amounts or were not able to access the uterine lumen, thus leading to questionable results. Alzet osmotic pumps provide a unique way to manipulate the uterine environment because they deliver constant rates of a chosen compound directly into the uterus via a catheter. These pumps have been used successfully in pigs to study effects of various compounds on endometrial gene expression without interrupting pregnancy (Joyce *et al.* 2007).

Since many PTGS inhibitors are available, choosing an effective agent is important. Numerous inhibitors have been studied with a wide range of effects on

PTGS1, PTGS2, and PG production. A major area of concern is the specificity of the inhibitor; while some compounds can target PTGS1 or PTGS2 specifically, others are considered non-selective and inhibit both PTGS isoforms (Landa *et al.* 2009). Moreover, many of these agents have only been tested *in vitro* (Hood *et al.* 2003, Uddin *et al.* 2005) and their *in vivo* effects are unknown. Several inhibitors have proven effective in sheep, including the selective PTGS2 inhibitors meloxicam (Rac *et al.* 2006, Rac *et al.* 2007, Shukla *et al.* 2007) and methanesulfonamide (NS-398) (Mertz *et al.* 2003), and non-selective PTGS inhibitors indomethacin and acetylsalicylic acid (Lacroix & Kann 1982, Weems *et al.* 2007). Of these inhibitors, NS-398 is relatively new to animal experimentation, while indomethacin and meloxicam have been used extensively in sheep (Lees *et al.* 2004). Importantly, indomethacin has non-specific effects on PTGS-induced PG synthesis. However, meloxicam specifically targets PTGS2, the inducible isoform responsible for reproductive functions, making it a prime candidate for studying PG production during early pregnancy in sheep.

Experiments with meloxicam have been shown to decrease PG production and uterine activity in sheep (McKeown *et al.* 2000, Rac *et al.* 2006). The effects of meloxicam treatment on PTGS1 and PTGS2 protein expression, as well as PTGS2 localization, were evaluated in the ovine endometria, myometria, amnions, placentae, and fetal tissues in a model of RU486-induced preterm labor (Rac *et al.* 2007). Pregnant ewes received an intravenous infusion of a high-maintenance-dose meloxicam, graded-maintenance-dose of meloxicam, or saline after induction of labor. All regimens of meloxicam treatment decreased PTGS2 proteins significantly in endometria, myometria, and amnions compared to saline treated ewes, but did not affect PTGS2 in placental or fetal tissues. Not only did PTGS1 remain constitutively expressed, but PGDH levels were also unaffected, confirming the specificity of meloxicam for PTGS2 (Rac *et al.* 2007). PTGS2 staining was strong in the cytoplasm of the uterine LE and weak in the GE and stroma in ewes receiving saline; however, after meloxicam treatment, strong staining was present in uterine LE but staining in the GE and stroma was not detectable (Rac *et al.* 2007).

In a similar sheep study, levels of meloxicam in fetal plasma were much lower than in maternal plasma (Rurak *et al.* 2001), probably due to the high polarity and low solubility of meloxicam and/or the permeability characteristics of the epitheliochorial placenta. Administration of meloxicam to ewes is associated with decreased levels of PGs such as fetal PGE2 and maternal PGFM, as well as cortisol and ACTH (McKeown *et al.* 2000). Collectively, available results indicate that meloxicam affects PTGS2 activity and decreases PTGS2 protein in a dose- and time-dependent manner, but does not influence the PG catabolic pathway (Rac *et al.* 2007).

Summary

Proper communication between the conceptus and the endometrium is required for successful establishment and maintenance of pregnancy. As the fertilized blastocyst enters into the uterus, hatches from the zona pellucida and elongates, the mononuclear cells of the trophectoderm produce large amounts of IFNT, the ruminant pregnancy recognition signal. IFNT not only blocks the luteolytic mechanism to allow continued production of P4 from the CL, but P4 also induces and IFNT further stimulates key genes that encode for secreted factors released into uterine histotroph that support conceptus development. The nutrient composition of histotroph is clearly important for conceptus elongation and pregnancy maintenance; therefore, genes that encode for secreted growth factors, binding proteins, enzymes, and adhesion proteins require further evaluation.

A family of binding proteins regulates the actions of IGFs, two of which have been implicated in development of ovine conceptuses during the peri-implantation implantation period. IGFBP1 is unique among its family members because its RGD binding sequence allows it to bind the α5β1 integrin heterodimer expressed constitutively by the conceptus trophectoderm and uterine LE to stimulate migration of trophectoderm cells. IGFBP3 has a high affinity for IGFs which extends the half-lives of IGFs in blood and alters IGF interactions with cell surface receptors to prevent apoptosis. Expression of both *IGFBP1* and *IGFBP3* expression is common to most

mammals, but *IGFBP1* expression is undetectable in uteri of pigs. Because both *IGFBP1* and *IGFBP3* are specific to endometrial LE/sGE in sheep and are induced by P4, it is hypothesized that these binding proteins stimulate elongation of ovine conceptuses.

Additional studies identified *HSD11B1*, another P4-responsive gene, expressed in ovine uterine GE during the estrous cycle and early pregnancy. HSD11B1 is one of two isoforms of 11β-hydroxysteroid dehydrogenase that modulate the actions of GCs by converting inactive cortisone to active cortisol (humans) or corticosterone (rodents). While HSD11B2 protects the fetus from excess GCs by generating inactive corticosterone from active cortisol, HSD11B1 found throughout the body acts primarily as a reductase to produce active cortisol. *HSD11B1* is often localized to the uterus during early pregnancy and both expression and reductase activity can be stimulated by P4, IFNT, and PGs in many species. Thus, *HSD11B1*, through cortisol production and regulation, may modulate the uterine environment to ensure a receptive environment for implantation and conceptus development, including elongation. Although HSD11B1 has been studied extensively in sheep and humans for its contributions to the induction of parturition, the importance of this enzyme in early pregnancy is unknown. Since cortisol influences both embryonic and postnatal development, interpreting expression patterns and activity of HSD11B1 may provide important insights into endometrial gene regulation, as well as growth and development of the conceptus.

GCs, such as cortisol and corticosterone, regulate a myriad of events required for successful implantation, followed by growth and development of the fetus and placenta. While increased levels of GCs induce maturational changes in the fetal organs and, in some species, stimulate labor during the peri-parturient period, GCs can also have adverse effects during the latter stages of pregnancy. Elevated cortisol levels, resulting from improper HSD11B activity or maternal stress, can lead to a variety of postnatal problems in both humans and farm animals. Furthermore, the presence of MR and/or NR3C1 affects tissue response to circulating GCs. Spatial and temporal expression of *MR* and *NR3C1* mRNA varies among species and is often tissue- and cell- specific.

While *NR3C1* is common in uteri of most species evaluated, the role(s) of *MR* has not been established. Because either *NR3C1* or *MR* have been identified in early pregnancy in several species, their response to cortisol may be more important than previously anticipated. GCs are known to influence uterine receptivity and can improve pregnancy rates in women undergoing assisted reproduction. Therefore, understanding effects of GCs, location and timing of receptor expression, and differences among species will contribute to understanding early pregnancy and means to improve pregnancy rates in both humans and animals.

PGs, particularly PGE2, PGF2α and PGI2, are hypothesized to influence events of early pregnancy. Three isoforms of the PTGS enzyme may be involved in synthesis of PGs from arachidonic acid, i.e., PTGS1, PTGS2 and PTGS3. While PTGS1 is constitutively expressed in a variety of cell types, PTGS2 is an inducible enzyme that is most often associated with reproduction. PTGS3 is a variant of PTGS1 and has no known function in reproduction. During early pregnancy in sheep, both PTGS1 and PTGS2 are expressed in the conceptus and endometrium, with PTGS1 being expressed constitutively. Further, PG secretion in sheep is coincident with elongation of conceptus trophectoderm. PGs regulate *IGFPB1* in human and rodent decidua and *HSD11B1* in the ovine placenta; thus, it is hypothesized that PGs stimulate these and possibly other implantation and conceptus elongation-related genes during early pregnancy. Review of current literature provides ample evidence for endometrial genes that likely support growth and development of the conceptus, including elongation of conceptus trophectoderm, and for effects of P4, IFNT, and/or PGs in regulation of key genes required for establishment and maintenance of pregnancy.

CHAPTER III
INSULIN-LIKE GROWTH FACTOR BINDING PROTEIN ONE IN THE RUMINANT UTERUS: POTENTIAL ENDOMETRIAL MARKER AND REGULATOR OF CONCEPTUS ELONGATION*

Introduction

Maternal support of blastocyst growth and development into an elongated conceptus is critical for pregnancy recognition signaling and implantation in ruminants (Spencer & Bazer 2004, Spencer *et al.* 2007). After hatching from the zona pellucida on Day 8 (sheep) or Days 9 to 10 (cattle), the blastocyst develops into an ovoid form by Day 11 (sheep) or Day 13 (cattle) and is termed a conceptus (Betteridge & Flechon 1988, Guillomot 1995, Spencer *et al.* 2004a). The ovoid conceptus begins to elongate to a tubular form on Day 12 (sheep) or Day 14 (cattle) and a filamentous conceptus of 14 cm or more in length by Day 16 (sheep) or Day 19 (cattle). Conceptus elongation involves exponential increases in length and weight and decrease in diameter of the trophectoderm, as well as differentiation of the extraembryonic membranes (Wales & Cuneo 1989), and requires substances secreted from the endometrial LE and GE (Flechon *et al.* 1986, Gray 2002). During early pregnancy in ruminants, endometrial functions are regulated primarily by P4 from the corpus luteum and secreted cytokines and hormones from the trophectoderm/chorion including IFNT (Bazer *et al.* 1979, Spencer *et al.* 2004a, Spencer *et al.* 2008). IFNT, produced during conceptus elongation, exerts antiluteolytic effects on the endometrium to maintain CL function and its production of P4 that, in turn, stimulates and maintains uterine endometrial functions necessary for conceptus growth, implantation, placentation and successful development

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of the fetus to term (Spencer *et al.* 2004b). Additionally, IFNT acts on the endometrium to induce or increase expression of many genes that potentially regulate conceptus growth and development (see (Spencer 2007, Bauersachs *et al.* 2008, Spencer *et al.* 2008) for review).

We recently reported results from an ovine model of accelerated blastocyst growth and conceptus development elicited by advancing the post-ovulatory rise in circulating levels of P4 during metestrus (Satterfield *et al.* 2006). That model was used to identify a number of candidate P4-regulated genes encoding secreted proteins (*LGALS15, GRP, IGFBP1,* and *IGFBP3*) implicated in peri-implantation conceptus elongation (Satterfield *et al.* 2006, Satterfield *et al.* 2008, Song *et al.* 2008). *IGFBP1* is expressed exclusively in the LE/sGE of the endometrium of both sheep and cattle (Osgerby *et al.* 1999, Robinson *et al.* 2000). *IGFBP3* is expressed predominantly in the LE/sGE of sheep endometria (Reynolds *et al.* 1997), but in stroma and GE of bovine endometria (Robinson *et al.* 2000). Limited or no information is available on effects of the conceptus, P4, or IFNT on *IGFBP1* and *IGFBP3* expression in endometria of sheep and cattle. IGFBP1 and IGFBP3 are among the 16 known members of the IGFBP superfamily that regulate IGF bioavailability and cellular actions (for reviews see (Wang & Chard 1999, Firth & Baxter 2002, Nayak & Giudice 2003, Holly & Perks 2006)). IGF1 and IGF2 possess both mitogenic and differentiative properties and are implicated in early embryonic and placental development in many species including sheep and cattle (Wathes *et al.* 1998, Irwin *et al.* 1999, Watson *et al.* 1999). IGFBPs can both enhance and retard IGF actions (Jones & Clemmons 1995, Clemmons 1997). IGFBP1 is a unique IGFBP as it contains a functional RGD integrin recognition domain (Irwin & Giudice 1998). Integrins expressed constitutively on both the conceptus trophectoderm and endometrial LE in sheep and cattle (Johnson *et al.* 2001, MacIntyre *et al.* 2002) are essential for blastocyst implantation, but require functional binding and cross-linking to regulate implantation (Burghardt *et al.* 2002, Armant 2005). The biological functions of IGFBP1 include stimulation of trophoblast cell migration (Irving & Lala 1995, Gleeson *et al.* 2001) and inhibition of trophoblast invasiveness (Irwin & Giudice 1998). IGFBP3

has a very high affinity for IGF1 and IGF2, prolongs their half-life in serum, alters their interaction with cell surface receptors, and may have IGF-independent actions to control the cell cycle and apoptosis (Rajaram *et al.* 1997, Lee & Cohen 2002).

The reported biological roles for IGFBP1 and IGFBP3 make these molecules excellent candidates to influence trophectoderm proliferation, migration and attachment to uterine LE that are essential processes modulating peri-implantation ruminant conceptus growth and development (Guillomot 1995, Spencer *et al.* 2004a, Spencer 2007). Thus, the working hypothesis for the present study is that IGFBP1 and IGFBP3 have biological roles in peri-implantation conceptus growth and development in ruminants. As a first step in testing this hypothesis, these studies determined: (1) effects of the estrous cycle and early pregnancy on *IGFBP1* and *IGFBP3* expression in ovine and bovine uteri; (2) effects of P4 and IFNT on *IGFBP1* and *IGFBP3* expression in the ovine uterus; and (3) effects of IGFBP1 on ovine trophectoderm cell proliferation, migration, and attachment.

Materials and Methods

Experimental Design

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.

Study One

At onset of estrus (Day 0), ewes were mated to either an intact or vasectomized ram and then hysterectomized (n=5 ewes/day) on either Day 3, 6, 10, 12, 14 or 16 of the estrous cycle or Day 10, 12, 14, 16, 18 or 20 of pregnancy. Uterine and/or conceptus tissues were processed as described previously (Spencer *et al.* 1999). At hysterectomy, several 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 and then dehydrated and embedded in Paraplast-

Plus (Oxford Labware, St. Louis, MO). The remaining endometrium was physically dissected from myometrium, frozen in liquid nitrogen, and stored at -80°C for subsequent RNA extraction. In monovulatory pregnant ewes, uterine tissue samples were only from the ipsilateral uterine horn to the ovary bearing the CL. The uterine lumen was flushed with 20 ml of sterile 10 mM Tris buffer (pH 7.2) on Days 10 to 16 of pregnancy and the estrous cycle and, in pregnant ewes, the flushing was examined for the presence of a morphologically normal conceptus.

Study Two

Crossbred nulliparous heifers were artificially inseminated (AI) with semen from a single bull following a timed AI synchronization protocol (Bridges *et al.* 2008) and then slaughtered on either Day 10, 13, 16 or 19 post-mating. The uterus was flushed with 20 ml of sterile 10 mM Tris buffer (pH 7.2). Heifers were classified as pregnant if the uterine flush contained a blastocyst/conceptus of the correct morphology and size or nonpregnant if the uterine flush did not contain a blastocyst/conceptus. The uterine horn ipsilateral to the CL was processed as described for Study One. Uterine tissues were collected from nonpregnant heifers on Days 10, 13, 16 and 19 (n=6 per day) and pregnant heifers on Days 13, 16 and 19 (n=6 per day).

Study Three

Cyclic ewes (n=20) were checked daily for estrus and then ovariectomized and fitted with indwelling uterine catheters on Day 5. Ewes were then assigned randomly (n=5 per treatment) to receive daily intramuscular (i.m.) injections of P4 and/or a PGR antagonist (RU486; Sigma Chemical Company) and intrauterine (i.u.) infusions of either control (CX) serum proteins and/or recombinant ovine IFNT as follows: (1) 50 mg P4 (Days 5 to 16) and 200 μg serum proteins (Days 11 to 16) [P4+CX]; (2) P4 and 75 mg RU486 (Days 11 to 16) and serum proteins [P4+RU+CX]; (3) P4 and IFNT (2 x 10^7 antiviral units, Days 11 to 16) [P4+IFN]; or (4) P4 and RU and IFNT [P4+RU+IFN]. Steroids were administered i.m. daily in corn oil vehicle. Both uterine horns of each ewe

received twice daily injections of either CX serum proteins (50μg/horn/injection) or recombinant IFNT ($5x10^6$ antiviral units/horn/injection). Recombinant ovine IFNT was produced in *Pichia pastoris* and purified as described previously (Van Heeke *et al.* 1996). Serum proteins and IFNT were prepared for intrauterine injections as described previously (Spencer & Bazer 1995). This regimen of P4 and IFNT mimics the effects of P4 and IFNT from the CL and conceptus, respectively, on endometrial expression of hormone receptors and IFNT-stimulated genes during early pregnancy in ewes (Bazer & Spencer 2006). All ewes were hysterectomized on Day 17, and uteri processed as described for Study One.

Slot Blot Hybridization Analysis

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. Steady-state levels of *IGFBP1* and *IGFBP3* mRNAs in endometria were assessed by slot blot hybridization using radiolabeled antisense *IGFBP1*, *IGFBP3* or *18S* cRNA probes as described previously (Spencer *et al.* 1999, Satterfield *et al.* 2008). 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*

Cell-specific expression of *IGFBP1* and *IGFBP3* mRNAs in ovine and bovine uteri was determined using radioactive *in situ* hybridization analysis methods as described previously (Spencer *et al.* 1999, Satterfield *et al.* 2008). All slides for each respective gene were exposed to photographic emulsion for the same period of time. 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.

Cell Proliferation Assay

An ovine trophectoderm cell line (oTr1) from a Day 15 conceptus reported previously (Farmer *et al.* 2008) was used to conduct proliferation assays as described previously (Dunlap *et al.* 2006, Farmer *et al.* 2008). Briefly, oTr1 cells were subcultured into 12-well plates (Corning Costar #3513, Corning, NY) to about 50% confluency in trophoblast growth medium for 6 to 8 h and then switched to serum and insulin-free DMEM for 24 h. In some experiments, cells were cultured under low serum (1%) or full serum (10%). After 24 h, the wells (n=4 per treatment) were treated with either increasing amounts of purified human IGFBP1 (Catalog 8-IGFBP; Advanced Immunochemical Inc., Long Beach, CA) in serum and insulin-free DMEM, trophoblast growth medium containing serum and insulin as a positive control, or DMEM alone as a negative control. After 48 h of culture, cell numbers were determined as described previously (Raspotnig *et al.* 1999). The entire experiment was repeated at least three times with different passages of oTr1 cells.

Cell Migration Assay

The oTr1 cells (100,000 per 100 µl serum-free DMEM) were seeded in a confluent layer on 8 µm pore transwell inserts (Corning-Costar, Corning, NY). Purified human IGFBP1 (Advanced Immunochemical Inc.) or bovine serum albumin (BSA; Sigma Chemical Company) was then added to separate wells in serum-free DMEM-F12 at either 1, 10, 100 or 1000 ng/ml (n=3 replicates/treatment). After 12 h, cells remaining on the top portion of the membrane were removed by scraping with a cotton swab and membranes were fixed in -20^{o}C methanol for 10 min. Membranes were removed, placed on slides and stained with 4',6'-diamidino-2-phenylindole (DAPI) (Invitrogen, Hercules, CA). Cells that migrated to the bottom surface of the membrane were counted in five non-overlapping sections of each membrane, which accounted for approximately 70% of the membrane area, using a Zeiss Axioplan 2 fluorescence microscope with an Axiocam HR digital camera and Axiovision 4.3 software. Cells incubated in DMEM-F12 containing 10% fetal bovine serum served as a positive control for migration.

Cell Attachment Assay

Cell attachment assays were conducted with oTr1 cells as described previously (Bayless & Davis 2001). Polystyrene microwells (Corning-Costar) were coated overnight at 4°C with two-fold serial dilutions (10 µg/ml to 20 ng/ml) of the following proteins (50 µl) in phosphate-buffered saline (PBS) (n=3 replicates/treatment): full-length recombinant human fibronectin (hFN; Sigma), purified human IGFBP1 (Advanced Immunchemical Inc.); or bovine serum albumin (BSA; Sigma). After blocking each well with 10 mg/ml BSA in PBS (100 µl), oTr1 cells (n=50,000) were added to the well and allowed to attach for 1 h (37°C, 5% CO2). Nonadherent cells were removed by washing in isotonic saline, and attached cells were fixed using 10% formalin. Plates were stained with 0.1% Amido black for 15 min, rinsed and solubilized with 2 N NaOH to obtain an absorbance reading at 595 nm which directly correlated with the number of cells stained in each well (Davis & Camarillo 1993).

Statistical Analyses

Data from slot blot hybridization analyses were subjected to least-squares analysis of variance using the General Linear Models procedures of the Statistical Analysis System (SAS Institute Inc., Cary, NC). Slot blot hybridization data were corrected for differences in sample loading by using the *18S rRNA* mRNA data as a covariate. In Study 3, preplanned orthogonal contrasts were used to determine effects of treatment (P4+CX vs P4+RU+CX, P4+CX vs P4+IFNT, and P4+RU+CX vs P4+RU+IFNT). In all analyses, error terms used in tests of significance were identified according to the expectation of the mean squares for error. Significance ($P<0.05$) was determined by probability differences of least squares means. Data are presented as least-squares means (LSM) with overall standard error (SE).

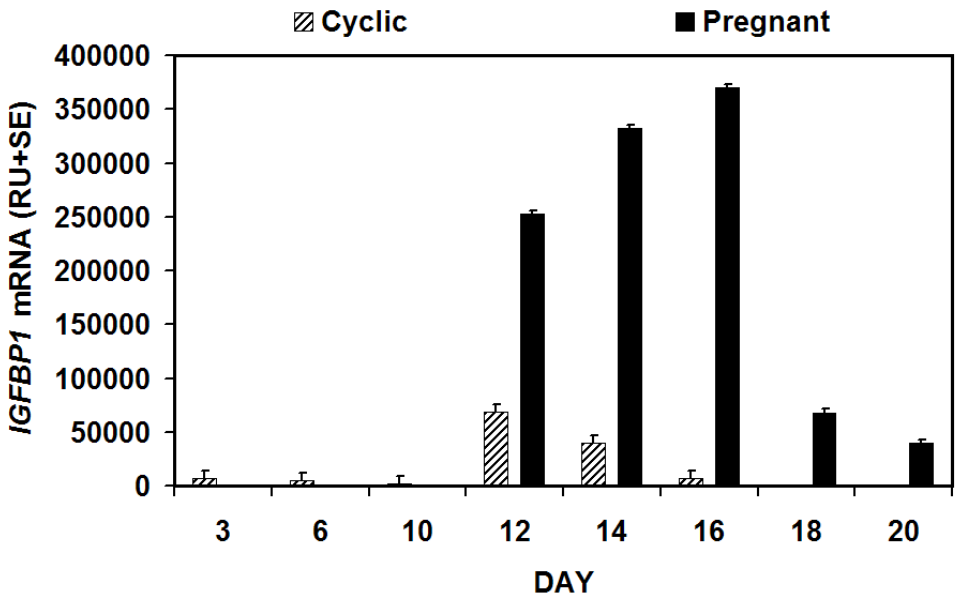
Results

IGFBP1 and IGFBP3 in the Ovine Uterus

Steady-state levels of *IGFBP1* mRNA in ovine uterine endometria are presented in Fig. 3.1A. In cyclic ewes, endometrial *IGFBP1* mRNA levels were low to undetectable on Days 3, 6 and 10, but increased 29-fold on Day 12 and then declined to Day 16 (cubic effect of day, $P<0.01$). Between Days 12 and 16, endometrial *IGFBP1* mRNA levels were greater in pregnant than cyclic ewes (day x status, $P<0.01$). Indeed, *IGFBP1* mRNA levels were about 4-fold higher on Day 12 and about 50-fold higher on Day 16 of pregnancy than for the same days of the estrous cycle. In pregnant ewes, endometrial *IGFBP1* mRNA levels increased from Days 12 to 16 and declined substantially to Days 18 and 20 (quadratic effect of day, $P<0.01$). *In situ* hybridization analysis found that *IGFBP1* mRNA was present specifically in endometrial LE and sGE of both cyclic and pregnant ewes and in the Day 18 embryo (Fig. 3.2). Interestingly, *IGFBP1* mRNA appeared to be more abundant in the intercaruncular endometrial LE and sGE as compared to LE covering the caruncles.

Steady-state levels of *IGFBP3* mRNA in ovine endometria are illustrated in Fig. 3.1B. In cyclic ewes, endometrial *IGFBP3* mRNA levels were lowest on Days 3 to 10, increased ~2.5-fold on Day 12 and increased further between Days 14 and 16 (cubic effect of day, $P<0.01$). On Days 12 to 16, endometrial *IGFBP1* mRNA levels were not different between cyclic and pregnant ewes (day x status, $P<0.10$). In pregnant ewes, endometrial *IGFBP3* mRNA levels were highest between Days 12 and 16 and then declined substantially to Day 20 (quadratic effect of day, $P<0.05$). *In situ* hybridization analysis found that *IGFBP3* mRNA was most abundant in the endometrial LE and sGE of both cyclic and pregnant ewes and was also present in the endothelium of blood vessels (Fig. 3.3).

[A]



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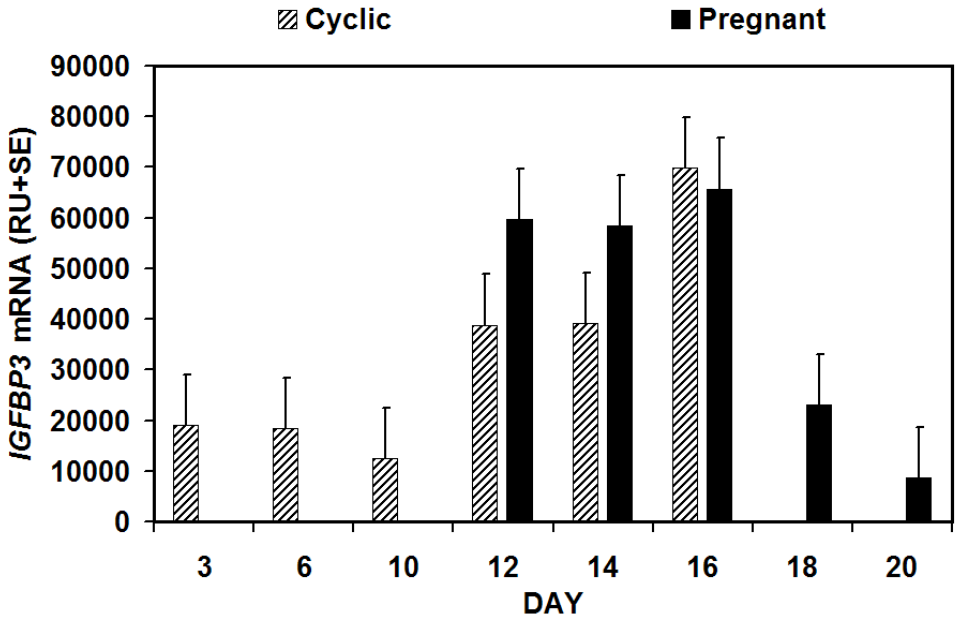


Fig. 3.1. Steady-state levels of *IGFBP1* [A] and *IGFBP3* [B] mRNAs in endometria of cyclic and pregnant ewes. Endometrial mRNA abundance was determined by slot blot hybridization analyses (see Materials and Methods). Data are presented as LSM with SE.

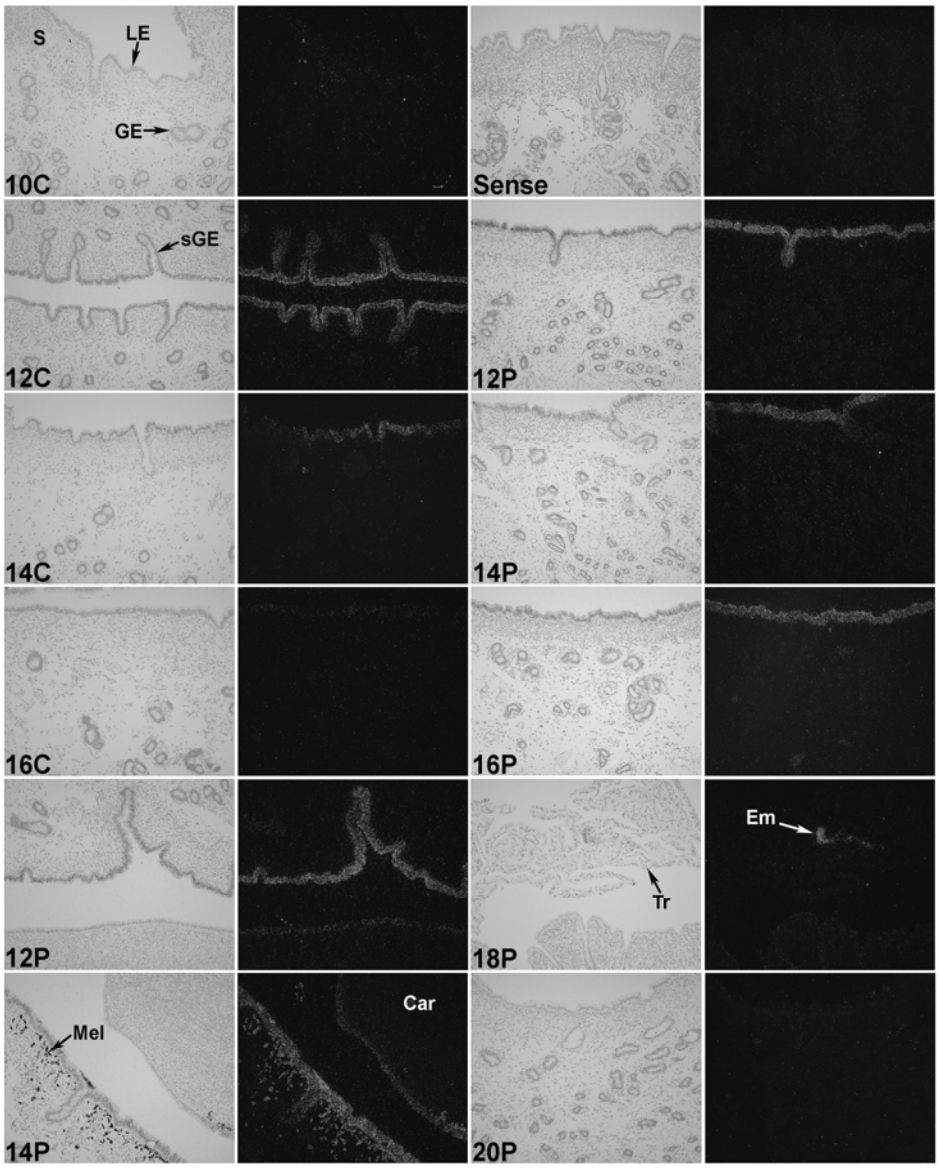


Fig. 3.2. *In situ* hybridization analysis of *IGFBP1* mRNA in uteri of cyclic and pregnant ewes. Cross-sections of the uterine wall from cyclic (C) and pregnant (P) ewes were hybridized with radiolabeled antisense or sense ovine *IGFBP1* cRNAs. Note that *IGFBP1* mRNA is most abundant in the endometrial LE and sGE. Legend: Car, caruncle; Em, embryo; LE, luminal epithelium; sGE, superficial glandular epithelium; GE, middle to deep glandular epithelium; Mel, melanocyte; S, stroma. All photomicrographs are displayed at the same width of field (560 μm).

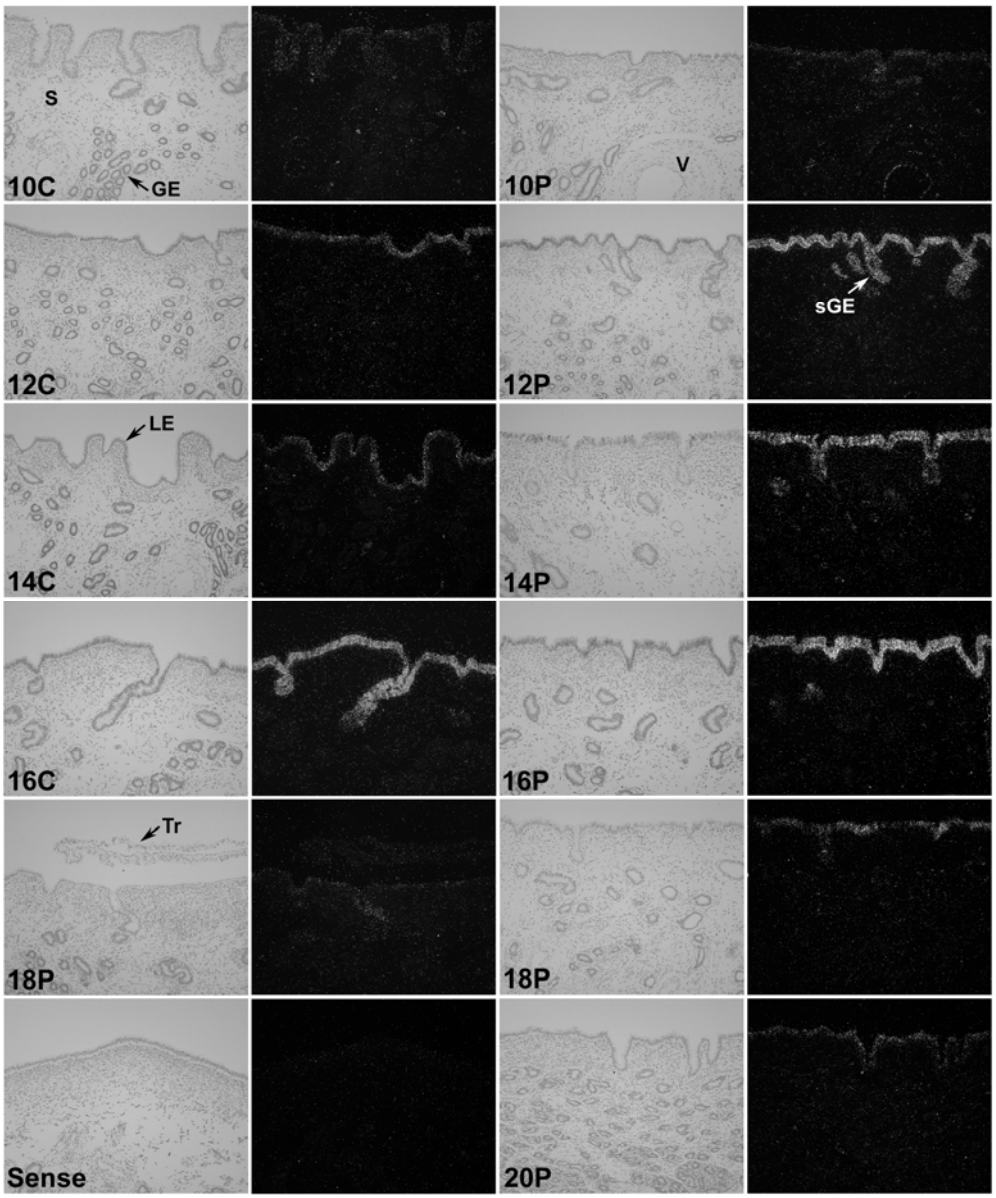


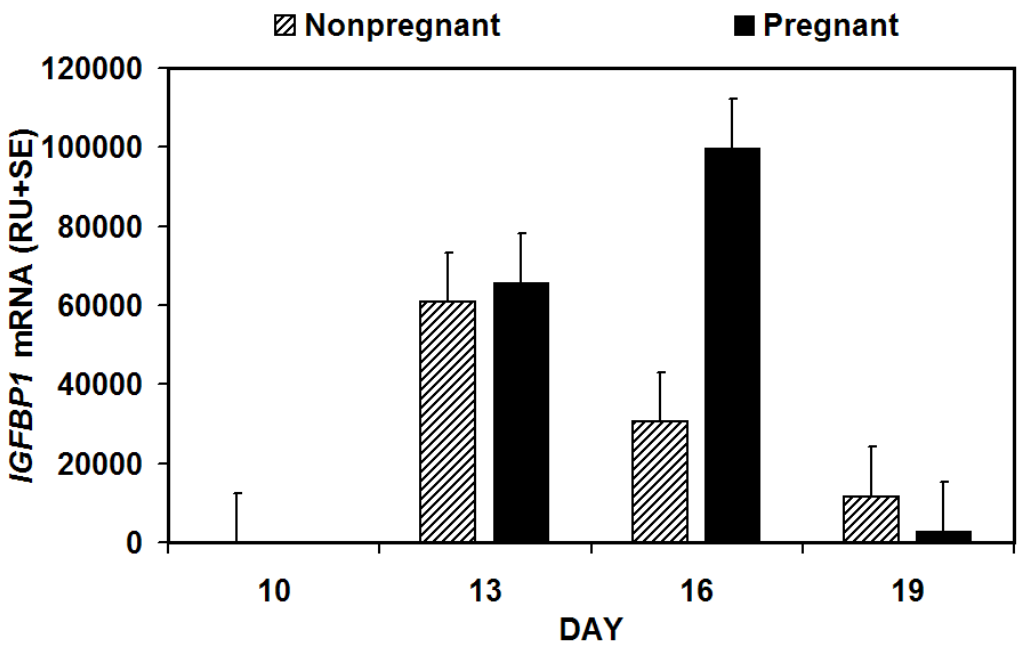
Fig. 3.3. *In situ* hybridization analysis of *IGFBP3* mRNA in uteri of cyclic and pregnant ewes. Cross-sections of the uterine wall from cyclic (C) and pregnant (P) ewes were hybridized with radiolabeled antisense or sense ovine *IGFBP3* cRNAs. Note that *IGFBP3* mRNA is most abundant in endometrial LE and sGE. Legend: LE, luminal epithelium; sGE, superficial glandular epithelium; GE, middle to deep glandular epithelium; S, stroma; Tr, trophectoderm; V, blood vessel. All photomicrographs are displayed at the same width of field (560 μm).

IGFBP1 and IGFBP3 in the Bovine Uterus

Steady-state levels of *IGFBP1* mRNA in bovine endometria are illustrated in Fig. 3.4A. In nonpregnant heifers, endometrial *IGFBP1* mRNA levels were very low or undetectable on Day 10, but increased about 228-fold on Day 13 and then declined about 5-fold to Day 19 (cubic effect of day, $P<0.01$). Between Days 13 and 19, endometrial *IGFBP1* mRNA levels were affected by pregnancy (day x status, $P<0.01$) in that they were not different on Day 13, but were 3.3-fold higher on Day 16 in pregnant than nonpregnant heifers. In pregnant heifers, *IGFBP1* mRNA levels increased between Days 13 and 16, were maximal on Day 16, and then declined substantially to Day 19 (cubic effect of day, $P<0.01$). *In situ* hybridization analysis found that *IGFBP1* mRNA was present specifically in the endometrial LE and sGE of both nonpregnant and pregnant heifers on Days 13 and 16 and also in the middle GE on Day 19 in pregnant heifers (Fig. 3.5).

Steady-state levels of *IGFBP3* mRNA in bovine endometria are illustrated in Fig. 3.4B. In nonpregnant heifers, endometrial *IGFBP3* mRNA levels did not change ($P>0.10$) between Days 10 and 19. Between Days 13 and 19, endometrial *IGFBP3* mRNA levels were not affected by pregnancy (day x status, $P<0.10$). Moreover, *IGFBP3* mRNA levels were not different ($P>0.10$) between Days 13 and 19 in pregnant heifers. *In situ* hybridization analysis was not conducted due to the lack of effects of day and pregnancy status on endometrial *IGFBP3* expression in heifers.

[A]



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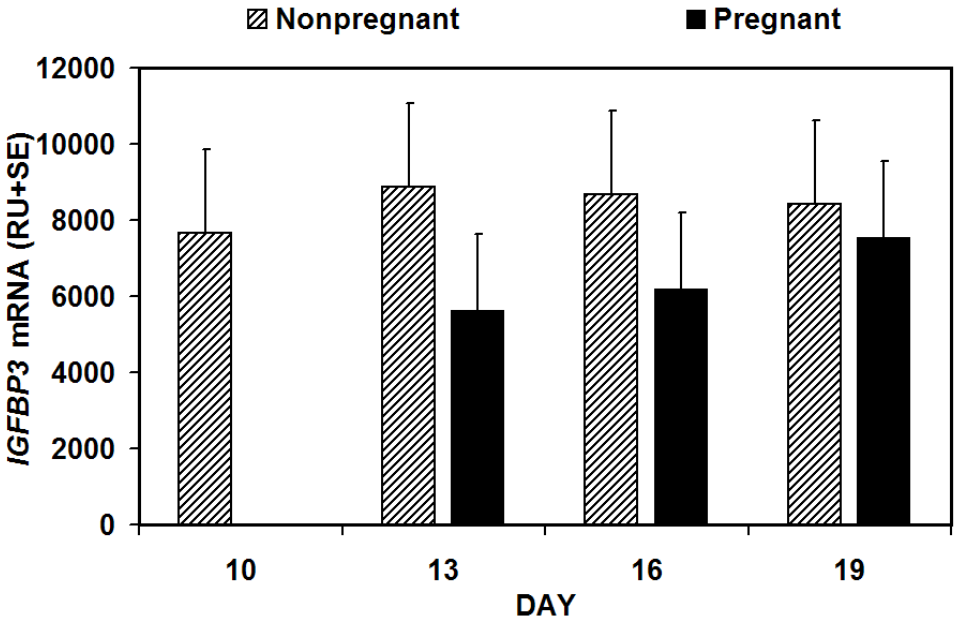

Fig. 3.4. Steady-state levels of *IGFBP1* [A] and *IGFBP3* [B] mRNAs in endometria of nonpregnant and pregnant heifers. Endometrial mRNA abundance was determined by slot blot hybridization analysis (see Materials and Methods). Data are presented as LSM with SE.

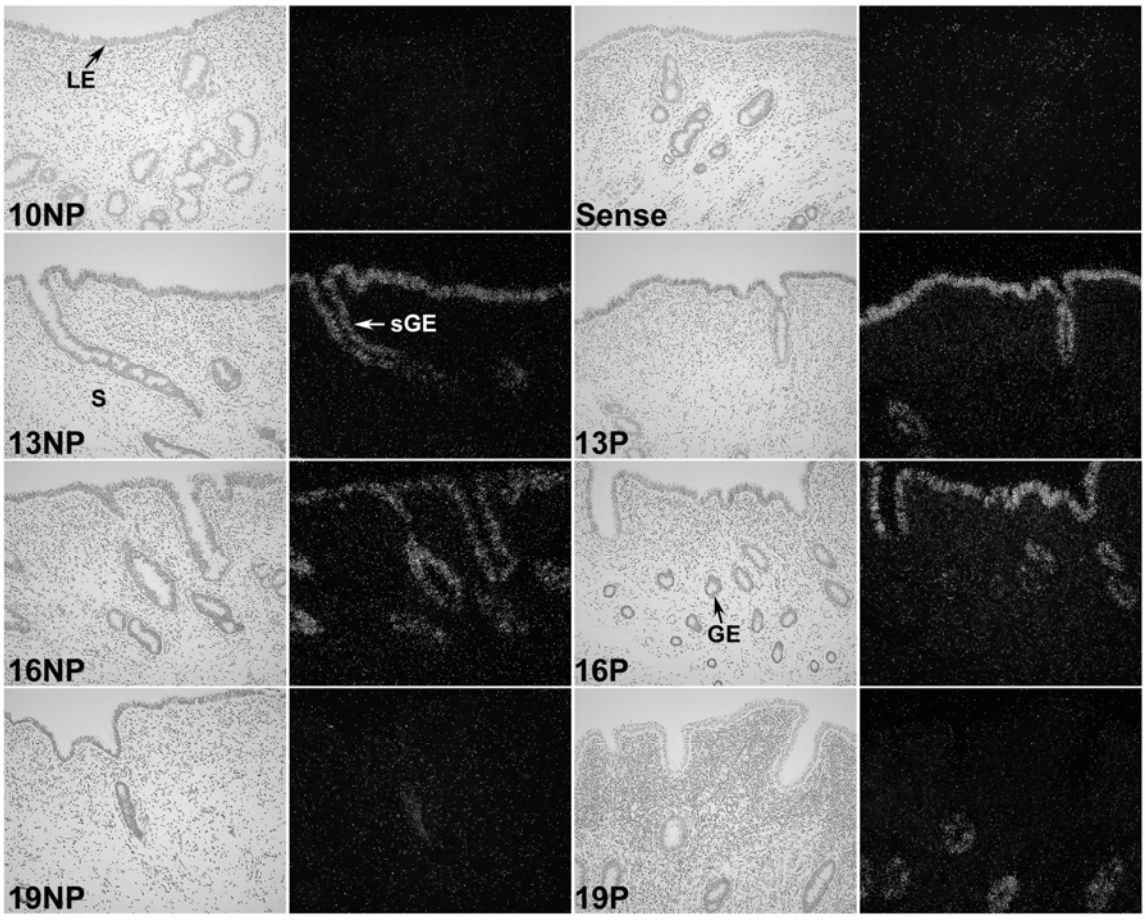

Fig. 3.5. *In situ* hybridization analysis of *IGFBP1* mRNA in uteri of nonpregnant and pregnant heifers. Cross-sections of the uterine wall from nonpregnant (NP) and pregnant (P) heifers were hybridized with radiolabeled antisense or sense ovine *IGFBP1* cRNAs. Note that *IGFBP1* mRNA is most abundant in the endometrial LE. Legend: LE, luminal epithelium; GE, middle to deep glandular epithelium; S, stroma. All photomicrographs are displayed at the same width of field (560 μm).

IGFBP1 is Induced by P4 and Stimulated by IFNT

The temporal changes in endometrial *IGFBP1* and *IGFBP3* mRNAs in uterine LE/sGE of cyclic ewes suggested that the *IGFBP1* and *IGFBP3* genes are regulated by ovarian P4, whereas the pregnancy-specific increase in endometrial *IGFBP1* mRNA in ovine and bovine uteri suggested regulation by a factor from the conceptus such as IFNT. Therefore, the effects of P4, RU486 and IFNT on endometrial *IGFBP1* and *IGFBP3* expression were studied in the ovine uterus (Study Three). As illustrated in Fig. 3.6A, treatment of ovariectomized ewes with P4 for 12 days increased endometrial *IGFBP1* mRNA abundance about 38-fold ($P<0.01$, P4+CX vs P4+RU+CX). Intrauterine infusions of IFNT increased endometrial *IGFBP1* mRNA levels about 2-fold in P4-treated ewes ($P<0.01$, P4+CX vs P4+IFNT), but had no effect in ewes receiving RU486 ($P>0.10$, P4+RU+CX vs P4+RU+IFNT). *In situ* hybridization analysis revealed that effects of P4 to induce and IFNT to stimulate *IGFBP1* expression in the endometrium were confined to the endometrial LE/sGE and GE (Fig. 3.7).

As illustrated in Fig. 3.6B, treatment with RU486 increased endometrial *IGFBP3* mRNA abundance about 3-fold ($P<0.01$, P4+CX vs P4+RU+CX). Intrauterine infusion of IFNT did not affect endometrial *IGFBP3* mRNA levels in P4-treated ewes ($P<0.01$, P4+CX vs P4+IFNT), whereas IFNT decreased *IGFBP3* mRNA abundance by about 2-fold in ewes receiving P4 and RU486 ($P<0.05$, P4+RU+CX vs P4+RU+IFNT). *In situ* hybridization analyses were not conducted given that IFNT is not produced in the absence of P4 action and RU486 is abortifacient in sheep as in other mammals.

IGFBP1 Stimulates Trophectoderm Cell Migration and Mediates Their Attachment But Does Not Affect Proliferation

The effect of IGFBP1 on trophectoderm migration was determined using oTr1 cells (Fig. 3.7A) that are mostly mononuclear and express *IFNT* (Dunlap *et al.* 2006, Hayashi *et al.* 2007). IGFBP1 stimulated migration of oTr1 cells in serum- and insulin-free medium compared to the BSA control. Relative to the BSA control, as little as 1 ng/ml IGFBP1 stimulated ($P<0.01$) oTr1 cell migration, but effects were maximal at 10

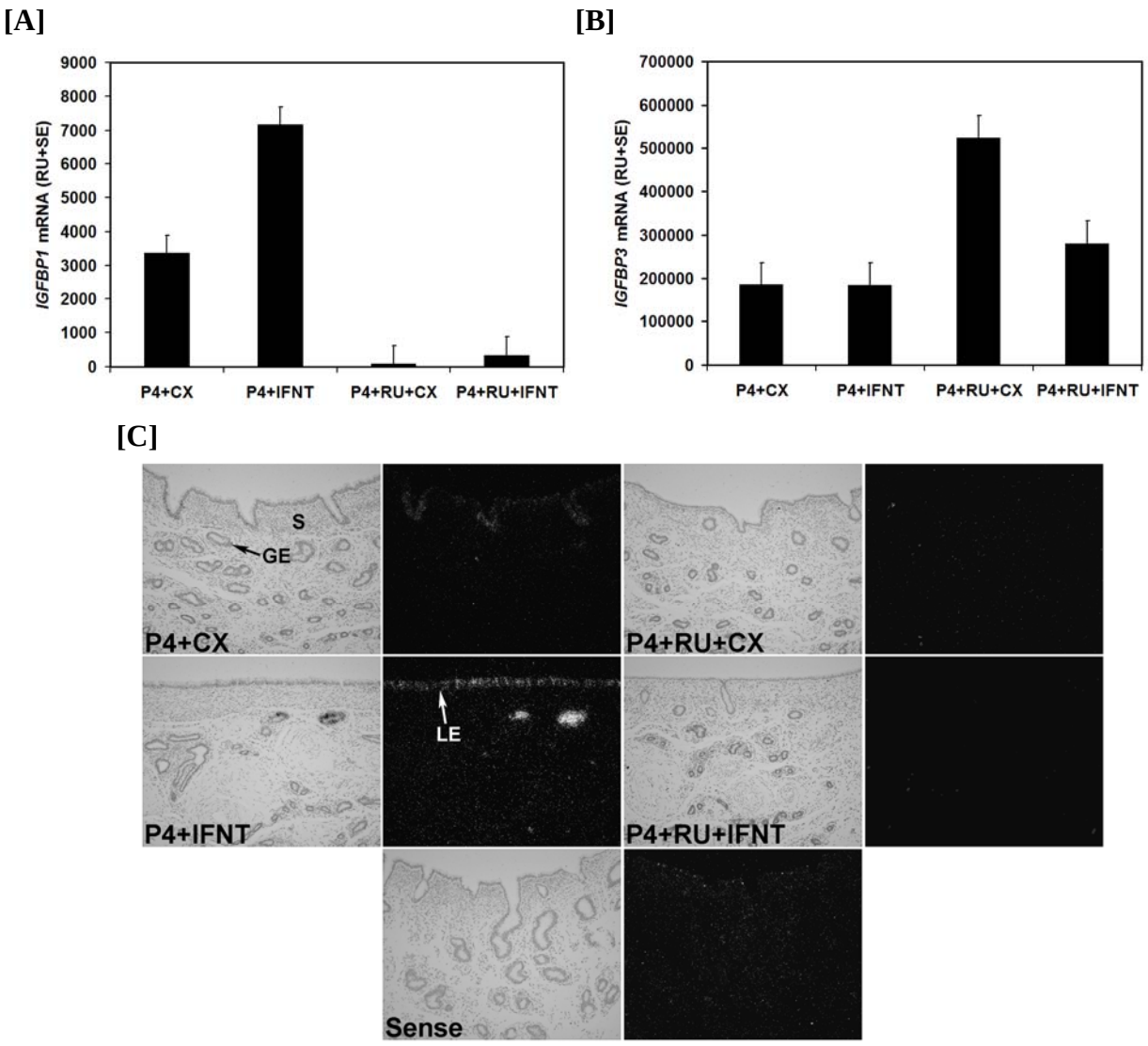

Fig. 3.6. Effects of progesterone and IFNT on *IGFBP1* and *IGFBP3* mRNA in the ovine uterus. [A] Steady-state levels of *IGFBP1* mRNA in endometria were determined by slot blot hybridization analysis. Treatment of ewes with progesterone (P4) increased (*, P<0.01) endometrial *IGFBP1* mRNA abundance as compared to ewes receiving P4 and the antiprogestin RU486. Intrauterine IFNT increased *IGFBP1* mRNA (P<0.01) in P4-treated ewes, but not in P4+RU486-treated ewes. [B] Steady-state levels of *IGFBP3* mRNA in endometria were determined by slot blot hybridization analysis. Treatment of ewes with progesterone (P4) and RU486 increased (*, P<0.01) endometrial *IGFBP3* mRNA abundance as compared to ewes receiving P4 alone. In ewes receiving P4+RU486, intrauterine IFNT decreased *IGFBP3* mRNA (P<0.01), but not in P4-treated ewes. [C] *In situ* hybridization analyses of *IGFBP1* mRNA in the ovine uterus. Cross-sections of the uterine wall from treated-ewes were hybridized with radiolabeled antisense or sense ovine *IGFBP1* cRNA probes. Note that the effects of P4 and IFNT on *IGFBP1* expression were manifest on the endometrial LE and upper GE. Legend: LE, luminal epithelium; GE, middle to deep glandular epithelium; S, stroma. All photomicrographs are displayed at the same width of field (560 μm).

and 100 ng/ml and then decreased at 1,000 ng/ml (quadratic effect of dose, $P<0.01$). IGFBP1 and fibronectin (FN) stimulated attachment of oTr1 cells in a dose-dependent manner (Fig. 3.7B) compared to BSA control wells. An increase in oTr1 cell attachment occurred in wells with as little as 80 ng/ml IGFBP1 and the effect of IGFBP1 was dose-dependent (cubic effect of dose, $P<0.01$). The attachment elicited by IGFBP1 was consistently greater (dose x treatment, $P<0.01$) than that elicited by similar concentrations of FN. In contrast, IGFBP1 (0.01-10 μg/ml) had no effect ($P>0.10$) on proliferation of oTr1 cells in insulin-free medium containing 0, 1 or 10% serum (data not shown).

Discussion

Results of the present study support previously published results for IGFBP1 in LE and sGE of sheep (Osgerby *et al.* 1999) and cattle (Reynolds *et al.* 1997), and suggest that IGFBP1 is a common endometrial marker of conceptus elongation and implantation in sheep and cattle that is regulated by ovarian P4 and a conceptus-derived factor. In contrast, expression of *IGFBP3* is different between sheep and cattle. *IGFBP3* is expressed by ovine endometrial LE/sGE (Fig. 3.3) (Reynolds *et al.* 1997), but predominantly by subepithelial stromal cells in bovine uteri (Robinson *et al.* 2000). These results agree with emerging evidence to indicate that, although both sheep and cattle are ruminants with considerable similarities in conceptus growth, development and implantation during early pregnancy, substantial differences exist in endometrial gene expression between these species. For instance, *LGALS15*, a member of the galectin superfamily, is one of the most abundant mRNAs present in ovine endometria during early pregnancy (Gray *et al.* 2004, Gray *et al.* 2006), but the *LGALS15* gene present in the bovine genome is not expressed in cattle (Lewis *et al.* 2007). These results highlight the importance of caution in translating research findings in sheep directly to cattle when seeking to identify common mediators of endometrial function and conceptus development in ruminants (Spencer *et al.* 2008).

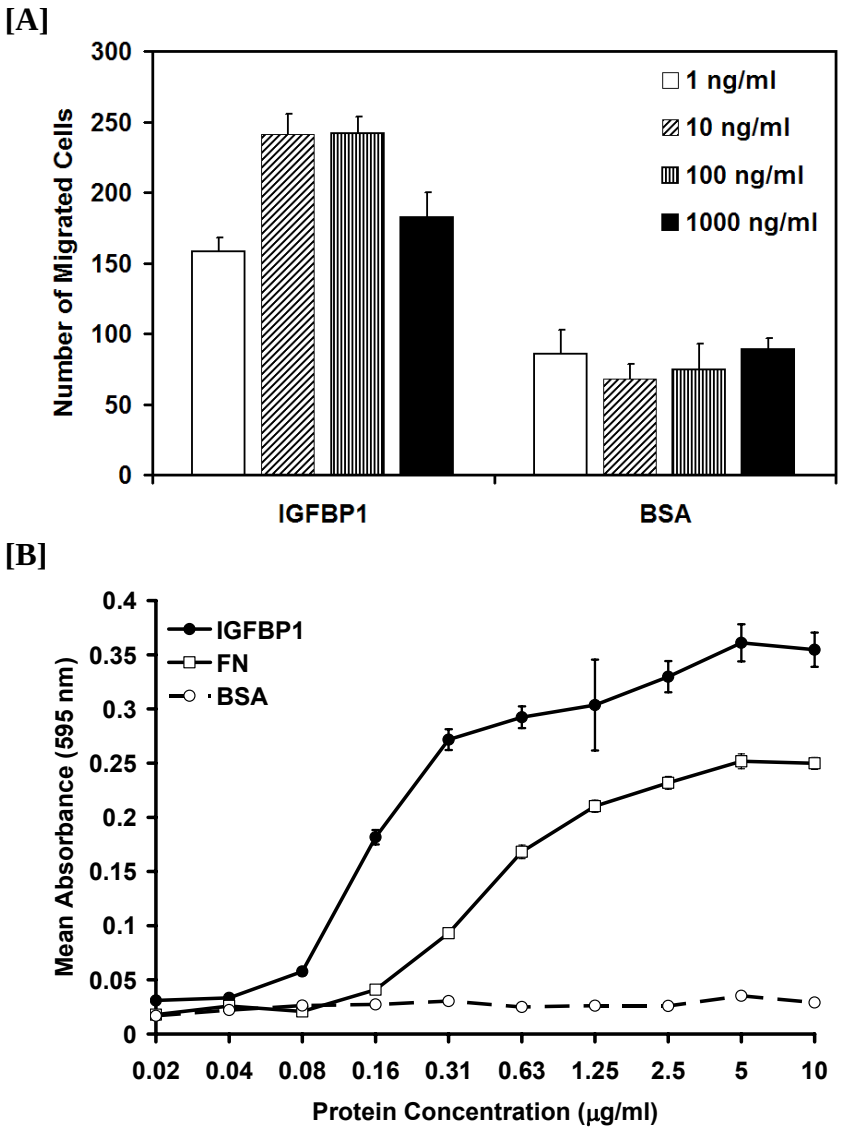

Fig. 3.7. Effects of IGFBP1 on migration and attachment of ovine trophectoderm cells. [A] Cell migration. Ovine trophectoderm cells (oTr1) were cultured in a Transwell plate in serum- and insulin-free medium and treated with IGFBP1 purified from human amniotic fluid or with bovine serum albumin (BSA) as a control. The number of cells that migrated was determined after 12 h of treatment. IGFBP1 increased (*, $P<0.01$) oTr1 cell migration relative to the BSA control. The graph is a compilation of three independent experiments with three replicates per treatment in each experiment. Data are presented as LSM with SE. [B] Cell attachment. Wells of suspension culture plates were pre-coated overnight with increasing amounts of either human IGFBP1, BSA (negative control) or human fibronectin (FN; positive control). Equal numbers of oTr1 cells were added to each well, and the number of attached cells determined after 1 h. A dose-dependent increase ($P<0.01$) in cell attachment was induced by both IGFBP1 and FN, but not BSA. The graph is a compilation of three independent experiments with three replicates per treatment in each experiment. Data are presented as LSM with SE.

In sheep, the induction in *IGFBP1* and increase in *IGFBP3* expression in endometrial LE/sGE between Days 10 and 12 of the estrous cycle and pregnancy is temporally associated with loss of *PGR* expression in these epithelia (Spencer & Bazer 1995). Similarly, the induction of *IGFBP1* expression in endometrial LE/sGE between Days 10 and 13 in nonpregnant and pregnant heifers is also associated with loss of *PGR* in those epithelia (Robinson *et al.* 1999, Kimmins & MacLaren 2001). Likewise, the decrease in *IGFBP1* mRNA in LE/sGE between Days 14 and 16 of the estrous cycle in sheep and Days 16 and 19 in cattle is coincident with the subsequent reappearance of *PGR* expression in those epithelia (Wathes & Hamon 1993, Spencer & Bazer 1995, Robinson *et al.* 2001). Although the cellular and molecular mechanism(s) are not clear, continuous exposure of the sheep uterus to P4 for 8 to 10 days is required for loss of *PGR* mRNA and PGR protein in endometrial LE and sGE, but not stroma or myometrium (Spencer & Bazer 1995, Johnson *et al.* 2000). Indeed, *PGR* expression remains undetectable in ovine endometrial epithelia throughout pregnancy (Spencer *et al.* 2004b). In the present Study Three, *IGFBP1* mRNA was induced by P4 in endometrial LE/sGE, but this effect was blocked by administration of the antiprogestin RU486. Treatment of ewes with antiprogestins results in reappearance of PGR in endometrial epithelia (Johnson *et al.* 2000, Satterfield *et al.* 2006), because they prevent P4 actions to down-regulate *PGR* expression and production of stromal-derived growth factors (Satterfield *et al.* 2008). In addition to being an antiprogestin, RU486 is a high affinity antagonist of the glucocorticoid receptor (Baulieu 1989), but little is known of glucocorticoid receptor expression and glucocorticoid effects within the ovine uterus during either the estrous cycle or pregnancy. *IGFBP3* expression in endometrial LE/sGE did not decline between Days 14 and 16 of the estrous cycle and increased in uteri of ewes treated with RU486 in Study Three, suggesting that *IGFBP3* expression is regulated by a different mechanism than *IGFBP1*.

In addition to induction by ovarian P4 during the estrous cycle and pregnancy, *IGFBP1* expression in endometrial LE/sGE was also increased by the presence of a conceptus in both sheep and cattle. During early pregnancy, the ruminant conceptus

synthesizes and secretes a number of different factors, but IFNT is the most abundant protein produced by the elongating ruminant conceptus (Roberts 2007). Indeed, infusion of recombinant ovine IFNT into uteri of P4-treated ewes increased *IGFBP1* mRNA abundance by almost two-fold. However, this stimulation by IFNT was rather modest and did not mimic the approximately 5- and 29-fold increases in endometrial *IGFBP1* expression observed on Days 12 and 16, respectively, in pregnant as compared to cyclic ewes. In bovine uteri, endometrial *IGFBP1* mRNA abundance was over 3-fold higher for Day 16 pregnant compared to nonpregnant heifers. Indeed, the spherical Day 12 conceptus of sheep produces little IFNT compared to large amounts produced by the elongating conceptus that is maximal on Days 15-16 (Ashworth & Bazer 1989). These results strongly suggest that another factor produced by the conceptus regulates endometrial *IGFBP1* expression in sheep and perhaps cattle, with prostaglandins being strong candidates.

Despite markedly different implantation schemes among primates, rodents, and ruminants, *IGFBP1* is upregulated in endometria of each of these species during early pregnancy and implicated as a regulator of blastocyst implantation and placental growth and development (Giudice & Saleh 1995, Fowler *et al.* 2000). In humans, *IGFBP1* is a highly up-regulated gene in the human secretory endometrium during the period of receptivity to implantation (Kao *et al.* 2002) and localized to endometrial LE, a subpopulation of stromal cells, and the decidua (Zhou *et al.* 1994, Han *et al.* 1996). Similarly, IGFBP1 is the primary secretory product of baboon decidua that is stimulated by hCG, the pregnancy recognition signal produced by primate conceptuses (Fazleabas *et al.* 1993). In the present study, IGFBP1 stimulated migration and mediated attachment of oTr1 cells, which are required for elongation and implantation of ruminant conceptuses (Guillomot 1995, Spencer *et al.* 2004a); however, IGFBP1 did not stimulate oTr1 cell proliferation, suggesting that the purified IGFBP1 used in the present studies was not contaminated with IGF1 or another mitogen. In addition to IGF ligand binding, IGFBP1 contains a conserved RGD sequence that can act as a ligand for the integrin heterodimer α5β1 (Irwin & Giudice 1998, Gleeson *et al.* 2001). Blocking antibodies

against the α5β1 integrin subunits inhibit trophoblast cell migration (Irving & Lala 1995), and IGFBP1 stimulated migration of trophoblast cells is attenuated by mutation of the RGD integrin binding sequence to WGD or pretreatment with an inhibitory peptide (Gleeson *et al.* 2001). In sheep, the α5 and β1 integrin subunits are constitutively expressed on the surface of uterine LE/sGE and conceptus trophectoderm (Johnson *et al.* 2001), which supports the hypothesis that IGFBP1 from uterine LE/sGE can stimulate migration and adhesion of trophectoderm cells to the uterine LE during the attachment phase of implantation. Indeed, the transient nature of *IGFBP1* expression in uterine LE/sGE is correlated with elongation of conceptuses of both sheep and cattle (Guillomot 1995, Spencer *et al.* 2004a, Satterfield *et al.* 2006, Spencer 2007). In the present studies, IGFBP1 mediated attachment of oTr1 cells, which is an essential element of blastocyst implantation and trophoblast differentiation in many species (Burghardt *et al.* 2002, Armant 2005). Indeed, integrins are proposed to be the dominant glycoproteins that regulate trophectoderm adhesion to endometrial LE during implantation in mammals (Aplin 1997, Armant 2005). During the peri-implantation period of pregnancy in sheep, integrin subunits αv, α4, α5, β1, β3 and β5 are constitutively expressed on apical surfaces of the conceptus trophectoderm and endometrial LE (Johnson *et al.* 2001). Thus, conceptus implantation in sheep does not appear to involve temporal or spatial changes in patterns of integrin expression (Johnson *et al.* 2001), but may depend primarily on changes in secreted integrin ligands, such as IGFBP1, LGALS15 and secreted phosphoprotein one (SPP1 or osteopontin) (Johnson *et al.* 2003, Spencer *et al.* 2004a, Joyce *et al.* 2005, Farmer *et al.* 2008). Adhesive LE ligands, normally masked by mucins, become exposed during the receptive period, and various adhesion molecules then function sequentially, or in parallel, to stabilize adhesion of the trophectoderm to the endometrial LE (Johnson *et al.* 2001, Burghardt *et al.* 2002, Johnson *et al.* 2003, Burghardt *et al.* 2009).

Although endometrial *IGFBP3* expression differed between sheep and cattle, its expression did increase in endometrial LE/sGE of both cyclic and pregnant ewes after Day 10 and was consistently detected in the bovine endometria during early pregnancy.

In sheep, the increase and decrease in endometrial *IGFBP3* expression was correlated with the period of rapid elongation of the conceptus. Although distinct differences exist between the cell types expressing IGFBP3 in bovine and ovine uteri, IGFBP3 is the predominant IGFBP in the uterine lumen during early pregnancy in both sheep and cattle (Keller *et al.* 1998, Peterson *et al.* 1998b). Indeed, substances present in the uterus are derived from synthesis and secretions of the endometrium as well as selective transport of serum components (Bazer & Roberts 1983), and IGFBP3 is the most abundant circulating IGFBP in serum. In serum, IGFBP3 regulates IGF bioavailability by sequestering IGFs in circulating ternary complexes, and it also competitively inhibits IGF action at the cellular level (Burger *et al.* 2005). IGFBP3 also has IGF-independent actions that appear to be mediated by a cell surface receptor and/or by direct nuclear action (Burger *et al.* 2005). Thus, IGFBP3 may have IGF-dependent and -independent activities that modulate conceptus growth and development during early pregnancy in ruminants.

IGF1 and IGF2 possess both mitogenic and differentiative properties and are components of uterine luminal histotroph in sheep and cattle (Ko *et al.* 1991, Keller *et al.* 1998, Kim *et al.* 2008). Both ovine and bovine pre-implantation embryos (Watson *et al.* 1999), as well as Day 15 elongated bovine conceptuses express *IGF1R* (Sawai *et al.* 2007). Indeed, IGF1 stimulates proliferation and inhibits apoptosis in cultured bovine embryos (Jousan & Hansen 2004), and IGF2 stimulates oTr cell migration (Kim *et al.* 2008). Thus, access of the blastocyst to IGF1 and IGF2 in the uterine lumen could be mitigated by upregulation of *IGFBP3* and perhaps *IGFBP1* in uterine LE/sGE between Days 10 and 12 of pregnancy. IGF-dependent and -independent activities of IGFBP3 are regulated by deactivation via several proteases (Burger *et al.* 2005). Indeed, treatment of ovariectomized ewes with P4 for 10 days resulted in the proteolysis of IGFBP3 in the uterine lumen which would theoretically increase bioactive IGF available to the blastocyst (Peterson *et al.* 1998b). Of particular interest, CTSL1 is a P4- and IFNT-stimulated protease expressed by ovine endometrial LE and sGE during early pregnancy that may act as an IGFBP protease (Song *et al.* 2005). Further, MMP2 and

MMP9 are secretory products of ovine endometria that increase from Days 12 to 20 of pregnancy and may regulate IGFBP cleavage and thus IGF bioavailability (Fowlkes *et al.* 1995, Bunn & Fowlkes 2003). The IGF-dependent and -independent effects of IGFBP3 on trophectoderm functions need to be investigated in order to understand the biological role(s) of this IGFBP in the uterine lumen.

In summary, the spatiotemporal alterations in *IGFBP1* mRNA in ovine and bovine uterine LE/sGE during pregnancy, combined with the functional aspects of IGFBP1 discovered in the present studies and in published results, support the hypothesis that IGFBP1 functions as a heterotypic cell adhesion molecule bridging integrins in the endometrial LE and conceptus trophectoderm, which stimulates trophectoderm migration and adhesion required for conceptus growth and elongation in ruminants prior to implantation *in utero*. Future experiments will be directed toward discerning the biological role(s) of conceptus-derived prostaglandins on expression of *IGFBP1* as well as other genes in the endometrium that are important for conceptus elongation and development as well as endometrial receptivity to implantation of the conceptus.

CHAPTER IV
HSD11B1, HSD11B2, PTGS2 AND NR3C1 EXPRESSION IN THE PERI-IMPLANTATION OVINE UTERUS: EFFECTS OF PREGNANCY, PROGESTERONE AND INTERFERON TAU

Introduction

Maternal support of blastocyst growth and development into an elongated conceptus is critical for pregnancy recognition signaling and implantation in ruminants (Spencer *et al.* 2004a, Spencer 2007). After hatching from the zona pellucida on Day 8, the blastocyst develops into an ovoid form by Day 11 and is then termed a conceptus (Guillomot 1995, Spencer *et al.* 2004a). The conceptus begins to elongate on Day 12 to form a tubular and then filamentous conceptus of 14 cm or more in length by Day 16. Conceptus elongation requires substances secreted from the endometrial LE and GE (Flechon *et al.* 1986, Gray 2002). During early pregnancy in ruminants, endometrial epithelia functions are regulated primarily by P4 from the CL and secreted factors from the trophectoderm/chorion such as IFNT (Bazer *et al.* 1979, Spencer *et al.* 2004b, Spencer *et al.* 2008). IFNT, produced by the mononuclear trophectoderm cells of the conceptus during elongation, exerts antiluteolytic effects on the endometrium to inhibit production of luteolytic pulses of PGF2α, thereby maintaining CL function for production of P4 that, in turn, stimulates and maintains uterine endometrial functions necessary for conceptus growth, implantation, placentation and successful development of the conceptus to term (Spencer *et al.* 2004b). In addition to antiluteolytic effects, IFNT induces or increases expression of many genes in the endometrial epithelia that regulate conceptus growth and development during the peri-implantation period of pregnancy (see (Spencer 2007, Bauersachs *et al.* 2008, Spencer *et al.* 2008) for review).

We recently reported results from an ovine model of accelerated blastocyst growth and conceptus development elicited by advancing the post-ovulatory rise in circulating levels of P4 during metestrus (Satterfield *et al.* 2006). That model was used

to identify a number of P4-regulated genes that are expressed in the endometrial epithelia (LE, sGE and/or GE) and encode secreted proteins (*LGALS15, GRP, IGFBP1,* and *IGFBP3*) which are hypothesized to regulate conceptus elongation via effects on trophectoderm proliferation, migration and/or attachment (see CHAPTER III and (Satterfield *et al.* 2006, Farmer *et al.* 2008, Satterfield *et al.* 2008, Song *et al.* 2008)). In addition to genes encoding secreted proteins, our microarray analysis identified HSD11B1 as a candidate P4-regulated gene (Satterfield MC et al., submitted). HSD11B1 is one of two isoforms of 11β-hydroxysteroid dehydrogenase that modulate the actions of GCs within potential target cells (see (Michael *et al.* 2003) for review). HSD11B1 acts predominantly as an NADP(H)-dependent reductase to generate active cortisol from inactive corticosterone, whereas HSD11B2 acts predominantly to generate inactive corticosterone from active cortisol. Cortisol can activate both the NR3C1 and the MR. Although the NR3C1 and NR3C2 expression has not been investigated in the ovine uterus during early pregnancy, HSD11B1 was previously found to be expressed in the endometrial LE and upper GE of the ovine uterus during the estrous cycle and early pregnancy (Yang *et al.* 1996). In fact, the pattern of expression during the estrous cycle and pregnancy led those investigators to conclude that *HSD11B1* expression was regulated by P4, although experiments were not presented to test that hypothesis. In the ovine placenta, PGs can stimulate HSD11B1 activity and GCs increase PG synthesis by up-regulating expression and activities PLA2 and PTGS2 (see (Michael & Papageorghiou 2008)), thereby establishing a positive feed-forward loop implicated in the timing of parturition (Challis *et al.* 2000). Recent studies found that prostaglandins regulate HSD11B1 activity in the bovine uterus (Lee *et al.* 2009). In sheep, elongation of the conceptus is coordinate with PTGS2 expression in the endometrial epithelia and prostaglandin production by the conceptus (Lewis & Waterman 1985, Charpigny *et al.* 1997a, Charpigny *et al.* 1997b).

Therefore, available evidence supports the working hypotheses that ovarian P4 and prostaglandins control *HSD11B1* expression in endometrial epithelia and that regenerated cortisol produced by HSD11B1 acts via NR3C1 to regulate endometrial

functions important for conceptus elongation and implantation during the peri-implantation period of pregnancy. As a first step in testing these hypotheses, the present studies determined effects of pregnancy, P4 and IFNT on *HSD11B1, HSD11B2*, PTGS2, and NR3C1 expression in the ovine uterus.

Materials and Methods

Experimental Design

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.

Study One

The experimental design and methods for Study One are the same as those described in Chapter III.

Study Two

In order to determine circulating levels of cortisol in blood during early pregnancy, cyclic ewes (n=4) were mated at estrus (Day 0) to a fertile ram. Blood samples were taken daily by jugular venipuncture until Day 20 post-mating. Serum was collected after clotting at 4^{o}C for 4 h and clarified by centrifugation (3000 x *g* for 30 min at 4^{o}C) before storage at -20^{o}C. All ewes were confirmed pregnant by transrectal ultrasonography.

Study Three

The effects of early administration of P4 on blastocyst development and uterine endometrial gene expression were evaluated using the following experimental design. As described previously (Satterfield *et al.* 2006), ewes were mated at estrus (Day 0) to intact 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); all ewes were

hysterectomized on Day 9. An additional group of ewes were mated and assigned randomly to receive daily intramuscular (i.m.) injections of either: (a) CO (n=8); (b) 25 mg P4 (Sigma Chemical Co., St. Louis, MO) from Days 1.5 to 12 (n=7); or (c) 25 mg P4 (from days 1.5 to 8, n=5) and 75 mg of RU486 (Sigma-Aldrich, Inc., St. Louis, MO), a PGR and NR3C1 antagonist (Baulieu 1989), from Days 8 to 12 (P4+RU486); all ewes were hysterectomized on Day 12. Uteri were processed as described for Study One. Pregnancy was confirmed by the recovery of a morphologically normal blastocyst or conceptus in the uterine flushing.

Study Four

The purpose, details of experimental design, tissue collection and analyses for Study Four are the same as described for Study Three in Chapter III.

Cortisol Radioimmunoassay

Concentrations of cortisol in serum were measured in samples from Study Two by solid phase radioimmunoassay (RIA) as per manufacturer's instructions (TKC02, Siemens Diagnostic Products Corporation; Deerfield, IL). The minimum detectable concentration of cortisol was 1.2 ng/ml and the intra-assay coefficient of variation was 3.75%. Standards, controls, and samples (25 μl each of standards and controls) were added to appropriately labeled coated tubes, followed by the addition of I^{125}-labeled cortisol (1 ml/tube). Tubes were incubated for 45 minutes in a 37°C water bath, after which supernatants were decanted, and tubes dried for at least 5 minutes. Tubes were placed in Packard Cobra II auto gamma counter to determine counts per minute (i.e., CPM bound). Concentrations of cortisol in serum were determined by comparison to a standard curve generated with known concentrations of cortisol (Assay Zap, Biosoft, Cambridge, United Kingdom). Data are presented as ng/ml cortisol in serum.

RT-PCR of Ovine HSD11B1 and HSD11B2 cDNAs

Partial cDNAs for ovine *HSD11B1* and *HSD11B2* mRNAs were amplified by RT-PCR using total RNA isolated from Day 18 pregnant ewes as described previously (Song *et al.* 2008). For *HSD11B1*, the sense primer (5'- CAT TCT GGG GAT CTT CTT GG -3') and antisense primer (5'- GAA TAG GCA GCA GCA AGT GG -3') were derived from the *Ovis aries HSD11B1* mRNA coding sequence (GenBank accession no. NM_001009395) and amplified a 534 bp product. For *HSD11B2*, the sense primer (5'- AGT TCA CCA AGG TCC ACA CC -3') and antisense primer (5'- TGC TCG ATG TAG TCC TCA CC -3') were derived from the *Ovis aries HSD11B2* mRNA coding sequence (GenBank accession no. NM_001009460) and amplified a 457 bp product. Partial ovine cDNAs were cloned into pCRII using a T/A Cloning Kit (Invitrogen), and sequences verified using an ABI PRISM Dye Terminator Cycle Sequencing Kit and ABI PRISM automated DNA sequencer (Perkin-Elmer Applied Biosystems).

Slot Blot Hybridization Analysis

Total cellular RNA was isolated from frozen endometria 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. Steady-state levels of *HSD11B1* mRNA were assessed by slot blot hybridization using radiolabeled antisense *HSD11B1* and *18S* cRNA probes as described previously (Spencer *et al.* 1999). Radiolabeled antisense cRNA probes were generated by *in vitro* transcription using linearized partial plasmid cDNA templates, RNA polymerases, and [α-^{32}P]-UTP. 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*

Cell-specific expression of *HSD11B1* and *HSD11B2* mRNAs in ovine uteri was determined using radioactive *in situ* hybridization analysis as described previously

(Spencer *et al.* 1999, Satterfield *et al.* 2008). Radiolabeled antisense and sense cRNA probes were generated by *in vitro* transcription using linearized partial plasmid cDNA templates, RNA polymerases, and [α-^{35}S]-UTP. All slides for each respective gene were exposed to photographic emulsion for the same period of time. 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.

Immunohistochemical Analyses

Immunoreactive PTGS2 and NR3C1 proteins were localized in ovine uteri as described previously using a rabbit anti-murine PTGS2 polyclonal antibody (Catalog 160106; Cayman Chemical) at a 1:250 final dilution, and a mouse anti-rat NR3C1 monoclonal antibody (Catalog MA1-510; Affinity Bioreagents) at 5 μg/ml final dilution. Antigen retrieval for both PTGS2 and NR3C1 was performed using the boiling citrate method. Negative controls included substitution of the primary antibody with purified non-immune rabbit IgG at the same final concentration. Sections were not counterstained prior to affixing coverslips.

Statistical Analyses

All quantitative data were subjected to least-squares analyses of variance (ANOVA) using the Statistical Analysis System (SAS Institute, Cary, NC). Slot blot hybridization data were corrected for differences in sample loading using the 18S rRNA data as a covariate. Data from ewes between Days 12 and 16 (Study One) were analyzed for effects of day, pregnancy status (cyclic or pregnant), and their interaction. Data from Study Two were analyzed for effects of day of pregnancy using regression analysis. Data from Studies Three and Four were analyzed using orthogonal contrasts (Day 9: CO vs P4, Day 12: CO vs P4, and Day 12: P4 vs P4+RU486 for Study Two; and P4+CX vs P4+IFN, P4+RU+CX vs P4+RU+IFN, P4+CX vs P4+RU+CX for Study Three) to elucidate effects of treatment. All tests of significance were performed using the

appropriate error terms according to the expectation of the mean squares for error. A P-value of 0.05 or less was considered significant. Data are presented as least-square means (LSM) with standard errors (SE).

Results

HSD11B1, HSD11B2, PTGS2 and NR3C1 in the Ovine Uterus During the Cycle and Early Pregnancy

HSD11B1. As illustrated in Fig. 4.1, endometrial *HSD11B1* mRNA levels were low to undetectable on Days 3, 6 and 10, increased 7.3-fold between Days 10 and 12, increased another 10.8-fold from Days 12 to 14, and remained elevated to Day 16 in cyclic ewes (cubic effect of day, $P<0.01$). Between Days 12 and 16, endometrial *HSD11B1* mRNA levels were greater in pregnant than cyclic ewes (day x status, $P<0.01$). As compared to cyclic ewes, *HSD11B1* mRNA levels were 11.3-fold higher on Day 12, 2.0-fold higher on Day 14, and 2.4-fold higher on Day 16 of pregnancy. In pregnant ewes, endometrial *HSD11B1* mRNA levels increased from Days 12 to 14, were maximal on Days 14 and 16, and then declined to Day 20 (quadratic effect of day, $P<0.01$).

In situ hybridization analysis revealed that *HSD11B1* mRNA was most abundant in the LE, superficial GE (sGE) and upper GE of endometria, although mRNA was observed at much lower abundance in the middle to deep GE, stroma and myometrium (Fig. 4.2A). Consistently, *HSD11B1* mRNA appeared to be more abundant in the uterine intercaruncular LE/sGE than the caruncular LE. In Day 18 and 20 conceptuses, *HSD11B1* mRNA was detected, but very low in abundance.

HSD11B2. *In situ* hybridization analysis found that *HSD11B2* mRNA was present at very low levels in all cell types of uteri from both cyclic and pregnant ewes (Fig. 4.2B); therefore, steady-state levels of *HSD11B2* mRNA in the endometria were not measured. In contrast to the uterus, *HSD11B2* mRNA was readily detected in the trophectoderm and endoderm of conceptuses on Days 18 and 20 of pregnancy.

PTGS2. In the uterus, immunoreactive PTGS2 protein was observed only in the LE/sGE and upper GE and distributed throughout the cytoplasm (Fig. 4.3). In cyclic ewes, PTGS2 was low in the endometrial LE/sGE on Day 10, increased to maximal abundance on Day 12, and then declined to Day 16. In uteri from pregnant ewes, PTGS2 was also low in the endometrial LE/sGE on Day 10, increased to maximal abundance on Day 14, and then steadily declined to nearly undetectable levels on Day 20. On both Days 14 and 16, PTGS2 was consistently more abundant in the endometrial LE/sGE and upper GE of pregnant as compared to matched cyclic ewes. In Day 18 and 20 conceptuses, PTGS2 was detected in the trophectoderm and was particularly abundant in the inner cell mass.

NR3C1. Immunoreactive NR3C1 (glucocorticoid receptor) protein was detected in the nuclei of most, if not all, cell types in the endometrium and myometrium (Fig. 4.3). In cyclic ewes, NR3C1 protein was most abundant on Days 10 and 12 in the endometrial epithelia. After Day 12, NR3C1 protein abundance decreased in the endometrium, particularly in the epithelia. In pregnant ewes, NR3C1 protein was most abundant on Day 10 in the endometrial epithelia. After Day 12, NR3C1 was most abundant in the middle to deep GE, stroma and myometrium and present at low abundance in the LE, sGE and upper GE of the endometrium. In Day 18 and 20 conceptuses, NR3C1 was also detected in the nuclei of trophectoderm cells.

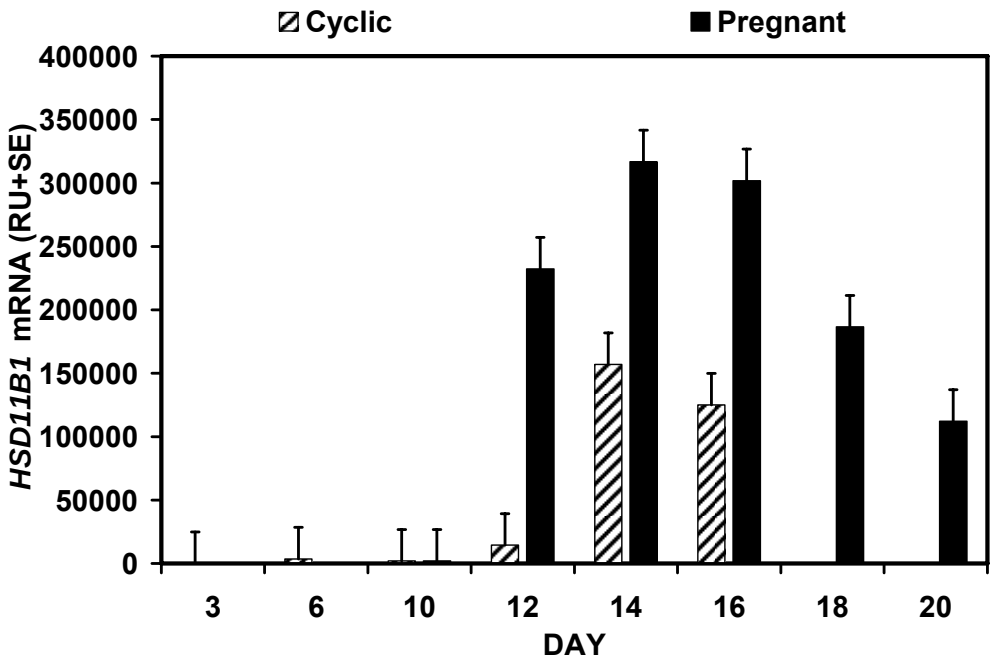

Fig. 4.1. Steady-state levels of *HSD11B1* mRNA in endometria of cyclic and pregnant ewes. Endometrial *HSD11B1* mRNA abundance was determined by slot blot hybridization analyses (see Materials and Methods). Between Days 12 and 16, endometrial *HSD11B1* mRNA levels were greater in pregnant than cyclic ewes (day x status, $P<0.01$).

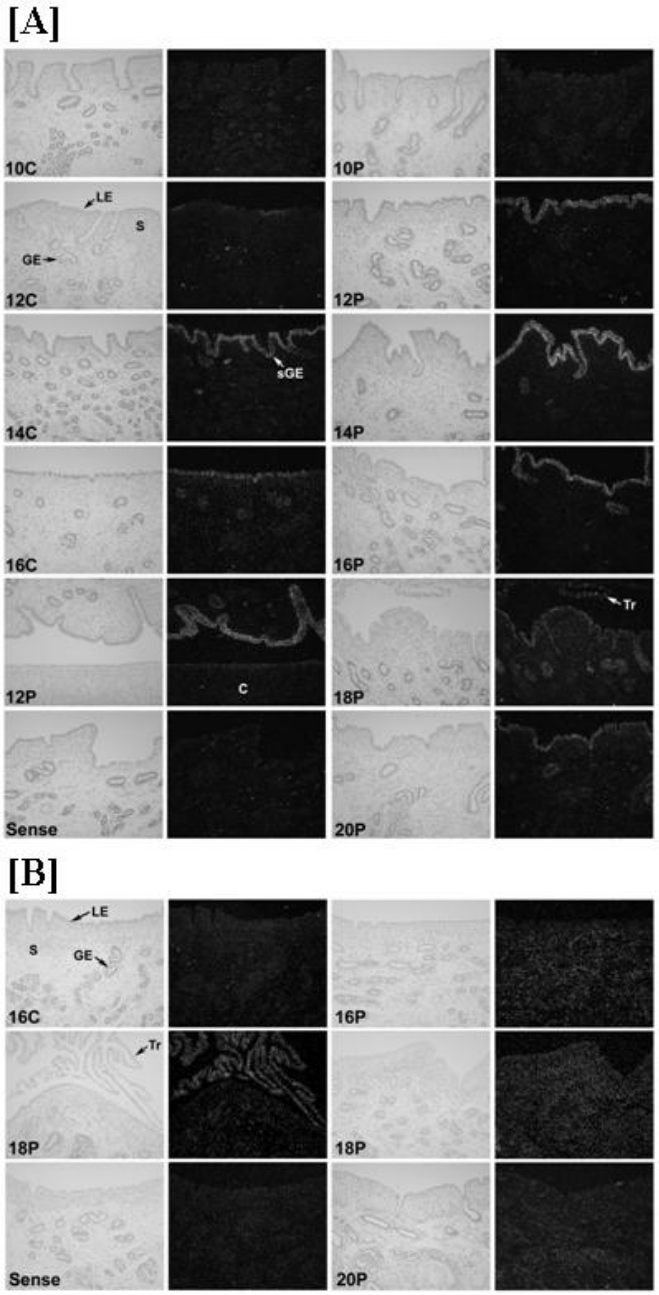

Fig. 4.2. *In situ* hybridization analysis of *HSD11B1* [A] and *HSD11B2* [B] mRNAs in uteri of cyclic and pregnant ewes. Cross-sections of the uterine wall from cyclic (C) and pregnant (P) ewes were hybridized with radiolabeled antisense or sense ovine cRNAs. Note that *HSD11B1* mRNA is most abundant in the endometrial LE and sGE, whereas *HSD11B2* mRNA is most abundant in the conceptus during early pregnancy. Legend: C, caruncle; LE, luminal epithelium; sGE, superficial glandular epithelium; GE, middle to deep glandular epithelium; S, stroma; Tr, trophectoderm. All photomicrographs are displayed at the same width of field (560 μm).

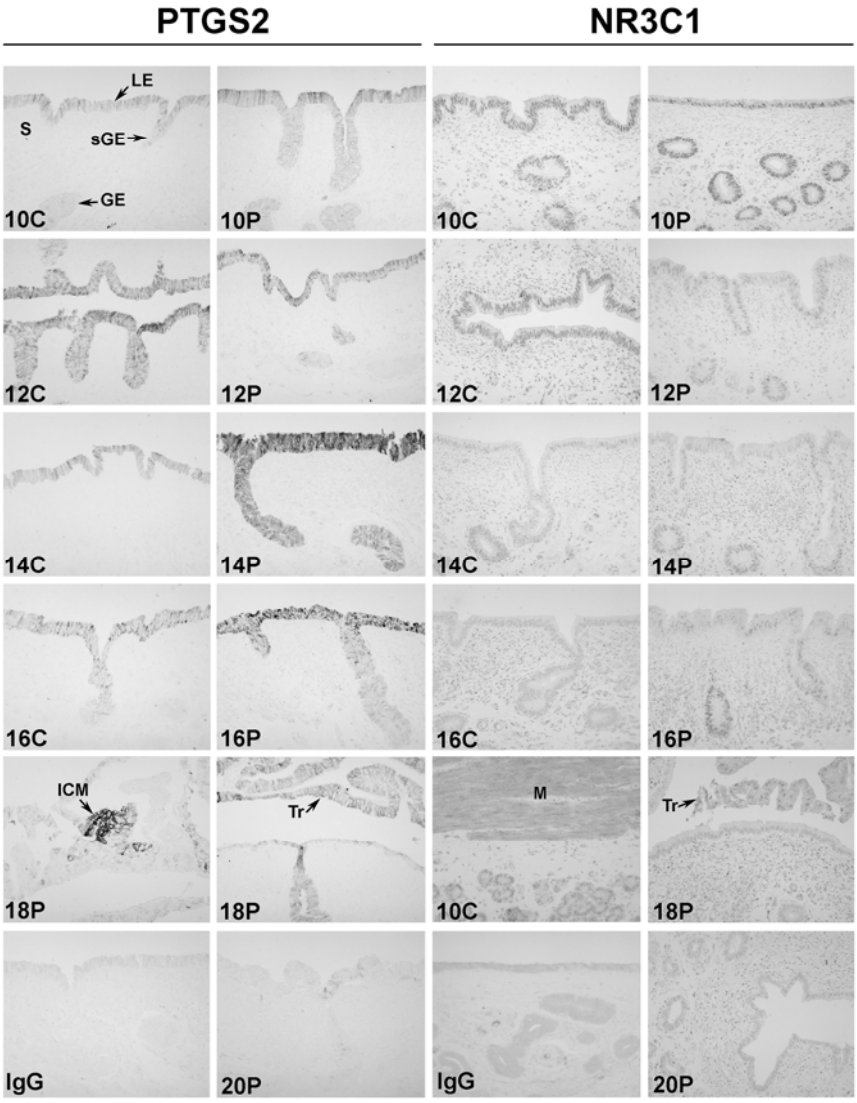

Fig. 4.3. Immunohistochemical analysis of PTGS2 and NR3C1 proteins in uteri of cyclic and pregnant ewes. Cross-sections of the uterine wall from cyclic (C) and pregnant (P) ewes were used for immunohistochemical using specific antibodies or non-immune IgG as a control (see Materials and Methods). Sections were not counterstained. Legend: LE, luminal epithelium; sGE, superficial glandular epithelium; GE, middle to deep glandular epithelium; ICM, inner cell mass; M, myometrium; S, stroma; Tr, trophectoderm. All photomicrographs are displayed at the same width of field (280 μm).

Circulating Levels of Cortisol During Early Pregnancy

As illustrated in Fig. 4.4, circulating levels of cortisol in serum were highest on Day 1 (~27 ng/ml) and then declined to between 5 and 15 ng/ml from Days 5 to 20 of pregnancy (quadratic effect of day, $P<0.01$).

HSD11B1 is Induced by P4 and Stimulated by IFNT

The temporal changes in endometrial *HSD11B1* mRNA in uterine epithelia of cyclic and early pregnant ewes observed in Study One supported the idea that its expression was regulated by ovarian P4 and a factor from the conceptus. Study Three utilized a sheep model in which circulating levels of P4 were prematurely elevated after estrus by treatment with exogenous P4 from Day 1.5 post-mating. This early P4 treatment is known to increase blastocyst size on Day 9 and conceptus growth into an elongated filamentous form on Day 12 that secreted more IFNT (Satterfield *et al.* 2006). As illustrated in Fig. 4.5A, *HSD11B1* mRNA abundance was two-fold (CO vs P4, $P<0.01$) greater in early P4-treated ewes on Day 9 and 2.4-fold (CO vs P4, $P<0.01$) greater on Day 12. Endometrial *HSD11B1* mRNA abundance was 23.6-fold (P4 vs P4+RU486, $P<0.001$) lower in P4 than P4+RU486-treated ewes on Day 12. *In situ* hybridization analysis revealed that *HSD11B1* mRNA was expressed most abundantly in the endometrial LE/sGE of CO- and P4-treated Day 12 ewes (Fig. 4.5B). The amount of PTGS2 protein in the endometrial LE/sGE was higher in P4-treated ewes on both Days 9 and 12, higher in Day 12 than Day 9 CO-treated ewes, but much lower to absent in Day 12 ewes receiving P4 and RU486 and Day 9 ewes receiving CO. NR3C1 protein was detected in all endometrial cell types and was more abundant in the endometrial LE/sGE of Day 9 and 12 early P4-treated ewes. Moreover, NRC3C1 protein was lower in uteri, particularly in the endometrial epithelia, of Day 12 ewes treated with P4 and RU486 as compared to uteri from Day 12 ewes receiving CO or early P4 treatment (Fig. 4.5B).

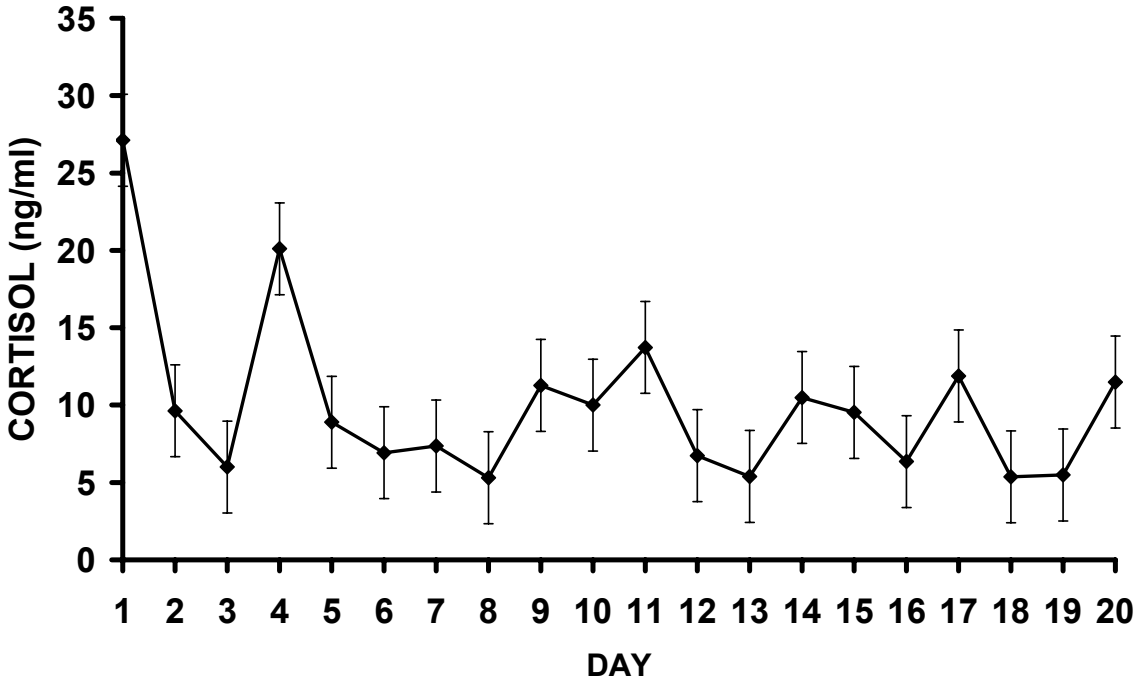

Fig 4.4. Circulating levels of cortisol in the serum of early pregnant ewes. Serum levels of cortisol were measured using a specific radioimmunoassay (see Materials and Methods) and were highest on Day 1, declined to Day 6 and remained constant thereafter (quadratic effect of day, $P<0.05$).

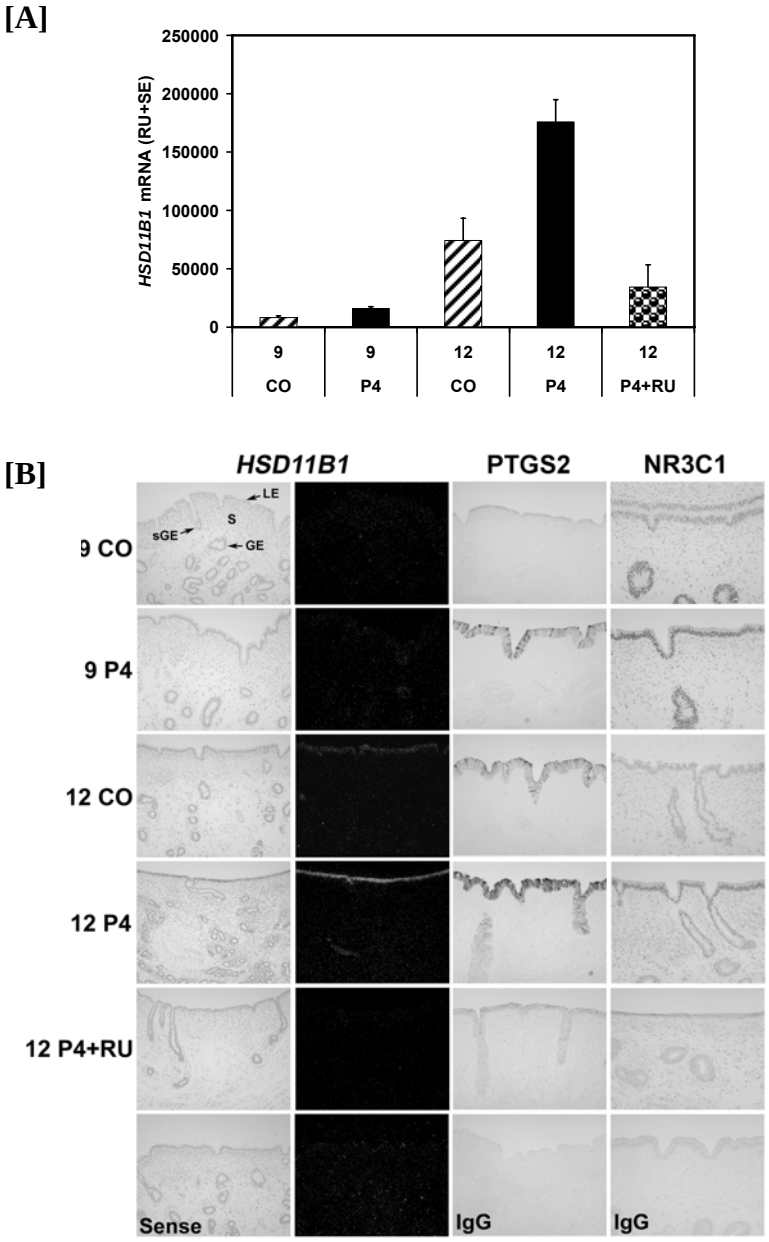

Fig. 4.5. Effects of progesterone during early pregnancy on *HSD11B1* mRNA, PTGS2 and NR3C1 in the ovine uterus. [A] Steady-state levels of *HSD11B1* mRNA in endometria determined by slot blot hybridization analysis. In ewes treated to Day 9, endometrial *HSD11B1* mRNA abundance was increased 2-fold by P4 (CO vs P4, P<0.01). In ewes treated to Day 12, *HSD11B1* mRNA was increased 2.3-fold by P4 (CO vs P4, P<0.01). Treatment of ewes with the anti-progestin RU486 from Days 8 to 12 reduced endometrial *HSD11B1* mRNA levels by 23.5-fold (P4 vs P4+RU486, P<0.01) compared to P4-treated ewes. The asterisk denotes an effect of treatment (*, P < 0.01). [B] *In situ* hybridization analysis of *HSD11B1* mRNA and immunohistochemical analysis of PTGS2 and NR3C1 protein. Legend: LE, luminal epithelium; sGE, superficial glandular epithelium; GE, glandular epithelium; S, stroma. Photomicrographs of in situ hybridization analysis are displayed at the same width of field (560 μm), whereas those of the immunohistochemical analysis are at 280 μm.

Study Four utilized a model extensively used by our laboratory to determine if genes are regulated by ovarian P4 and conceptus IFNT (Bazer & Spencer 2006). As illustrated in Fig. 4.6A, endometrial *HSD11B1* mRNA abundance was 3.3-fold ($P<0.01$, P4+CX vs P4+RU+CX) greater in ovariectomized ewes treated with P4 for 12 days. Further, endometrial *HSD11B1* mRNA levels were 2.2-fold greater ($P<0.01$, P4+CX vs P4+IFNT) in P4-treated ewes receiving intrauterine infusions of IFNT, but not in ewes receiving P4 and RU486 ($P>0.10$, P4+RU+CX vs P4+RU+IFNT). *In situ* hybridization analysis revealed that effects of P4 to induce and IFNT to stimulate *HSD11B1* expression in the endometrium were confined to the endometrial LE/sGE and upper GE (Fig. 4.6B). In ewes receiving P4 and RU486, *HSD11B1* mRNA observed in the subepithelial stroma was localized primarily to immune cells.

As observed in Study Three, PTGS2 and NR3C1 abundance paralleled *HSD11B1* mRNA in the endometrial LE/sGE and upper GE of uteri from P4-treated ewes (Fig. 4.6B). In P4-treated ewes, PTGS2 protein and NR3C1 protein was more abundant in the endometrial LE/sGE of ewes receiving IFNT. In ewes receiving intrauterine infusion of CX proteins, PTGS2 and NR3C1 proteins were much higher in the endometrial LE/sGE of ewes receiving P4 than P4 and RU486.

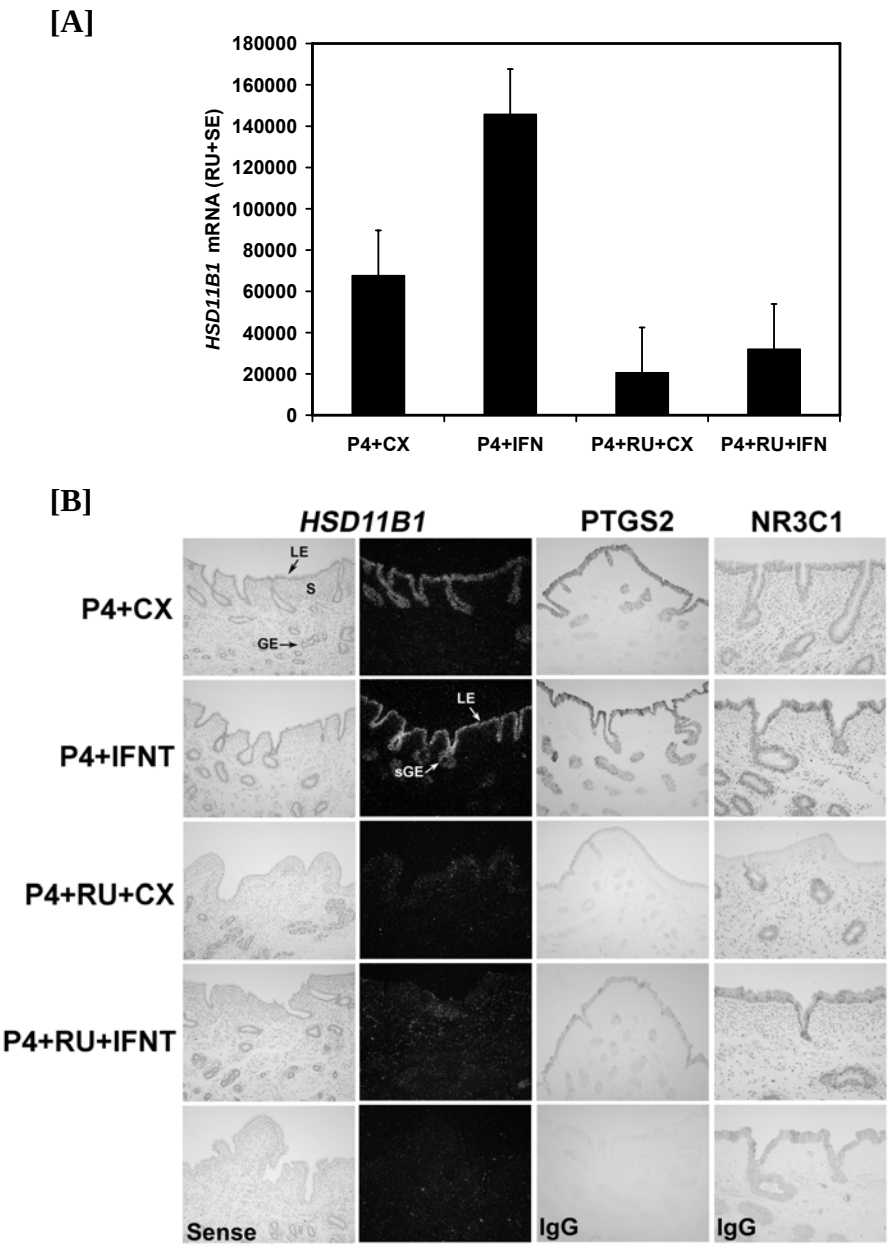

Fig. 4.6. Effects of progesterone and IFNT on *HSD11B1* mRNA, PTGS2 and NR3C1 in the ovine uterus. [A] Steady-state levels of *HSD11B1* mRNA in endometria were determined by slot blot hybridization analysis. Treatment of ewes with progesterone (P4) increased (*, $P<0.01$) endometrial *HSD11B1* mRNA abundance as compared to ewes receiving P4 and the antiprogestin RU486. Intrauterine IFNT increased *HSD11B1* mRNA ($P<0.01$) in P4-treated ewes, but not in P4+RU486-treated ewes. [B] *In situ* hybridization analysis of *HSD11B1* mRNA and immunohistochemical analysis of PTGS2 and NR3C1 protein. Legend: LE, luminal epithelium; sGE, superficial glandular epithelium; GE, glandular epithelium; S, stroma. Photomicrographs of *in situ* hybridization analysis and PTGS2 Immunolocalization are displayed at the same width of field (560 μm), whereas those of NR3C1 Immunolocalization are at 280 μm.

Discussion

Results of the present studies support the idea that ovarian P4, conceptus IFNT and PTGS2-derived prostaglandins regulate *HSD11B1* expression in the endometrial LE/sGE. In Studies Three and Four, *HSD11B1* mRNA was induced by P4 in endometrial LE/sGE, but this effect was blocked by administration of RU486, a PGR and NR3C1 antagonist (Baulieu 1989). In both studies, *HSD11B1* mRNA and PTGS2 protein were absent from the endometrial epithelia of ewes treated with RU486. Progesterone upregulation of PTGS2 expression in the ovine uterine endometrium was reported previously (Charpigny *et al.* 1997b). Although postulated previously (Yang *et al.* 1996), results of the present studies are the first to demonstrate that P4 induces *HSD11B1* expression in the ovine endometrial LE/sGE. Paradoxically, the increase in *HSD11B1* and PTGS2 expression in endometrial LE/sGE between Days 10 and 12 of the cycle and pregnancy is temporally associated with loss of *PGR* expression in the same epithelia (Wathes & Hamon 1993, Spencer & Bazer 1995, Satterfield *et al.* 2006). Continuous exposure of the sheep uterus to P4 for 8 to 10 days is required for loss of PGR in endometrial epithelia, which does not occur in the stroma or myometrium (Spencer & Bazer 1995, Johnson *et al.* 2000). Loss of PGR in the endometrial LE/sGE between Days 10 and 12 is associated with the onset of expression of *HSD11B1* and a number of other different genes, including PTGS2, LGALS15, GRP, IGFBP1, SLC2A1, SLC5A1, and solute carrier family 7 (cationic amino acid transporter, y+ system), member 2 (SLC7A2), that are implicated as regulators of cellular processes involved in conceptus elongation (see CHAPTER III and (Charpigny *et al.* 1997b, Gray *et al.* 2004, Satterfield *et al.* 2006, Satterfield *et al.* 2008, Song *et al.* 2008, Gao *et al.* 2009a, Gao *et al.* 2009b)). In addition to being an antiprogestin, RU486 is also a high affinity antagonist of NR3C1 (Baulieu 1989), and loss of NR3C1 was observed along with *HSD11B1* mRNA in endometria of ewes treated with RU486 in both Studies Three and Four. It is possible that effects of P4 on *HSD11B1* and PTGS2 expression are mediated by NR3C1, because P4 regulates gene expression in human trophoblasts via an interaction with NR3C1 (Karalis *et al.* 1996, Patel *et al.* 2003). The effects of a specific

NR3C1 antagonist on uterine gene expression and function during early pregnancy has not been reported in sheep, although the RU486 treatment used in Study Three elicited embryo loss in sheep (Satterfield *et al.* 2006).

In Study One, *HSD11B1* mRNA and PTGS2 abundance in the endometrial LE/sGE was clearly increased by the presence of a conceptus as early as Day 12. IFNT, a novel member of the type I IFN family, is the most abundant protein secreted by the elongating conceptus (Roberts 2007). In Study Three, endometrial *HSD11B1* and PTGS2 expression was greater in early P4-treated Day 12 ewes that contained an elongating, filamentous conceptus producing more IFNT as compared to control ewes containing a much smaller ovoid conceptus producing little or no IFNT (Satterfield *et al.* 2006). In Study Four, infusion of recombinant ovine IFNT into uteri of P4-treated ewes increased abundance of *HSD11B1* mRNA in the endometrial LE/sGE. Collectively, results of the present studies indicate that *HSD11B1* is another novel P4-induced and IFNT-stimulated gene along with LGALS15, GRP, IGFBP1, SLC2A1, SLC5A1, and SLC7A2 (Gray *et al.* 2004, Song *et al.* 2008, Gao *et al.* 2009a, Gao *et al.* 2009b). The mechanism whereby IFNT stimulates expression of those genes in the LE/sGE and upper GE of the endometrium is not known. The canonical JAK-STAT signaling pathway used by type I IFNs is not active in the endometrial LE/sGE and upper GE of the ovine uterus during early pregnancy, because several essential transcription factors (STAT1, STAT2 and IRF9 that form the ISGF3 transactivator complex) that mediate canonical type I IFN signaling are not expressed in those epithelia due to expression of IRF2, a potent transcriptional repressor (Choi *et al.* 2001, Fleming *et al.* 2009). Thus, IFNT likely regulates gene expression in endometrial LE/sGE using a non-canonical signaling pathway, such as MAPK p38 cascade and the phosphatidylinositol 3-kinase cascade reported for other IFNs (Platanias 2005).

When comparing results of Studies One and Four, it was apparent that the two-fold increase in endometrial *HSD11B1* mRNA levels elicited by intrauterine infusions of IFNT into uteri of P4-treated ewes was rather modest compared to the 11.3-fold higher level of *HSD11B1* mRNA observed in the endometria of Day 12 pregnant as compared

to Day 12 cyclic ewes. The ovoid to slightly tubular Day 12 ovine conceptus produces little IFNT compared to large amounts produced by the elongating Day 14 and 16 conceptuses (Ashworth & Bazer 1989). Thus, another factor produced by the ovine conceptus likely regulates endometrial *HSD11B1* expression with prostaglandins being a strong candidate. The trophectoderm cells of the ovine conceptus contain abundant levels of PTGS2 (Charpigny *et al.* 1997a) and synthesize and secrete a number of different prostaglandins including PGE2 and PGF2α (Lewis & Waterman 1983, Lewis & Waterman 1985). In pregnant ewes, the increase in *HSD11B1* expression in endometrial LE/sGE between Days 10 and 14 and the subsequent decline in expression between Days 16 and 20 is temporally associated with PG production by the ovine conceptus and PG levels in the uterine lumen (Marcus 1981, Lewis 1989, Charpigny *et al.* 1997b). In the endometrial LE/sGE, PTGS2 abundance was coordinate with *HSD11B1* expression in the present studies and in agreement with other studies (Charpigny *et al.* 1997b). In the ovine placenta, *HSD11B1* expression and/or bioactivity is stimulated by a positive feedback loop involving cortisol and prostaglandins as well as proinflammatory cytokines (IL1B and TNFA) and P4 (see (Michael *et al.* 2003, Michael & Papageorghiou 2008) for review), thereby establishing a positive feed-forward loop implicated in the timing of parturition (Challis *et al.* 2000). Given that circulating levels of cortisol did not increase in ewes during early pregnancy (Study Four), it is unlikely that extrauterine cortisol is involved in stimulating endometrial *HSD11B1* expression. Collectively, available results support the idea that *HSD11B1* expression in ovine endometrial LE/sGE and upper GE during early pregnancy is regulated by IFNT and prostaglandins from the elongating conceptus and HSD11B1-regenerated cortisol within the endometrial epithelia.

In addition to conceptus-derived prostaglandins, it is plausible that endometrial-derived prostaglandins regulate *HSD11B1* expression, because changes in *HSD11B1* mRNA and PTGS2 protein were coordinate in the endometrial LE/sGE and upper GE during the estrous cycle. Both pregnant and nonpregnant endometria have similar abilities to synthesize prostaglandins (Silvia *et al.* 1984, Zarco *et al.* 1988a, Zarco *et al.*

1988b). In non-pregnant bovine endometria, PGF2α stimulated HSD11B1 activity (Lee *et al.* 2009). In placentae, cortisol increases prostaglandin synthesis (Sun and Myatt 2003; Li, Gao et al. 2006) by stimulating PTGS2 transcription (Whittle, Patel et al. 2001). Thus, the increased abundance of PTGS2 on Days 12, 14 and 16 of the estrous cycle and early pregnancy in endometrial LE/sGE in Study One could be due to higher levels of prostaglandins, HSD11B1 and cortisol acting in an autocrine manner on those epithelia. Moreover, active cortisol generated by HSD11B1 in uterine LE/sGE could activate NR3C1 and regulate expression of target genes, particularly since PGR are absent from the same epithelia. Future studies should determine the biological roles of cortisol and prostaglandins in regulation of HSD11B1 and PTGS2 gene expression and bioactivity in the ovine uterus during the estrous cycle and early pregnancy. Finally, it is possible that other factors within the endometrium and from the conceptus regulate *HSD11B1* and PTGS2 expression in the ovine uterus.

The present studies established the presence of both HSD11B1 and NR3C1 in the ovine uterus during early pregnancy; however, the biological roles of HSD11B1-regenerated cortisol and NR3C1 mediated actions are unknown. Despite extensive studies of the developmental consequences of increased glucocorticoid exposure in mid- to late pregnancy, relatively little is known regarding the significance of HSD11B1 and glucocorticoids in early pregnancy in any species (see (Michael *et al.* 2003, Michael & Papageorghiou 2008). Clear physiological roles for placental HSD11B1 in parturition and HSD11B2 in protection of the fetus from excess cortisol have been established (see (Michael & Papageorghiou 2008) for review). Moreover, HSD11B2 is expressed and functional in first trimester trophoblasts of the human placenta where it has been implicated in successful embryo attachment and implantation (Arcuri *et al.* 1998). Glucocorticoids are hypothesized to exert many actions that could impact both negatively and positively on key aspects of early pregnancy (see (Michael & Papageorghiou 2008) for review). The positive effects would promote pregnancy (e.g. stimulation of hCG secretion, immunosuppression, promotion of trophoblast growth/invasion, and increased amino acid transport), whereas the negative effects

would compromise the pregnancy (e.g. inhibition of cytokine-prostaglandin signaling, restriction of trophoblast invasion, induction of apoptosis, and inhibition of embryonic and placental growth). Of particular interest, pregnancy rates are increased in women undergoing standard *in vitro* fertilization who receive synthetic glucocorticoids prior to or immediately after embryo transfer (Boomsma *et al.* 2007).

In summary, the present studies established that: HSD11B1 is expressed predominantly in ovine uterine epithelia, whereas HSD11B2 is expressed predominantly in the conceptus during early pregnancy; HSD11B1 and PTGS2 expression are coordinate in the endometrium and during conceptus elongation; P4 induces and IFNT modestly stimulates HSD11B1 expression in the endometrium; and NR3C1 is expressed in most uterine and conceptus cell types during early pregnancy. Collectively, these results and others support our working hypotheses that ovarian P4 and prostaglandins control *HSD11B1* expression in endometrial epithelia and cortisol produced by HSD11B1 acts via NR3C1 to regulate endometrial functions important for conceptus elongation and implantation during the peri-implantation period of pregnancy.

CHAPTER V
INTRAUTERINE INFUSION OF MELOXICAM RETARDS OVINE CONCEPTUS ELONGATION AND ALTERS ENDOMETRIAL GENE EXPRESSION AND FUNCTION

Introduction

In ruminants, pregnancy recognition signaling and implantation is dependent on maternal support of blastocyst growth and development into an elongated conceptus (embryo/fetus and associated membranes) (Bazer 1992, Spencer *et al.* 2007). The ovine blastocyst hatches from the zona pellucida on Day 8, develops into an ovoid or tubular form by Day 11, and is termed a conceptus. The conceptus begins to elongate on Day 12 and becomes filamentous, reaching 14 cm or more in length by Day 16. Conceptus elongation involves exponential increases in length and weight and a decrease in diameter of the trophectoderm, differentiation of the extraembryonic membranes (Wales & Cuneo 1989), as well as transient attachment of the conceptus trophectoderm to the uterine LE for outgrowth and migration. IFNT, produced by the elongating conceptus, exerts antiluteolytic effects on the endometrium to maintain CL function and its production of P4. Progesterone, in turn, stimulates and maintains uterine endometrial functions necessary for conceptus growth, implantation, placentation and successful development of the fetus to term (Spencer *et al.* 2004b). Additionally, IFNT acts on the endometrium to induce or increase expression of many genes that potentially regulate conceptus growth and development (Spencer *et al.* 2008, Bazer *et al.* 2009). These genes encode for various secreted enzymes, binding proteins, and growth factors from the endometrial LE and GE that are presumed to support the conceptus throughout early development (Bazer 1975, Bazer *et al.* 1979) and are hypothesized to play a role in successful growth of the conceptus to term.

We recently reported results from an ovine model of accelerated blastocyst growth and conceptus development that advanced circulating levels of P4 during

metestrus (Satterfield *et al.* 2006). That model has been used to identify numerous P4-regulated genes, many of which are expressed in the endometrial LE/sGE consistent with a role in pre-implantation blastocyst growth, development, and conceptus elongation (Satterfield *et al.* 2006, Satterfield *et al.* 2008, Song *et al.* 2008). For example, SLC2A1 and SLC5A1 mediate the transfer of glucose across the plasma membrane (Pantaleon & Kaye 1998). Glucose has a recognized role in pre-implantation embryonic development (Devreker & Englert 2000) and can amplify trophoblast cell growth and proliferation during pregnancy (Wen *et al.* 2005). Other genes encode for secreted proteins and function in cell migration and attachment (*IGFBP1* and *LGALS15*), cell proliferation (*GRP*), or regulate IGFs (*IGFBP3*). Enzymes and their inhibitors (*CST3* and *CTSL*) process proteins and are perhaps involved in endometrial remodeling and placentation in sheep (Song *et al.* 2007). Other genes encode enzymes (*HSD11B1* and *PTGS2*) that produce factors implicated in endometrial function and/or conceptus development. Expression of these endometrial genes is associated with loss of the *PGR* in the LE/sGE between Days 10-11 and the GE between Days 12-13 post-mating (Wathes & Hamon 1993, Spencer & Bazer 1995). Exposure of the endometrium to P4 for at least 8 to 10 days downregulates *PGR* expression in the endometrial epithelia, but not stroma or myometrium (Spencer & Bazer 2002). IFNT from the elongating conceptus acts on the endometrium to induce or enhance expression of a myriad of genes, collectively termed ISGs (Hansen *et al.* 1999, Spencer *et al.* 2004a, Spencer 2007, Bazer *et al.* 2008). Two classical ISGs, *RSAD2* and *ISG15*, are hypothesized to regulate conceptus development. IFNT enhances expression of a number of the aforementioned genes that encode for secreted proteins (*IGFBP1*, *IGFBP3*, *LGALS15*, *CST3*, *CTSL1*, *GRP*), transporters for glucose (*SLC2A1*), or enzymes (*HSD11B1*, *PTGS2*), further implicating these genes in the events of early pregnancy.

Effects of P4 and IFNT on endometrial genes in the ovine uterus have been well documented (Spencer *et al.* 2008). However, recent work in cattle has implicated prostaglandins (PGs) in the events of early pregnancy. In the bovine endometrium, IFNT from the conceptus upregulates endometrial *PTGS2* gene expression, PTGS2

enzymatic activity, and PGE2 production via the p38 MAPK pathway (Asselin *et al.* 1997, Arosh *et al.* 2004b, Emond *et al.* 2004, Krishnaswamy *et al.* 2009). In other species, PGs regulate numerous reproductive events including ovulation, implantation, parturition, and luteolysis (Poyser 1995, Dubois *et al.* 1998). In sheep, it is well established that the conceptus and endometria synthesize and secrete PGs during early pregnancy, particularly PGE2 and PGF2α (Lewis & Waterman 1985). PTGS2, the predominant enzyme responsible for the production of PGs, is transiently expressed in the ovine endometrium from Days 12 to 15 of the estrous cycle and Days 12 to 17 of pregnancy (Charpigny *et al.* 1997b). Furthermore, *PTGS2* mRNA and protein is localized to the endometrial LE and GE in cyclic and pregnant ewes (Kim *et al.* 2003). Studies from our lab have further implicated a role for PGs in endometrial gene expression (CHAPTERS III and IV). P4 induced and IFNT stimulated *IGFBP1* and *HSD11B1* mRNA expression in sheep uterine epithelia; however, IFNT stimulation was modest in pregnant ewes compared to corresponding day of the estrous cycle. Further, *HSD11B1* and PTGS2 expression was coordinate in the endometrial epithelia, and NR3C1 was present in all endometrial cell types (CHAPTER IV). Collectively, these results support ideas that *IGFBP1* and *HSD11B1* expression is regulated by P4, IFNT and prostaglandins, and that HSD11B1-regenerated cortisol acts via NR3C1 to regulate endometrial functions during early pregnancy in the ovine uterus. Thus, our working hypothesis is that PGs regulate endometrial expression and function of genes associated with ovine conceptus growth, development and elongation. In order to test this hypothesis, a study was conducted to determine effects of intrauterine infusion of meloxicam, a selective PTGS2 inhibitor, on (1) peri-implantation conceptus development and (2) endometrial function in sheep.

Materials and Methods

Experimental Design

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.

Study One

Ewes (n=10) were mated at estrus (Day 0) to rams of proven fertility. On Day 8 post-mating, ewes underwent a mid-ventral laparotomy, and the uterine horn ipsilateral to the ovary with a visible corpus luteum received a catheter connected to a preloaded and equilibrated Alzet 2ML1 Osmotic Pump (see Fig. 5.1). The Alzet pump was affixed to the intercornual ligament between the uterine horns, oviduct and ovary using medical adhesive and then secured using suture. Ewes (n=5 per treatment) received pumps containing either: (1) vehicle control (2 ml of 2% ethanol saline); or (2) meloxicam (1225 ng in 2 ml vehicle; Boehringer Ingelheim Pharmaceuticals), a partially selective PTGS2 inhibitor (13.1 times more inhibitory for PTGS2 than PTGS1) (Pairet *et al.* 1998) that is effective in sheep (Rac *et al.* 2007, Shukla *et al.* 2007). The Alzet 2ML1 Osmotic Pump delivers 10 µl/h (240 µl/day) for 7 days. All ewes were necropsied on Day 14 post-mating. The uterine lumen was flushed with 20 ml sterile 10 mM Tris buffer (pH 7.2) on Day 14 of pregnancy and the flushing was examined for the presence of a morphologically normal conceptus. At hysterectomy, several 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 and then dehydrated and embedded in Paraplast-Plus (Oxford Labware, St. Louis, MO). The remaining endometrium was physically dissected from myometrium, frozen in liquid nitrogen, and stored at -80°C for subsequent RNA extraction. In monovulatory pregnant ewes, uterine tissue samples were only from the ipsilateral uterine horn to the ovary bearing the CL.

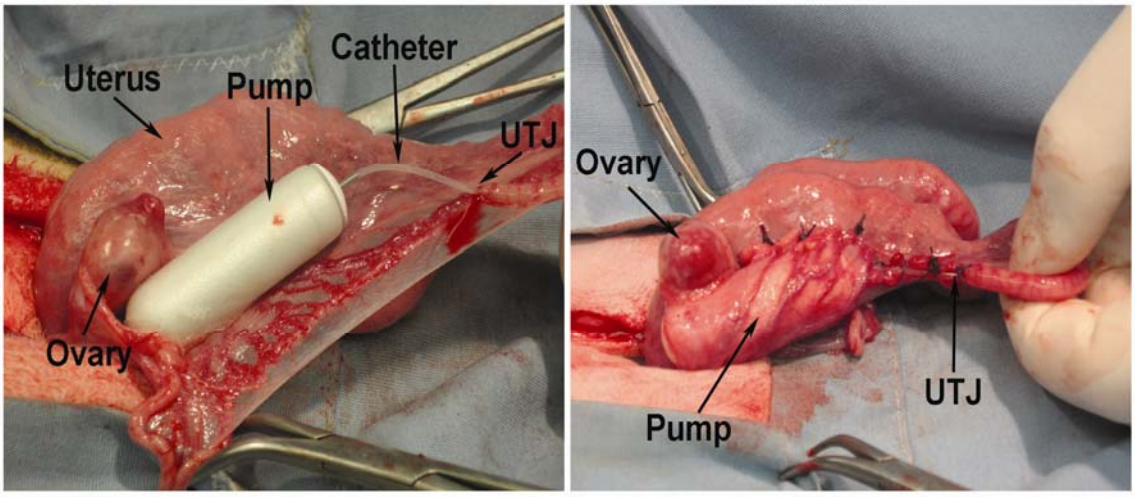

Fig. 5.1. Implantation of an Alzet osmotic pump and placement of catheter into the uterine lumen of a ewe. The Alzet osmotic pump was affixed to the intercornual ligament between the uterine horns, oviduct and ovary using medical adhesive. The catheter and pump were then secured using suture.

Slot Blot Hybridization Analysis

Total cellular RNA was isolated from frozen ipsilateral endometrium using Trizol reagent (Gibco-BRL, Bethesda, MD) according to manufacturer's instructions. The quantity and quality of total RNA was determined by spectrometry and denaturing agarose gel electrophoresis, respectively. Steady-state levels of *IGFBP1*, *IGFBP3*, *LGALS15, HSD11B1, SLC2A1*, *SLC5A1*, *RSAD2*, *ISG15*, *GRP*, *CST3*, and *CTSL* mRNAs and *18S* cRNA in endometria were assessed by slot blot hybridization as described previously (Spencer *et al.* 1999, Satterfield *et al.* 2008). Radioactivity associated with slots was quantified using a Typhoon 8600 MultiImager (Molecular Dynamics, Piscataway, NJ). Data are expressed as relative units (RU).

Statistical Analyses

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

Results

Elongating and filamentous conceptuses (12 to >14 cm) were recovered from all 5 control ewes implanted with a pump releasing control vehicle. In contrast, ovoid (~2 mm; n=3), tubular (~1 cm; n=1), and a very fragmented conceptus (n=1) were recovered from the ewes implanted with a pump releasing the PTGS2 inhibitor meloxicam (data not shown).

Steady-state levels of endometrial *IGFBP1*, *IGFBP3*, *LGALS15*, *HSD11B1*, *SLC2A1*, *GRP*, and *CST3* mRNAs were reduced ($P<0.05$) in endometria from meloxicam-infused compared to vehicle-control ewes (Table 5.1). Consistent with

retarded conceptus elongation, expression of two classical IFNT-stimulated genes (*RSAD2* and *ISG15*) were lower ($P<0.05$) in endometria of meloxicam-infused ewes. Interestingly, *SLC5A1* mRNA levels were increased ($P<0.01$) by meloxicam infusion, whereas *CTSL* mRNA levels were not affected ($P>0.10$).

Table 5.1. Effect of intrauterine infusion of meloxicam on endometrial mRNA levels in the ovine uterus

GENE	CONTROL	TREATMENT[a]
IGFBP1	100%	45%$^{+}$
IGFBP3	100%	62%$^{+}$
LGALS15	100%	61%$^{+}$
HSD11B1	100%	61%$^{+}$
SLC2A1	100%	54%$^{+}$
SLC5A1	100%	181%*
RSAD2	100%	15%*
ISG15	100%	25%*
GRP	100%	55%$^{+}$
CST3	100%	55%*
CTSL	100%	130%

[a]Data are shown as percentages of mRNA levels in meloxicam-treated endometria relative to that in endometria of control ewes (designated as 100%). $^{+}P<0.05$; $^{*}P<0.01$

Discussion

The present study supports our working hypothesis that PTGS2-derived PGs regulate conceptus elongation and influence endometrial gene expression during early pregnancy. In 1982, LaCroix and Kann (Lacroix & Kann 1982) reported that treatment of pregnant ewes from Days 7 to 22 post-mating with two non-selective PTGS inhibitors, indomethacin (300 mg subcutaneous daily) and aspirin (1 g intravenous daily), decreased endometrial and conceptus production of PGs as assessed on Day 23. Pregnancy was not affected by those treatments, because a morphologically normal conceptus was present on Day 23. Thus, they concluded ovine blastocyst implantation and establishment of pregnancy were not dependent upon PGs as had been demonstrated in the rodent model. While the levels of indomethacin and aspirin used should have exceeded the half maximal inhibitory concentration (IC_{50}) for each compound, it was not determined if these compounds were able to access the uterine lumen and effectively inhibit the high levels of PTGS2 in trophectoderm of the elongating conceptus. From Days 12-13, blood flow to the uterus is relatively low (Reynolds *et al.* 1984) and the endometrial LE is intact, whereas blood flow is higher on Day 23 and much of the LE has been removed by fusion with trophoblast giant binucleate cells (BNC) (Wooding 1984). Thus, results of the LaCroix and Kann (Lacroix & Kann 1982) experiment could be due to inadequate levels of the inhibitors within the lumen during early pregnancy.

IGFBP1, *IGFBP3*, *HSD11B1*, *LGALS15*, and *GRP* are P4-regulated genes implicated in peri-implantation conceptus elongation (Satterfield *et al.* 2006, Song *et al.* 2008). We have previously determined *IGFBP1* to be a marker of conceptus elongation in ruminants (CHAPTER III) while *IGFBP3* expression was only coordinate with elongation in sheep but not in cattle. HSD11B1 provides a source of active cortisol (Stewart & Krozowski 1999) to bind its nuclear receptor subfamily 3, group C, member 1 (glucocorticoid receptor) (NR3C1) and regulate gene expression in the ovine endometrium. Previous studies from our laboratory have shown *IGFBP1*, *HSD11B1* and PTGS2 expression are coordinate in the endometrial epithelia during early pregnancy (CHAPTER IV), further supporting a role for PGs during conceptus elongation. Both

LGALS15 and *GRP* encode for secreted proteins that, collectively, regulate trophectoderm proliferation, migration, and adhesion (Patel *et al.* 2006, Farmer *et al.* 2008). Thus, IGFBP1, IGFBP3, LGALS15, HSD11B1 and GRP may all contribute to conceptus elongation during early pregnancy. Interference with PG production reduced gene expression and function, contributing to failed conceptus elongation.

Endometrial genes, such as *RSAD2* and *ISG15*, are classical IFNT-stimulated genes implicated in the events of implantation and/or conceptus elongation. RSAD2 is an antiviral protein produced in response to Type I Interferons that may modulate local immune cells to ensure acceptance of the allogenic conceptus and stimulate cytokine production (Hansen 1995, Croy *et al.* 2003). *ISG15* is expressed in uteri of ruminants in response to IFNT and its activity increases during pregnancy, making it another candidate gene for regulating peri-implantation (Joyce *et al.* 2005). As expected, expression of these genes was lower in endometria of meloxicam-infused ewes, consistent with retarded conceptus elongation. All of the conceptuses in the meloxicam-treated ewes failed to elongate properly, indicating reduced IFNT production since the spherical Day 12 conceptus produces little IFNT (Ashworth & Bazer 1989). These results imply indirect effects on endometrial genes, since lack of PGs alter gene expression imperative for conceptus elongation and subsequent IFNT production, which then affects genes classically stimulated by IFNT.

Both *SLC2A1* and *SLC5A1* mRNA and proteins are abundant in the ovine LE, sGE, and conceptus (Gao *et al.* 2009a). While SLC2A1 is a P4 induced and IFNT stimulated gene, SLC5A1 is induced by P4 but not further stimulated by IFNT (Gao *et al.* 2009a). In the present experiment, PG inhibition decreased *SLC2A1*, but increased endometrial *SLC5A1* levels. Both genes are localized to the same cell types, but clearly each responds differently to the hormones of pregnancy. Because *SLC2A1* expression is dependent on IFNT, conceptuses from ewes receiving meloxicam infusion failed to elongate properly and, thus, produced less IFNT. Lack of IFNT, caused by reduced PG production, explains decreased endometrial *SLC2A1* mRNA. The increase in *SLC5A1* mRNA can be rationalized in at least two ways. Perhaps IFNT and/or PGs inhibit

SLC5A1 which peaks on Day 12 of pregnancy (Gao *et al.* 2009a), i.e., before significant amounts of IFNT are being produced (Ashworth & Bazer 1989) and declines thereafter. In addition, large amounts of PGs are being produced by both the endometrium and conceptus during this period (Lewis & Waterman 1985, Lewis 1989). Thus, a combination of increasing IFNT and PGs may contribute to declining levels of SLC5A1 during normal pregnancy. The disruption of IFNT and PG production in the present experiment may account for the increase in *SLC5A1* mRNA levels. Alternatively, other transporters not dependent on IFNT or PG stimulation may be upregulated to compensate for decreased glucose transport.

While *CST3* levels decreased in meloxicam-infused ewes, *CTSL* mRNA levels did not change. Because CST3 is an inhibitor of CTSL (Charpigny *et al.* 1997a, Charpigny *et al.* 1997b, Spencer *et al.* 2008), downregulation of endometrial *CST3* mRNA may have permitted unregulated expression of *CTSL*. Both genes are induced in the presence of P4 and further stimulated by IFNT (Song *et al.* 2005, Song *et al.* 2006). Why expression of *CST3* is reduced, but *CTSL* expression is unaffected remains unclear. It is possible that the decrease in production of PGs in meloxicam-treated ewes fails to adequately stimulate *CST3* mRNA expression. However, *CTSL* remains constitutively expressed in uterine endometria, suggesting that *CTSL* regulation is more complex than other genes evaluated.

While many endometrial genes were downregulated from meloxicam treatment, no gene expression was completely ablated. It is important to note that meloxicam preferentially inhibits PTGS2 within the endometrium and conceptus; however, PTGS1 is constitutively expressed in the ovine endometrium during early pregnancy (Charpigny *et al.* 1997a, Charpigny *et al.* 1997b). Therefore, while meloxicam infusion decreased PG production by PTGS2, the uterine endometrium could still produce moderate levels of PGs via PTGS1, thus maintaining low levels of endometrial gene expression. Moreover, different tissues throughout the body express varying levels of PTGS1 and PTGS2 (Sales & Jabbour 2003). Meloxicam infusion targeted locally produced PGs within the uterus, but circulating PGs may have also influenced endometrial gene

expression. Thus, PGs may be derived from the endometrium, conceptus, circulation, or perhaps a combination of all three sources. While results from this experiment demonstrate the importance of PGs for ovine conceptus elongation, the source of these PGs remains to be determined.

In summary, PG production stimulates endometrial gene expression and promotes proper conceptus elongation. Conceptuses that fail to elongate produce little IFNT, which further impacts gene expression of classical ISGs. The effects of PTGS2 inhibition and subsequent decline in PG production on conceptus elongation and endometrial gene expression support the hypothesis that PGs are important regulators of peri-implantation development in sheep. Both conceptuses and endometria of sheep and cattle synthesize and secrete a variety of PGs during early pregnancy (Lewis & Waterman 1985, Lewis 1989). While PGF2α and PGE2 are well studied, little information exists to define the roles of other PGs in early pregnancy. Moreover, identifying the source of PGs as conceptus and/or endometrial in origin may also provide valuable insight into gene regulation and elongation. It is probable that additional genes are involved in conceptus elongation and are perhaps regulated, in part, by PGs. Identifying novel genes or better characterizing existing ones would greatly improve the existing knowledge of conceptus elongation. Understanding the biological function of PGs and their interactions with endometrial genes may further our understanding of early pregnancy events and perhaps contribute to solutions for early pregnancy loss.

CHAPTER VI
CONCLUSIONS

Conceptus elongation and implantation require effective cross-talk between the uterus and developing conceptus. This is a complex process which involves a myriad of endocrine cues and subsequent gene responses, both by the uterine endometrium and/or conceptus trophectoderm. While certain aspects of regulatory pathways governing changes in gene expression have been well characterized, little work has been done to explore novel methods of gene regulation and how affected genes influence conceptus elongation. Results discussed in this thesis are the first to provide evidence for the involvement of PGs in conceptus elongation via regulation of endometrial gene expression.

Among factors hypothesized to regulate ovine endometrial gene expression and subsequent conceptus elongation and implantation, ovarian P4 and conceptus-derived IFNT have been well described in the literature (see (Spencer & Bazer 2002, Spencer *et al.* 2008) for reviews). The objective of the present study was to enhance the body of knowledge regarding conceptus elongation through identification and evaluation of genes that may be influenced by P4 and IFNT. In fact, P4 induced and IFNT further stimulated expression of genes specific to the LE and sGE, including *IGFBP1* and *HSD11B1* (CHAPTERS III and IV). It is important to note that the upregulation in gene expression following stimulation by IFNT in experimentally manipulated ewes was only modest compared to the greater degree of upregulation detected in pregnant vs. cyclic ewes on the same day after onset of estrous. These results provide a rationale for the hypothesis that factors other than IFNT upregulate endometrial genes during the period of conceptus elongation.

The coordinate expression of PTGS2, *IGFBP1* and *HSD11B1* in the ovine endometrium during early pregnancy also implicates PGs in gene regulation. To better characterize the effects of PGs *in vivo*, ewes were fitted with indwelling Alzet osmotic pumps which provided intrauterine infusion of either meloxicam, a selective PTGS2

inhibitor, or vehicle control. Ewes with less access to PGs, due to the meloxicam exposure, had reduced expression of many genes hypothesized to be important for conceptus implantation and elongation, including *IGFBP1*, *IGFBP3*, and *HSD11B1*. Importantly, all conceptuses recovered from ewes treated with meloxicam failed to elongate properly (CHAPTER V). These results suggest that PGs are required for upregulation of *IGFBP1*, *IGFBP3*, and *HSD11B1* as well as conceptus elongation. However, determining a functional role for those genes in early pregnancy and their involvement in conceptus elongation is an important area of investigation that remains to be addressed.

The biological significance of *IGFBP1* and *IGFBP3* in ruminant conceptus elongation was evaluated in this study because expression of both *IGFBP1* and *IGFBP3* is concomitant with conceptus elongation; however, only *IGFBP1* expression is coincident with the period of conceptus elongation in cattle. Furthermore, *IGFBP3* expression was downregulated in the presence of P4 and did not change with the addition of IFNT, indicating its expression is regulated in an alternate fashion (CHAPTER III). Therefore, *IGFBP1*, but not *IGFBP3*, may be a marker of conceptus elongation in ruminants. Similar to sheep and cattle, *IGFBP1* is upregulated in endometria of both primates and rodents during early pregnancy, and is implicated as a regulator of blastocyst implantation and placental growth and development (Giudice & Saleh 1995, Fowler *et al.* 2000). Thus, despite differences in implantation schemes, *IGFBP1* is likely imperative for success in early pregnancy and may serve as a universal requirement for pregnancy.

The prevalence of research involving IGFBP1 within the literature begs the question of what is known about IGFBP1 in reproductive biology across species, and how does that relate to our working knowledge of IGFBP1 in regulating implantation in sheep? Molecularly, IGFBP1 is unique within the family of IGFBPs as it contains a functional RGD integrin recognition domain that specifically binds the α5β1 integrin heterodimer (Irwin & Giudice 1998). This distinction is of particular importance as integrins are proposed to be the dominant glycoproteins regulating trophectoderm

adhesion to endometrial LE during implantation in mammals (Aplin 1997, Armant 2005). Functional studies have demonstrated that blocking antibodies against the α5β1 integrin subunits (Irving & Lala 1995) or mutation of the RGD integrin binding sequence (Gleeson *et al.* 2001) inhibit human trophoblast cell migration. In sheep, a variety of integrin subunits, including α5 and β1, are constitutively expressed on apical surfaces of the conceptus trophectoderm and endometrial LE during the peri-implantation period (Johnson *et al.* 2001) and similar to humans, IGFBP1 stimulates migration and mediates attachment of ovine trophectoderm cells (CHAPTER III). This affect may be due to the interaction of the internal RGD site with integrins (such as α5β1) expressed by the conceptus trophectoderm cells and LE (Fig. 6.1). However, additional experiments using ovine cells are required to confirm if IGFBP1 binds the α5β1 integrin specifically, and if mutation of the RGD site negates trophectoderm cell migration. This information will provide valuable insight into the similarities and/or differences in IGFBP1 functionality across species.

In humans, other primates, and rodents, IGFBP1 levels can influence conceptus invasion. Excess IGFBP1 is often detected in cases of shallow trophoblast invasion which leads to pre-eclampsia and intra-uterine growth restriction of offspring (Giudice *et al.* 1997). In rodents, overexpression of *Igfbp1* in transgenic animals may result in impaired fertility and implantation, interrupted or prolonged labor, and fetal or neonatal death (Gay *et al.* 1997). While decreased endometrial *IGFBP1* expression contributed to failed conceptus elongation in sheep (CHAPTER V), an excess of IGFBP1 may also be detrimental to establishment of pregnancy in ruminants. The infusion of excess levels of IGFBP1 into the ovine uterine lumen via Alzet osmotic pumps may further elucidate its effects during early pregnancy. Realizing that sheep embryos utilize a process of elongation as opposed to invasion for establishment of pregnancy and a large surface area for uptake of nutrients and gases, it is still likely that in ruminants, as in other species, IGFBP1 levels must be closely regulated for successful implantation.

In comparison to the relatively clear influence of IGFBP1 on conceptus elongation, the role(s) of IGFBP3 remain largely undefined. Despite the relative paucity

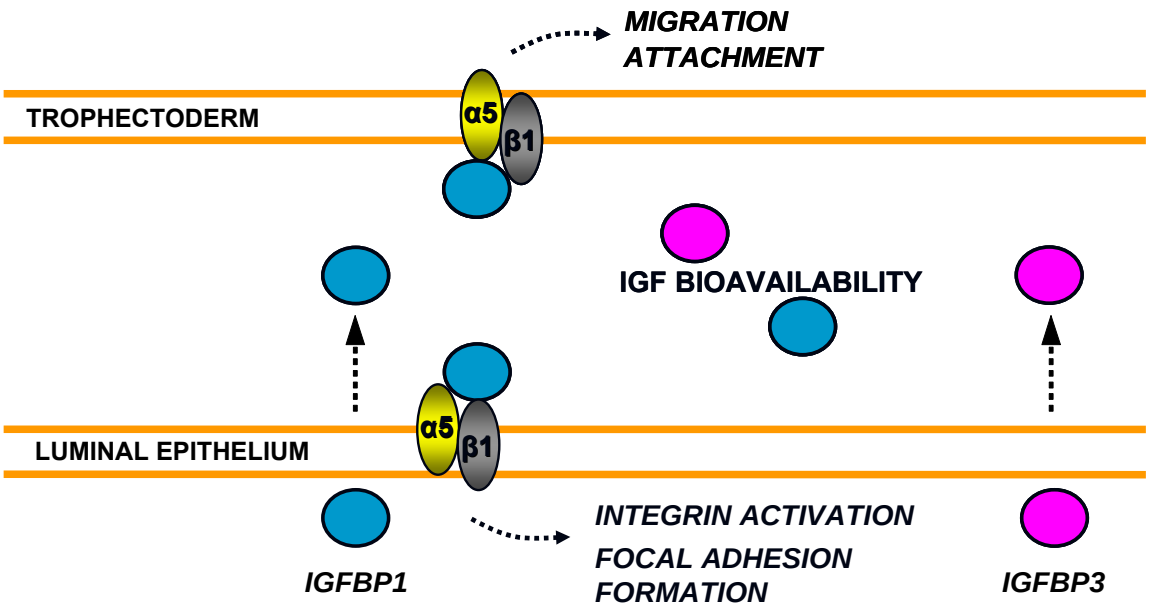

Fig. 6.1. Schematic illustration of the proposed roles of IGFBP1 and IGFBP3 during ovine conceptus elongation. IGFBP1 and IGFBP3 are produced by the endometrial epithelia and secreted into the uterine lumen. IGFBP1 can bind α5β1 integrin heterodimers expressed by the conceptus trophectoderm to promote cell migration and adhesion, or to α5β1 integrin heterodimers expressed by the uterine LE, resulting in integrin activation and the formation of focal adhesions. Within the uterine lumen, both IGFBP1 and IGFBP3 regulate the bioavailability of IGFs. IGFPB3, however, is the most abundant binding protein in the uterine lumen, making it more influential in controlling IGF levels than IGFBP1.

of information available, it is important not to dismiss IGFBP3s importance during early pregnancy due to its high affinity for IGF1 and IGF2. Expression patterns of *IGFBP3* are very different between sheep and cattle. Relative changes in expression of *IGFBP3* in cyclic and pregnant ewes are equivalent (CHAPTER III). Conversely, there is elevated expression of *IGFBP3* in non-pregnant as compared to pregnant heifers. It is interesting to note that the overall levels of *IGFBP3* were much lower in non-pregnant and early pregnant heifers as compared to sheep (CHAPTER III). Thereby in sheep and cattle, it is possible that levels of *IGFBP3* increase, even in cyclic animals, to prepare for a potential pregnancy by sequestering IGFs within the uterine lumen.

In terms of conceptus elongation, *IGFBP3* expression increased coincidentally with that event in sheep, but *IGFBP3* expression did not increase after Day 10 of pregnancy in cattle. Moreover, *IGFBP3* is expressed in the endometrial LE and sGE in the ovine uterus, but is predominant in the subepithelial stroma in the bovine uterus (CHAPTER III). Perhaps less IGFBP3 allows greater utilization of IGFs by the bovine conceptus. Although proteolysis of IGFBP3 has not been studied in the bovine uterine lumen, IGFBP3 exists primarily in a fragmented form between Days 12 to 15 of pregnancy in sheep, purportedly to increase availability of IGFs for the elongating conceptus (Peterson *et al.* 1998b). Because IGFBP3 is known to inhibit IGF-induced mitogenesis, it has also been hypothesized that reduced *IGFBP3* expression may increase the stimulatory effects of IGFs on the endometrial stroma and influence caruncular development (Cerro & Pintar 1997, Robinson *et al.* 2000). IGFPB3 may also be important for conceptus elongation in sheep, as there was decreased endometrial *IGFBP3* mRNA detected in ewes with conceptuses that failed to elongate (CHAPTER V).

In addition to IGFPB1 and IGFBP3, the enzyme HSD11B1 is an attractive candidate for influencing pregnancy. HSD11B1 primarily converts inactive corticosterone to active cortisol which serves as a ligand for two nuclear receptors, MR and NR3C1. The binding of cortisol to activate NR3C1 leads to transactivation of numerous genes implicated in the events of implantation and conceptus elongation.

Treating ewes with RU486, a high affinity antagonist of both PGR and NR3C1 (Baulieu 1989), decreased NR3C1 protein and *HSD11B1* mRNA in the endometrium (CHAPTER IV). Similarly, P4 regulates gene expression via an interaction with NR3C1 in human trophoblast cells (Karalis *et al.* 1996, Patel *et al.* 2003). Thus, many P4-induced and IFNT stimulated genes may also be regulated by cortisol. The relative binding affinity of RU486 to *PGR* is more than twice that of P4 and its relative binding affinity for *NR3C1* is more than 10 times that of cortisol (Heikinheimo *et al.* 2003). Because RU486 can influence the effects of both P4 and cortisol, it cannot be determined from these studies whether the decreases in *IGFBP1*, *IGFBP3*, and *HSD11B1* mRNAs (CHAPTERS III and IV) were the result of RU486 antagonizing *PGR*, *NR3C1* or both. However, the increase in *IGFBP1*, *IGFBP3*, and *HSD11B1* mRNA expression in the endometrial LE and GE between Days 10 and 12 of the cycle and pregnancy is temporally associated with loss of *PGR* expression (Wathes & Hamon 1993, Spencer & Bazer 1995, Satterfield *et al.* 2006). Thus, cortisol activation of *NR3C1* may be regulating expression of target genes since *PGR* are absent from the same epithelia. Development of a specific antagonist for the *PGR* and *NR3C1* would clarify the precise effects of P4 and cortisol on endometrial gene expression.

Extensive studies document the developmental consequences of increased GC exposure in mid- to late pregnancy, including intra-uterine growth restriction, increased risk of pre-term labor, and adverse effects on fetal brain development (see (Michael *et al.* 2003, Michael & Papageorghiou 2008) for reviews). Thus, cortisol production by placental *HSD11B1* and cortisol inactivation by placental *HSD11B2* is highly regulated in late pregnancy. However, *HSD11B1*, *HSD11B2*, and GCs in early pregnancy are a more recent area of interest since GCs can benefit pregnancy. Positive effects of GCs include stimulation of hCG secretion, immunosuppression for maternal acceptance of the conceptus allograft, promotion of trophoblast growth/invasion, and increased amino acid transport (see (Michael & Papageorghiou 2008) for review). Interestingly, pregnancy rates are increased in women undergoing standard *in vitro* fertilization who receive synthetic GCs prior to or immediately after embryo transfer (Boomsma *et al.* 2007). Our

sheep model utilizing meloxicam treatment further supports the need for GCs during the peri-implantation period, since uteri of treated ewes expressed less *HSD11B1* mRNA (CHAPTER V), which likely resulted in decreased intrauterine cortisol production.

While these results suggest biological roles for GCs in early pregnancy, the source of cortisol remains questionable. Cortisol is the most potent GC produced by the adrenal cortex and is often released in response to physical or psychological stress (Weber 1998). However, circulating levels of cortisol did not increase in ewes during early pregnancy (CHAPTER IV); therefore, intrauterine production of cortisol by *HSD11B1* is likely responsible for regulating endometrial gene expression, and subsequent conceptus elongation.

As previously mentioned, stimulation of *IGFBP1* and *HSD11B1* in ovine endometria by IFNT was modest compared to the much larger increase in expression noted in Day 12 pregnant compared to Day 12 cyclic ewes (CHAPTERS III and IV). Because relatively little IFNT is produce by the spherical Day 12 ovine conceptuses (Ashworth & Bazer 1989), we hypothesized that another conceptus-derived factor must be important for the regulation of gene expression. In rodents PGs are critical for successful pregnancies as they regulate blastocyst implantation, decidualization, and uterine angiogenesis (Dey *et al.* 2004, Wang & Dey 2006, Kennedy *et al.* 2007). While it is well established that conceptuses and endometria of sheep and cattle synthesize and secrete various PGs during early pregnancy (Ellinwood *et al.* 1979, Marcus 1981, Lewis *et al.* 1982, Lewis & Waterman 1985), production of PGF2α is more closely associated with luteolysis (McCracken *et al.* 1999) than regulation of conceptus elongation. However, the coordinate expression of *IGFBP1* and *HSD11B1* in ovine endometrial LE/sGE with PTGS2 (CHAPTERS III and IV) suggests that PGs may be biologically relevant to the process of implantation and conceptus elongation in ruminants (Kraeling *et al.* 1985, Harney & Bazer 1989). Previous research has shown IFNT stimulates PGE2 production via the p38 MAPK pathway in bovine uterine endometria (Krishnaswamy *et al.* 2009) and that PGF2α can stimulate HSD11B1 enzyme bioactivity and protein expression in bovine endometrial stromal cells (Lee *et al.* 2009). PGs also serve to

regulate *HSD11B1* in the ovine placenta (Strakova *et al.* 2000, Michael & Papageorghiou 2008).

Our studies also found that interruption of PG synthesis via PTGS2 inhibition is detrimental for implantation and expression of elongation-related genes such as: *IGFBP1*, *IGFBP3*, *LGALS15*, *HSD11B1*, *ISG15*, *RSAD2*, *GRP*, *SLC5A1*, and *CST3* (CHAPTER V). In addition to playing a role in conceptus elongation, many of those PG regulated genes are hypothesized to be involved in the cascade of events which occur during early pregnancy. Specifically, endometrial receptivity for implantation (*ISG15*), contribution to conceptus elongation by nutrient transport (*SLC5A1*), or encoding enzymes producing factors implicated in endometrial function and/or conceptus development (*HSD11B1*). Other genes affected by PGs are known to encode secreted proteins that function as trophectoderm migration and attachment factors (*IGFBP1*, *LGALS15*), regulate cell proliferation (*GRP*) or IGF bioavailability (*IGFBP3*), and process proteins that regulate conceptus invasion (*CST3*, *CTSL*).

While PGs are undoubtedly important during peri-implantation, the source of these PGs remains to be determined. The ovine blastocyst and endometrium both express PTGS2 (Charpigny *et al.* 1997a, Charpigny *et al.* 1997b), thereby meloxicam infusion likely targeted both sources of the enzyme. This poses a question as to whether the source of PGs necessary for regulation of gene expression and subsequent conceptus elongation are of conceptus or endometrial origin. Upregulation of *IGFPB1*, *HSD11B1*, and numerous other genes during pregnancy supports the hypothesis that PGs are derived from the conceptus. However, expression of *IGFBP1* and *HSD11B1* are coordinate with PTGS2 protein in endometrial LE/sGE and upper GE during the estrous cycle, suggesting that presence of a conceptus is not required, ergo the endometrium is the source of PGs. Moreover, both pregnant and nonpregnant endometria can synthesize PGs (Silvia *et al.* 1984, Zarco *et al.* 1988a, Zarco *et al.* 1988b). At the onset of parturition, cortisol increases PG synthesis (Sun & Myatt 2003) by stimulating *PTGS2* transcription in the placenta (Whittle *et al.* 2001); thus, the increased abundance of *PTGS2* in endometrial LE/sGE observed in the estrous cycle and early pregnancy

(CHAPTER IV) could be due to higher levels of *HSD11B1*, cortisol, and upregulation of *PTGS2* mRNA in those same tissues.

Summarily, our working hypothesis implicates both endometrial and conceptus-derived factors in the regulation of gene expression to facilitate conceptus elongation (Fig. 6.2). We propose that ovarian P4 and endometrial and/or conceptus-derived PGs can stimulate the activity of HSD11B1, leading to an increase in levels of active cortisol that can then bind either *MR* to stimulate ion and water transport, or *NR3C1* to activate genes containing a GRE or PRE. Potentially activated genes include *LGALS15*, *IGFBP1*, *PTGS2*, *SLC5A1*, and many others. The stimulation of *PTGS2* expression is notable, as that would allow for enhanced PG synthesis from arachidonic acid and increased HSD11B1 activity, thus propagating a feed forward loop that is important for the events of early pregnancy, specifically the process of conceptus elongation.

Based on the results presented in this thesis as well as established information in the literature, we propose the central hypothesis that peri-implantation conceptus growth and development in ruminants is modulated by secretions of the endometrium that are regulated by factors produced by the ovary (P4), the conceptus (IFNT and PGs), and the endometrium itself (PGs) (Fig. 6.3). In the conceptus trophectoderm and uterine LE/sGE, arachidonic acid is liberated from the cell membrane by PLA2 and then converted into PGH2 by PTGS2. PGH2 is then converted into different primary PGs by cell-specific isomerases and synthases. Two PGs, PGI2 and a variant of PGJ2, 15-Δ-PGJ2, can activate nuclear PPARs (PPARD and PPARG), which can then dimerize with retinoid X receptors and regulate transcription of genes. PGs can also be transported into the uterine lumen by PG transporters (SLC20A1 and MRP4) where they can interact with their G protein-coupled receptors expressed by the LE/sGE and stimulate the release of intracellular calcium (Ca^{2+}) or increase cAMP production. These messengers activate the cAMP response element binding (CREB) protein, leading to regulation of elongation- and implantation-related genes. IFNT produced be the elongating conceptus can bind its receptor (IFNAR) on the LE/sGE and upregulate PTGS2, and thus PG pro-

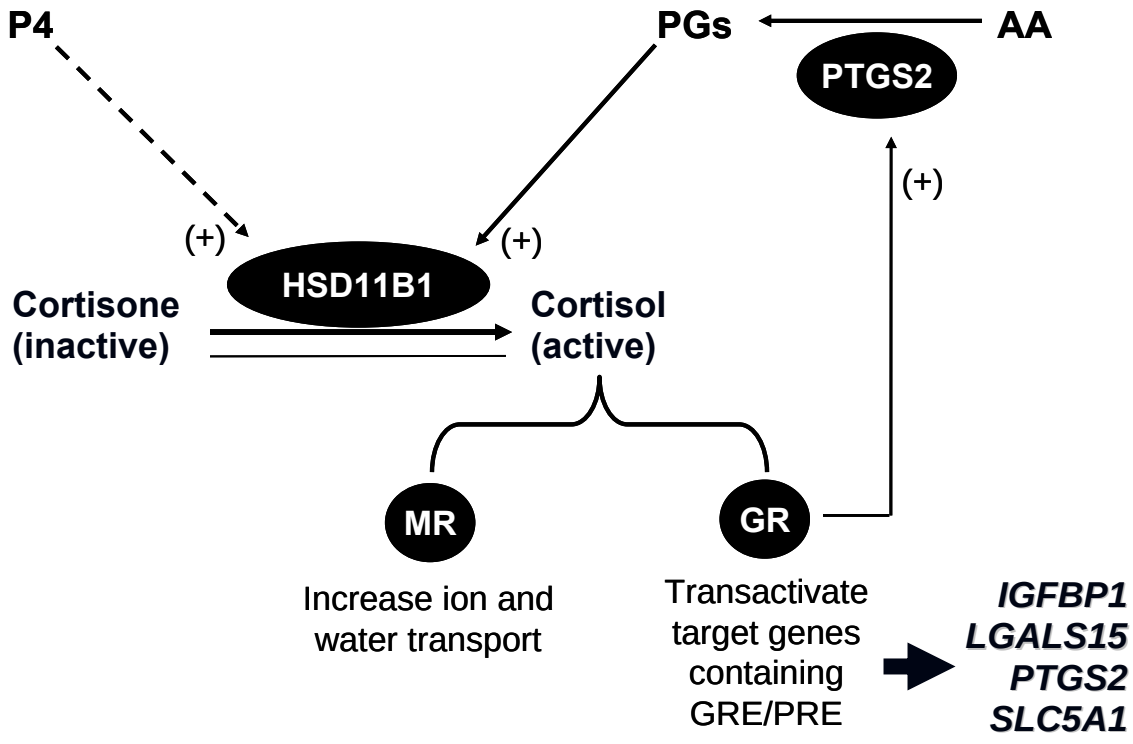

Fig. 6.2. Proposed model for the interactions of prostaglandins, HSD11B1, and cortisol during elongation in sheep. Ovarian P4 and endometrial and/or conceptus-derived PGs can stimulate the activity of HSD11B1 to increase production of active cortisol. Cortisol can then bind nuclear MR or NR3C1, leading to ion and water transport or activation of genes, respectively. Stimulation of PTGS2 increases PG synthesis from arachidonic acid (AA), increasing HSD11B1 to propagate a feed-forward mechanism that contributes to conceptus elongation.

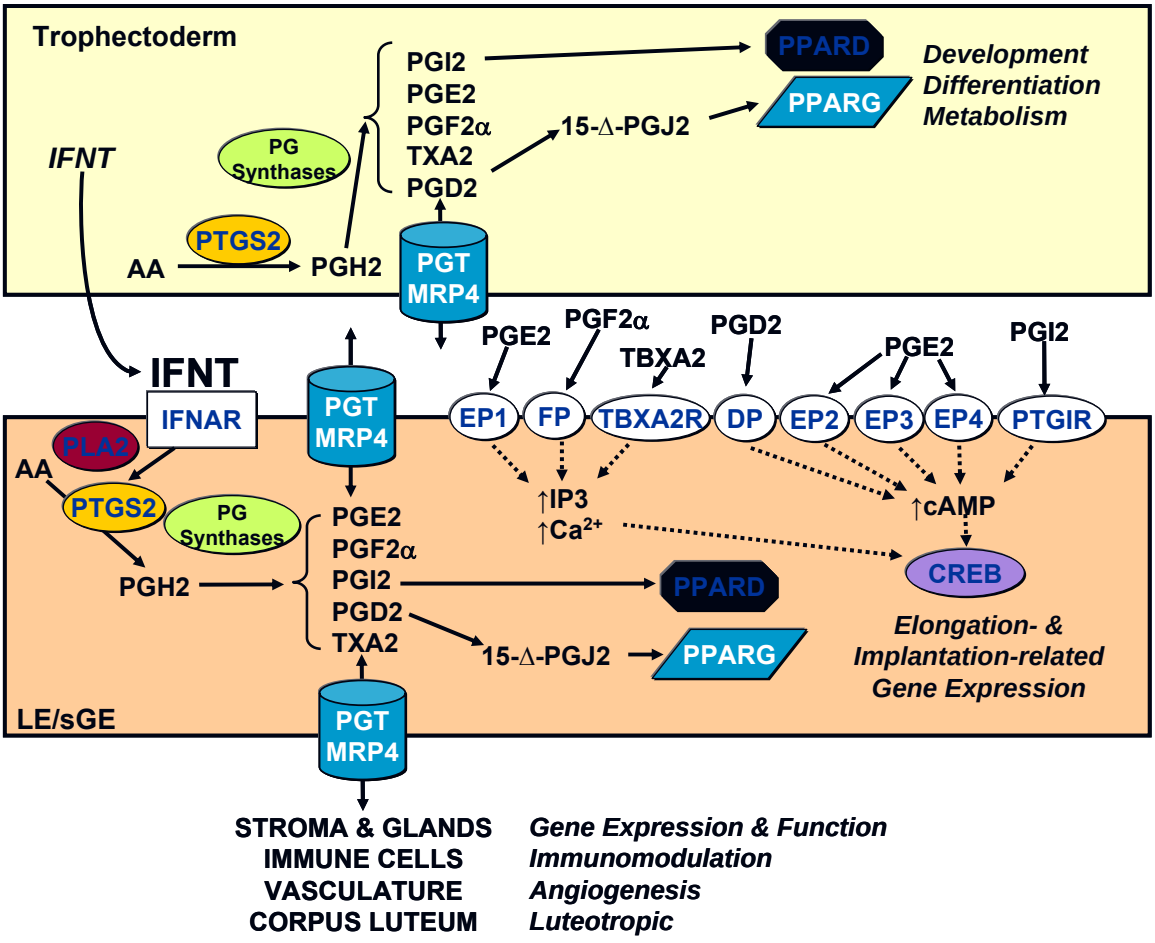

Fig. 6.3. Schematic of central hypothesis. In the conceptus trophectoderm and uterine LE/sGE, arachidonic acid is liberated from the cell membrane by phospholipase A2 (PLA2) and then converted into PGH2 by prostaglandin-endoperoxide synthase 2 (PTGS2). PGH2 is then converted into different primary PGs by cell-specific isomerases and synthases. Two PGs, prostacyclin (PGI2) and PGJ2 can activate nuclear peroxisome proliferator-activating receptors (PPARs), which can then dimerize with retinoid X receptors (RXRs) and regulate transcription of genes. PGs can also be transported into the uterine lumen by PG transporters (SLC20A1 and MRP4) where they can interact with their G protein-coupled receptors expressed by the LE/sGE and stimulate inositol triphosphate (IP3) and the release of intracellular calcium (Ca^{2+}), or increase cyclic adenosine monophosphate (cAMP). These messengers activate the cAMP response element binding (CREB) protein, leading to regulation of elongation- and implantation-related genes. Interferon tau (IFNT) produced be the elongating conceptus can bind its receptor (IFNAR) on the LE/sGE and upregulate PTGS2, and thus PG production by uterine endometria.

duction by uterine endometria. PGs produced in uterine LE/sGE can also be transported throughout the body to regulate gene expression and function in the stroma and glands, immune response, blood vessel formation, or development of the CL.

Experiments in this thesis have contributed toward the body of knowledge regarding regulation of ruminant endometrial gene expression and the roles of PGs during conceptus elongation. These analyses have also determined that the utilization of Alzet osmotic pumps is a viable method for administration of pharmacological reagents to the ovine uterine lumen during gestation without interrupting pregnancy, as control samples were undisturbed. These findings suggest that this technology would be suitable for use in investigation of other relevant signaling pathways within the uterus of sheep or potentially in other animals. Importantly, these analyses have also determined that *IGFBP1*, *IGFPB3*, and *HSD11B1* are implantation and elongation-related genes that are regulated by PGs, in addition to P4 and IFNT. Future experiments are needed to determine the relative importance of individual PGs and endometrial genes on conceptus elongation in sheep, as well as in other ruminants. Such experiments should include (1) characterization of the spatial and temporal expression of additional genes that may be important during conceptus elongation, as identified by microarray analysis; (2) determination of the regulatory effects of P4, IFNT, and PGs on individual gene expression and localization; and (3) utilization of osmotic pumps to deliver reagents targeting individual PGs for inhibition, thus allowing elucidation of roles for specific PGs on conceptus elongation. Such experiments will expand the body of knowledge associated with the onset and regulation of events in early pregnancy and subsequent conceptus elongation in sheep, perhaps providing a comparative model for evaluation of the implantation process among other species.

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