IDENTIFICATION OF ENDOMETRIAL GENES IMPORTANT FOR CONCEPTUS SURVIVAL AND DEVELOPMENT IN SHEEP

A Dissertation

by

CATHERINE ALLISON GRAY

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

DOCTOR OF PHILOSOPHY

May 2005

Major Subject: Physiology of Reproduction

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ABSTRACT

Identification of Endometrial Genes Important for Conceptus
Survival and Development in Sheep. (May 2005)
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Chair of Advisory Committee: Dr. Thomas E. Spencer

Recurrent early pregnancy loss in the ovine uterine gland knockout (UGKO) ewe model manifests on Day 14 of pregnancy, indicating that endometrial secretions are critical for peri-implantation conceptus development. Therefore, the following studies were conducted with fertile ewes and infertile UGKO ewes to identify candidate endometrial factors essential for normal conceptus survival, utilizing both genomics and proteomics approaches.

The first study used transcriptional profiling of endometrium from Day 14 cyclic, pregnant, and bred UGKO ewes, as well as ewes treated with interferon tau (IFNτ) and progesterone, to identify genes important for conceptus development. A number of novel and previously known IFNτ-stimulated genes, as well as progesterone-stimulated genes were identified that are higher in fertile ewes, such as galectin-15. Interactive effects of progesterone and IFNτ regulate endometrial gene expression in a temporal and cell-type specific manner.

The second study characterized the endometrial expression and hormonal regulation of galectin-15, a member of the galectin family of secreted β-galactoside lectins. Galectin-15 was secreted into the uterine lumen by the lumenal (LE) and superficial glandular epithelium (sGE), where it may promote adhesion during implantation, as well as was phagocytosed by the trophectoderm and formed intracellular crystals.

The third study determined the endometrial expression of galectin-15 throughout gestation. Galectin-15 was secreted into the uterine lumen, where it was phagocytosed

by the trophectoderm/chorion, transferred through placental vasculature to the fetus, and cleared through the fetal kidney to be stored in allantoic fluid.

The fourth study utilized proteomic analysis of uterine flushes and endometrial explant cultures from Day 14 cyclic, pregnant and UGKO ewes to identify differences in uterine secretions. Analyses identified several genes that were expressed by the LE and sGE and may be involved in prostaglandin production and/or pH regulation.

Collectively, results of these studies suggest that transcriptional profiling and analysis of uterine secretions are effective tools to determine genes important for early pregnancy. Further, identified genes are expected to reveal novel endometrial factors and metabolic pathways for support of conceptus survival and implantation, as well as provide improvements for embryo culture methods and diagnose endometrial dysfunctions leading to infertility.

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CHAPTER I
INTRODUCTION

Establishment of normal uterine histoarchitecture early in life undoubtedly affects fertility and prolificacy later in life. Organogenesis of the uterus occurs during both fetal and neonatal life in many species, including domestic animals (Bartol et al. 1999; Gray et al. 2001a). After birth, endometrial glands develop in the uterus, a process that involves budding and tubulogenesis, followed by coiling and branching morphogenesis. Postnatal uterine morphogenesis is dependent upon many factors, such as proper epithelial-mesenchymal interactions modulated by communication between growth factors and their receptors, as well as the composition and distribution of the extracellular matrix (ECM) and expression of epithelial hormone receptors (Bartol et al. 1999; Bartol et al. 1993).

The peri-implantation period of pregnancy in sheep is characterized by conceptus (embryo/fetus and associated extraembryonic placental membranes) elongation and production of conceptus-derived interferon-tau (IFNτ), which serves as the signal for maternal recognition of pregnancy in ruminants. IFNτ is antiluteolytic and suppresses development of the endometrial luteolytic mechanism, in order to sustain the corpus luteum (CL) and production of progesterone. Additionally, IFNτ induces or increases expression of interferon stimulated genes (ISGs) by the endometrium. Gestation in ruminant and other domestic livestock species is characterized by an extended pre-implantation period of development during which time the conceptus receives nutrients exclusively from uterine secretions or histotroph (Heap et al. 1979). The nutrients in these secretions must be sufficient to stimulate conceptus survival, elongation and production of IFNτ, which are prerequisite for implantation. The uterus must also be synchronized with the conceptus in order to provide the most beneficial environment for conceptus development (Rowson and Moor, 1966a). Therefore, histotroph produced by

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This dissertation follows the style of *Molecular Reproduction and Development*.

the endometrial epithelia is hypothesized to be particularly important for peri-implantation conceptus development and growth.

Both lumenal (LE) and glandular epithelial (GE) cells (Miller and Moore 1983) synthesize and/or transport uterine secretions into the lumen during gestation (Bazer 1975). Histotroph is composed of a variety of transport proteins, enzymes, adhesion molecules, cytokines, amino acids, ions, and growth factors (Bazer 1975; Bazer and First 1983; Carson et al. 2000; Kane et al. 1997; Martal et al. 1997; Roberts and Bazer 1988; Simmen and Simmen 1990). A specific role for secretions of GE has been established in rodents, wherein the absence of specific glandular secretory proteins, such as leukemia inhibitory factor and calcitonin, compromises embryo survival due to disruptions in establishment of uterine receptivity and embryo development (Stewart et al. 1992; Zhu et al. 1998a). In humans, histotroph appears to be the primary source of nutrition for conceptus development during the first trimester, when hematotrophic nutrition is not yet established (Burton et al. 2002). Similarly, peri-implantation pregnancy loss was observed in the uterine gland knockout (UGKO) ewe model (Gray et al. 2000a; Gray et al. 2002; Gray et al. 2001c).

The UGKO ewe is a novel animal model in which endometrial gland morphogenesis has been epigenetically ablated by inappropriate exposure of neonatal ewes to a progestin during the critical period of endometrial gland morphogenesis (Gray et al. 2000a; Spencer et al. 1999c). The progestin specifically ablated development of the glands (both shallow and deep glands) within the endometrium without altering development of the uterine myometrium or other Müllerian duct-derived female reproductive tract structures (Gray et al. 2001b; Gray et al. 2000b). Additionally, UGKO ewes have smaller uteri with short uterine horns and decreased LE cells, due to a lack of endometrial folding (Gray et al. 2001b). Despite repeated matings to fertile rams, adult UGKO ewes are unable to establish pregnancy (Gray et al. 2000a; Gray et al. 2002; Gray et al. 2001c). To date, there have been no differences detectable in temporal gene expression by the LE and stroma of UGKO ewes compared with normal cyclic and pregnant ewes, indicating that the pregnancy defect is specifically due to histotrophic insufficiency (Gray et al. 2002). Normal blastocysts can be found in the uterine flushings of bred UGKO ewes on Days 6 or 9 post-mating, but not on Day 14 (Gray et al. 2001b;

Gray et al. 2002; Gray et al. 2001c). On Day 14, the uterine flushings of bred UGKO ewes either contain no conceptuses or severely growth-retarded conceptuses that have failed to properly elongate (Gray et al. 2001c). Therefore, UGKO ewes exhibit a peri-implantation pregnancy defect whose timing correlates with the majority of embryo loss that occurs during pregnancy in livestock as well as humans (Bazer 1975; Kane et al. 1997; Norwitz 2001).

Adhesion during superficial implantation is mediated through the binding of bridging ligand secretory proteins from the GE and/or LE to receptors on the apical surface of the conceptus trophectoderm and LE. In sheep, osteopontin (OPN) and glycosylated cell adhesion molecule-1 (GlyCAM-1) are secreted by the epithelia and may bind to integrin subunits and L-selectin, respectively, to promote adhesion (Johnson et al. 2003a; Spencer et al. 1999a). Another family of proteins that may mediate implantation are galectins, which contain conserved carbohydrate recognition domains (CRD) that bind β-galactosides, thereby cross-linking glycoconjugate receptors on the surface of cells and initiating biological responses (Cooper 2002; Yang and Liu 2003). Fourteen mammalian galectins have been identified to date in a wide variety of tissues from different species (Cooper 2002; Cooper and Barondes 1999). Many galectins exhibit dual localization, being found in both intracellular (cytoplasm and, in some cases, the nucleus) and extracellular (cell surface and medium) compartments (Hughes 1997). Although galectins are often present on cell surfaces or in ECM, they are secreted in a non-classical manner, as they lack signal peptide sequences. Functional studies on the extracellular and intracellular roles of galectins have implicated them in cell growth, differentiation and apoptosis, in addition to cell adhesion, chemoattraction and cell migration (Leffler et al. 2004). All of these biological effects are critical for the apposition, attachment and adhesion stages of conceptus implantation.

Knowledge of the complex, precisely orchestrated interplay between secretions of the endometrial epithelia and trophectoderm that influence conceptus development could provide new targets to improve embryo culture and diagnose endometrial dysfunction that leads to infertility. Therefore, identification of useful molecular and cellular markers of endometrial function and receptivity to the embryo are of utmost importance. Studies were designed to determine differences between Day 14 cyclic, pregnant, and bred

UGKO ewes in endometrial gene expression and composition of uterine secretions. Utilization of both genomics and proteomics approaches will potentially identify candidate endometrial factors essential for conceptus survival and elongation in ruminants. Results from this research will facilitate the development of markers for evaluation, regulation and enhancement of fertility, particularly methods of screening endometrial function in women prior to the initiation of costly infertility treatments (Westergaard et al. 1998).

CHAPTER II
LITERATURE REVIEW

Uterine Morphogenesis

Morphogenesis of the uterus occurs during both fetal and neonatal life in many species, including livestock (cattle, swine, and sheep). Common to mammals with long gestation periods, uterine organogenesis is completed prior to birth (Bartol et al. 1993; Marion and Gier 1971; Wiley et al. 1987). In all mammals, the uterus develops as a specialization of the paramesonephric or Müllerian ducts, which gives rise to infundibula, oviducts, uterus, cervix and anterior vagina (Mossman 1987). The mature uterine wall is comprised of two functional compartments, the endometrium and myometrium, as illustrated in Fig. 2.1. The endometrium is the inner lining of the uterus and is derived from the inner layer of ductal mesenchymal cells. The endometrium consists of two epithelial cell types, LE and GE, two stratified stromal compartments, a densely organized zone of fibroblasts (stratum compactum) and a more loosely organized zone in the deeper or basal endometrium (stratum spongiosum), blood vessels and immune cells. The myometrium is the smooth muscle component of the uterine wall that includes an inner circular layer, derived from the intermediate layer of ductal mesenchymal cells, and an outer longitudinal layer, derived from subperimetrial mesenchyme.

Sequential morphogenetic events common to development of all mammalian uteri include: (1) differentiation and growth of the myometrium; (2) organization and stratification of endometrial stroma; and (3) coordinated development of the endometrial glands (Bartol et al. 1999; Bartol et al. 1993). Genesis of uterine glands involves differentiation and budding of GE from LE. The GE then form tubes which penetrate the stroma to form extensive coiled and branched structures embedded deeply within the endometrial wall. In humans and livestock species, endometrial adenogenesis is completed postnatally and involves extensive coiling and branching morphogenesis.

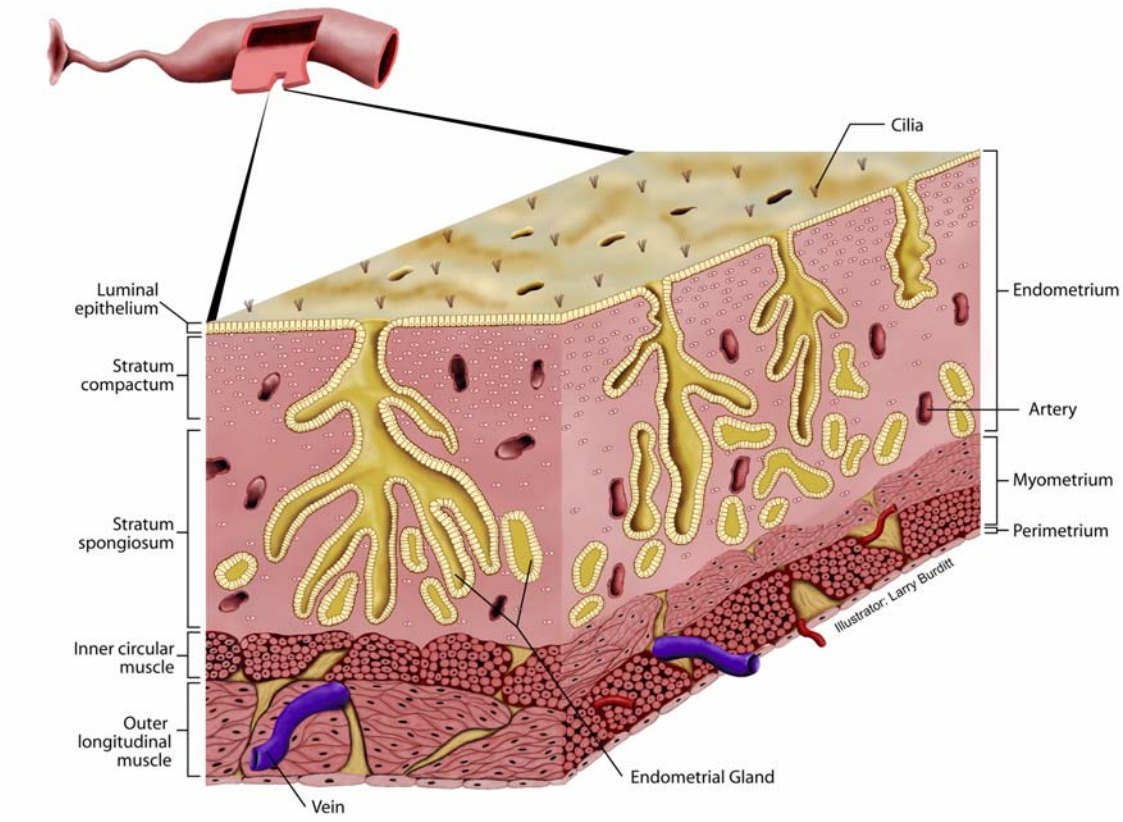

Fig. 2.1. Schematic illustration of uterine histoarchitecture. The uterus is divided into endometrial and myometrial compartments. The myometrium consists of an inner circular and outer longitudinal smooth muscle layers. The endometrium consists of lumenal epithelial cells lining the lumen of the uterus, 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)

Consequently, neonatal ungulates (e.g., sheep, cattle, and pigs) provide attractive models for the study of mechanisms regulating these processes (Bartol et al. 1999; Gray et al. 2001a; Spencer and Bazer 2004). Although adenogenesis is also a postnatal event in rodents, the adult rodent uterus does not contain the tightly coiled, branched glands characteristic of endometria in most other mammals.

Sheep Uterine Development

The endometrium in adult sheep consists of a large number of raised aglandular caruncles and glandular intercaruncular areas (Atkinson et al. 1984; Wimsatt 1950). Caruncular areas are the sites of superficial implantation and placentation (Mossman 1987; Wimsatt 1950). Domestic ruminants have a synepitheliochorial type of placentation in which placental cotyledons fuse with endometrial caruncles to form placentomes which serve a primary role in fetal-maternal gas exchange and derivation of micronutrients by the placenta (Mossman 1987; Wimsatt 1950; Wooding 1984). Intercaruncular endometrial areas contain large numbers of branched, coiled uterine glands which synthesize and secrete or transport a variety of substances, collectively termed “histotroph” (Amoroso 1951; Bazer 1975).

The dichotomous nature of the adult ruminant endometrium, consisting of both aglandular caruncular areas and glandular intercaruncular areas, makes it an excellent model for the study of mechanisms underlying establishment of divergent structural and functional areas within a single, mesodermally derived organ (Wiley et al. 1987). Uterine morphogenesis has been described in sheep (Bartol et al. 1988a; Bartol et al. 1988b; Bryden 1969; Davies 1967; Gray et al. 2000b; Kennedy et al. 1974; Taylor et al. 2001; Taylor et al. 2000; Wiley et al. 1987). The ewe has a gestation length of about 147 days. Paramesonephric duct fusion occurs between gestational day (GD) 34 and 55 in sheep, is partial and produces a bicornuate uterus (Bryden 1969; Davies 1967; Wiley et al. 1987). By GD 90, raised aglandular nodules are present that are destined to become caruncles (Bartol et al. 1988b; Wiley et al. 1987). Endometrial gland

development is first observed as shallow invaginations of LE in internodular clefts between GD 135 and 150 in sheep (Wiley et al. 1987).

Postnatal uterine morphogenesis in sheep involves the emergence and proliferation of endometrial glands, development of endometrial folds and, to a lesser extent, growth of endometrial caruncular areas and myometrium (Atkinson et al. 1984; Bartol et al. 1988a; Bartol et al. 1999; Kennedy et al. 1974; Marion and Gier 1971; Taylor et al. 2000; Wiley et al. 1987). The progressive development of endometrial GE from the LE to the inner circular layer of myometrium is a coordinated event that involves bud formation, tubulogenesis, and coiling and branching morphogenesis. In sheep, endometrial gland genesis is initiated between birth (postnatal day or PND 0) and PND 7, when shallow epithelial invaginations appear along the LE in presumptive intercaruncular areas (Bartol et al. 1988b; Taylor et al. 2000). Between PNDs 7 and 14, nascent, budding glands proliferate and invaginate into the stroma, forming tubular structures that coil and branch by PND 21 (Taylor et al. 2000). After PND 21, the majority of glandular morphogenetic activity involves branching morphogenesis of tubular and coiled endometrial glands to form terminal end bud-like structures in deeper stroma. By PND 56, the caruncular and intercaruncular endometrial areas are histoarchitecturally similar to those of the adult uterus. In UGKO ewes, the endometrium lacks a recognizable stratum spongiosum within the stroma that is characteristic of the normal stroma in intercaruncular glandular areas of the uterus (Gray et al. 2000a; Gray et al. 2001b; Gray et al. 2000b; Gray et al. 2001c; Spencer et al. 1999c). Thus, in sheep, development of GE appears to direct or permit differentiation of uterine stroma into subluminal stratum compactum and stratum spongiosum in intercaruncular areas of the endometrium.

Although the ovine uterine wall is histoarchitecturally mature by eight weeks after birth, final maturation and growth may not occur until puberty (Kennedy et al. 1974), or even the first pregnancy (Stewart et al. 2000; Wimsatt 1950). Extensive endometrial gland hyperplasia and hypertrophy occurs during each pregnancy (Stewart et al. 2000; Wimsatt 1950), presumably in response to increasing demands for

histotrophic support by the growing fetoplacental unit (Bazer 1975). After parturition in the ewe, intercaruncular endometrial LE remains intact, but contraction of many glands occurs in the days following parturition (Gray et al. 2003; O'Shea and Wright 1984). Glandular regeneration commences by Day 8 postpartum, and the glands were substantially regenerated by Day 15. In caruncles, regeneration of LE commenced after Day 8 and is not complete until Days 28 to 31 postpartum (Gray et al. 2003; van Wyk et al. 1972a; van Wyk et al. 1972b). Caruncular LE appear to emanate from epithelia in the intercaruncular areas of the endometrium (van Wyk et al. 1972a; van Wyk et al. 1972b).

Overall, patterns of endometrial gland genesis and development in the neonatal and adult ovine uterus during pregnancy and after parturition are very similar to GE morphogenesis characteristic of the stages of mammogenesis, lactogenesis and involution of the mammary gland (Houdebine et al. 1985). However, the precise mechanisms and factors regulating endometrial gland morphogenesis and regeneration are likely to be different than those in the mammary gland given the differences in organ histoarchitecture and embryonic origin.

Peri-Implantation Events in Sheep

The ruminant conceptus undergoes an extended period of growth and development prior to superficial implantation. Implantation marks the beginning of the process of placentation, or formation of the extraembryonic membranes. Implantation, in sheep, is not initiated until Day 15 (Day 0=mating) and involves the trophectoderm coming into contact with discrete caruncular areas of the uterine epithelium, as well as extension of villous interdigitations into the mouths of the endometrial glands (Boshier 1969; Guillomot et al. 1981; Guillomot and Guay 1982; King et al. 1980; King et al. 1981; Spencer et al. 2004). Prior to this period, the conceptus is bathed in and hypothesized to be dependent upon secretions produced by the uterus. These secretions must provide sufficient nutrients for the conceptus to simultaneously undergo major cellular restructuring during elongation, differentiation prior to placentation, as well as production of the biochemical signal for maternal recognition of pregnancy. IFNτ is the signal produced by the ruminant conceptus that acts in a paracrine manner on the maternal endometrium to elicit pregnancy recognition (Spencer and Bazer 2004; Spencer et al. 2004). IFNτ maintains the CL by inhibiting estrogen receptor (ER) and oxytocin receptor (OTR) gene expression and, therefore, endometrial production of luteolytic pulses of prostaglandin $(PGF)_{2\alpha}$. IFNτ may also protect the fetal allograft from maternal immune response and induce endometrial histotroph production to aid in conceptus growth and development. During the peri-implantation period of conceptus development the trophoblast plays several major roles to promote embryonic survival including: 1) undergoing rapid elongation; 2) producing the signal for the maternal recognition of pregnancy; and 3) absorbing histotroph as a nutrient source.

Ruminant Conceptus Development

Stages of Pre-Implantation Conceptus Growth

Development of the pre-implantation conceptus can be divided into several phases: 1) cleavage; 2) compaction; 3) shedding of the zona pellucida; and 4) rapid elongation. In a natural breeding situation, ewes mate with intact rams at estrus.

Ovulation of the ovum occurs approximately 30 h following estrus as it enters the ampulla of the oviduct (Fig. 2.2). The first phase of conceptus development is initiated with fertilization of the ovum at the ampullary-isthmic junction of the oviduct. At this point, the fertilized ovum (zygote) is surrounded by the zona pellucida, a mucopolysaccharide membrane of the ovum that protects the zygote for the first 7 to 9 days (Bindon 1971). As the zygote travels through the isthmus of the oviduct, it undergoes cleavage to the 2-, 4-, and 8-cell stages as cleavage mitotically divides the zygote into progressively smaller blastomeres. These blastomeres are spherical cells in a loose mass that are not in close contact with one another. The cleavage of cells occurs approximately every 12 h to 22 h in sheep (Clark 1934) with little increase in size of the embryo, due to restrictions of the surrounding zona pellucida. The diameter of the embryo prior to shedding the zona pellucida is approximately 145 μm at all stages between 2-cell and morula on Day 6 (Clark 1934). The first cleavage to the 2-cell stage occurs approximately 39 to 52 h post-breeding, the second to the 4-cell stage at 52 to 75 h and the third to the 8-cell stage at 75 h or 3 days post-breeding (Chang and Rowson 1965). By Days 4 to 5, the embryo has reached the morula stage, where it becomes difficult to count individual blastomeres, and has entered the uterine lumen (Bindon 1971; Chang 1952; Chang and Rowson 1965; Clark 1934; Rowson and Moor 1966a).

Beginning on Day 5, the embryo differentiates into a blastocyst, characterized by the compaction of the blastomeres and cavitation, or the formation of a fluid-filled cavity called the blastocoele (Bindon 1971; Chang 1952; Chang and Rowson 1965). The process of blastocyst differentiation involves polarization and flattening of cells to maximize contact and formation of junctional complexes to increase communication and coordination between cells (Ziomek and Johnson 1980). Bovine trophoblastic cells contain adherent and tight junctions involved in the organization of epithelial differentiation and adhesion (Barcroft et al. 1998). The abundance of junctional complexes between the trophoblastic cells ensures regulation of the embryonic microenvironment inside the blastocoele (Enders 1971). At this point, the blastocyst

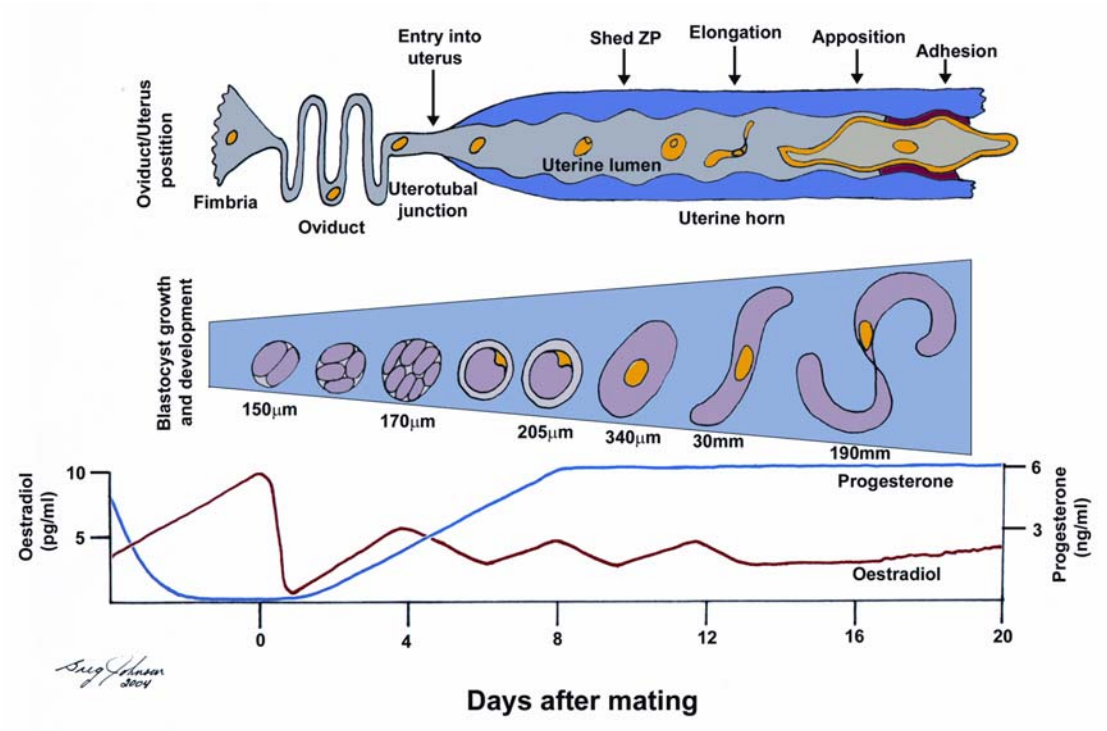

Fig. 2.2. Schematic of events occurring during early pregnancy in sheep. Position of the conceptus in the female reproductive tract, stages of blastocyst development and circulating levels of estradiol and progesterone are illustrated. Following ovulation, the oocyte enters the oviduct, is fertilized 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 (Spencer et al. 2004).

contains approximately 3000 blastomeres and is 150 to 200 μm in diameter (Bindon 1971; Wintenberger-Torres and Flechon 1974).

On Days 8 to 9, shedding of the zona pellucida occurs, leaving the blastocyst surrounded by a fine, monolayer of trophoblastic cells lined internally by a layer of endodermal cells separated by a basement membrane (Bindon 1971; Chang 1952; Rowson and Moor 1966a; Wintenberger-Torres and Flechon 1974). On Day 9, the conceptus is approximately 200-400 μm in diameter. However, once the zona pellucida is shed, rapid expansion and growth of the conceptus occurs. This event marks the beginning of the elongation phase of development by the ovine conceptus. By Day 11, the blastocyst has become spherical in shape and is 430 μm in diameter. The conceptus continues to expand and grow from spherical to tubular form on Day 12 measuring 11 mm on Day 13. At this same time, very little growth or increase in size of the inner cell mass has occurred (Chang, 1952; Chang and Rowson, 1965; Bindon, 1971; Wintenberger-Torrés and Fléchon, 1974). By Day 14, extraordinary trophoblastic growth and remodeling has occurred so that the conceptus appears as a long, filamentous strand and is approximately 100 mm in length and 150 to 190 mm on Day 15 (Bindon 1971; Chang 1952; Chang and Rowson 1965; Rowson and Moor 1966a; Wintenberger-Torres and Flechon 1974). The width remains approximately 1 to 1.5 mm throughout this phenomenal elongation, resulting in a filamentous appearance. It should also be noted that conceptus elongation is correlated with the period of maternal recognition of pregnancy, but precedes the initiation of firm adhesion of the trophoblast and the endometrial LE (Ashworth and Bazer 1989a; Boshier 1969; Chang 1952; Rowson and Moor 1966a).

Contributions of the Uterine Environment

The uterine environment must be synchronized to the conceptus for normal elongation and development to occur (Lawson and Cahill 1983). In sheep, blastocyst development prior to Day 11 is not entirely dependent on the uterus (Averill et al. 1955; Rowson and Moor 1966b). Ovine embryos undergo normal cleavage and compaction in

rabbit oviducts (Averill et al. 1955). When 2-cell through morula stage ovine zygotes were transferred into the oviduct of pseudopregnant does, morula and blastocyst stage conceptuses were recovered from the uteri five days later. These blastocysts were then transferred into a Day 6 non-pregnant ewe resulting in normal embryonic development for an additional 16 days (Averill et al. 1955). However, ovine trophoblast elongation does not appear to require the uterus (Flèchon et al. 1986). Elongation of the ovine trophoblast can occur *in vivo* in the absence of the embryonic disc, creating trophoblastic vesicles. These trophoblastic vesicles continue to function normally by secreting IFNτ (Heyman et al. 1984; Martal et al. 1979). However, trophoblastic vesicles, derived from Day 12 conceptuses survive, but are unable to undergo elongation *in vitro*. When these trophoblastic vesicles are transferred for 5 days *in utero* the trophoblast elongates and produces IFNτ (Flèchon et al. 1986). This dependence on the uterus for trophoblastic elongation may be species-specific, because in rodents, the presence of the inner-cell mass is required for trophectoderm growth (Heyman et al. 1984). Similarly, Day 4 ovine conceptuses transferred into Day 7 recipient ewes demonstrated a 100% survival rate, but were unable to undergo elongation (Lawson and Cahill 1983). Indeed, these conceptuses stopped growing after Day 11. Overall, these experiments suggest that the maternal uterine environment regulates conceptus survival and development after Day 11, during the pregnancy recognition period.

Maternal Recognition of Pregnancy

*IFN*τ

IFNτ is the pregnancy recognition hormone in ruminants (Short 1969) that acts on the endometrium to prevent development of the luteolytic mechanism, thereby maintaining the CL and production of progesterone (Bazer et al. 1998; Spencer et al. 2004). The antiluteolytic signal originates from the conceptus, as the transfer of trophoblastic vesicles into cyclic cows and ewes also results in maintenance of the CL (Heyman et al. 1984; Martal et al. 1979; Rowson and Moor 1967). IFNτ is a Type I interferon with potent antiviral (Pontzer et al. 1988), antiproliferative (Fillion et al. 1991;

Roberts 1989), antitumor (Pontzer et al. 1990) and immunomodulatory biological activities (Fillion et al. 1991; Roberts 1989). Conceptus-produced IFNτ may play multiple roles in early pregnancy in ruminants by serving as the signal for maternal recognition of pregnancy, inhibiting viral infection of the conceptus or uterus, as well as modulating the maternal immune response to allow the fetal allograft to avoid rejection (Pontzer et al. 1988). In sheep, evidence for a signal was originally proposed by Rowson and Moor (Rowson and Moor 1967). A protein originally named trophoblastin (Martal et al. 1979) and ovine trophoblast protein-1 (Bazer et al. 1986) was later renamed IFNτ by the International Interferon Society.

IFNτ produced by different livestock ruminant species (cow, sheep, goat) share high amino acid and DNA sequence homology, as well as mechanism of action (Imakawa et al. 1987; Roberts et al. 1992a; Roberts et al. 1992b; Stewart et al. 1987). In fact, introduction of either ovine trophoblastic vesicles or recombinant ovine (ro)IFNτ into the uterine lumen of a cow will prevent luteolysis and extend the interestrous interval (Heyman et al. 1984; Meyer et al. 1992). However, there are differences in IFNτ between species. Secreted ovine IFNτ (oIFNτ) is not glycosylated (Nephew et al. 1993), bovine IFNτ is glycosylated (Roberts et al. 1992a), and caprine IFNτ is a combination of both glycosylated and non-glycosylated variants (Baumbach et al. 1990).

*Conceptus Production of IFN*τ

IFNτ is produced by the mononuclear trophoblast cells of the conceptus between Days 8 to 21 in the sheep (maximally on Days 13 to 16) and acts in a paracrine manner on the endometrium (Ashworth and Bazer 1989b; Bartol et al. 1985; Bazer 1992; Farin et al. 1990; Godkin et al. 1982; Godkin et al. 1984a). Onset of maximal oIFNτ secretion by the trophoblast is directly related to morphological transition of the conceptus from spherical to filamentous form (Farin et al. 1990; Nephew et al. 1991). Secretion of IFNτ is also specifically related to length or stage of development of the blastocyst, as concentrations in the uterine lumen increase with day of pregnancy (Ashworth and Bazer 1989b; Nephew et al. 1991).

IFNτ Signaling

Type I IFN receptors are present in all endometrial cell types (Rosenfeld et al. 2002), and are known to classically activate the Jak-signal transducers and activators of transcription (Stat) signaling pathway to stimulate or repress gene transcription. The ability of IFNτ to induce gene transcription of endometrial ISGs is dependent upon the presence of progesterone (Johnson et al. 2000a; Spencer et al. 2004). Additionally, exogenous administration of roIFNτ does not result in activation of IFN responsive genes (Spencer et al. 1999d), indicating that it is binding to Type I IFN receptors in the endometrium. However, because the Jak-Stat pathway is inactive in the LE and superficial GE (sGE), IFNτ signals through the receptors in the stroma and deeper GE. To date, Wnt7a, is the only ISG that is expressed by the signal transducer and activator of transcription (Stat)1-negative LE and sGE cells (Kim et al. 2003a). The classical ISGs, such as Stat1, Stat2, IFN regulatory factor (IRF)-9, 2'5'-oligoadenylate synthetase (OAS), ISG15, major histocompatibility complex (MHC) class I, and β2-microglobulin (β2M) are upregulated in the stroma and deep GE cells (Choi et al. 2001; Choi et al. 2003; Johnson et al. 2002; Johnson et al. 2000a; Johnson et al. 1999c; Johnson et al. 2001b). This is due to the presence of IRF-2 in the LE and sGE, which is a potent repressor of ISG transcription (Choi et al. 2001). Thus, IFNτ is not signaling through the traditional Jak-Stat pathway in the LE and shallow GE, but must be utilizing another signal transduction pathway, such as the p38 Map kinase (Platanias 2003), protein kinase C (Thatcher et al. 2001), or nuclear factor-kappa B pathways (Hiscott et al. 2003).

Action of IFNτ

Although IFNτ prevents the luteolytic mechanism from occurring, it is not luteotrophic (Godkin et al. 1984b). This is in direct contrast to primates, in which the trophoblastic-derived chorionic gonadotropin acts directly on luteal cells via the lutenizing hormone (LH) receptor to sustain CL function (Hearn et al. 1991). In sheep, IFNτ does not act to stabilize progesterone receptor (PR) expression in the endometrial epithelia during pregnancy (Spencer and Bazer 1995; Spencer and Bazer 1996; Spencer

et al. 1995a). Rather, IFNτ acts in a paracrine fashion on endometrial LE and sGE to suppress transcription of ERα and oxytocin receptor OTR genes (Fleming et al. 2001; Spencer and Bazer 1996), thereby abrogating development of the endometrial luteolytic mechanism by preventing production of luteolytic $PGF_{2\alpha}$ pulses. Progesterone and expression of PR are key to the temporal expression of PR, ERα, and OTR in the uterine endometrium (Spencer et al. 2004). During the estrous cycle in sheep, progesterone initially blocks synthesis of ERα and OTR in the endometrial epithelium and PR is expressed by the epithelia at highest levels on Days 1 through 6 and remains throughout diestrus. The PR is expressed by stromal cells throughout the estrous cycle and pregnancy; however, the epithelial PR is negatively autoregulated by exposure of progesterone, causing an absence of PR expression in the LE after Day 11 (Spencer and Bazer 1995). During pregnancy the high levels of progesterone prevent the upregulation of the PR in the epithelia. Thus, once PR expression decreases in LE and shallow GE during the estrous cycle, ERα and OTR expression increase, presumably in response to follicular estrogen or simply the absence of repressive effects of progesterone (Spencer et al., 1995a). Indeed, the increases in ERα, PR and OTR gene expression detected in endometrial LE and GE between Days 11 to 17 post-estrus in cyclic sheep do not occur in pregnant sheep (Spencer and Bazer 1995) or in cyclic sheep infused with IFNτ (Spencer et al. 1995b). Thus, the antiluteolytic actions of IFNτ are to prevent increases in epithelial ERα, PR, and OTR gene expression, which are all estrogen responsive genes, by directly inhibiting transcription of the ERα gene, thereby maintaining secretion of progesterone by the CL (Fleming et al. 2001). The antiestrogenic actions of IFNτ on the epithelia prevent increases in PR expression, a prototypical estrogen-responsive gene.

In addition to preventing development of the luteolytic mechanism, IFNτ also acts on the endometrium to induce or increase a number of genes that are proposed to play a role in conceptus-endometrial interactions (Spencer and Bazer 2002). Amongst these genes, a chemokine, IFNγ-inducible protein 10 kDa (IP-10), is expressed in monocytes of the endometrium, is increased by IFNτ in the endometrium and appears to

regulate the establishment of apical interactions between trophoblast and epithelial cells during early gestation (Nagaoka et al. 2003a; Nagaoka et al. 2003b). In addition to effects on gene transcription, the conceptus appears to increase the secretion of several adhesion molecules from the endometrial epithelium, including GlyCAM-1 and OPN (Gray et al. 2002; Johnson et al. 1999a; Spencer et al. 1999a). However, the mechanism underlying this conceptus effect is not understood.

Superficial Implantation

Unlike primates and rodents, ruminant conceptuses do not invade into the maternal endometrium. Instead, the ruminant conceptus superficially attaches to the endometrium and ultimately obtains hematotrophic nutrition from placentomes. Placentomes are formed by interdigitation of a maternal caruncle and placental cotyledon. Implantation in ruminants is a highly coordinated process that involves apposition, attachment, and adhesion of the endometrial LE and conceptus trophectoderm (Guillomot 1995), as illustrated in Fig. 2.3. In the sheep, apposition of conceptus trophectoderm and LE is initiated on Day 14, followed quickly by adhesion on Day 15, and attachment on Days 16 to 18 (Guillomot et al. 1981). The process of apposition involves a transient contact between conceptus and LE. During attachment, a cohesive contact is initiated between integrins and glycosylated membrane proteins. Attachment involves interdigitation of the microvilli between the two epithelial layers. During synepitheliochorial placentation in sheep, trophoblast giant binucleate cells begin to differentiate between Days 14 and 16 and then fuse apically with the endometrial LE to form syncytia, thereby assimilating and replacing the endometrial LE (Wimsatt 1950; Wooding 1984).

Integrins

Implantation of the conceptus trophectoderm to the endometrial LE is temporally regulated by non-adhesive and adhesive factors on the apical surface of the LE (Burghardt et al. 1997; Burghardt et al. 2002; Lessey 1998; Lessey 2002). Initially, non-adhesive factors, such as mucin one (Muc-1), are hypothesized to sterically impair

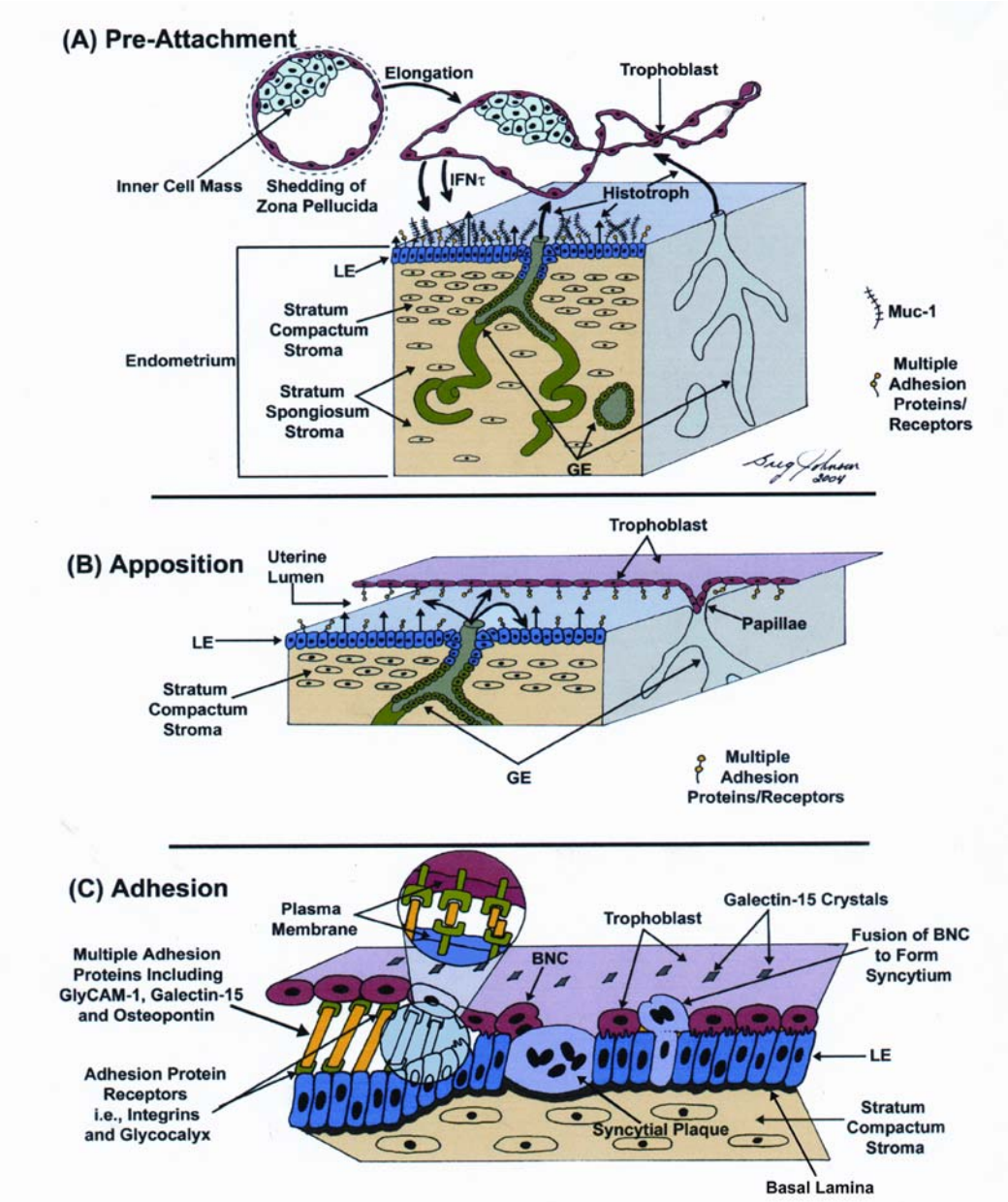

Fig. 2.3. Schematic illustration of the pre-attachment, apposition, and adhesion stages of superficial implantation in sheep. Pre-attachment (A) involves shedding of the zona pellucida, followed by conceptus expansion, as well as precontact and blastocyst orientation. Muc-1 expression prevents contact with integrin subunits and/or carbohydrate receptors. Apposition (B) involves a decrease in Muc-1 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 OPN and GlyCAM-1, binding to receptors. Trophoblast binucleate cells fuse with the LE cells to form syncytial plaques, but are not invasive beyond this single cell layer (Spencer et al. 2004).

interactions between adhesive glycoproteins expressed on the apical surfaces of conceptus trophectoderm and LE due to the extensive glycosylation and extended extracellular structure of Muc-1 (Carson et al. 2000; Johnson et al. 2001a). In sheep, immunoreactive Muc-1 expression by LE progressively decreases between Days 9 and 17 of early pregnancy, presumably to unmask adhesive glycoproteins on the LE for interaction with the trophectoderm (Johnson et al. 2001a; Johnson et al. 2003a). Integrins are thought to be the dominant glycoproteins that regulate trophectoderm adhesion to endometrial LE (Lessey 1998; Lessey 2002). During the peri-implantation period of pregnancy in sheep, integrin subunits αv, α4, α5, β1, β3 and β5 are constitutively expressed on the conceptus trophectoderm and apical surface of the endometrial LE (Johnson et al. 2001a). In sheep, receptivity to implantation does not appear to involve changes in temporal or spatial patterns of integrin expression, but may depend on expression of ECM or secretory proteins, such as OPN, which are ligands for heterodimers of these integrins (Johnson et al. 2003a).

Carbohydrates and ECM

Most cell-surface carbohydrates are present as attached oligosaccharides on glycoproteins, glycolipids, or ECM molecules (i.e., glycosaminoglycans). Carbohydrate recognition systems appear to mediate cell-cell interactions in peri-implantation mouse embryos (Bird and Kimber 1984), as well as adhesion between cells of the endometrial epithelium (Dutt and Carson 1990; Dutt et al. 1987), and between the conceptus and endometrial epithelium (Lindenberg et al. 1988). Changes in cell surface glycoconjugates have been detected by lectin binding and biochemical studies of mouse, rabbit, pig, horse and sheep uteri (Chavez and Anderson 1985; Kimber et al. 1988; Whyte and Allen 1985; Whyte and Robson 1984). Using monoclonal antibodies of

defined carbohydrate specificity, Kimber and colleagues (Kimber et al. 1988) found that expression of specific Galβ1-3(4)GlcNAc related oligosaccharide structures showed independent changes in staining intensity in the mouse uterus during the peri-implantation period of pregnancy. Similar findings have been reported for the expression of Galβ1-3(4)GlcNAc related oligosaccharide structures in the goat (Powell et al. 2000). In an *in vitro* model of mouse blastocyst adhesion and trophoblast outgrowth on endometrial epithelial monolayers, lacto-N-fucopentose I, produced a significant reduction in the percentage of attached and outgrown blastocysts after co-culture (Lindenberg et al. 1988). Additionally, in both sheep and pigs, the apical surface of elongating conceptus trophectoderm abundantly express glycocongugates composed of fucose (Whyte and Robson 1984). Trophectodermal expression of N-acetyl-D-glucosamine saccharides appears between Days 14 and 17 in sheep. However, only weak expression of N-acetyl-D-glucosamine and D-glucopyranosyl are detectable at the apical surface of endometrial epithelia on Days 14 and 17 of pregnancy (Whyte and Robson 1984). In a number of species, fucosylated carbohydrate antigens carried on related N-linked blood group glycans are expressed by the endometrium and their expression is changed during the peri-implantation period (Woldesenbet et al. 2002). In sheep, one of these carbohydrate antigens, H-type 1, is expressed by the LE and GE during pregnancy recognition and is increased by progesterone and IFNτ *in vitro* (Woldesenbet et al. 2002).

In addition to the presence of specific oligosaccharides at the fetal-maternal interface, there are also a number of glycoproteins that contain carbohydrate residues, such as laminin and fibronectin, two major ECM components. These may be important for attachment and adhesion during implantation. In the rhesus monkey, laminin protein

is detected on the uterine LE and cytotrophoblast on Day 25 of pregnancy (Qin et al. 2003). In the pig, fibronectin and vitronectin are present on the trophectoderm, but only vitronectin was detected on uterine epithelia (Bowen et al. 1996). In that study, vitronectin and fibronectin were detected at sites of attachment between uterine epithelial cells and trophectoderm on Days 12 to 15 of pregnancy. Similarly, oncofetal fibronectin is detected on both the uterine epithelium and trophectoderm during pregnancy in the pig (Tuo and Bazer 1996). Although little is currently known of the distribution of ECM molecules in the sheep uterus, oncofetal fibronectin is expressed at the apical surface of the LE, GE, and the conceptus trophectoderm (G.A. Johnson, unpublished observations). Additionally, the glycosaminoglycan content of the ovine endometrium is high and is regulated by steroid hormone levels, although the spatial patterns of expression of many of these ubiquitous ECM molecules is unknown (Tellbach et al. 2002). Therefore, a number of glycocongugates are present at the fetal-maternal interface during the peri-implantation period and may be involved in promoting attachment and adhesion of the conceptus to LE.

Role of Endometrial Secretions During Gestation

Histotroph Produced by LE, sGE, and GE

Endometrial epithelia, both LE and GE, produce secretions, termed histotroph (Amoroso 1951; Bazer 1975). As demonstrated in Table 2.1, histotroph is complex and contains numerous binding and nutrient transport proteins, ions, mitogens, cytokines, lymphokines, glucose, enzymes, hormones, growth factors, protease inhibitors and many other substances (Bazer 1975; Bazer and First 1983; Carson et al. 2000; Kane et al. 1997; Martal et al. 1997; Roberts and Bazer 1988; Simmen and Simmen 1990). Histotroph is proposed to be involved in trophoblast growth regulation, conceptus attachment and implantation, and perhaps immunological protection of the fetus (Flèchon et al. 1986; Geisert et al. 1992; Lee et al. 1998; Roberts and Bazer 1988).

The components found in histotroph have been demonstrated to be essential in many species to support peri-implantation conceptus survival and growth. In addition, histotroph from the endometrial glands has also been hypothesized to be an important supplement to hematotrophic nutrition during mid- to late pregnancy in domestic livestock (Heap et al. 1979; Stewart et al. 2000; Wimsatt 1950), as well as humans (Burton and Jaunaiux 2001; Burton et al. 2002). Compared to domestic livestock, which exhibit superficial implantation and an epitheliochorial type of placentation, implantation in the human establishes a precocious and intimate apposition between the maternal and fetal tissues (Burton and Jaunaiux 2001). In the past it has been assumed that this relationship permits early onset of hematotrophic exchange. However, Burton and Jauniaux (Burton and Jaunaiux 2001) suggest that human pregnancy comprises two contrasting periods. During the first trimester there is little maternal blood flow to the placenta, the oxygen tension within the feto-placental unit is low, and the uterine glands may provide much of the nutrient supply. At the start of the second trimester the maternal circulation within the intervillous space becomes fully established, the oxygen tension rises and hematotrophic nutrition becomes dominant. In addition, the

Table 2.1. Components of ovine histotroph (proteins synthesized and/or transported by the endometrium, not including conceptus secretory proteins) and temporal patterns of secretion during the estrous cycle and pregnancy.

Substance	Cycle	Pregnancy	Reference
(1) Proteins			
β2M		16	(Vallet and Lamming 1991)
Gastrin-releasing peptide	16	17-20, 115-145	(Giraud et al. 1993; Whitley et al. 1998)
GlyCAM-1	1-11	13-19	(Spencer et al. 1999a)
Endothelin	12-16	15-20	(Riley et al. 1994)
OPN		13-120	(Johnson et al. 2003b; Johnson et al. 1999a; Johnson et al. 1999b)
ISG15		13-120	(Johnson et al. 1999c; Joyce et al. 2005)
UTMP (uterine serpins)	13-15	15-150	(Moffatt et al. 1987a; Stewart et al. 2000)
Albumin	12-17	12-17	(Lee et al. 1998)
Immunoglobulins	12-17	12-17	(Lee et al. 1998)
Apolipoprotein A1		17	(Lee et al. 1998)
Serum albumin	12-17	12-17	(Lee et al. 1998)
Unknown 14-kDa protein (Galectin-15)	12-14	12-17	(Kazemi et al. 1990; Lee et al. 1998) (CHAPTER IV)
Megasuppressin		60,100,140	(Skopets and Hansen 1993; Stephenson et al. 1989)
(2) Enzymes (by activity)			
OAS		16	(Mirando et al. 1991)
α-D-glucosidase		13-15	(Roberts et al. 1976)

Table 2.1 Continued

Substance	Cycle	Pregnancy	Reference
(2) Enzymes (by activity; cont)			
α-L-fucosidase	13	17-18	(Roberts et al. 1976)
β-D-galactosidase	13-15	13-15	(Roberts et al. 1976)
β-N-acetylgalactos-aminidase	13	7-18	(Roberts et al. 1976)
β-N-acetylglucos-aminidase	13	7-18	(Roberts et al. 1976)
Cluster Differentiation Antigen-26		60-140	(Liu and Hansen 1995)
(3) Growth factors			
Insulin-like Growth Factor (IGF)-I	3-16	3-22	(Cann et al. 1998)
IGF Binding Protein-3 (proteolyzed forms)	12-15	12-15	(Peterson et al. 1998)
Transforming growth factor (TGF)-β1	13-16	14-30	(Chene et al. 1991; Dore et al. 1996)
TGF-β2	13-16	14-30	(Dore et al. 1996)
TGF-β3	13-16	16-30	(Dore et al. 1996)
Uterine Derived Growth Factor		120	(Bird et al. 1988)
(4) Cytokines			
Granulocyte-Macrophage Colony Stimulating Factor		17	(Imakawa et al. 1993)
Leukemia Inhibitory Factor	4-16	4-20	(Vogiagis et al. 1997)

Table 2.1 Continued

Substance	Cycle	Pregnancy	Reference
(4) Cytokines (cont)			
Platelet Activating Factor	14-15		(Battye et al. 1996)
(5) Others			
Calcium		30-144	(Harrison et al. 1976; Moffatt et al. 1987b)
Glucose		140	(Bazer et al. 1979a)
Prostaglandin $F_{2\alpha}$	13-15	30-144	(Harrison et al. 1976; Moffatt et al. 1987b)

endometrial glands remain functional in the human uterus throughout gestation (Graham J. Burton, personal communication).

Histotroph is produced by both the LE and GE; however, the majority is produced by the endometrial GE (Miller and Moore 1983). The functions of these secretions may differ depending upon the type of epithelium from which it originates. Secretions from the LE appear to assist in implantation, while those from the GE may aid more in development and nutrition of the post-implantation conceptus (Martal et al. 1997; Roberts and Bazer 1988; Spencer et al. 1999c).

Crystalline Inclusions in the Endometrium and Conceptus

Progesterone-induced crystal structures are present in the endometrial epithelium and/or conceptus trophectoderm of a number of species, including sheep (Kazemi et al. 1990; Wintenberger-Torres and Flechon 1974), mouse (Calarco and Szollosi 1973), rabbit (Daniel and Kennedy 1978; Nakoa et al. 1971), and human (Nakoa et al. 1971). In human and rabbit endometrium, the crystalline inclusions are membrane limited and localized near the nucleus (Nakoa et al. 1971). The presence of crystals is regulated during the menstrual cycle in women and these crystals are secreted into the uterine lumen, after which they may be phagocytosed by the embryo (Nakoa et al. 1971). In the rabbit, crystals are present in embryos by Day 4¼ and are abundant by Day 4½ post-mating. The presence of the crystals is dependent upon the uterus, as crystals are absent in Day 5 embryos restricted to the oviduct and present when those same embryos are then transplanted to the uterus (Daniel and Kennedy 1978). A similar event occurs with bovine embryos developed within a sheep uterus. Normally, crystals are not present in bovine trophectoderm or endometrial epithelium, but are present in bovine trophoblasts when Day 7 blastocysts are transplanted into sheep uteri for 7 to 9 days (Talbot et al. 2000). The crystalline structures are present in the ovine trophoblast beginning on Day 10 post-mating and increase to Day 14. After Day 16 the abundance of crystals begins to decrease in the proximity of the embryo, as the protein is potentially being utilized (Wintenberger-Torres and Flechon 1974). An unknown 14-kDa protein is associated with crystals in the LE and conceptus trophectoderm on Day 16 post-mating in sheep.

This protein is apparently synthesized and secreted from the endometrial epithelia and accumulated by the conceptus (Kazemi et al. 1990), as crystals are also present in uterine histotroph at the fetal-maternal interface (Amoroso 1952). Therefore, the presence of uterine-dependent, progesterone-induced crystals in the conceptus and endometrial epithelium is conserved in a number of species, but the function and identity of these crystals is currently unknown.

Mechanisms Regulating Histotroph Production

Production of histotroph by the endometrium can be regulated by several different mechanisms. Primary regulators of histotroph production are the ovarian steroid hormones, estrogen and progesterone. In rodents, estrogen induces the production of many secretory proteins from the epithelium (Julian et al. 1992). Signals from the conceptus can also modulate the production of secretions by the uterus. In sheep, interactions between the maternal endometrium and the production of IFNτ by the trophectoderm cells of the conceptus results in simultaneous stimulation and inhibition of the secretion of proteins (Godkin et al. 1984a; Vallet et al. 1987). Similarly, in the sow a large "dumping" of histotrophic secretions into the lumen of the endometrial glands occurs immediately following the release of estrogens from the conceptus on Day 11 of pregnancy (Fazleabas et al. 1983; Geisert et al. 1982). The hormonal, cellular and molecular mechanisms regulating uterine gland morphogenesis and function during gestation are not well understood in any species. In both the rabbit and pig, interactions between lactogenic hormones and ovarian steroids are proposed to constitute a "servomechanism" regulating endometrial function (Chilton et al. 1988; Young et al. 1990). Similarly, a hormonal servomechanism also appears to be operative in the ovine uterus that regulates endometrial gland differentiation and function during gestation (Noel et al. 2003; Spencer et al. 1999b).

Endometrial glands undergo a major period of remodeling and growth during pregnancy in sheep. The uterine glands increase in length (four-fold) and width (ten-fold) (Stewart et al. 2000; Wimsatt 1950). Thus, the GE undergoes intense cellular

proliferation and greatly increases the secretory surface area. Established endometrial glands produce additional side-branchings during early pregnancy to increase glandular surface area for the production of histotroph (Wimsatt 1950). During pregnancy in sheep, the endometrium is exposed sequentially to estrogen, progesterone, IFNτ, placental lactogen (oPL) and growth hormone (oGH), that may activate and maintain endometrial remodeling, secretory function, and uterine growth (Spencer et al. 1999b). Estrogen from ovulatory and non-ovulatory follicular waves increases expression of the PR in the endometrial epithelia (Bazer et al. 1998; Spencer and Bazer 1995). Progesterone from the newly formed corpus luteum increases the expression of a number of unique progesterone-responsive genes in the endometrial LE and/or GE (Spencer et al. 1999c). As mentioned previously, continuous exposure of the endometrium to progesterone down-regulates expression of the PR in the endometrial LE and then GE (Spencer and Bazer 1995; Spencer et al. 1999b). Between Days 11 and 15 of pregnancy, PR expression is down-regulated in the LE and superficial GE followed closely by the middle to deep GE. IFNτ is produced by mononuclear cells of the conceptus trophectoderm between Days 8 to 21 in sheep and acts in a paracrine manner on the adult endometrium (Ashworth and Bazer 1989b).

The paradigm of loss of PR in uterine epithelia immediately prior to implantation is common across mammals, including sheep (Spencer and Bazer 1995), cattle (Kimmins and MacLaren 2001), pigs (Geisert et al. 1994), western spotted skunks (Mead and Eroschenko 1995), baboons (Hild-Petito et al. 1992), rhesus monkeys (Okulicz and Scarrell 1998), humans (Okulicz and Scarrell 1998), and mice (Tan et al. 1999). Recent results strongly support the hypothesis that progesterone down-regulation of PR gene expression in the endometrial GE is required for progesterone induction of secretory gene expression, such as the uterine serpins, or uterine milk proteins (UTMP) and OPN (Johnson et al. 2000b; Spencer et al. 1999b). Administration of estrogen with progesterone induced PR expression in endometrial GE and concomitantly ablated effects of progesterone alone to induce UTMP and OPN mRNA expression in GE (Spencer et al. 1999b). Similarly, administration of the PR antagonist ZK136,317 (ZK)

Table 2.2. Hormones secreted by the ovine conceptus involved in a servomechanism. Sequential production of these placental hormones is necessary for maintenance of pregnancy in the sheep (Gray et al. 2001a).

<table>
<tr><th>Hormone</th><th>IFNτ</th><th>Placental Lactogen</th><th>Growth Hormone</th></tr>
<tr><td>Day</td><td>8-20</td><td>16-147</td><td>35-70</td></tr>
<tr><td>Source</td><td>Mononuclear cells</td><td>Binucleate cells</td><td>Syncytial cells</td></tr>
<tr><td>Receptor</td><td>Type I IFN receptor</td><td>Homodimer PRLR
Heterodimer PRLR/GHR</td><td>Homodimer GHR</td></tr>
<tr><td rowspan="3">Function</td><td>Signal for maternal recognition of pregnancy</td><td colspan="2" rowspan="2">Stimulation of GE proliferation and differentiated function</td></tr>
<tr><td>Maintain corpus luteum (progesterone production)</td></tr>
<tr><td colspan="3">Increased secretion of histotroph</td></tr>
</table>

along with progesterone ablated effects of progesterone alone to induce OPN mRNA expression in GE (Johnson et al. 2000b). In that study, the ZK anti-progestin prevented progesterone from down-regulating PR expression.

As shown in Table 2.2, secretion of oPL by the binucleate cells of the conceptus trophectoderm begins on Day 16 of pregnancy, with peak secretion occurring from Days 120 to 130 of gestation (Kelly et al. 1974; Wooding 1992). PL can bind to the long form of the prolactin receptor (PRLR) (Schuler et al. 1997) as well as to a heterodimer of a PRLR and GH receptor (GHR) (Herman et al. 2000; Noel et al. 2003), which is expressed exclusively by GE and increases throughout gestation (Stewart et al. 2000). GH is secreted by the ovine placenta on Days 35 to 70 of gestation and binds to endometrial GHR (Lacroix et al. 1996). Sequential treatment of ewes first with daily injections of progesterone, followed by intrauterine infusion of oIFNτ from Days 11 to 15 post-estrus and then oPL from Days 21 to 25, increased proliferation of GE in the deep stratum spongiosum. Further, infusion of oGH into the uterine lumen from Days 21 to 25 increased uterine gland density in the deep stratum spongiosum, and increased the size of endometrial glands in the shallow stratum spongiosum (Spencer et al. 1999b). Results of these studies indicate that a developmentally programmed sequence of events, mediated by specific paracrine-acting factors at the conceptus-endometrial interface, ultimately supports both endometrial remodeling and induction of increases in uterine secretory activity (i.e., UTMP and OPN) during ovine gestation (Spencer et al. 2004). Whether similar gestational servomechanisms regulate uterine gland development and function in other species remains to be determined. However, strategic manipulation of such mechanisms may offer therapeutic schemes designed to improve uterine capacity, conceptus survival and reproductive health (Spencer et al. 2004).

Animal models illustrating functional importance of endometrial glands

In the rodent, the period of pre-implantation embryonic development is extremely short in comparison with domestic livestock. The murine blastocyst enters the uterus, hatches from the zona pellucida and begins to implant on Day 4 (Day

0=fertilization) (Finn and McLaren 1967; Orcini and McLaren 1967). Although the pre-implantation period is short, at least two factors produced only by the endometrial glands are required for successful establishment of uterine receptivity and conceptus implantation, leukemia inhibitory factor (LIF) and calcitonin (Bhatt et al. 1991; Zhu et al. 1998a). In the sheep, findings from the UGKO ewe model indicate that components of histotroph are also required for peri-implantation conceptus survival and development (Gray et al. 2000a; Gray et al. 2002; Gray et al. 2001c). In humans and domestic livestock, models are needed to address the role of endometrial glands in uterine support of conceptus growth and development during mid to late gestation.

Leukemia Inhibitory Factor

One substance required for conceptus development that is present in mouse uterine secretions is LIF (Carson et al. 2000; Stewart and Cullinan 1997; Vogiagis and Salamonsen 1999). LIF is a pleiotropic cytokine that exhibits a multitude of biological effects including modulation of cell proliferation and differentiation. In mice, LIF is expressed only in the endometrial glands and exhibits distinct temporal changes in expression during early pregnancy. A burst of LIF expression is detected on Day 1 of pregnancy that declines by Day 3 (Bhatt et al. 1991). A second burst of LIF expression occurs in the endometrial glands on Day 4 which is the beginning of implantation. After Day 4, LIF expression is detected at low levels; a decline that is likely the result of gland ablation by decidualization. The increased circulating levels of estrogen on Days 3 to 4 of pregnancy is suggested to stimulate uterine LIF expression (Bhatt et al. 1991; Shen and Leder 1992). Studies using LIF knockout (LIF-KO) mice have demonstrated that maternal LIF is necessary for normal implantation (Stewart et al. 1992). LIF-KO mice are viable and ovulate normally, but blastocysts fail to implant. Although LIF is expressed normally in pseudopregnant mice, the endometrium of LIF-KO mice is unable to undergo a decidual response naturally, or following artificial stimulation with intrauterine injection of paraffin oil (Stewart et al. 1992). Embryos from LIF-KO mice are viable and will implant in wild type mice. Recent evidence indicates that LIF from the endometrial glands is important for preparation of the uterus that is required for

blastocyst activation (Carson et al. 2000). In particular, expression of heparin-binding epidermal growth factor is not detected in LIF-KO mice (Song et al. 2000).

In humans, maximal LIF expression is detected in the endometrial glands during the secretory phase of the menstrual cycle (Cullinan et al. 1996). LIF has also been identified in uterine flushings from rabbits (Yang et al. 1995), pigs (Anegon et al. 1994), sheep (Vogiagis et al. 1997), and western spotted skunk (Hirzel et al. 1999). LIF mRNA is expressed throughout the ovine estrous cycle and exhibits an increase at the beginning of implantation (Day 16). Passive immunization of ewes against LIF to neutralize its activity decreased pregnancy rates to 33.5%, even though LIF activity was only reduced and not ablated. Thus, LIF may be obligatory or facilitory for implantation in sheep (Vogiagis et al. 1997). Available evidence supports an essential role for LIF in embryo implantation across a wide variety of species.

Calcitonin

Calcitonin is a 32-amino acid peptide hormone previously thought to be produced exclusively by the parafollicular C cells of the thyroid gland as a regulator of calcium in bone and kidney cells (Foster 1968). Calcitonin has been identified as a gene expressed transiently by the endometrial glands of the uterus during the peri-implantation period (Ding et al. 1994). In rats, calcitonin expression increases in the endometrial glands on Day 2 of pregnancy and reaches a peak on Day 4 which is the day before implantation. On the day of implantation (Day 5), calcitonin expression declines and is undetectable by Day 6. In contrast to LIF expression in the endometrial glands, calcitonin is progesterone-dependent (Zhu et al. 1998a). Using a delayed implantation rat model, Zhu et al. (Ding et al. 1994; Zhu et al. 1998a; Zhu et al. 1998b) demonstrated that levels of calcitonin remained high past Day 6, until the administration of estrogen to induce blastocyst implantation. Intrauterine injection of antisense oligonucleotides against calcitonin mRNAs suppressed calcitonin expression and dramatically reduced the number of implantation sites in rats (Zhu et al. 1998b). Therefore, calcitonin is an essential component of histotroph that is required for blastocyst implantation. Calcitonin may modulate intracellular calcium oscillations in the embryo (Armant et al. 2000),

which may serve as a signaling event in embryonic development to regulate trophoblast adhesion and/or integrin trafficking (Wang et al. 1998).

In the human, calcitonin expression is restricted to the endometrial epithelium of the midsecretory phase of the cycle, which closely overlaps the window of implantation (Kumar et al. 1998). In addition, progesterone regulates expression of calcitonin in human endometrium. Therefore, calcitonin is a unique marker of uterine receptivity in both the mouse and human. Calcitonin expression in the sheep uterus is unknown.

Ovine Uterine Gland Knockout (UGKO) Model

The UGKO ewe is a novel model in which endometrial gland morphogenesis is epigenetically ablated by administration of a synthetic progestin during the critical period of endometrial gland morphogenesis in the developing neonate (Gray et al. 2000a; Spencer et al. 1999c). The progestin specifically ablates development of the GE within the endometrium (Fig. 2.4), without altering development of the uterine myometrium or other Müllerian duct-derived female reproductive tract structures (Gray et al. 2001b; Gray et al. 2000b). This suggests that morphogenesis of extra-uterine reproductive tract structures is completed prior to birth in sheep. UGKO ewes fail to cycle normally (Gray et al. 2000a). During normal luteolysis, pulses of oxytocin released from the posterior pituitary and CL bind to OTR present in LE and sGE to induce luteolytic pulses of prostaglandin (PG)$F_{2\alpha}$ (McCracken et al. 1999). In the UGKO ewe, expression of hormone receptors (PR, ERα and OTR) in the uterine LE is not different from that observed in normal cyclic ewes. Oxytocin challenges to measure uterine release of $PGF_{2\alpha}$ produced subluteoltyic pulses of $PGF_{2\alpha}$ in UGKO ewes (Gray et al. 2000a). Therefore, available evidence suggests that expression of OTR by the LE and sGE of the uterine endometrium are necessary for cyclicity.

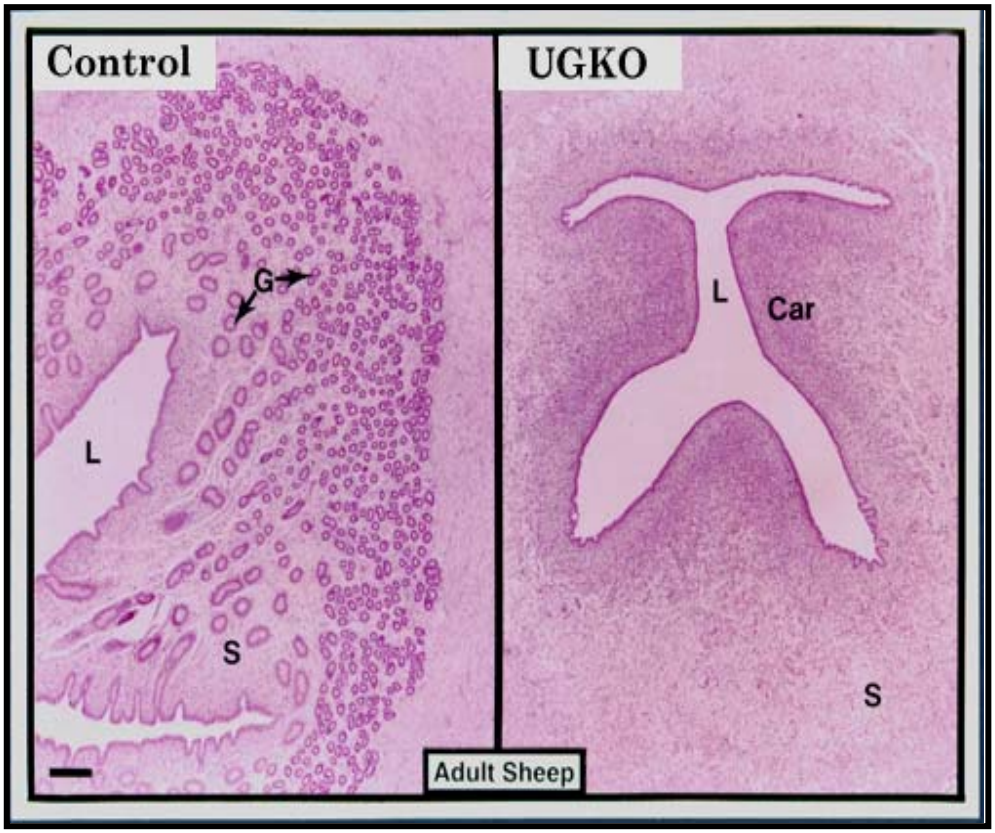

Fig. 2.4. Representative photomicrographs of uterine histology of adult control and UGKO ewes. The endometria from control, untreated ewes contain numerous uterine glands. The endometria from UGKO ewes contain no endometrial glands; however, the LE appears ruffled in the presumptive intercaruncular regions. Additionally, the endometrial area and LE length are reduced in the UGKO ewes compared with the controls. (Bartol et al. 1999)

Despite repeated matings to fertile rams, adult UGKO ewes are unable to establish pregnancy (Gray et al. 2000a; Gray et al. 2002; Gray et al. 2001c). Transfer of normal hatched blastocysts into the uteri of timed recipient UGKO ewes fails to ameliorate the pregnancy defect (Gray et al. 2001a). Normal blastocysts can be found in the uterine flushes of bred UGKO ewes on Days 6 or 9 post-mating, but not on Day 14 (Gray et al. 2001a; Gray et al. 2001b; Gray et al. 2001c). On Day 14, the uterine flush of bred UGKO ewes either contains no conceptus or a severely growth-retarded conceptus that has failed to elongate (Gray et al. 2001c).

As mentioned previously, adhesion of the conceptus trophectoderm to the LE is regulated by both non-adhesive and adhesive factors (Burghardt et al. 1997; Johnson et al. 2001a). The non-adhesive property of the LE may be partially due to apical Muc-1, that could sterically impair interactions between the trophectoderm and adhesive glycoproteins, such as integrins or carbohydrates. In the UGKO ewes, expression of Muc-1 on the LE is not different from that found in normal ewes (Gray et al. 2002). Therefore, failure of conceptus elongation in UGKO ewes is not due to aberrant expression of anti-adhesive proteins, but could be due to decreased expression of these genes, as the UGKO have less LE and no sGE cells, as compared with control ewes.

Integrins are thought to be the dominant glycoproteins that regulate trophectoderm adhesion to the LE in both caruncular and intercaruncular areas. However, integrin expression in the Day 14 UGKO uterus is not different in the LE as compared to normal Day 14 pregnant ewes (Gray et al. 2001c). Therefore, available

evidence supports the hypothesis that the inability of the conceptus to survive and develop within the UGKO uterus is due to a lack of certain adhesion proteins and perhaps growth factors normally derived from the endometrial LE and GE.

The endometrial glands of the ovine uterus produce several adhesion molecules, such as OPN (Johnson et al. 1999a; Johnson et al. 1999b; Johnson et al. 2000b) and GlyCAM-1 (Spencer et al. 1999a). These proteins are synthesized and secreted by the endometrial epithelium during the peri-implantation period and are thought to aid in conceptus elongation, adhesion, and attachment of conceptus to the LE (Johnson et al. 2001a). Immunoreactive levels of OPN and GlyCAM-1 are much lower or absent in the uterine flushings of Day 14 bred UGKO ewes (Gray et al. 2001c). Previous studies have found no differences in LE of steroid hormone receptors (Gray et al. 2000a) and LE-specific genes (Gray et al. 2001c) in UGKO compared to normal ewes. The LE does not appear to be aberrantly programmed by neonatal progestin exposure in the UGKO. However, there is less LE and no sGE or deeper GE. Thus, the absence of uterine secretions emanating from the GE and/or LE appears to be responsible for the inability of the UGKO uterus to support conceptus survival and development.

Galectins

Galectins are a superfamily of β-galactoside binding lectins. Family members bind β-galactosides via a CRD that contains many conserved sequence elements (Barondes et al. 1994a). Galectins were first discovered as proteins that bound to β-galactoside affinity columns, but novel galectin family members are being discovered rapidly by mass sequencing and differential gene expression analyses. Members of this family must fulfill two key criteria: 1) binding affinity for β-galactosides; and 2) conserved sequence elements in the carbohydrate-binding site (Barondes et al. 1994a). However, most of the novel galectin superfamily members discovered by sequence similarity have not been shown to be expressed or active as lectins. Galectins are widely distributed across mammalian species, as well as have been identified in many non-mammalian species, including birds, amphibians, fish, worms, sponges and fungi (Cooper and Barondes 1999). Databases of genomic DNA sequences and ESTs predict the presence of more putative galectins, in animal, plant and virus systems (Cooper and Barondes 1999).

Galectin Domain Organization

Each member of the galectin family contains at least one conserved domain of about 130 amino acids, the carbohydrate recognition domain (CRD), which is responsible for carbohydrate-binding activity (Cooper 2002; Cooper and Barondes 1999; Gabius et al. 2002). In 1994, galectins were defined as a family of proteins sharing this conserved domain, as well as the ability to specifically bind β-galactoside sugars without requiring metal ions for activity (Barondes et al. 1994a; Barondes et al. 1994b; Rabinovich 1999). The three-dimensional structures of the CRDs derived from galectin-1, -2, -3, -7, -10 and -13 have been elucidated by X-ray crystallography. The CRD is tightly folded with two anti-parallel β-pleated sheets forming a sandwich-like structure (Rini and Lobsanov 1999) that recognizes the basic structure of N-acetyllactosamine (Galβ1-4GlcNAc) (Rabinovich 1999). The interaction between a galectin CRD and the monosaccharide ligand galactose is weak with a dissociation constant in the micromolar

range, but the disaccharide lactose binds with about 100-fold higher affinity than galactose alone (Leffler and Barondes 1986). Some larger oligosaccharides exhibit even higher affinity than lactose, suggesting that the carbohydrate binding site extends beyond the core binding site for galactose. The amino acids in this extended binding site are much less conserved among the galectins than those in the core binding site. Indeed, galectins exhibit different affinities and specificity for longer oligosaccharides. For instance, galectins bind to N-acetyllactosamine with relatively low affinity [dissociation constant (K_d) in the range of 90-100 μM], but they bind to glycoproteins containing polylactosamine sequences with high affinity ($K_d \approx 1$ μM) (Cho and Cummings 1995).

Galectin Types

Most galectins are divalent, either by homodimerization or by including two CRDs within one protein (Brewer and Dam 2002; Gabius et al. 2002; Hirabayashi et al. 2002; Rabinovich et al. 2002b). All galectins can be structurally classified into three basic types (Hirabayashi and Kasai 1993), which achieve divalence or higher valency by distinct mechanisms. Multimerization is a common feature across various families of carbohydrate-binding proteins. This feature may be due to the binding interactions of individual CRDs with their most favored carbohydrate ligands and multimerization can greatly increase binding avidity for multivalent or clustered ligands. Further, the biological significance of multimerization may be that it provides the ability to crosslink ligands. Considerable evidence exists that galectins regulate association of their glycoconjugate ligands on cell surfaces, in extracellular matrices, or both (Brewer and Dam 2002). Indeed, the evolution of galectins into distinct structural classes may have been driven by each class achieving distinct crosslinking properties (Cooper 2002).

Fourteen mammalian galectins have been identified in a wide variety of tissues from different species (Cooper 2002; Cooper and Barondes 1999). "Prototype" galectins (galectin-1, -2, -5, -7, -10, -11, -13 and -14) exist as monomers or noncovalent homodimers, consisting of one domain, the CRD. Most of these galectins, such as galectin-1, self-associate to form homodimers. However, rat galectin-5 is monomeric

(Gitt et al. 1995). Dimerization involves self-association of monomer subunits at sites opposite their CRDs. Thus, the CRD pockets and any unbound ligands face away from each other. These dimers can effectively crosslink separated ligands, to form chains or crystalline networks of the divalent lectin bound to di- or multivalent ligands (Bourne et al. 1994; Brewer and Dam 2002; Sacchettini et al. 2001). "Tandem-repeat" galectins (galectin-4, -6, -8, -9 and -12) contain two non-identical CRDs joined either directly or via a linker peptide of variable length. No crystal structures of these galectins have been reported. The possibility exists that some tandem-repeat galectins have their CRDs positioned to allow simultaneous binding to multivalent ligands of perhaps two distinct types, as opposed to homofunctional crosslinking by dimeric prototype galectins (Arata et al. 1997; Hirabayashi et al. 2002). "Chimeric" galectins (galectin-3) contain an unusual proline- and glycine-rich domain fused onto the CRD. In vertebrates, the only known chimeric galectin is galectin-3 which, similar to other galectins, forms multimers and displays positive cooperativity in binding to immobilized ligand clusters (Lahm et al. 2000).

Galectin Expression

Galectin expression is regulated in a developmental and physiological manner that is highlighted by three important facts: (1) organisms usually expresses multiple members of the galectin family; (2) different cells within an organism usually contain a different complement of galectins; and (3) almost all cells have at least one galectin (Liu et al. 2002). Some galectins, such as galectin-1 and galectin-3, are expressed in many tissues and cell types, including the mid- to late gestation ovine placenta (Iglesias et al. 1998a; Iglesias et al. 1998b). Others are restricted to specific tissues, e.g., galectin-2 and galectin-7 are expressed only in the gastrointestinal tract (Gitt et al. 1992; Oka et al. 1999) and stratified epithelia (Magnaldo et al. 1998; Sato et al. 2002), respectively. Even the widely expressed galectins have distinct expression patterns with marked developmental and physiological regulation (Colnot et al. 1996). Marked induction or

increases in expression of specific galectins have also been noted in many cancers, suggesting their involvement in cancer progression (Danguy et al. 2002).

Intracellular Localization of Galectins

In addition to binding galactose-containing glycoconjugates, some members of the galectin family share another property in terms of their cell biology (Liu et al. 2002). They exhibit dual localization, being found in both intracellular (cytoplasm and, in some cases, the nucleus) and extracellular (cell surface and medium) compartments (Hughes 1997). Although galectins are often present on cell surfaces or in ECM, they are secreted through nonclassical intracellular trafficking, to avoid binding to carbohydrates present in the endoplasmic reticulum and Golgi. Galectins are also localized at the cell surface to mediate cell-to-cell cross-linking through carbohydrates present on the same and different cells (Daniels et al. 2002; Hughes 2001; Rabinovich et al. 2002a). Most galectins have characteristics typical of cytoplasmic proteins, such as an acetylated N-terminus, free sulfhydryls, and lack of glycosylation. The subcellular location of galectins appears to be cytosolic, submembranous, or sometimes nuclear, but not compartmentalized inside classical secretory compartments. Nevertheless, there is strong experimental evidence that galectins are secreted by novel nonclassical mechanisms (Boulianne et al. 2000; Hughes 1999). In this regard, galectins may belong to a small category of proteins, including fibroblast growth factors one and two (FGF-1 and -2) and interleukin-1 (IL-1) that must be secreted by mechanisms distinct from classical vesicle-mediated exocytosis. Prior to secretion, galectins become concentrated under the plasma membrane and in plasma membrane evaginations, which appear to pinch off to form galectin-enriched extracellular vesicles (Cooper 1997).

Biological Functions of Galectins

Galectins function to regulate (promote, strengthen or, in some cases, suppress) the association of recognized glycoconjugate ligands. As shown in Fig. 2.5, functional studies have implicated these proteins as mediators of cell-cell and cell-extracellular

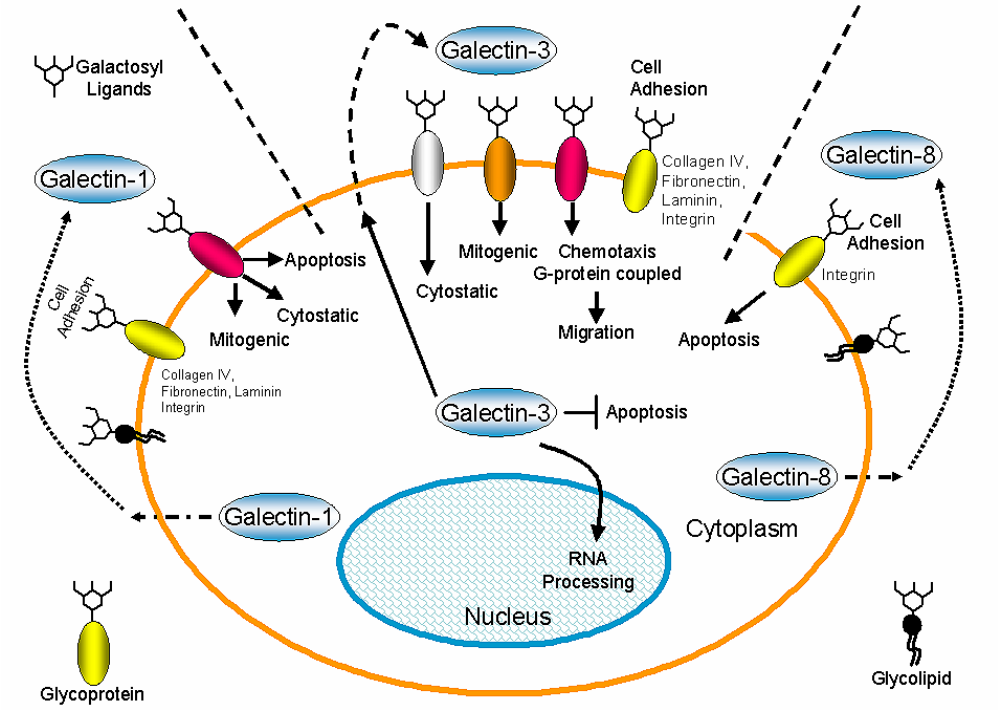

Fig. 2.5. Illustration of the biological functions mediated by three of the most well characterized galectin family members, galectins-1, -3, and -8. Secreted galectin-1 promotes cell adhesion by binding to glycolipid and glycoprotein receptors to induce mitogenesis, cytostasis, and apoptosis. Secreted galectin-3 also binds to cell-surface glycoconjugates to promote cell adhesion, as well as induction of mitogenesis, chemotaxis and cell migration. Intracellular galectin-3 also promotes RNA processing and is anti-apoptotic. Secreted galectin-8 binds to glycoconjugates to promote cell adhesion and is apoptotic.

matrix interactions, and cell growth, differentiation, migration and apoptosis (Hughes 2001; Moiseeva et al. 1999; Perillo et al. 1998; Yang and Liu 2003).

Cell Adhesion

Galectin-1 has been implicated in cellular interactions with the ECM through recognition of poly-N-acetyllactosamine residues on ECM glycoproteins such as laminin and fibronectin (Zhou and Cummings 1993). The adhesive aspects of galectin-1 are controversial, because of both positive and negative effects on cell adhesion to ECM glycoproteins. Indeed, it is possible that this protein promotes cell attachment or detachment according to the type, activation status, or developmental stage of a cell (Cooper 1997). The binary actions of galectin-1 may also be associated to the high or low concentrations of galectin-1 in the ECM or the glycosylation state of counter-receptors. Although this lectin inhibits myoblast interactions with laminin by blocking $\alpha 7\beta 1$ integrin, it has proadhesive effects toward other cell types, such as melanocytes, tetracarcinoma cells, olfactory neurons, rhabdomiosarcoma cells, and fibroblasts (Cooper 1997; Rabinovich 1999).

Controversial results have been found regarding galectin-3 (Rabinovich 1999) which promotes neutrophil adhesion to laminin in the context of an inflammatory response (Kuwabara and Liu 1996). However, galectin-3 has a strong inhibitory effect on melanoma cell adhesion to ECM (Ochieng et al. 1998). Finally, galectin-3 mediates dendritic cell adhesion to lymphocytes following entry via high endothelial venules and after activation via L-selectin (Swarte et al. 1998). Galectin-8 modulates integrin interactions with the ECM (Hadari et al. 2000) and forms complexes with various integrin family members to inhibit cell adhesion. The roles of other galectin family members on cell adhesion and trafficking are unknown.

Cell Growth and Proliferation

Different members of the galectin family exert critical but contradictory effects on cell growth and proliferation. Depending on the study, both negative and positive effects have been reported for galectin-1 on cell proliferation (Chiariotti et al. 1991). Biphasic modulation of cell growth has been reported for recombinant galectin-1 as high

doses inhibited cell proliferation independent of its carbohydrate binding activity, while low doses induced cell proliferation in a lactose-inhibitable fashion (Adams et al. 1996). Galectin-3 promotes cell growth and proliferation and acts as a mitogenic signal for several cell types (Bao and Hughes 1995; Hikita et al. 2000; Honjo et al. 2001; Yoshii et al. 2001). Little is known about the mechanism of growth modulation by galectins. However, the "double-edged" effects of galectins on cell growth and proliferation might be regulated by their relative levels in the extracellular milieu and the equilibrium between their monomeric and dimeric forms. Further, their effects are highly dependent on cell type, cell-activation status, environmental signals, and the availability of ligands.

In contrast to the substantial information on galectins-1 and -3, little is known about the influence of other members of the galectin family on cell growth. Galectin-12 suppresses growth of cells and may play a role in the growth arrest associated with cellular differentiation (Yang et al. 2001). Because targeted disruption of galectins-1 and -3 in mice does not result in major phenotypic abnormalities (Colnot et al. 1998), it might be speculated that other galectin family members compensate for the absence of those proteins.

Cell Migration

Galectins play an important role in modulating different steps in the metastatic cascade (Akahani et al. 1997), as increased expression of galectin-1 and galectin-3 correlates with the malignant potential of many tumor types, possibly by affecting cell motility and invasion of ECM (Bresalier et al. 1998; Bresalier et al. 1997; Ellerhorst et al. 1999; Honjo et al. 2001; Rorive et al. 2001; van den Brule et al. 2001; Xu et al. 1995). The antimetastatic effect of single low-dose cyclophosphamide involves modulation of galectin-1 and Bcl-2 expression by lymphoma and spleen cells (Rabinovich et al. 2002c). Galectin-1 is expressed on tumor cells and appears to increase adhesion of cancer cells to ECM and/or also induce homotypic or heterotypic adhesion between cells through interactions with complementary glycoconjugates (van den Brule et al. 1995). Other galectins also appear to be involved in the metastatic process (Lahm et al. 2001). Galectin-7 is found in human basal and spinous-cell

carcinomas and an early transcriptional target of the tumor suppressor protein p53 (Bernerd et al. 1999). Galectin-8 is found in 59 tumor cell lines, while galectins-2 and -4 are restricted to colorectal and neural tumors, and galectin-9 to colorectal carcinoma cells lines (Lahm et al. 2001). Galectin-8 binds to $\alpha 3\beta 1$ and $\alpha 6\beta 1$ integrins and regulates adhesion and survival of carcinoma cells in the context of the ECM (Hadari et al. 2000; Levy et al. 2001). Galectin-9 is widely distributed, its expression is developmentally regulated (Tureci et al. 1997; Wada and Kanwar 1997; Wada et al. 1997), and it is a well recognized chemoattractant for eosinophils (Matsumoto et al. 1998; Matsushita et al. 2000). Microarray analysis of melanoma cell lines identified galectin-9 as a novel Type I ISG (Chiariotti et al. 1991; Leaman et al. 2002).

Cell Apoptosis

Evidence now suggests that the balance between T-cell proliferation and apoptosis can be influenced by galectins (Rabinovich et al. 2002b). Exogenous galectin-1 induces apoptosis in activated human T cells and certain human leukemia T cell lines (Perillo et al. 1995). Resting T cells also bind galectin-1, but do not undergo apoptosis. The mechanism of galectin-1-induced apoptosis is not known. Galectin-1 is produced by thymic epithelial cells, and certain subsets of thymocytes are susceptible to galectin-1-induced apoptosis (Baum et al. 1995; Hafer-Macko et al. 1996). Galectin-1 binds CD45, CD43 and CD7 on the surface of T cells (Walzel et al. 1999), and galectin-1 regulates CD45-induced signaling in Burkitt lymphoma B cells. High levels of galectin-1 will induce apoptosis of activated T cells and shut off T-cell effector functions after the completion of an immune response (Blaser et al. 1998; Rabinovich et al. 1996; Rabinovich et al. 1998; Zuniga et al. 2001).

Galectins may have effects on cell survival with apoptotic activity being tissue-specific (Rabinovich et al. 2002b). Galectin-7 is only expressed in human epidermis and rapidly induced in skin keratinocytes in response to ultraviolet B irradiation (Bernerd et al. 1999; Magnaldo et al. 1995; Magnaldo et al. 1998). Overexpression of galectin-7 in a squamous cell line induces apoptosis, apparently by increasing *c-Jun* N-terminal kinase activity. The galectin-9 gene is expressed in thymic epithelial cells, and exogenous

recombinant galectin-9 protein induced apoptosis in thymocytes, but not hepatocytes, in a lactose-inhibitable manner (Wada and Kanwar 1997). Finally, galectin-12 is expressed by adipocytes and may induce cell-cycle arrest and apoptosis (Yang et al. 2001).

Immune Response

Galectin family members play a role in both innate and adaptive immune response through mediation of parasite infection, allergic inflammation, and regulation of immune cells (i.e., B cells, T cells, monocyte/macrophages, neutrophils, eosinophils, basophils, and mast cells) (Rabinovich et al. 2004; Young and Meeusen 2004). One galectin family member, galectin-1, is able to induce a shift from Th1 to Th2 immune response by inhibiting IL-2 and IFN-γ production while increasing or maintaining IL-5 and IL-10 production (Rabinovich et al. 2004; Young and Meeusen 2004). This shift to a Th2 response is also beneficial in cases of parasite infection (Young and Meeusen 2004). In contrast, galectin-3, is a pro-inflammatory molecule that promotes cell adhesion, inhibits T-cell apoptosis and activates basophils (Rabinovich et al. 2004). Galectins-1 and -3, co-purified from ovine placental tissue exert antagonistic inhibitory and stimulatory effects on cell proliferation and apoptosis, which are neutralized reciprocally in a natural mixture of these proteins (Iglesias et al. 1998a; Iglesias et al. 1998b). Thus, the interplay between these two carbohydrate-binding proteins might be critical in the establishment of immune homeostasis.

Galectins-1,-3, and -9 are all capable of regulating T cells (Rabinovich et al. 2002a). Because galectin-1 is highly expressed in sites of "immune privilege", such as the placenta (Iglesias et al. 1998b; Maquoi et al. 1997), testis (Wollina et al. 1999), and retina (Maldonado et al. 1999), Rabinovich and coworkers (Rabinovich et al. 2002b) speculate that it ensures rapid elimination of inflammatory T cells to protect these vulnerable sites from tissue damage. Accordingly, galectin-1 is present mainly in trophoblast and endometrium from first-trimester human samples (Maquoi et al. 1997).

Intracellular Functions

Many galectins are predominantly intracellular proteins. Following synthesis on cytoplasmic ribosomes, there appears to be selective intracellular targeting of specific

galectins to subcompartments of the cytosol, to distinct subcellular organelles, and even to membranes and membrane-bound organelles (Liu et al. 2002). Galectin-1 has been localized to both the cytoplasm and nucleus, and appears to be a component of the nuclear matrix with a role in RNA processing (Park et al. 2001). In addition, galectin-1 interacts with the oncogene H-ras to mediate Ras membrane anchorage and cell transformation (Paz et al. 2001) Galectin-3 is concentrated in the nuclei of differentiated colonic epithelial cells, and the progression from normal mucosa to adenoma to carcinoma is characterized by the absence of galectin-3 in the nuclei of adenoma and carcinoma cells (Lotz et al. 1993). Proteins that interact with galectin-3 include Bcl-2, Gemin4, CBP70, and cytokeratin (Goletz et al. 1997). In addition, galectin-7, -10, -11 and -12 are present in both the nucleus and cytoplasm. Established roles for intracellular galectins include regulation of cell transformation, nuclear splicing of pre-mRNA, and regulation of the cell cycle through G1 or G2/M arrest and anti-apoptosis (Liu et al. 2002). Galectins, as a family of phylogenetically conserved proteins, may have initially served a carbohydrate recognition function outside of cells and then become adapted for protein-protein interactions for functions within cells. Alternatively, galectins may have been intracellular proteins that evolved into carbohydrate recognition molecules outside of cells.

CHAPTER III
TRANSCRIPTIONAL PROFILING OF THE OVINE ENDOMETRIUM DURING THE PERI-IMPLANTATION PERIOD

Introduction

Conceptus survival and growth is dependent on endometrial responses to pregnancy. During early pregnancy, the endometrium synthesizes and secretes, as well as selectively transports a variety of substances, collectively termed histotroph, into the uterine lumen (Amoroso 1951; Bazer 1975). Histotroph is a complex mixture of transport proteins, adhesion proteins, cytokines, growth factors, hormones, protease inhibitors, amino acids and ions (Kane et al. 1997). In laboratory animals, several components of uterine histotroph, including leukemia inhibitory factor and calcitonin, are necessary for conceptus survival and growth and establishment of uterine receptivity (Carson et al. 2000). In humans, histotroph appears to be the primary source of nutrition for conceptus development during the first trimester before hematotrophic nutrition is established (Burton et al. 1999; Burton et al. 2002). Uterine secretions are hypothesized to be of particular importance for conceptus survival and growth in domestic animals due to the prolonged length of the peri-implantation period and superficial nature of implantation and placentation (Bazer 1975; Bazer et al. 1979b; Roberts et al. 1987; Spencer et al. 2004). However, the components of histotroph, for the most part, remain poorly defined.

The UGKO ewe model was developed to study the role of histotroph in conceptus survival and development. In this model, postnatal endometrial gland morphogenesis is inhibited by exposing neonatal ewes to a 19-norprogestin from birth to at least 8 weeks of age (Gray et al. 2000a; Spencer et al. 1999c). Progestin exposure specifically ablates development of the endometrial glands in the uterus without altering development of the uterine myometrium or other Müllerian duct-derived female reproductive tract structures (Gray et al. 2001b; Gray et al. 2000b). In addition to the absence of superficial and deep endometrial glands, the endometrium contained less LE

due to the lack of endometrial folds (Gray et al. 2001b). Adult UGKO ewes are unable to establish pregnancy (Gray et al. 2000a; Gray et al. 2001b; Gray et al. 2002; Gray et al. 2001c). Transfer of hatched blastocysts, recovered from superovulated normal donor ewes, into the uteri of timed recipient UGKO ewes failed to ameliorate the pregnancy defect (Gray et al. 2001c). Morphologically normal blastocysts were found in the uterine flushings of bred UGKO ewes on Days 6 and 9 post-mating, but not on Day 14 (Gray et al. 2001b; Gray et al. 2001c). On Day 14, uterine flushings from bred UGKO ewes contained either no conceptus or a severely growth-retarded conceptus that had failed to elongate from a tubular to filamentous form (Gray et al., 2001c). In sheep, the peri-implantation period is marked by rapid transition of conceptuses from a tubular to filamentous forms that is initiated on Days 11 to 12 (Guillomot 1995; Spencer et al. 2004) and production of IFNτ, a Type I IFN that is antiluteolytic and the signal for maternal recognition of pregnancy in ruminants (Bazer et al. 1997). Available results indicate that endometrial insufficiency in UGKO ewes leads to the absence of or a reduction in histotroph of endometrial epithelial origin, resulting in recurrent pregnancy loss (Gray et al. 2002; Spencer et al. 1999c). Indeed, Flèchon et al. (1986) surmised that histotroph from the endometrium is required for conceptus elongation in sheep, as it does not occur *in vitro*.

The purpose of this study was to create and use a custom ovine endometrial cDNA array to gain insight into hormonally regulated genes crucial for endometrial support of early conceptus survival and growth in sheep and perhaps other mammals. Specific objectives were to utilize the custom array to profile gene expression in endometria of: (1) Day 14 cyclic and pregnant fertile ewes and UGKO infertile ewes; and (2) ewes treated with progesterone and/or IFNτ.

Materials and Methods

Animals

Experimental and surgical procedures complied with the Guide for Care and Use of Agriculture Animals and were approved by the University Laboratory Animal Care

and Use Committee as well as the Institutional Agricultural Animal Care and Use Committee of Texas A&M University.

UGKO ewes were produced as described previously (Gray et al. 2000a; Spencer et al. 1999c) by implanting crossbred Rambouillet ewe lambs with a single Synchromate B® (Sanofi, Overland Park, KS) implant within 12 h of birth and every two weeks thereafter for a total of eight weeks. Implants were inserted subcutaneously in the periscapular area and released approximately 6 mg of norgestomet (17α-acetoxy-11β-methyl-19-norpreg-4-ene-3,20-dione), a potent synthetic 19-norprogestin, over a 14 day period (Bartol et al. 1988a). Control ewes did not receive implants.

Study One

In order to synchronize estrus, adult UGKO (n=4) and normal crossbred ewes (n=8) were given two intramuscular (i.m.) injections (0700h and 1700h) of 10 mg PGF2α (Lutalyse, Upjohn, Kalamazoo, MI) nine days apart. Ewes were monitored daily for estrous behavior using vasectomized rams. All UGKO and some of the normal ewes (n=4) were bred when detected in estrus (Day 0) and 12 h and 24 h later by intact rams of proven fertility. The remaining normal ewes (n=4) were assigned to cyclic status, and time of onset of estrus was determined by vasectomized rams. On Day 14 post-estrus/mating, all ewes were subjected to mid-ventral laparotomy, and the uterine lumen flushed with 20 ml sterile saline followed by ovariohysterectomy. Uterine flushes were analyzed using a dissecting microscope to recover conceptuses, if any, and determine their morphology.

Study Two

In Study Two, cyclic ewes (n=20) were checked daily for estrus and then ovariectomized and fitted with indwelling uterine catheters on Day 5 as described previously (Spencer et al. 1999b). Ewes were then assigned randomly (n=5 per treatment) to receive daily i.m. injections of progesterone and/or a PR antagonist (ZK 136,317; Schering AG, Germany) and intrauterine (i.u.) infusions of control serum

proteins and/or recombinant ovine IFNτ protein as follows: (1) 50 mg progesterone (P, Days 5 to 16) and 200 μg control (CX) serum proteins (Days 11 to 16) [P+CX]; (2) P and 75 mg ZK 136,317 (ZK; Days 11 to 16) and CX proteins [P+ZK+CX]; (3) P and IFNτ (2 x 10^7 antiviral units, Days 11 to 16) [P+IFN]; or (4) P and ZK and IFNτ [P+ZK+IFN]. Steroids were administered daily in corn oil vehicle. Both uterine horns of each ewe received twice daily injections of either CX proteins (50 μg/horn/injection) or roIFNτ (5 x 10^6 antiviral units/horn/injection). roIFNτ was produced in *Pichia pastoris* and purified as described previously (Van Heeke et al. 1996). Proteins were prepared for intrauterine injection as described previously (Spencer et al. 1999b). This regimen of progesterone and IFNτ mimics the effects of progesterone and the conceptus on endometrial expression of hormone receptors and ISGs during early pregnancy in ewes (Johnson et al. 2001b; Kim et al. 2003a; Spencer et al. 1995a). All ewes were hysterectomized on Day 16.

For both studies, several sections (~1 cm) from the middle of each uterine horn were snap frozen in Tissue-Tek OCT compound (Miles, Oneonta, NY) and stored at -80°C. Several sections from the middle region of each uterine horn were also fixed in 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 endometrium was physically dissected from myometrium for the remainder of each uterine horn ipsilateral to the ovary bearing the corpus luteum and then snap-frozen in liquid nitrogen and stored at -80C for RNA extraction.

Preparation of Day 14 Pregnant Ovine Endometrial cDNA Microarray

A cDNA library was prepared by Clontech (Palo Alto, CA) from total endometrial RNA pooled from the four Day 14 pregnant ewes in Study One. The directional library was synthesized using the λTriplEx2 phage vector and cDNA generated by random and oligo-dT priming of purified polyadenylated mRNA. The library possessed >2x10^6 clones with an average insert size of 1.2 kb and less than 5% of clones from genomic, mitochondrial or ribosomal origin. The pTriplEx2 plasmid vector

containing the endometrial cDNAs was excised using the Cre-lox system. Library clones were plated onto Q-trays (Genetix, Queensway, United Kingdom) containing LB agar and carbenecillin (50 μg/ml) and grown overnight. Individual clones were picked and inoculated into the wells of 384-well plates using a Q-bot (Genetix). The cDNA clones were stored at -80^{o}C and subsequently grown in four 96-well plates for plasmid production. Approximately 5,000 clones from the library were selected, sequenced and subjected to PCR amplification for microarray printing. These plasmids were isolated from liquid bacterial cultures using a Qiagen BioRobot 3000 system and Qiagen R.E.A.L. Prep 96 Biorobot kits (Valencia, CA).

Plasmids obtained from the library were used as templates for DNA sequencing reactions. Sequencing reactions were carried out using 5'-TCCGAGATCTGGACGAGC-3' primer and Big Dye Terminator Cycle Sequencing Ready Reactions (Applied Biosystems, Foster City, CA) in MJ PTC-200 96 well thermocyclers (Waltham, MA). Salts and unicorporated dye terminators were removed using Montage 96 Sequencing Reaction Cleanup kits (Millipore; Billerica, MA) and reaction products were analyzed on a MegaBACE automated DNA sequencer (Amersham Biosciences; Piscataway, NJ).

Phred was used for base calling of sequences that were vector trimmed upstream of the EcoRI site (Ewing and Green 1998; Ewing et al. 1998). Phrap was used for clustering/contiging with the following command line: phredPhrap –penalty –15 –shatter_greedy –bandwidth 30 –minscore 100 (www.phrap.org). Contigs were edited using Consed. BLAST searches were performed on all sequences (Altschul et al. 1990; Altschul et al. 1997), and output was parsed into tabular form. Sequences of ≥50 bp with Phred 20 quality after vector clipping were deposited in GenBank with Accession Nos. CD285971-CD289358.

The first 4,992 cDNAs were used to construct the microarray. The cDNAs were amplified by PCR using 5'-TCCGAGATCTGGACGAGC-3' as a forward primer and 5'-TAATACGACTCACTATAGGG-3' as a reverse primer. PCR conditions were as follows: 95^{o}C for 5 min, 35 cycles of 95^{o}C for 30 sec, 54^{o}C for 30 sec, and 72^{o}C for 30

sec, and a final extension of 72°C for 7 min. PCR products were purified by ethanol precipitation, verified using agarose gel electrophoresis and used to create Day 14 pregnant ovine endometrial cDNA microarrays. The concentration of these cDNAs was approximately 100 ng per µl in 3X saline sodium citrate (SSC). An OmniGrid microarrayer (GeneMachines, San Carlos, CA) equipped with 8 Telechem (Sunnyvale, CA) SMP3 pins was used to spot the PCR products onto poly-L-lysine coated glass slides (CEL Associates, Houston, TX) in duplicate. Slides were dried and stored dessicated until used. Glass slide microarrays were rehydrated over steam and snap dried at 95°C prior to UV cross-linking the DNA to the slides using a Stratalinker (Stratagene, La Jolla, CA).

Microarray Probe Labeling and Hybridization

Total RNA was isolated from endometrial samples using Trizol reagent according to manufacturer's instructions (Gibco BRL, Grand Island, NY). The polyadenylated mRNA was obtained using an Oligotex mRNA Kit (Qiagen), amplified with a MessageAmp aRNA Kit (Ambion, Austin, TX), and quality and quantity were determined with an Agilent 2100 Bioanalyzer (Agilent Technologies, Palo Alto, CA). Amplified RNA from ewes in each status group was pooled into cyclic (C), pregnant (PX), and UGKO (U) for Study One and pooled into P+CX, P+IFN, P+ZK+CX and P+ZK+IFN for Study Two. A universal control sample from the pooled Day 14 pregnant RNA was utilized to compare against each of the different groups in both studies. From Study One, Groups C and U were compared without the universal control sample included. Approximately 200 ng of RNA from each group was labeled with 3DNA Array350RP Cy3 and Cy5 kits (Genisphere, Hatfield, PA) for dual labeling (PX vs. U, PX vs. C, C vs. U, PX vs. P+CX, PX vs. P+IFN, PX vs. P+ZK+CX, and PX vs. P+ZK+IFN), according to manufacturer's instructions. Each combination of fluorescent dual labeling was performed in triplicate, including one dye-swap. Probes were added to slides, cover-slipped, and incubated overnight in a humidified hybridization chamber that was submerged in a 65°C water bath. Slides were washed and visualized following

a second hybridization with the capture reagent. Fluorescent intensities of Cy3 and Cy5 were measured with an Affymetrix 428 (Santa Clara, CA) array scanner at 532 and 635 nm, respectively. After scanning, spots were aligned and analyzed with GenePix Pro 3.0 microarray analysis software (Axon Instruments, Union City, CA).

Microarray Analysis

GeneSpring 7.1 (Silicon Genetics, Redwood City, CA) was used for analysis of the microarray data. To account for dye swap, the signal channel and control channel measurements for dye swapped samples were reversed. A Lowess curve was fit to the log-intensity versus log-ratio plot. Twenty percent of the data were used to calculate the Lowess fit at each point. This curve was used to adjust the control value for each measurement. If the control channel was lower than 10 then 10 was used instead. To eliminate any effect of the dye swap, all genes were fit to a flat line (0.90). This eliminated approximately one-half of the genes in the microarray analyses. Lists were created from genes that passed the students t-test with a p-value of 0.05 or less and variances were assumed equal.

Slot Blot Hybridization Analysis

Galectin-15, IP-10, connective tissue growth factor (CTGF), ferritin, prothymosin-α, thioredoxin and 1-8U cDNAs were amplified by PCR using the primers and conditions described above and T/A cloned into pCRII (Invitrogen; Carlsbad, CA). The β2M, MHC Class I, Stat1 and carbonic anhydrase 2 (CA2) cDNAs were utilized in previous studies (Choi et al. 2001; Choi et al. 2003; Hu et al. 2004). Steady-state levels of galectin-15, IP-10, Stat1, β2M, MHC Class I, CTGF, and 1-8U mRNA were assessed by slot blot hybridization as described previously (Spencer et al. 1999c). Radiolabeled antisense cRNA probes were generated from linearized cDNA by in vitro transcription with [α-^{32}P]UTP. Denatured total endometrial RNA (20 μg) from ewes in Studies One and Two were hybridized with radiolabeled cRNA probes. To correct for variation in total RNA loading, duplicate RNA slot membranes were hybridized with a radiolabeled

antisense 18 rRNA cRNA (pT718S; Ambion). After washing, the blots were digested with ribonuclease A. The radioactivity associated with each slot was quantified using a Typhoon 8600 MultiImager (Molecular Dynamics, Piscataway, NJ) and expressed as relative units (RU).

In Situ *Hybridization Analysis*

Localization of mRNA in uterine tissue sections (5 μm) was conducted by *in situ* hybridization analysis as described previously (Spencer et al. 1999c). Briefly, deparaffinized, rehydrated, and deproteinated uterine tissue sections were hybridized with radiolabeled antisense or sense cRNA probes generated from linearized ovine cDNAs using in vitro transcription with $\alpha[^{35}S]$-uridine triphosphate. After hybridization, washing, and ribonuclease A digestion, slides were dipped in NTB-2 liquid photographic emulsion (Eastman Kodak, Rochester, NY), stored at 4°C for 1 to 2 weeks, and developed in Kodak D-19 developer. Slides were then counterstained with Gill's hematoxylin stain (StatLab, Lewisville, TX), dehydrated through a graded series of alcohol to xylene, and protected with a coverslip.

Photomicroscopy

Photomicrographs were taken using a Nikon Eclipse E1000 photomicroscope (Nikon Instruments, Inc., Melville, NY). Digital images were captured with ACT-1 2.11 (Nikon Instruments, Inc.) and assembled using Adobe Photoshop 7.0 (Adobe Systems, Seattle, WA).

Statistical Analyses

Data from slot blot hybridization were subjected to least-squares ANOVA (LS-ANOVA) using the General Linear Models procedures of the Statistical Analysis System (Cary, NC). Slot blot hybridization data were corrected for differences in sample loading using the 18S rRNA data as a covariate in LS-ANOVA. Data from Study One were analyzed for effect of status (C, PX, or U). Data from Study Two were analyzed

for effects of treatment (P+CX vs P+IFN, P+IFN vs P+ZK+IFN, P+CX vs P+ZK+CX). 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 0.1 or less was considered to be significant. Data are presented as the least-square means (LSM) with overall standard errors (SE).

Results

Sequence Analysis

A total of 5,145 cDNAs were sequenced in the 5' direction from a Day 14 pregnant ovine endometrial cDNA library. Bioinformatic analyses indicated that the 5' ESTs consisted of 291 clusters and 4,131 singletons, giving a total of 4,422 non-redundant sequences (80%). However, a number of sequences were abundantly present in the sequencing analyses, such as galectin-15 and 6-16 protein. All cDNAs were arrayed onto glass slides to create a custom 5K ovine endometrial cDNA array.

Study One

Uterine flushes from Day 14 bred normal ewes contained an elongated filamentous conceptus, whereas those from Day 14 bred UGKO and cyclic ewes did not contain a conceptus.

Microarray analyses of endometrium from Day 14 PX, C, and U ewes were performed using the custom 5K array. As summarized in Tables 3.1 and 3.2 and illustrated in Fig. 3.1, microarray analyses of endometrial samples from Study One detected 20 genes that were at least 2-fold different ($P<0.05$) between Day 14 PX and C ewes (15 higher in PX and 5 higher in C), 38 genes were different between PX and U ewes (19 higher in PX and 19 higher in U), and 24 genes were different between C and U ewes (16 higher in C and 8 higher in U). As expected, a greater number of genes were higher in PX, as compared with either C or U endometria, than in the reverse comparisons. The number of genes different between groups takes into account genes with no homology and replicates of genes.

Study Two

As summarized in Table 3.2 and Fig. 3.2, microarray analyses of endometrial samples from Study Two detected 75 genes that were at least 2-fold different ($P<0.05$) between P+CX and P+IFN (36 higher in P+CX and 39 higher in P+IFN), 100 genes between P+ZK+CX and P+ZK+IFN (36 higher in P+ZK+CX and 64 higher in P+ZK+IFN), 180 genes between P+IFN and P+ZK+IFN (83 higher in P+IFN and 97 higher in P+ZK+IFN), and 125 genes between P+CX and P+ZK+CX (58 higher in P+CX and 67 higher in P+ZK+CX).

Validation of Microarray Results

Many of the genes determined to be expressed higher in endometrium from Px (Study One) or IFNτ-treated ewes (Study Two) were ISGs previously known to be induced or increased by IFNτ from the conceptus during early pregnancy in sheep or by Type I IFNs (Tables 3.1 and 3.2). Known ISGs identified by microarray include β2M (Choi et al. 2003), 1-8U, Leu-13/9-27 (Pru et al. 2001), galectin-15 (CHAPTER IV), Mx (Ott et al. 1998), ISG12 (Kim et al. 2003a), IP-10 (Nagaoka et al. 2003b), IFN-induced protein with tetratricopeptide repeats 1 (Lee et al. 1994), Stat1 (Choi et al. 2001), and MHC class I protein (Choi et al. 2003). Differential expression of those ISGs in endometria from PX and C ewes and P+CX and P+IFN ewes validates, in part, the microarray results.

Slot Blot Hybridization

Steady-state levels of galectin-15 and IP-10 mRNA in endometria from ewes in Study One were determined by slot blot hybridization analysis (Fig. 3.2). Expression of galectin-15 mRNA was higher ($P<0.01$) in pregnant than cyclic or pregnant than UGKO endometria, but not different ($P>0.10$) between endometria from cyclic and

Table 3.1. Genes identified by microarray analysis to be 2-fold different between pregnant (PX) and cyclic (C) or pregnant (PX) and UGKO (U) in Study One (P<0.05).

PX/C[1]	PX/U[1]	Both[2]	GenBank	Score[3]	Species	Gene Description	Gene Symbol
11.88	5.93	*	X03205	331	*H.sapiens*	18S ribosomal RNA	
5.45	4.84	*	AF156929	198	*S.scrofa*	Inflammatory response protein 6	IRG6
4.68	3.17	*	AB098889	394	*B.taurus*	40S ribosomal protein S18	RPS18
4.62	3.22	*	BC007091	119	*H.sapiens*	Interferon-induced protein with tetratricopeptide repeats	IFIT1
4.19	2.13	*	AY549962	214	*O.aries*	Beta-2 microglobulin	B2M
4.09	2.14	*	AY570551	406	*B.taurus*	Aut2b2	LOC408002
3.89	1.63		AF272041	452	*B.taurus*	Interferon-induced protein 1-8U	IFITM3
3.77	2.66	*	BN000212	896	*O.aries*	6-16 protein	
3.67	4.20	*	AL137315	137	*H.sapiens*	Unknown	LOC116068
3.45	3.30	*	NM_005529	111	*H.sapiens*	Heparan sulfate proteoglycan 2 (perlecan)	HSPG2
3.40	3.21	*	AJ237937	184	*B.taurus*	Stat5A gene	
3.26	3.04	*	NM_007242	880	*H.sapiens*	DEAD box polypeptide 19	DDX19
3.26	2.98	*	AF252548	785	*O.aries*	Galectin-15 (formerly known as galectin-11)	OVGAL11
2.95	2.17	*	BC070484	325	*H.sapiens*	General transcription factor II	GTF2I
2.95	2.94	*	BC037303	287	*H.sapiens*	Protein kinase, AMP-activated,alpha 1	PRKAA1
2.75	2.82	*	X66093	1063	*O.aries*	Mx homologue	
2.64	2.83	*	BX538162	272	*H.sapiens*	Collaborates/cooperates with alternate reading frame (ARF)	CARF
2.58	2.02	*	S75723	305	*O.aries*	Gastrin-releasing peptide	GRP
2.53	2.33	*	NM_004396	686	*H.sapiens*	DEAD box polypeptide 5	DDX5
2.52	1.01		D13666	444	*H.sapiens*	osteoblast specific factor 2	POSTN
2.51	2.67	*	AF297553	954	*B.taurus*	Seryl-tRNA synthetase	SARS
2.42	0.95		BN000238	1041	*O.aries*	ISG12(a) protein	
1.91	6.00		AY265350	852	*S.scrofa*	18S ribosomal RNA	LOC448984
1.83	2.89		AB058743	365	*H.sapiens*	Unknown	FLJ21439
1.80	2.30		U54800	724	*O.aries*	Ferritin heavy-chain	LOC443355
1.53	0.47		AY438817	749		16S ribosomal RNA gene	
1.41	2.69		AC150540	379	*B.taurus*	Unknown	
1.29	2.65		AF212233	293	*H.sapiens*	Signal peptidase complex (18kD)	LOC90701
1.26	3.87		AF038140	343	*O.aries*	Immunoglobulin lambda-1c light chain variable region	
1.23	2.03		AC150752	168	*B.taurus*	Unknown	
1.09	0.49		CR597617	856	*H.sapiens*	Unknown	
1.02	2.86		Z49188	270	*O.aries*	Immunoglobulin germline heavy chain VH	

Table 3.1 Continued

PX/C[1]	PX/U[1]	Both[2]	GenBank	Score[3]	Species	Gene Description	Gene Symbol
1.01	2.38		NM_174472	210	*B.taurus*	Tissue inhibitor of metalloproteinase-2	TIMP2
0.95	2.97		AF038139	297	*O.aries*	Immunoglobulin lambda-1a light chain variable region	
0.87	4.18		AF038144	559	*O.aries*	Immunoglobulin lambda-2c light chain variable region	
0.87	0.49		BC014944	256	*H.sapiens*	Unknown	FLJ13213
0.84	0.49		AB016798	509	*S.scrofa*	Ribosomal protein L29/cell surface heparin binding protein HIP	RPL29/HIP
0.83	0.37		BX323046	706	*H.sapiens*	Unknown	
0.80	0.46		NM_174743	515	*B.taurus*	Spondin 1, (f-spondin) extracellular matrix protein	SPON1
0.78	0.50		AB099048	955	*B.taurus*	Ribosomal protein L9	RPL9
0.75	0.42		AB081095	539	*B.taurus*	Craniofacial development protein 1	CFDP1
0.74	0.34		M28630	690	*R.norvegicus*	SV40 infected cell line 14B, segment	
0.71	0.42		AB098959	886	*B.taurus*	Ribosomal protein L10a	
0.69	0.46		AB060107	369	*B.taurus*	Eukaryotic translation elongation factor 1 alpha 1	EEF1A1
0.68	0.47		AC147677	450	*C.Familiaris*	Unknown	
0.66	0.46		AY563025	638	*O.aries*	Ribosomal protein S25	OVRPS25
0.62	0.40		AB099028	872	*B.taurus*	H3 histone, family 3A	H3F3A
0.61	0.44		L47641	1227	*B.taurus*	Type III pro-collagen	
0.60	0.49		AY641848	882	*G.japonicus*	Unknown	
0.60	0.50		AC150855	793	*B.taurus*	Unknown	
0.53	2.03		AY734681	337	*O.aries*	Immunoglobulin lambda light chain constant region segment 1	
0.47	0.64		AF010406	957	*O.aries*	Complete mitochondrial genome	
0.45	0.85		X04098	611	*H.sapiens*	Actin, gamma 1	ACTG1
0.42	0.47	*	J03233	813	*B.taurus*	Secreted protein, acidic, cysteine-rich (osteonectin)	SPARC
0.42	0.92		AL031121	52	*H.sapiens*	Unknown	
0.41	0.31	*	X60732	230	*O.cuniculus*	Alpha smooth muscle actin	
0.38	0.47	*	AB008683	977	*B.taurus*	Alpha2(I) collagen	COL1A2
0.37	0.47	*	X13839	410	*H.sapiens*	Smooth muscle alpha-actin 2	ACTA2
ND[4]	3.15		AB070717	1074	*O.aries*	IP-10	IP-10

[1]The two left columns indicate the fold difference of gene expression in pregnant (PX) over cyclic (C) or PX over UGKO (U)
[2]If genes were 2-fold different in both comparisons, they contain an asterisk (*) in the "both" column
[3]All genes in this table have a sequence similarity score of greater than 100
[4]ND, not detected

Table 3.2. Genes identified by microarray analysis to be 2-fold different between cyclic (C) and UGKO (U) in Study One ($P<0.05$).

C/U[1]	GenBank	Score[2]	Species	Gene Description	Gene Symbol
4.16	BC015042	117	*H.sapiens*	Unknown	FLJ11301
3.66	X03205	331	*H.sapiens*	18S ribosomal RNA	
3.64	AF268198	250	*H.sapiens*	Esophagus cancer-related gene-2	ECG2
3.23	BC041624	339	*H.sapiens*	Transgelin	TAGLN
3.17	AB046774	176	*H.sapiens*	Unknown	C17orf27
3.14	AF038139	297	*O.aries*	Immunoglobulin lambda-1a light chain variable region	
3.14	AF038144	559	*O.aries*	Immunoglobulin lambda-2c light chain variable region	
3.11	Z49188	416	*O.aries*	Immunoglobulin germline heavy chain VH gene	
2.96	AF038140	339	*O.aries*	Immunoglobulin lambda-1c light chain variable region	
2.96	X69797	973	*O.aries*	Immunoglobulin gamma1 chain secreted form	
2.93	AY734681	111	*O.aries*	Immunoglobulin lambda light chain constant region segment 1	
2.83	AF040916	323	*O.aries*	Immunoglobulin V lambda chain gene	
2.57	CR615142	212	*H.sapiens*	Unknown	
2.45	AF000137	837	*B.taurus*	Connective tissue growth factor	CTGF
2.10	L38929	912	*H.sapiens*	Protein tyrosine phosphatase delta	PTPRD
2.04	AF396698	258	*B.taurus*	Immunoglobulin lambda light chain (IgL)	
0.48	AF181119	161	*S.scrofa*	RNA helicase	RHIV-1
0.45	BC007091	119	*H.sapiens*	Interferon-induced protein with tetratricopeptide repeats 1	IFIT1
0.44	AF133425	484	*H.sapiens*	Tetraspanin TM4-C	TSPAN-1
0.43	AF272041	432	*B.taurus*	Interferon induced transmembrane protein 3 (1-8U)	IFITM3
0.42	AF272042	438	*B.taurus*	Interferon induced transmembrane protein 1 (9-27)	IFITM1
0.39	X52945	168	*B.taurus*	Pleiotrophin	PTN
0.39	AF271924	283	*B.taurus*	Unknown	
0.38	M63838	123	*H.sapiens*	Interferon-gamma inducible protein 16	IFI16

[1]The left column indicates the fold difference in gene expression in cyclic (C) over UGKO (U) endometrium
[2]All genes in this table have a sequence similarity score of greater than 100

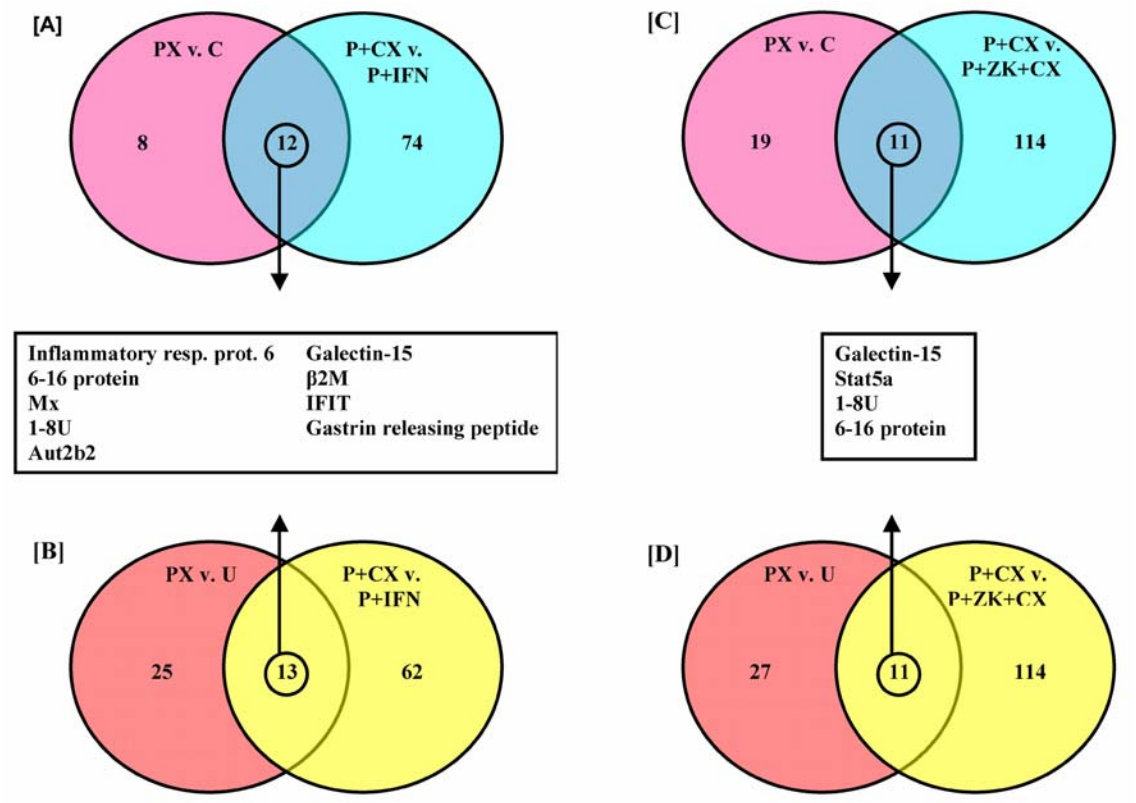

Fig. 3.1. Venn diagram illustration of genes in common between the two microarray experiments. Number of genes that differed 2-fold or more between Day 14 pregnant (PX) versus cyclic (C) and P+IFN versus P+CX [A] and PX versus UGKO (U) and P+IFN versus P+CX [B]; between PX versus C and P+CX versus P+ZK+CX [C] and PX versus U and P+CX versus P+ZK+CX [D]; between PX versus C and P+IFN versus P+ZK+IFN [E] and PX versus U and P+IFN versus P+ZK+IFN [F]; and between PX versus C and P+ZK+CX versus P+ZK+IFN [G] and PX versus U and P+ZK+CX versus P+ZK+IFN [H]. The list of genes is from genes in common between the two Venn diagrams.

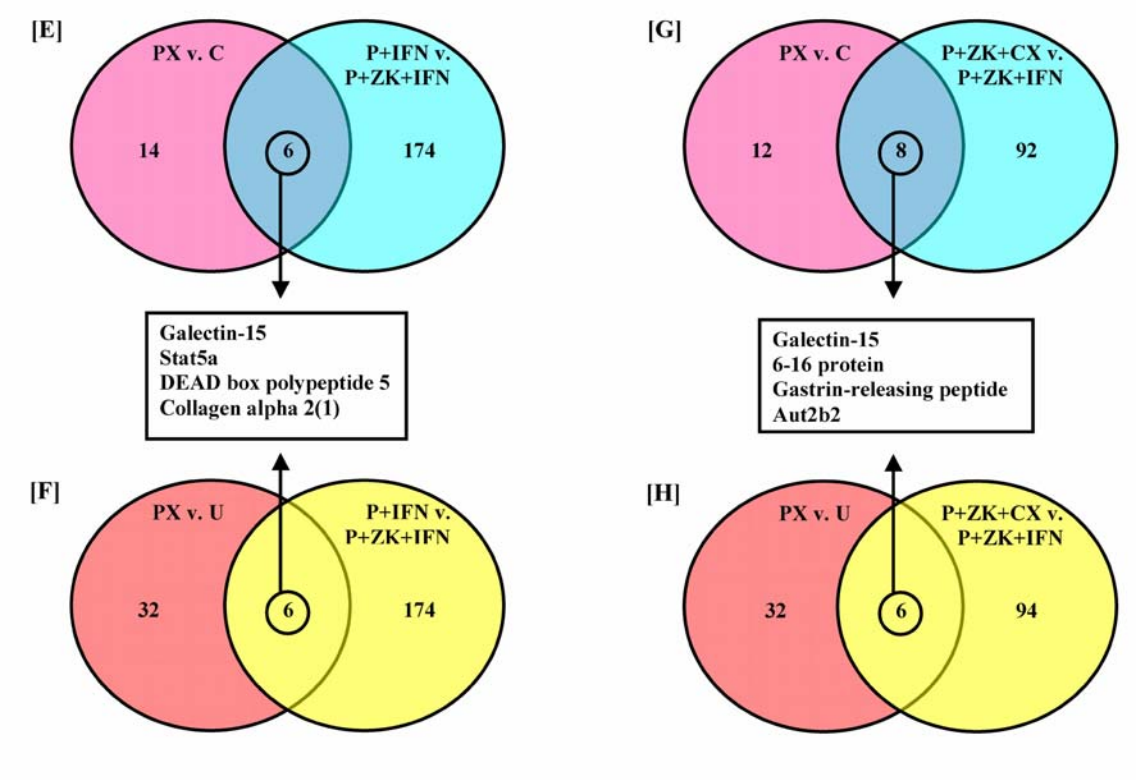

Fig. 3.1 Continued

Table 3.3. Genes identified by microarray analysis to be 2-fold or more different between treatment groups in Study Two (P<0.05). Ewes received daily i.m. injections (Days 5 to 16) of progesterone (P) and/or a PR antagonist (ZK) and i.u. infusions (Days 11 to 16) of control serum proteins and/or recombinant ovine IFNτ protein as follows: (1) P and control (CX) serum proteins [P+CX]; (2) P and ZK and CX proteins [P+ZK+CX]; (3) P and IFNτ [P+IFN]; or (4) P and ZK and IFNτ [P+ZK+IFN].

PI/PC[1]	PC/PZC[1]	PI/PZI[1]	PZI/PZC[1]	GenBank	Score[2]	Species	Gene Description	Gene Symbol
8.22	0.58	2.47	1.94	AF156929	198	*S.scrofa*	Inflammatory response protein 6	IRG6
7.25	1.50	0.92	11.88	AB070717	1074	*O.aries*	IFN-gamma-inducible protein-10	IP-10
5.76	0.26	1.60	0.95	BC007091	119	*H.sapiens*	IFN-induced protein with tetratricopeptide repeats 1	IFIT1
5.72	0.24	0.70	1.96	BN000238	1074	*O.aries*	ISG12(a)	
5.50	1.04	1.71	3.40	S75723	305	*O.aries*	Gastrin-releasing peptide	GRP
4.65	ND[3]	1.03	ND[3]	NM_002473	710	*H.sapiens*	Myosin, heavy polypeptide 9	MYH9
4.60	0.26	1.06	1.14	X66093	1063	*O.aries*	Mx homologue	
4.10	0.20	0.37	2.24	XM_220256	355	*R.norvegicus*	Unknown	LOC302998
3.85	0.28	1.46	0.73	BC065829	690	*H.sapiens*	Transgelin	TAGL
3.85	0.15	2.70	0.23	BN000212	936	*O.aries*	6-16 protein	
3.66	0.70	1.00	2.57	AB116564	323	*S.scrofa*	Stat1	STAT1
3.65	0.60	1.51	1.47	AY570551	406	*B.taurus*	Aut2b2	LOC408002
3.26	0.48	0.56	2.77	AF272041	452	*B.taurus*	IFN-induced transmembrane protein 3 (1-8U)	IFITM3
2.97	ND[3]	2.52	ND[3]	NM_022913	525	*H.sapiens*	Vasculin	DKFZp761C169
2.67	0.78	1.27	1.63	AC105910	159	*H.sapiens*	Unknown	
2.59	0.69	0.97	1.82	U40845	389	*S.scrofa*	Medium-chain acyl-CoA dehydrogenase	ACADM
2.45	0.36	0.97	0.90	BN000255	955	*O.aries*	ISG12(b)	
2.42	0.46	2.31	0.48	AL136934	353	*H.sapiens*	Unknown	FLJ21827
2.35	0.99	0.97	2.41	AB032826	172	*B.taurus*	Selenoprotein P	SEPP1
2.33	0.94	2.81	0.79	NM_033648	113	*M.musculus*	FXYD domain-containing ion transport regulator 4	FXYD4
2.32	1.16	3.52	0.77	AY626238	730	*S.scrofa*	Melanoma antigen family D1	MAGED1
2.28	0.57	1.24	2.05	M34676	361	*O.aries*	MHC class I protein	
2.22	0.79	3.08	0.57	XM_496384	232	*H.sapiens*	Gm566	LOC440603
2.04	17.11	8.75	0.35	AF252548	785	*O.aries*	Galectin-15 (formerly known as galectin-11)	OVGAL11
2.01	0.99	0.82	2.41	AY549962	724	*O.aries*	Beta-2 microglobulin	B2M

Table 3.3 Continued

PI/PC[1]	PC/PZC[1]	PI/PZI[1]	PZI/PZC[1]	GenBank	Score[2]	Species	Gene Description	Gene Symbol
1.69	3.41	0.75	7.66	U54800	724	*O.aries*	Ferritin heavy-chain	LOC443355
1.66	1.10	2.25	0.81	AB098895	769	*B.taurus*	Matrix Gla protein	MGP
1.64	0.26	0.22	1.94	AF000137	658	*B.taurus*	Connective tissue growth factor	CTGF
1.50	0.65	2.34	0.41	AY192438	924	*B.taurus*	Thymosin beta 4	LOC444862
1.32	ND[3]	2.80	ND[3]	AF513721	684	*B.gruniens*	Myosin regulatory light chain	
1.31	4.13	4.26	1.27	AJ237937	184	*B.taurus*	Stat5A	
1.31	1.49	3.23	0.60	NM_004396	686	*H.sapiens*	DEAD box polypeptide 5	DDX5
1.27	2.21	1.98	1.42	CR605177	652	*H.sapiens*	Unknown	
1.25	4.20	7.18	0.73	NM_178320	932	*B.taurus*	Peptidylprolyl isomerase A	PPIA
1.14	1.86	2.40	0.88	AF472577	341	*O.aries*	Regulator of G-protein signaling 2	RGS2
1.12	3.57	4.61	0.87	J03191	523	*H.sapiens*	Profilin 1	PFN1
1.11	ND[3]	2.51	ND[3]	BC031334	334	*H.sapiens*	Cystatin E/M	
1.08	0.63	0.38	1.77	U85962	755	*H.sapiens*	CREB-binding protein	CREBBP
1.08	1.61	4.56	0.38	AF014805	301	*F.catus*	Beta-N-acetylhexosaminidase	HEXB
0.94	3.34	1.19	2.74	AK074795	535	*H.sapiens*	Unknown	CIRH1A
0.91	3.72	4.07	0.83	AB098889	394	*B.taurus*	40S ribosomal protein S18	RPS18
0.81	1.17	0.39	2.39	BC015031	577	*H.sapiens*	Matrin 3	MATR3
0.73	0.64	0.26	1.80	L13263	722	*B.bovis*	Vimentin	VIM
0.55	2.76	1.89	0.81	AL834184	408	*H.sapiens*	Unknown	FLJ14490
0.49	1.75	1.3	0.66	AL832747	438	*H.sapiens*	Unknown	
0.48	2.48	0.49	2.44	AY570553	874	*B.taurus*	Light chain 3	LOC408001
0.48	1.31	0.47	1.33	BC017386	670	*H.sapiens*	Ribosomal protein S19	RPS19
0.45	1.18	0.18	2.66	AB099017	910	*B.taurus*	Ribosomal protein S3a	RPS3A
0.44	3.50	0.86	1.81	D13666	444	*H.sapiens*	Osteoblast specific factor 2 (periostin)	POSTN
0.26	1.42	0.19	1.96	NM_005978	240	*H.sapiens*	S100 calcium binding protein A2	S100A2
0.25	2.12	0.90	0.59	L47641	1227	*B.taurus*	Type III pro-collagen	

[1]The four left columns indicate the fold difference in gene expression in P+IFN (PI) over P+CX (PC), PC over P+ZK+CX (PZC), PI over P+ZK+IFN (PZI) or PZI over PZC

[2]All genes in this table have a sequence similarity score greater than 100

[3]ND, not detected

UGKO ewes (Fig. 3.2A). Similarly, IP-10 mRNA was higher ($P<0.10$) in pregnant than cyclic or pregnant than UGKO endometria (Fig. 3.2B), but not different ($P>0.10$) in endometria from cyclic compared to UGKO ewes.

Slot blot hybridization analyses revealed steady-state levels of IP-10 (Fig. 3.3A), 1-8U (Fig. 3.3B), β2M (Fig. 3.3C), MHC Class 1 (Fig. 3.3D), Stat1 (Fig. 3.3E) and CTGF (Fig. 3.3F) mRNAs in Study Two. All of these genes, except for CTGF, were 2-fold or greater in microarray analyses of endometrium from P+IFN over the P+CX ewes, as well as in endometria from P+ZK+IFN over the P+ZK+CX ewes. Accordingly, IP-10, 1-8U, β2M, MHC class I, and Stat1 mRNAs had similar patterns of expression. However, the pattern of CTGF mRNA expression was quite different from the others, which matched the microarray analyses (see Table 3.3). Steady-state levels of IP-10, 1-8U, β2M, MHC class I, and Stat1 mRNA were higher ($P<0.10$) in endometria of ewes receiving intrauterine infusions of IFNτ in both P and P+ZK treatment groups. In contrast, CTGF mRNA was most abundant in the P+ZK+IFN group (P+ZK+IFN vs. P+IFN, $P<0.06$).

Localization of Genes from Microarray

In situ hybridization analyses revealed which cell type(s) in the Day 14 ovine uterus expressed eight of the candidate genes identified by microarray analyses (Fig. 3.4). Galectin-15 mRNA was expressed exclusively by LE and superficial ductal glandular epithelium (sGE). CTGF mRNA was detected in GE as well as immune cells within the stroma. IP-10 mRNA was detected only in immune cells throughout the emdp,etroa; stroma. Both CA2 and thioredoxin mRNAs were expressed at low levels by LE and sGE. Both 1-8U and prothymosin-α mRNA were expressed predominantly in the stratum compactum area of the endometrial stroma adjacent to the LE. Ferritin mRNA was expressed by a number of cell types, including LE, GE, immune cells, and stroma, but was most abundant in the LE.

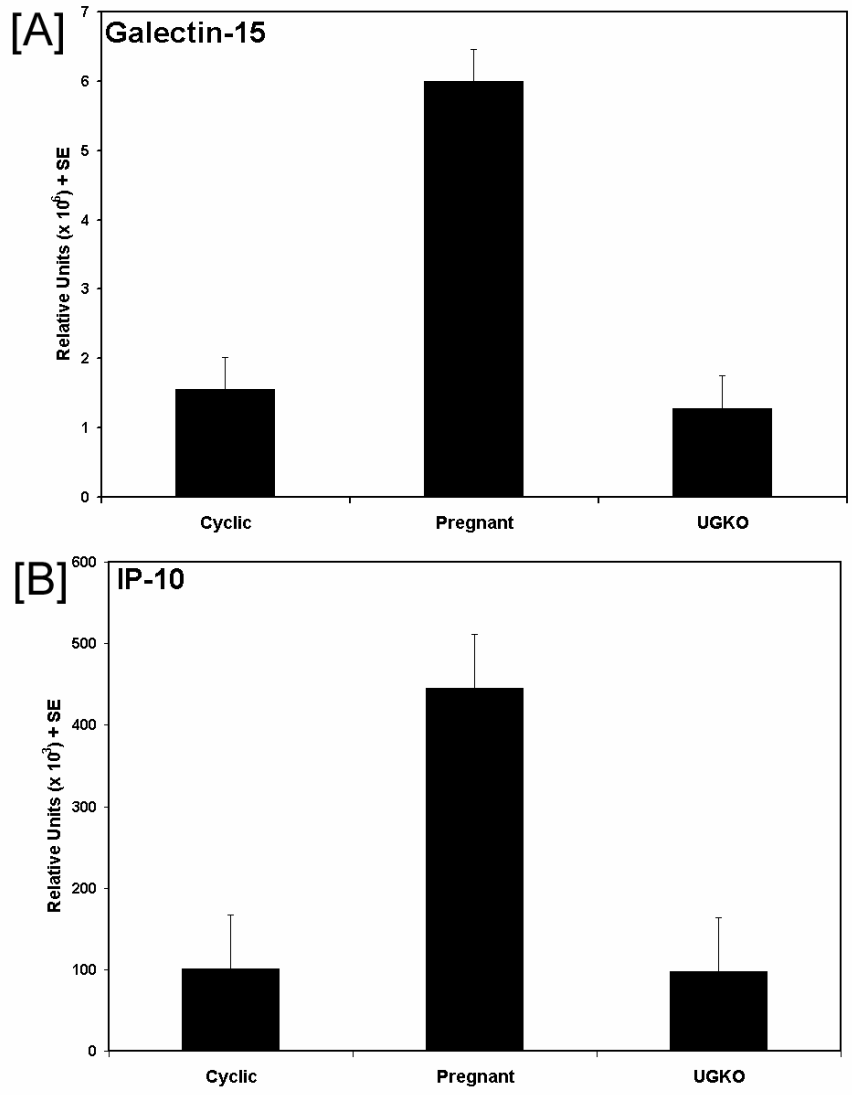

Fig. 3.2. Steady-state levels of galectin-15 [A] and IP-10 [B] mRNAs in endometrium from Day 14 pregnant, cyclic and UGKO ewes from Study One as determined by slot blot hybridization analysis.

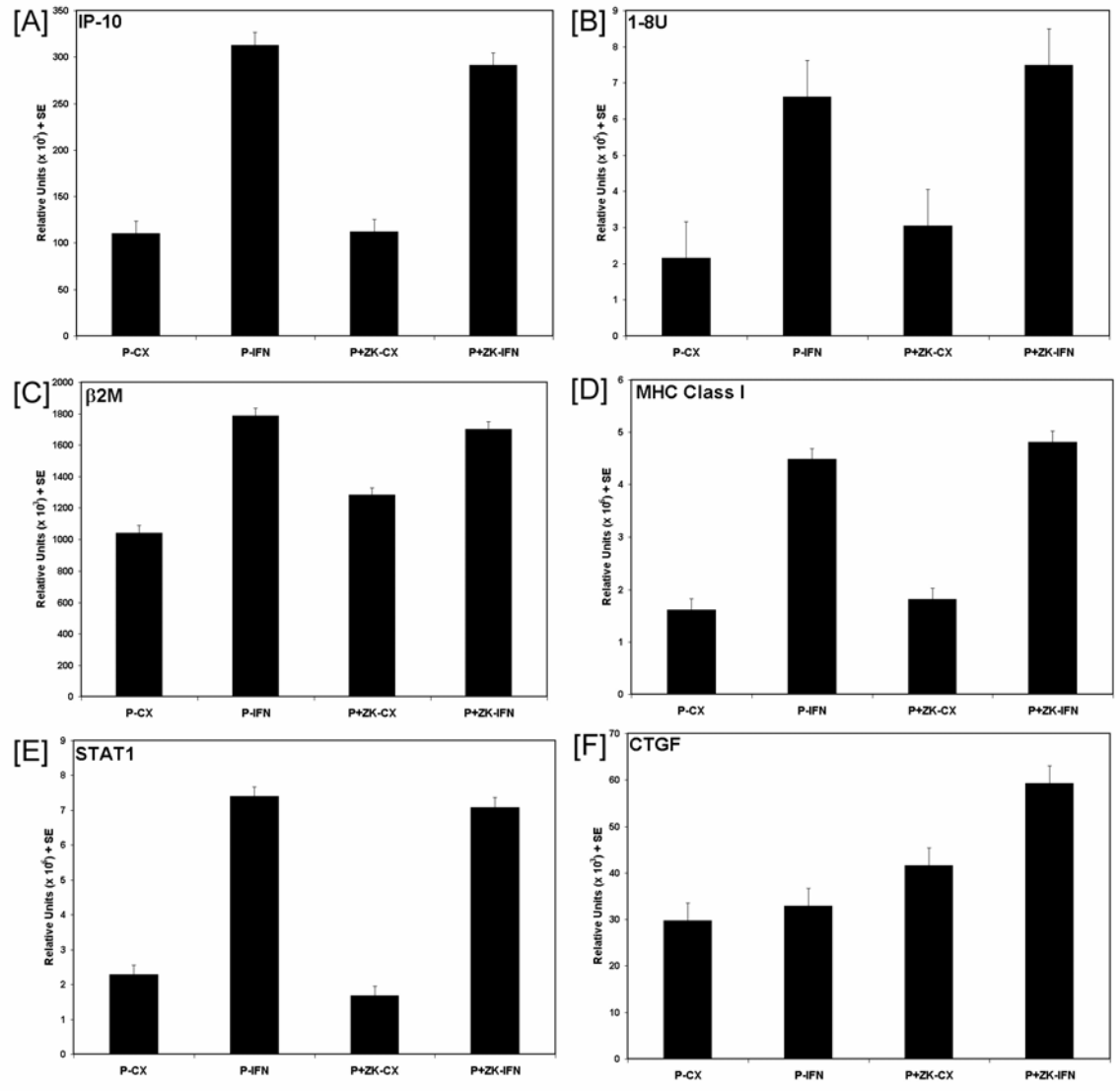

Fig. 3.3. Steady-state levels of several of the genes from Study Two by slot blot hybridization. The mRNAs for IP-10 [A], 1-8U [B], β2M [C], MHC Class I [D], Stat1 [E], and CTGF [H] were evaluated in ewes treated with P+CX, P+IFN, P+ZK+CX, or P+CX+IFN.

Discussion

Recurrent pregnancy loss in the UGKO ewe model indicates that histotroph from the endometrial LE and GE is required for peri-implantation conceptus survival and growth in sheep (Gray et al. 2000a; Gray et al. 2002; Gray et al. 2001c). The timing of recurrent early pregnancy loss in UGKO ewes correlates with the majority of embryo loss that occurs during pregnancy in livestock, as well as humans (Bazer 1975; Bazer and First 1983; Norwitz 2001). Therefore, identification of genes differentially expressed in endometria of normal fertile and infertile UGKO ewes should elucidate factors and pathways regulating conceptus survival and growth in mammals, which are not well defined. A previous study utilized differential display and PCR-based suppression subtraction hybridization techniques to compare gene expression in fertile ewes and infertile UGKO ewes (Spencer et al. 1999c). In that study, a number of epithelial-specific genes were identified, but very few encoding secreted proteins, presumably due to the predominance of clones for the endogenous Jaagsiekte sheep retroviruses (enJSRVs) that are among the most abundant genes in endometrial epithelia of the ovine uterus (Palmarini et al. 2001; Spencer et al. 1999c). In the present study, a custom endometrial cDNA array was used to identify a number of candidate genes regulating conceptus survival and growth. In Study One, transcriptional profiling analyses revealed that the UGKO endometrium was deficient in esophagus cancer-related gene-2 (ECRG2), as well as a number of immunoglobulin genes, such as lambda-1a light chain variable region, lambda-2c light chain variable region, lambda light chain constant region segment 1, germline heavy chain VH, and gamma1 chain secreted form. The role of these genes in the endometrium is largely unknown; however, in esophageal cancer, ECRG2 suppresses tumor growth by decreasing cell proliferation and increasing apoptosis (Cui et al. 2003). Although the precise role and regulation of immunoglobulin genes in the endometrium has not yet been elucidated, there are large numbers of immune cells populating the endometrium (Hansen 1995). In fact, endocrine disruption of ovine endometrial gland development with estradiol valerate during the first two weeks of neonatal life resulted in reduced numbers of immunoglobulin V lambda chain-

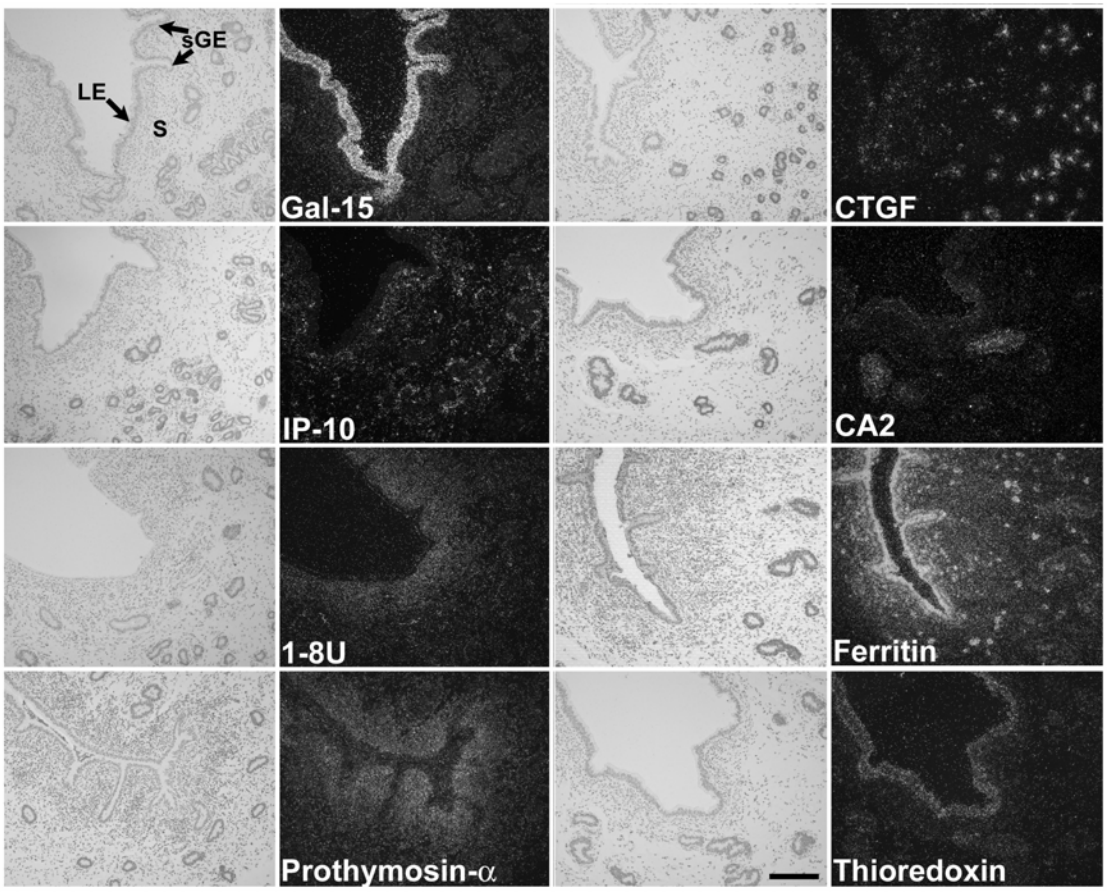

Fig. 3.4. *In situ* hybridization analysis of galectin-15, CTGF, IP-10, CA2, 1-8U, ferritin, prothymosin-α, and thioredoxin mRNA expression in the endometrium from the uterus of Day 14 or 16 pregnant ewes. Galectin-15, CA2, and thioredoxin mRNAs were expressed by the LE and sGE; 1-8U and prothymosin-α mRNAs were expressed by stratum compactum stromal cells; IP-10 mRNA was expressed by immune cells in the stroma; CTGF mRNA was expressed by deep GE and immune cells in the stroma; and ferritin mRNA was expressed by LE, sGE, immune cells and stromal cells. Cross-sections of the uterine wall were hybridized with radiolabeled antisense or sense cRNA probes generated from linearized plasmid cDNA clones. Hybridized sections were digested with RNase A, and protected transcripts were visualized by liquid emulsion autoradiography. Developed slides were counterstained lightly with hematoxylin, and photomicrographs were taken under brightfield or darkfield illumination. Legend: LE, luminal epithelium; sGE, superficial ductal glandular epithelium; S, stroma. Scale bar represents 100 μm.

positive lymphocytes in the stroma (Hayashi, K. and Spencer, T.E., unpublished observations). Perhaps the absence of uterine glands alters immune cell populations, and thus, immunoglobulin gene expression, in the endometrium. On the other hand, several genes were higher in UGKO endometrium than in either cyclic or pregnant endometria, such as pleiotrophin (U over C), smooth muscle alpha-actin and collagen (U over P). Interestingly, these genes may be correlated to the abnormal presence of very dense stroma throughout the endometrium of UGKO ewes (Bany and Schultz 2001; Gray et al. 2000a; Gray et al. 2001b). The identification of a relatively small number of genes from Study One could be due to the fact that endometrial support of peri-implantation conceptus survival and growth is a multifactorial process involving many components of histotroph. Thus, the recurrent early pregnancy loss in UGKO ewes is due to the cumulative effects of a reduction in histroph rather than the complete absence of a singular factor.

In Study One, expression of many genes was higher in endometria of Day 14 pregnant ewes compared to either Day 14 cyclic or UGKO ewes. Most of those genes were novel ISGs, because endometria of cyclic and bred UGKO ewes did not contain an elongated conceptus producing IFNτ. However, in comparing cyclic and UGKO ewes (Table 3.2), some ISGs were higher in UGKO compared to cyclic endometria (i.e., 1-8U, 9-27, and IFN-induced with tetratricopeptide repeats). This suggests that some of the UGKO ewes were initially pregnant, but unable to support conceptus development which resulted in decreased expression of ISGs. As illustrated in Fig. 3.1, combinatorial analysis of genes from Studies One and Two identified a number of known IFNτ-stimulated genes, including 6-16, galectin-15, β2M, 1-8U, and IFN-induced with tetratricopeptide repeats. That analysis also identified novel ISGs in the ovine endometrium, including inflammatory response protein 6, gastrin releasing peptide, aut2b2, and DEAD box polypeptide 5. A better understanding of effects of pregnancy and IFNτ on these genes will enhance our knowledge of early endometrial responses to the conceptus. Indeed, ISGs are common to pregnancy in many mammals, including

humans, primates, rats, mice, ruminants and pigs (Carson et al. 2000; Spencer and Bazer 2002; Spencer et al. 2004).

During the peri-implantation period of pregnancy, endometrial gene expression is programmed by hormones from the ovary (progesterone) and conceptus (IFNτ, PL, and placental GH) and changes in the PR (Bagchi et al. 2003; Spencer and Bazer 2002; Spencer et al. 2004). Results of Study Two highlight the individual and combinatorial effects of progesterone and IFNτ to program endometrial responses during pregnancy in a tissue and cell-type specific manner. Progesterone is unequivocally required for maternal support of conceptus survival and development (Bagchi et al. 2003). In Study Two, 125 genes were found to be regulated by progesterone, with 58 increased and 67 decreased. The complex responses of individual endometrial cell types to progesterone are regulated by PR expression. In both cyclic and pregnant ewes, continuous progesterone exposure for 8 to 10 days negatively autoregulates PR gene expression in the endometrial epithelia. During the estrous cycle and pregnancy, the PR is expressed in all uterine cell types on Day 6, but restricted to stromal cells throughout pregnancy after PR is lost from LE and GE on Days 11 and 13, respectively (Spencer and Bazer 1995; Spencer et al. 2004). The loss of PR in the endometrial LE and then GE reprograms gene expression patterns in the endometrium (Spencer et al. 2004). For example, galectin-15 is induced by progesterone in the endometrium in Study Two, and the expression of galectin-15 mRNA appears in the endometrial LE and sGE between Days 10 and 12 (CHAPTER IV), which is tightly correlated with the loss of PR in those epithelia (Spencer and Bazer 1995). Thus, many progesterone-induced genes in the ovine endometrium actually appear as a result of loss of PR, suggesting that many epithelial genes are repressed by liganded PR. Indeed, only enJSRVs are directly increased by liganded PR in the ovine endometrium (Palmarini et al. 2001).

IFNτ, the signal for maternal recognition of pregnancy in ruminants, is secreted by the ovine conceptus trophectoderm between Days 10 and 20 of pregnancy, with maximum levels produced on Days 15 and 16 (Ashworth and Bazer 1989b; Bazer et al. 1998; Farin et al. 1989). IFNτ stimulates expression of a number of genes in

endometrial stroma and deep GE, such as Stat1 (Choi et al. 2001), MHC Class I, β2M (Choi et al. 2003), and OAS (Johnson et al. 2001b). Currently, the only known IFN-regulated genes not restricted in expression to stroma and deep GE are galectin-15 (CHAPTER IV), ISG 12, Wnt7a (Kim et al. 2003a), and Mx (Ott et al. 1998), which are also expressed by LE and/or superficial GE. Both studies identified other ISGs, including IP-10 expressed by immune cells and 1-8U expressed by stroma and deep GE of the endometrium as shown previously (Nagaoka et al. 2003b; Pru et al. 2001), as well as inflammatory response protein 6 and gastrin-releasing peptide, 6-16, and DEAD box polypeptide 5. The timing of expression of these IFNτ-stimulated genes and their hormonal regulation have not been reported, but they may be involved in endometrial support of conceptus implantation and development.

Several genes, including galectin-15, were found to be expressed specifically and exclusively in endometrial LE and sGE, which also produce histotroph. Galectin-15, a novel member of the galectin family of galactoside-binding lectins (Dunphy et al. 2000), is a particularly abundant mRNA in the endometrium, because it represented ~1% of the 5' EST sequences in this study. In sheep, galectin-15 mRNA was detected only in endometrial LE and sGE after Day 10 of the cycle or pregnancy (CHAPTER IV). Galectin-15 is induced by progesterone and expression is further stimulated by IFNτ (CHAPTER IV). Immunoreactive galectin-15 protein is concentrated near and on the apical surface of the endometrial LE and sGE and also localized to crystalline structures within the conceptus trophectoderm (CHAPTER IV). Galectin-15 protein, an abundant protein in histotroph recovered from the uterine lumen in increased amounts on Days 14 and 16 of pregnancy, is functional in binding beta-galactosides (CHAPTER IV). The temporal and spatial alterations in galectin-15 mRNA and protein in ovine endometrial epithelia and uterine lumen during early pregnancy, combined with the functional aspects of other galectin family members (Hsu and Liu 2004), suggest that it may be a mediator of conceptus-endometrial interactions during implantation (CHAPTER IV). Thus, histotroph produced by endometrial LE and sGE may be more important during conceptus elongation and apposition and that produced by the GE may be more

important in the attachment and adhesion stages of implantation. Indeed, the present study did not identify osteopontin and uterine serpins, which are expressed only in the middle to deep GE after Days 14 to 16 of pregnancy (Johnson et al. 2003a; Stewart et al. 2000), because the endometrium used to generate the cDNA array was from Day 14 pregnant ewes.

As mentioned previously, PR is expressed by stromal cells throughout the estrous cycle and pregnancy; however, the epithelial PR is negatively autoregulated by progesterone, causing an absence of PR expression specifically in the epithelia (Spencer and Bazer 1995). Administration of the ZK compound, a PR antagonist (Study Two), abolished the ability of either progesterone or IFNτ to induce galectin-15 expression (CHAPTER IV), but not IP-10, 1-8U, β2M, or a number of other genes in Table 1 and Fig. 3.2. Similarly, galectin-1 expression is induced by progesterone, and this induction is blocked by exposure to RU486 (Choe et al. 1997). Thus, expression of LE-specific ISGs may be regulated through the action of progesterone binding to stromal PR (eg, galectin-15), whereas expression of classical, stromal- and deep GE-specific IFN-stimulated genes (eg, Stat1, MHC class I and β2M) are unaffected by the absence of stromal PR action. The hormonal regulation of CTGF was quite different from the genes mentioned previously, as it is only stimulated by IFN in the absence of a functional PR. The regulation of gene expression by IFNτ in the ovine endometrium appears to be very complicated and closely related to that of progesterone and the presence of PR. Further, many ISGs identified in Study Two were not found in comparisons to endometrium from Study One, because most ISGs are not different between Day 14 pregnant and cyclic endometria, but are different by Day 16 of pregnancy due to high levels of IFNτ secretion by the conceptus (Choi et al. 2001; Choi et al. 2003; Kim et al. 2003a).

Perhaps the effect of progesterone requires paracrine communication by a "progestamedin" that allows for epithelial-mesenchymal interactions, rather than an autocrine effect (Chen et al. 2000a; Chen et al. 2000b). In primates, keratinocyte growth factor (KGF) is expressed by endometrial stromal cells and mediates actions of progesterone on GE cell proliferation (Koji et al. 1994; Spencer and Bazer 2002).

Another option involves the specificity for IFN regulatory factor 2 (IRF-2), a potent repressor of ISG transcription, which restricts expression of ISGs to endometrial stroma and GE of the ovine uterus. This repression occurs through the ISG factor 3 (ISGF3), composed of Stat1, Stat2, and IRF9, portion of the JAK/Stat pathway by binding to IFN-stimulated response elements (Choi et al. 2001). This suggests that genes expressed specifically by the LE and sGE may not be in response to classical JAK-Stat signaling, but more likely related to IFNτ influencing multiple signal transduction pathways, such as p38 MAP kinase pathway (Platanias 2003), protein kinase C (Thatcher et al. 2001), or nuclear factor-kappa B pathways (Hiscott et al. 2003)..

These microarray experiments, in particular Study One, were designed to identify genes expressed by GE that would be components of histotroph, however galectin-15, CA2, and thioredoxin were expressed only by LE and sGE, whereas IP-10 was expressed by immune cells, 1-8U and prothymosin-α were expressed by the stratum compactum stroma, and CTGF and ferritin were expressed by a number of cell types, including GE and immune cells. The cell-specific effects of progesterone, the conceptus and IFNτ indicates the dynamic nature of endometrial responses to the conceptus. This is the first study to localize ferritin, CA2, CTGF, 1-8U and prothymosin-α to the ovine endometrium, but expression of galectin-15 (CHAPTER IV) and IP-10 (Nagaoka et al. 2003b) have been reported. These patterns of gene expression in the endometrium during early pregnancy are indicative of the complexity of endometrial responses to hormones and growth factors from the ovary and conceptus that are important regulators of conceptus survival and growth.

The novel results from these studies are expected to help unravel the hormonal, cellular and molecular mechanisms regulating IFN stimulation of genes, the process of implantation and uterine support of conceptus survival and development. These experiments identified a number of genes not previously identified or evaluated in the ovine endometrium that may influence immune functions, environmental modulation, adhesion, inhibition of apoptosis, etc. Therefore, transcriptional profiling of the ovine endometrium is effective in identifying genes and gene networks that regulate

endometrial function to support conceptus survival, development and implantation. Knowledge of these genes will likely lead to the discovery of new methods for diagnosing infertility and improving embryo culture systems and the embryotrophic potential of the uterus.

CHAPTER IV
DISCOVERY AND CHARACTERIZATION OF AN EPITHELIAL-SPECIFIC GALECTIN IN THE ENDOMETRIUM THAT FORMS CRYSTALS IN THE TROPHECTODERM

Introduction

Epithelia of the uterine endometrium synthesize and secrete or selectively transport a variety of substances necessary for conceptus survival, growth and implantation (Bazer 1975; Carson et al. 2000; Fazleabas et al. 1997; Kane et al. 1997; Roberts and Bazer 1988). Histotroph is a rather undefined, complex mixture of transport proteins, ions, cytokines, growth factors, lymphokines, hormones, protease inhibitors, and other molecules (Bazer 1975; Roberts and Bazer 1988). In rodents, colony-stimulating factor one, LIF, and calcitonin are specific histotroph proteins that mediate establishment of uterine receptivity and stimulate conceptus development (Pollard et al. 1991; Stewart et al. 1992; Zhu et al. 1998a). In humans, histotroph appears to be the primary source of nutrition for conceptus development during the first trimester, when mechanisms for hematotrophic nutrition are being established (Burton et al. 2002). Uterine secretions are of particular importance for conceptus survival and growth in domestic animals due to their protracted peri-implantation period and noninvasive types of placentation (Bazer 1975; Roberts and Bazer 1988; Roberts et al. 1987).

Available evidence from humans, laboratory animals and domestic animals indicate that a reduction in uterine histotroph compromises peri-implantation survival and growth of conceptuses, which can lead to infertility and intrauterine growth retardation (Burton et al. 2002; Gray et al. 2002; Herrler et al. 2003). Despite decades of research, the biochemical aspects of uterine histotroph remain undefined in most mammals. Recent studies correlate inadequate expression of cell-surface and secretory proteins with retarded endometrial differentiation and defective endometrial secretory phases (Hambartsoumian 1998; Thornburgh and Anderson 1997; Westergaard et al. 1998). Therefore, identification of useful molecular and cellular markers of endometrial

function and receptivity to the conceptus are of utmost importance, because they can be used for evaluation, regulation and enhancement of fertility (Westergaard et al. 1998).

In order to understand the peri-implantation pregnancy defect, the UGKO sheep model (Gray et al. 2002) was used for a gene expression profiling project using an endometrial cDNA library from the uterus of Day 14 pregnant sheep. Interestingly, approximately 1% of the 5,000 ESTs sequenced from the cDNA library was highly similar to *ovgal11*, a novel member of the galectin family of secreted animal lectins (Dunphy et al. 2000). OVGAL11 was originally shown to be induced in gastrointestinal tissue and secreted into the intestinal lumen in response to inflammation and eosinophil infiltration after infection of sheep with the helminth, *Haemonchus contortus* (Dunphy et al. 2000). The sequence of OVGAL11 protein displayed highest similarity to human galectin-10 (also known as Charcot-Leyden Crystal protein) (Dvorak 1996; Weller et al. 1984) and human galectin-13 (also known as placental protein-13 or PP13) (Bohn et al. 1983). Galectins are proteins with a conserved CRD that bind β-galactosides, thereby cross-linking glycoproteins as well as glycolipid receptors on the surface of cells and initiating biological responses (Cooper 2002; Yang and Liu 2003). Functional studies on the extracellular and intracellular roles of galectins have implicated them in cell growth, differentiation and apoptosis, in addition to cell adhesion, chemoattraction and cell migration. Since OVGAL11 from the intestine and endometrium of sheep does not have a known orthologue, it is proposed to be a new family member and renamed galectin-15. Of particular interest is the fact that galectin-15 appears to be the long sought after 14K protein from sheep endometrium initially characterized as a progesterone-modulated protein associated with crystalline inclusion bodies in uterine epithelia and conceptus trophectoderm (Kazemi et al. 1990).

Materials and Methods

Animals

Experimental and surgical procedures on crossbred Suffolk sheep (*Ovis aries*)

complied with the Guide for Care and Use of Agriculture Animals and were approved by the Institutional Animal Care and Use Committees of Texas A&M University.

Study One

In Study One, cyclic ewes were mated at estrus to either a vasectomized or an intact ram and were hysterectomized (n=5 sheep/day) on Days 10, 12, 14 or 16 of the estrous cycle or on Days 10, 12, 14, 16, 18 or 20 of pregnancy (gestation period is 147 days). On Days 10 to 16, the uterine lumen was flushed with saline and examined for the presence of a morphologically normal conceptus to confirm pregnancy. Flushes were not possible on Days 18 or 20, because the conceptus is firmly adhered to the endometrial LE and basal lamina. Cross-sections of the uterine horn ipsilateral to the ovary bearing the CL were fixed in 4% paraformaldehyde in PBS (pH 7.2) for 24 h, dehydrated in 70% ethanol, and then embedded in Paraplast-Plus (Oxford Labware). The remaining endometrial tissues were dissected from myometrium and frozen at -80^{o}C. Uterine flushes were clarified by centrifugation (3000 x *g* for 30 min at 4°C) and frozen at -80°C.

Study Two

In Study Two, cyclic ewes (n=20) were checked daily for estrus and then ovariectomized and fitted with indwelling uterine catheters on Day 5 as described previously (Spencer et al. 1999b). Sheep were then assigned randomly (n=5 per treatment) to receive daily i.m. injections of progesterone and/or ZK (Schering AG), a PR antagonist, and i.u. infusions of CX serum proteins and/or roIFNτ protein as follows: (1) 50 mg P (Days 5 to 16) and 200 μg CX serum proteins (Days 11 to 16) [P+CX]; (2) P and 75 mg ZK 136,317 (Days 11 to 16) and CX proteins [P+ZK+CX]; (3) P and IFNτ (2×10^7 antiviral units, Days 11 to 16) [P+IFN]; or (4) P and ZK and IFNτ [P+ZK+IFN]. Steroids were administered daily in corn oil vehicle. Both uterine horns of each ewe received twice daily injections of either CX proteins (50 μg/horn/injection) or roIFNτ (5×10^6 antiviral units/horn/injection). roIFNτ was produced in *Pichia pastoris* and purified as described previously (Van Heeke et al. 1996). Proteins were prepared for i.u. injection as described previously (Spencer et al. 1999b). This regimen of progesterone

and roIFNτ mimics the effects of progesterone and the conceptus on endometrial expression of hormone receptors and ISGs during early pregnancy in ewes (Johnson et al. 2001b; Kim et al. 2003a; Spencer et al. 1995a). All ewes were hysterectomized on Day 16, and the uterus and endometrium processed as described in Study One.

Study Three

In Study Three, uterine milk was collected from the non-gravid uterine horn of unilateral pregnant sheep (n=4) on Day 80 of pregnancy using methods described initially by Bazer et al. (Bazer et al. 1979a). Uterine milk was clarified by centrifugation and stored at -80°C.

RNA Analysis

Total cellular RNA was isolated from frozen endometrial tissue using Trizol reagent (Gibco-BRL). Steady-state levels of galectin-15 mRNA were assessed in the endometrium from Studies One and Two by slot blot hybridization as described previously (Spencer et al. 1999c). Radiolabeled antisense cRNA probe was generated from a linearized ovine endometrial galectin-15 cDNA by *in vitro* transcription with [α-^{32}P]-UTP and hybridized with denatured endometrial total RNA (20 μg) from each ewe affixed to a slot blot membrane. To correct for variation in total RNA loading, a duplicate total endometrial RNA slot blot membrane was hybridized with a radiolabeled antisense 18 rRNA cRNA (pT718S Ambion). Following washing, the blots were digested with ribonuclease A. The radioactivity associated with each slot was quantified using a Typhoon 8600 MultiImager (Molecular Dynamics) and is expressed as RU.

In Situ *Hybridization Analysis*

Galectin-15 mRNA was localized in uterine tissue sections (5 μm) by *in situ* hybridization analysis as described previously (Spencer et al. 1999c). Deparaffinized, rehydrated and deproteinated uterine tissue sections were hybridized with radiolabeled antisense or sense galectin-15 cRNA probes generated by *in vitro* transcription with [α-^{35}S]-UTP. After hybridization, washing and ribonuclease A digestion, slides were

dipped in NTB-2 liquid photographic emulsion (Kodak), stored at 4°C for one week, and developed in D-19 developer. Sections were then counterstained with Gill's hematoxylin (StatLab), and protected with a coverslip.

Immunohistochemistry

Immunoreactive galectin-15 protein was detected in uterine tissue cross-sections from Study One with rabbit anti-OVGAL11 polyclonal antibody (Dunphy et al. 2000) at a 1:2,000 dilution and a Super ABC Rabbit IgG Kit (Biomeda, Foster City, CA) using methods described previously (Spencer et al. 1999a). Antigen retrieval was performed using Pronase E digestion as described previously (Spencer and Bazer 1995). Negative controls included substitution of the primary antibody with purified rabbit IgG at the same final concentration.

Western Blot Analyses

Uterine flushes from Study One were concentrated using Centricon-3 columns (Amicon, Inc. Beverly, MA). Protein content of concentrated flushes (Study One) and uterine milk (Study Three) was determined using a Bradford protein assay (Bio-Rad, Hercules, CA) with bovine serum albumin (BSA) as the standard. Proteins were denatured, separated by 15% SDS-PAGE, and Western blots conducted as described previously (Spencer et al. 1999a) using enhanced chemiluminescence detection. Immunoreactive galectin-15 was detected using the rabbit anti-ovine OVGAL11 antibody (Dunphy et al. 2000) at a 1:10,000 dilution. Negative control blots were performed in which primary antibody was replaced by rabbit IgG at the same concentration.

Carbohydrate Binding Analysis

This analysis was a modified version of methods for the *in vitro* analysis of saccharide binding by galectin-10 (Dyer and Rosenberg 1996). All binding experiments were performed in Dulbecco's PBS (Sigma, St. Louis, MO). Briefly, uterine flush

proteins from Day 16 pregnant ewes (200 μg) were incubated overnight at 4°C with a 50 μl bed volume of lactose-conjugated agarose (Sigma). For competition, lactose, mannose, and fucose (Sigma) sugar was added at a final concentration of 500 mM. After incubation, the resin was washed three times in 1 ml PBS and the bound protein eluted with SDS-PAGE reducing sample buffer. The relative amount of galectin-15 protein bound to lactose was determined by 15% SDS-PAGE and Western blot analysis using the rabbit anti-ovine OVGAL11 antibody.

Immunoprecipitation

Proteins (40 μg) from uterine flushings from Day 16 pregnant ewes (Study One) or uterine milk (Study Three) were incubated overnight at 4°C with 1 μl of either normal rabbit serum (Sigma), rabbit anti-OVGAL11 antibody (Dunphy et al. 2000), or rabbit anti-ovine trophoblast protein 1 (oTP-1/oIFNτ) (Godkin et al. 1984a) (kindly provided by James Godkin, University of Tennessee) and 10 μl Protein A/G plus-agarose (Santa Cruz Biotechnology, Inc.; Santa Cruz, CA) in immunoprecipitation (IP) lysis buffer (Spencer et al. 1999a). Proteins bound to the washed beads were separated on an 8-16% SDS-PAGE gradient gel (Bio-Rad) and detected by Western blot analysis. Antibodies used in the analyses were rabbit anti-OVGAL11 (1:10,000) or rabbit anti-oTP-1/oIFNτ (1:1000). As a positive control, each gel contained 5 μg of total protein from a Day 16 pregnant uterine flush and from uterine milk.

Statistical Analyses

Data from slot blot hybridization and protein slot blot analyses were subjected to LS-ANOVA using the General Linear Models procedures of the Statistical Analysis System. Slot-blot hybridization data were corrected for differences in sample loading using the 18S rRNA data as a covariate. Data from Study One were analyzed for effects of day, pregnancy status (cyclic or pregnant), and their interaction. Data from Study Two were analyzed using preplanned orthogonal contrasts (P+CX versus P+IFN; P+CX

versus P+ZK+CX; and P+IFN versus P+ZK+IFN). Data are presented as the LSM with overall SE.

Results

Endometrial Ovine Galectin-15 Sequence Analysis

During the course of sequencing ESTs from a Day 14 pregnant ovine endometrial cDNA library, approximately 1% of the 5,000 sequences were determined to be highly similar (95% identity) to OVGAL11 (GenBank AF252548) (Dunphy et al. 2000). Based on the nucleotide sequence of the coding region (414 nucleotides), ovine endometrial galectin is composed of 137 amino acids predicted to yield a 15.4 kDa protein with a pI of 5.24. It displayed the greatest similarity/identity to human galectin-13 (~44% identity) followed by human galectin-10 (~40% identity) (Fig. 4.1). Subsequent FASTA searches against human, mouse and rat genome sequences detected no other similar genes or proteins. Given that it has no known orthologue, OVGAL11 from sheep intestine and endometrium is considered a new galectin family member and named galectin-15.

The amino acid sequence of endometrial galectin-15 was searched for conserved domains which revealed the presence of a CRD characteristic of galectins (Hirabayashi et al. 2002). The CRD is a consensus motif that consists of 13 amino acids (Oda et al. 1993), of which eight (H.N.R.V.N.W.E.R) play a critical role in sugar binding (Dyer and Rosenberg 1996; Hirabayashi and Kasai 1994). Comparison of the galectin-15 CRD with the conserved CRD of other galectins indicates that four residues are identical (V62, N64, W71, E74) and two are conservatively substituted (R53, K76). The C57 is different from prototypical galectins, but appears to allow for binding of mannose in galectin-10 (Swaminathan et al. 1999). Consistent with other galectins, galectin-15 had no apparent or predicted signal peptide, transmembrane domain, or glycosylation sites. A PROSITE search revealed two putative cell attachment sequences at positions 123 (LDV) and 126 (RGD) that are integrin binding domains (Ruoslahti 1996).

```
Galectin-15    1 MDSLPNPYLQ SVSLTVCYMV KIKANLLSPF 30
Galectin-13    1 MSSLPVPYKL PVSLSVGSCV IIKGTPIHSF 30
Galectin-10    1 MSLLPVPYTE AASLSTGSTV TIKGRPLACF 30

                                       * * *
Galectin-15   31 GKNPELQVDF GTGTGQGGDI PFRFWYCDG- 59
Galectin-13   31 INDPQLQVDF YTDMDEDSDI AFRFRVHFGN 60
Galectin-10   31 LNEPYLQVDF HTEMKEESDI VFHFQVCFGR 60

                  * *          *  * *
Galectin-15   60 IVVMNTLKDG SWGKEQKLHT EAFVPGQPFE 89
Galectin-13   61 HVVMNRREFG IWMLEETTDY VPFEDGKQFE 90
Galectin-10   61 RVVMNSREYG AWKQQVESKN MPFQDGQEFE 90

Galectin-15   90 LQFLVLENEY QVFVNNKPIC QFAHRLPLQS 119
Galectin-13   91 LCIYVHYNEY EIKVNGIRIY GFVHRIPPSF 120
Galectin-10   91 LSISVLPDKY QVMVNGQSSY TFDHRIKPEA 120

Galectin-15 120 VKMLDVRGDI VLTSVDTL---- 137
Galectin-13 121 VKMVQVSRDI SLTSVCVCN--- 138
Galectin-10 121 VKMVQVWRDI SLTKFNVSYLKR 140
```

Fig. 4.1. CLUSTALW alignment of the amino acid sequences of ovine galectin-15 with human galectin-10 and -13. The asterisks denote the conserved residues forming the CRD found in prototypical galectin family members. The underlined amino acids indicate a predicted cell attachment sequence that is a conserved integrin binding site.

Steady-state Levels of Galectin-15 mRNA

Northern blot analysis of total RNA from endometria of cyclic and pregnant ewes detected a single transcript of ~0.8 kb (data not shown). Steady-state levels of galectin-15 mRNA were very low on Day 10, but increased about 13-fold between Days 10 and 14 in both cyclic and pregnant ewes (Fig. 4.2A). However, galectin-15 mRNA levels decreased between Days 14 and 16 in cyclic ewes, but increased in pregnant ewes (day x status, $p<0.001$). Galectin-15 mRNA levels in endometrium of Day 16 pregnant ewes were about 7-fold greater than for Day 16 cyclic ewes. In pregnant ewes, galectin-15 mRNA levels declined between Days 16 and 18 of pregnancy, but remained detectable to Day 20.

Localization of Galectin-15 mRNA

Galectin-15 mRNA was specifically expressed in endometrial LE and sGE) in both cyclic and pregnant ewes (Fig. 4.2B). In cyclic ewes, expression of galectin-15 mRNA was low in endometrial LE and sGE of cyclic ewes on Day 10, increased to Day 14, and then markedly decreased on Day 16. Galectin-15 mRNA was not detected in the endometrium on Days 1, 3, 5, 7 or 9 of the estrous cycle (data not shown). In pregnant ewes, galectin-15 mRNA was very low or undetectable in endometrial LE and sGE on Day 10 and increased between Days 10 and 16 (Fig. 4.2B). Galectin-15 mRNA expression was abundant in endometrial LE and sGE on Days 18 and 20 of pregnancy, but was not detected in the conceptus. Indeed, the decline in galectin-15 mRNA between Days 16 and 20 of pregnancy is most likely due to ablation of LE by the conceptus as binucleate cells form a syncytium with the LE, which occurs as the conceptus elongates and attaches along the length of the uterine horns (Guillomot 1995). After syncytium formation with the LE, the binucleate cells migrate and adhere to the epithelial basement membrane.

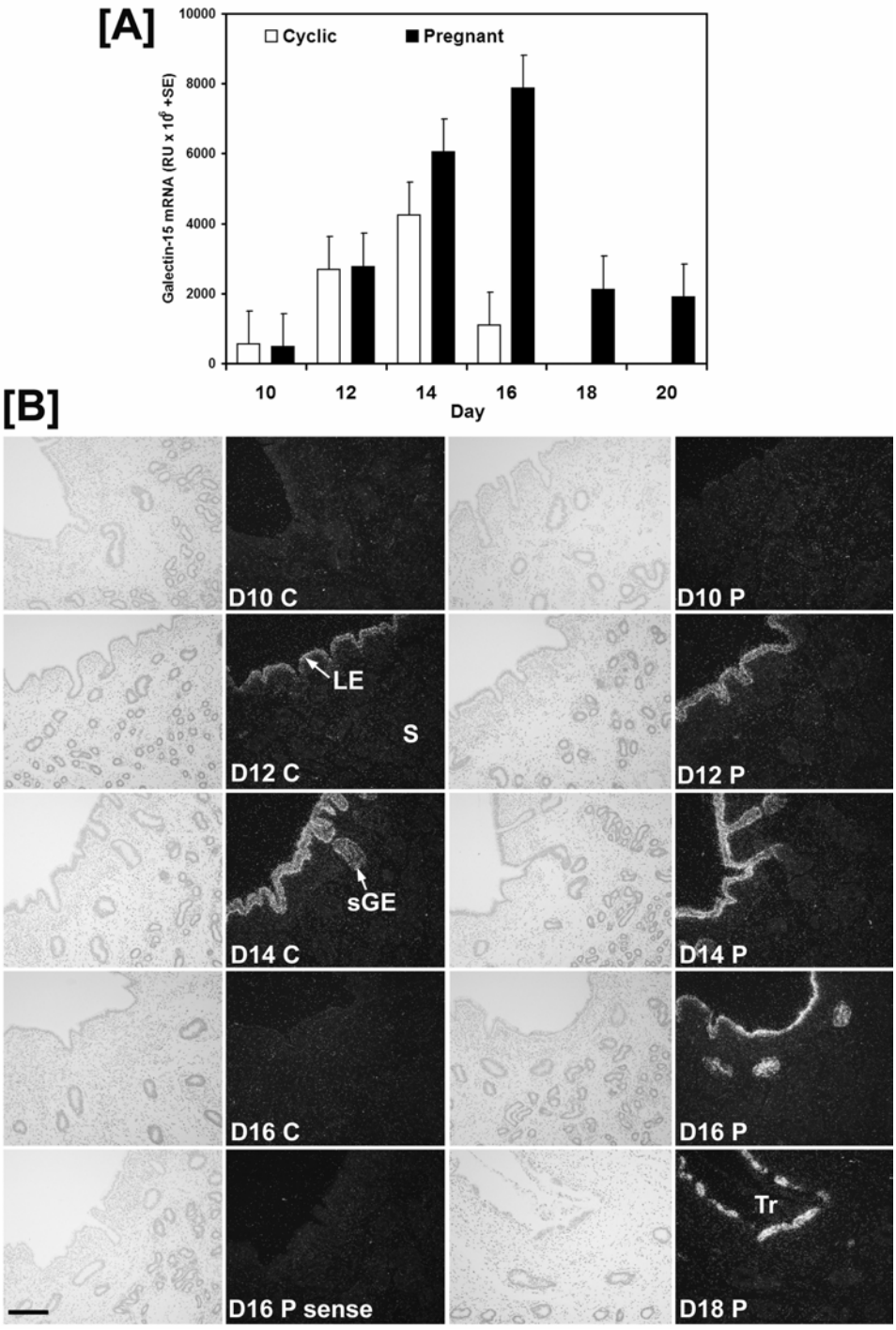

Fig. 4.2. Galectin-15 mRNA expression in the uterus (Study One). [A] Steady-state levels of galectin-15 mRNA in the endometrium as determined by slot-blot hybridization analysis. [B] *In situ* hybridization analysis of galectin-15 mRNA in the uterus. C, cyclic; D, day postmating; P, pregnant; LE, luminal epithelia; sGE, superficial glandular epithelia; S, stroma. Scale bar represents 100 μm.

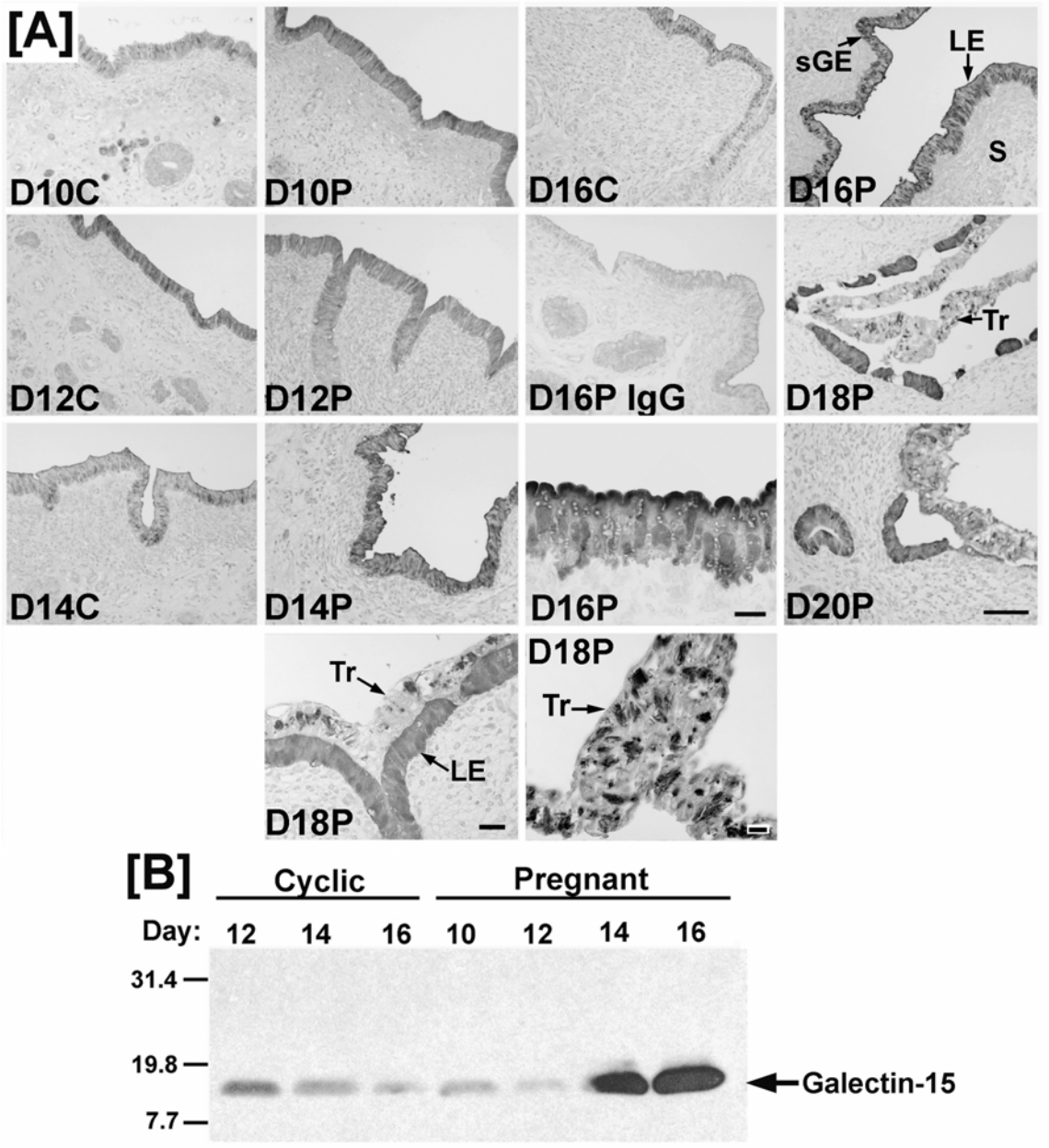

Fig. 4.3. Galectin-15 protein in the endometrium and lumen of the uterus (Study One). [A] Immunohistochemical localization of galectin-15 protein in the endometrium. Sections were not counterstained. [B] Western blot analysis of proteins in the lumen of the uterus. C, cyclic; D, day postmating; P, pregnant; LE, luminal epithelia; sGE, superficial glandular epithelia; S, stroma; Tr, trophectoderm. Scale bar represents 100 μm.

Localization of Galectin-15 Protein

Overall changes in immunoreactive galectin-15 protein abundance in endometrial LE/sGE paralleled changes in galectin-15 mRNA in cyclic and pregnant ewes (Fig. 4.3A). On Days 10 and 12 of the cycle and pregnancy, galectin-15 protein was localized primarily in the cytoplasm of epithelial cells. However on Days 14 to 20 of pregnancy, galectin-15 protein was readily apparent near and on the apical surface of endometrial LE. Further, galectin-15 protein was localized within discrete cytoplasmic structures in the conceptus trophectoderm on Days 16, 18 and 20 of pregnancy.

Presence of Galectin-15 in Uterine Flushings

Western blot analyses of uterine flushings revealed a single immunoreactive protein of ~15 kDa (Fig. 4.3B), which is the predicted size based on the inferred amino acid sequence and similar to OVGAL11 in sheep intestine (Dunphy et al. 2000). Galectin-15 protein levels were low in the uterine lumen of cyclic ewes on Days 10 and 12 of pregnancy, but abundant in the uterine lumen on Days 14 and 16, which corresponds to the onset of conceptus implantation and firm adherence of the trophectoderm to LE.

Hormonal Regulation of Galectin-15 mRNA

The regulation of galectin-15 gene expression in the endometrium by progesterone and ovine IFNτ was determined in Study Two (Fig. 4.4). In ewes receiving i.u. injections of CX proteins, progesterone increased galectin-15 mRNA in the endometrium (P+CX vs P+ZK+CX, $P<0.01$). In progesterone-treated ewes, i.u. injections of roIFNτ increased galectin-15 mRNA levels about 4-fold (P+CX vs P+IFN, $P <0.03$). However, this effect of IFNτ was dependent on progesterone (P+ZK+CX vs P+ZK+IFN, $P >0.10$). Galectin-15 mRNA was detected in LE and sGE of endometria from P+CX and P+IFN ewes (Fig. 4.4B); however, not in ewes receiving the ZK, PR antagonist, regardless of i.u. protein treatment.

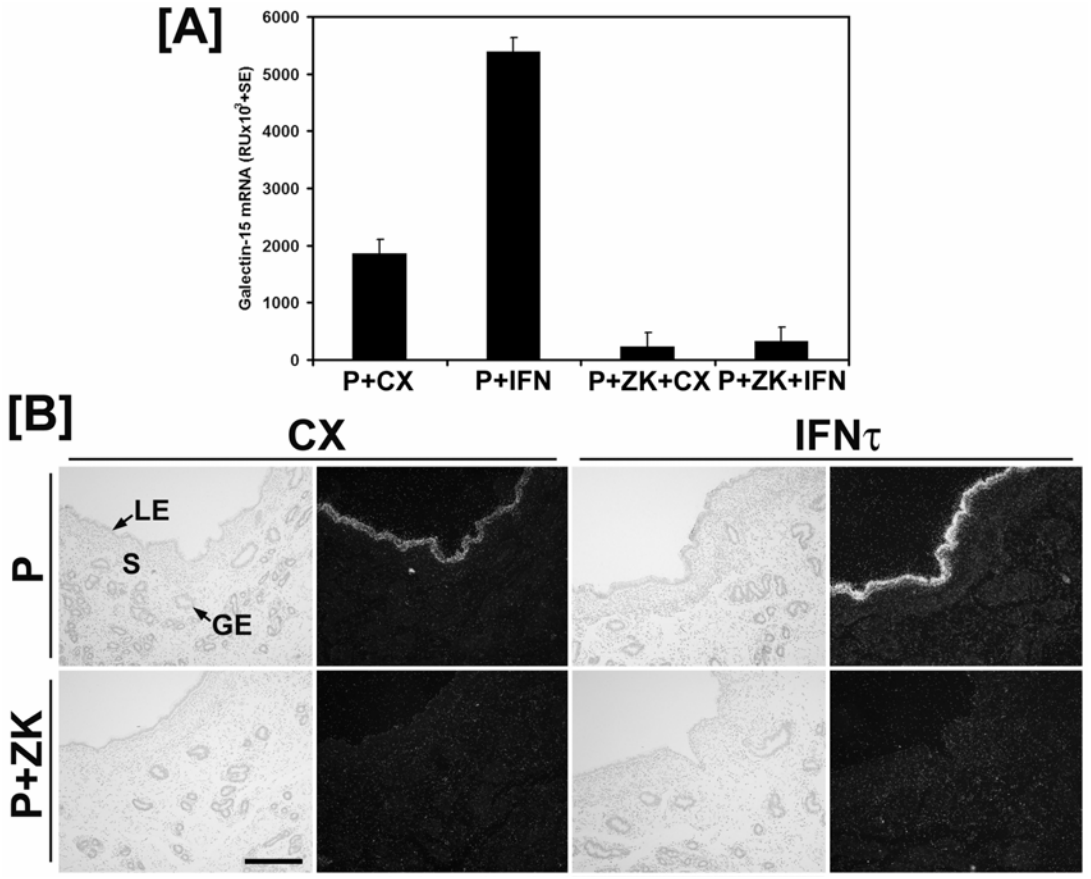

Fig. 4.4. Effects of progesterone and IFNτ on galectin-15 mRNA in the uterus (Study Two). [A] Steady-state levels of galectin-15 mRNA in the endometrium as determined by slot-blot hybridization analysis. [B] *In situ* hybridization localization of galectin-15 mRNA in the endometrium. LE, luminal epithelia; GE, glandular epithelia; S, stroma. Scale bar represents 100 μm.

Functional Ability of Galectin-15 to Bind to Carbohydrates

The carbohydrate binding ability of ovine endometrial galectin-15 was assessed using lactose-agarose (Fig. 4.5A). Galectin-15 from Day 16 pregnant uterine flushes bound lactose (lane 1), which could be partially competed with free lactose (lane 2), mannose (lane 3), and fucose (lane 4). Specific binding of galectin-15 from uterine flushes and uterine milk was detected for both mannose-agarose and fetuin-agarose (data not shown).

Identification of Galectin-15 as the Unknown 14K Protein

The homology of galectin-15 to galectin-10, also known as Charcot-Leyden Crystal protein in eosinophils and basophils (Weller et al. 1982), and its apparent molecular weight suggested that galectin-15 may be the 14K endometrial protein initially characterized as a progesterone-modulated, low-molecular-weight protein from the sheep uterus associated with crystalline inclusion bodies in uterine epithelia and conceptus trophectoderm (Kazemi et al. 1990). The 14K protein was originally identified as a contaminant of native ovine trophoblast protein one (oTP-1/oIFNτ) protein that was purified from conceptus cultures and used to generate antiserum in rabbits ~14 kDa (Kazemi et al. 1990). Indeed, galectin-15 from uterine flushes of Day 16 pregnant ewes and from uterine milk (Study Three) can be immunoprecipitated with rabbit antiserum to oTP-1/oIFNτ (Figs. 4.5B and 4.5C). Similarly, the rabbit antiserum to oTP-1/IFNτ also immunoprecipitated galectin-15 from the Day 16 pregnant uterine flushings and uterine milk. In the uterine flushing from a Day 16 pregnant ewe, the rabbit anti-oTP-1/oIFNτ antibody recognized two immunoreactive proteins that are oTP-1/oIFNτ (19 kDa) and galectin-15 (15 kDa). Only galectin-15 is present in uterine milk, because oTP-1/oIFNτ is only produced by the mononuclear trophectodermal cells of the ovine conceptus between Days 10 and 21 to 25 of gestation.

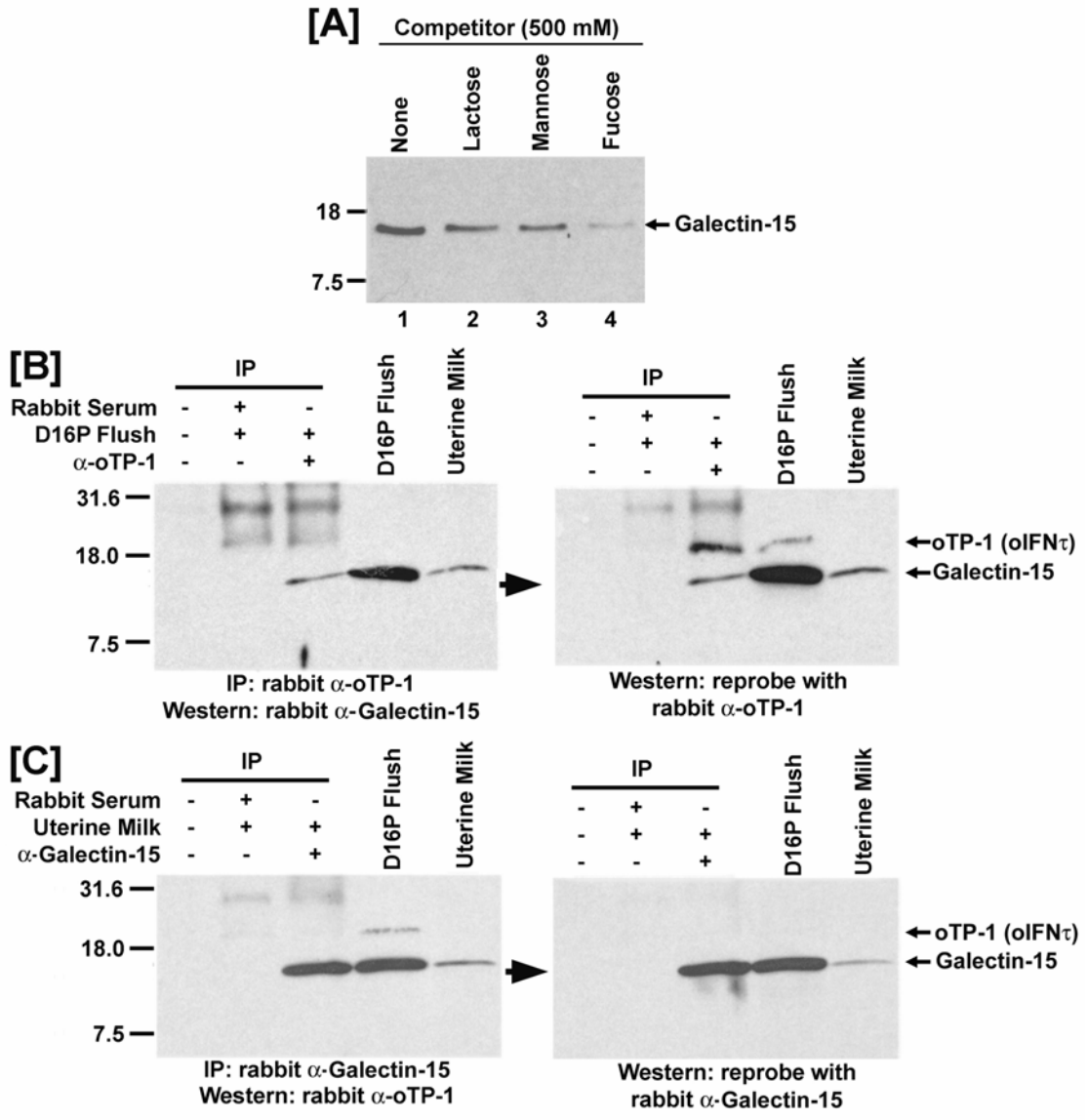

Fig. 5. Biochemical characterization of ovine endometrial galectin-15. [A] Carbohydrate-binding analysis of galectin-15 from the uterine lumen of Day 16 pregnant ewes by using lactose-agarose and competition with lactose, mannose, or fucose. [B] Immunological identification of galectin-15 from the uterine lumen of Day 16 pregnant ewes (Study One) and as the previously uncharacterized 14K protein. [C] Immunological identification of galectin-15 from uterine milk (Study Three) as the 14K protein. The anti-oTP-1/oIFNτ antibody crossreacts with the 14K protein (Kazemi et al. 1990), which is ovine endometrial galectin-15.

Discussion

The temporal changes in expression of endometrial galectin-15 mRNA support the hypothesis that ovarian progesterone and conceptus IFNτ regulate transcription of the galectin-15 gene in endometrial epithelia (Fig. 4.2A). The increase in galectin-15 mRNA in LE and sGE, between Days 10 and 12 post-estrus/mating, is coincident with the disappearance of PR mRNA and protein in the same epithelia (Spencer and Bazer 1995). Similarly, the decrease in galectin-15 mRNA between Days 14 and 16 of the cycle is coincident with the reappearance of PR protein in endometrial LE. Galectin-15 mRNA was expressed in endometrial LE and sGE of ovariectomized ewes treated with progesterone for 12 days (Fig. 4.4), but this expression was prevented by the PR antagonist ZK. Continuous exposure of the sheep uterus to progesterone for 8 to 10 days down-regulates PR expression in endometrial LE and sGE, but not stroma or myometrium (Spencer et al. 1995a). PR are present in the endometrial epithelia of P+ZK-treated sheep (Johnson et al. 2000b), because PR antagonists prevent the inhibitory effects of progesterone on the PR gene expression. These results support the hypothesis that progesterone binds to PR which represses or silences transcription of the galectin-15 gene in the endometrial LE/sGE before Day 10. Consequently, the progesterone modulation of galectin-15 mRNA may be attributed, at least in part, to down-regulation of the PR by progesterone that occurs in the LE and sGE between Days 10 and 12 of the cycle and pregnancy. Thus, PR loss in the endometrial epithelia may reprogram these cells, allowing them to express genes associated with terminal differentiated function (Spencer et al. 2004).

Galectin-15 is also an ISG. IFNτ is the pregnancy recognition hormone in sheep that acts on the endometrium to prevent development of the luteolytic mechanism, thereby maintaining the CL and production of progesterone (Spencer et al. 2004). The increase in galectin-15 expression in endometrial LE and sGE between Days 12 and 16 of early pregnancy parallels the increase in production of IFNτ by the conceptus, which is maximal between Days 14 and 16 (Farin et al. 1989). Intrauterine administration of IFNτ increased galectin-15 mRNA, but only in P-treated ewes (Fig. 4.3). One

hypothesis is that IFNτ can only stimulate transcription of the galectin-15 gene in the absence of repression by liganded PR. Alternatively, the PR-positive stroma may produce a 'progestamedin' that is required for the LE to respond to IFNτ (Chen et al. 2000a; Chen et al. 2000b; Spencer et al. 2004). The signaling pathway whereby IFNτ regulates transcription of the galectin-15 gene is not known, but it certainly does not involve the classical JAK-Stat signaling pathway (Choi et al. 2001; Kim et al. 2003a). To date, Wnt7a is the only other gene identified in endometrial LE that is increased by IFNτ (Kim et al. 2003a). Thus, the diverse actions of IFNτ on the endometrium include repression of genes involved in the luteolytic mechanism and stimulation of genes apparently important for conceptus survival, growth and implantation.

The present studies identified galectin-15 as the novel 14K progesterone-modulated protein from the sheep uterus associated with crystalline inclusion bodies in endometrial LE and conceptus trophectoderm (Kazemi et al. 1990). The 14K protein was originally identified as a component of conceptus-conditioned culture medium and uterine flushes (Salamonsen et al. 1984). Release of the 14K protein was attributed to the cellular breakdown of conceptuses in culture (Kazemi et al. 1990). As in the present studies, a previous study found that the 14K protein was confined to the endometrial LE and sGE, but absent from the deep GE in the uteri of Day 16 pregnant sheep and from sheep treated with progesterone for 14 or 30 days. In the uterine epithelia, immunoreactive 14K protein was most strongly detected over crystal-like structures, but was also uniformly present over the cytoplasm and nucleoplasm. Immunogold electron microscopy revealed that within trophectoderm, the 14K protein was localized to large, membrane-bound rhomboidal or needle-shaped crystal structures, but not in the endoplasmic reticulum and Golgi body. Thus, Kazemi and coworkers (Talbot et al. 2000) suggested that the protein was secreted by the endometrial epithelia and taken up by the conceptus from uterine histotroph. Interestingly, development of *in vitro* produced bovine blastocysts transferred into the sheep uterus resulted in the presence of crystals in trophectodermal cells (Rexroad and Powell 1999). The presence of crystals in sheep uterine milk (or histotroph) present between maternal and fetal

intercotyledonary membranes is well documented (Hoffman and Olson 1984; Wimsatt 1951). Reports of crystals in sheep trophectoderm and uterine epithelia are numerous (Hoffman and Olson 1984). Similar progesterone-induced crystal proteins are present in endometrium and conceptus trophectoderm of many mammals, including rabbit, mouse, pig and human (Calarco and Szollosi 1973; Daniel and Chilton 1978; Daniel and Kennedy 1978; Nakoa et al. 1971). Accordingly, galectin-15 related family members are likely to be expressed in the endometrium of many mammals to facilitate conceptus-endometrial interactions. Although the biological role(s) of galectin-15 crystals in the conceptus is not known, the intracellular role of other galectins include modulation of cell growth, differentiation and apoptosis through functioning as pre-mRNA splicing factors and interacting with specific intracellular ligands such as Ras and Bcl-2 (Hernandez and Baum 2002; Liu et al. 2002).

Galectin-15 is the newest member of the multifunctional galectin family. Members of this family must fulfill two key criteria: (1) conserved sequence elements forming a CRD; and (2) binding affinity for β-galactosides without requiring metal ions for activity. The present studies indicate that galectin-15 fulfills the two key criteria to be considered a *bona fide* galectin family member, because it contains the CRD and binds lactose and mannose (Figs. 4.1 and 4.5). Although the CRD of galectin-15 differs slightly from that in the prototypical galectins, it does possess the "jellyroll" structural fold similar to that of the prototypical galectins-1 and -2 and found in galectins-10 and -13 (Barondes et al. 1994a), which share similarity with galectin-15. Galectins bind β-galactosides via the CRD, but the carbohydrate binding specificity for each galectin appears to be different (Cho and Cummings 1995). In addition to the CRD, galectin-15 also contains predicted cell attachment sequences (LDV and RGD) that could mediate binding to integrins in ECM proteins (Kimber and Spanswick 2000; Wang and Armant 2002). Integrins are thought to be the dominant glycoproteins that regulate trophectoderm adhesion to endometrial LE during implantation (Johnson et al. 2001a). During the peri-implantation period of pregnancy in sheep, integrin subunits αv, α4, α5, β1, β3 and β5 are constitutively expressed on conceptus trophectoderm and apical

surface of endometrial LE. In sheep, receptivity to implantation does not appear to involve changes in temporal or spatial patterns of integrin expression, but may depend on changes in expression of ECM proteins which are ligands for heterodimers of these integrins (Hughes 2001). Other galectins bind integrins, fibronectin, and laminin, because these ECM proteins are modified with β-galactoside sugars (Cooper 2002; Yang and Liu 2003). Functional studies of other galectins have implicated these proteins in cell growth, differentiation and apoptosis as well as in cell adhesion, chemoattraction and migration (Barondes et al. 1994b; Hughes 2001; Yang and Liu 2003). The temporal and spatial alterations in galectin-15 mRNA and protein in endometrial LE and lumen of the ovine uterus during pregnancy, combined with the functional aspects of galectin-15 and its family members, make it a strong candidate for a mediator of conceptus-endometrial interactions during implantation. Therefore, the proposed extracellular role of galectin-15 in the uterine lumen is to functionally bind and cross-link β-galactosides on glycoproteins, such as integrins, fibronectin, and laminin, and glycolipids, thereby allowing it to function as a heterophilic cell adhesion molecule bridging the endometrial LE and conceptus trophectoderm. The biological responses of the trophectoderm to galectin-15 may also include migration, proliferation, and differentiation, which are critical for successful conceptus implantation. Understanding the extracellular and intracellular functions of galectin-15 in endometrial epithelia and conceptus trophectoderm will broaden our knowledge of the roles of galectins in basic biological processes and facilitate development of therapeutic applications for galectins in infertility and cancer.

CHAPTER V
GALECTIN-15 IN OVINE UTEROPLACENTAL TISSUES

Introduction

The endometrium of the uterus synthesizes and secretes or selectively transports a variety of substances, collectively termed histotroph, necessary for conceptus survival, growth and implantation in mammals (Bazer 1975; Burton et al. 2002; Carson et al. 2000; Fazleabas et al. 1997; Kane et al. 1997; Roberts and Bazer 1988). Histotroph is a rather undefined, complex mixture of adhesion proteins, transport proteins, ions, growth factors, hormones, protease inhibitors, amino acids and other molecules (Bazer 1975; Roberts and Bazer 1988). Uterine secretions are of particular importance for conceptus survival and growth in domestic animals due to their protracted peri-implantation period and non-invasive types of placentation (Bazer 1975; Roberts and Bazer 1988; Roberts et al. 1987). Analysis of recurrent pregnancy loss in the UGKO ewe model indicates that histotroph emanating from the endometrial LE and GE is clearly required for peri-implantation conceptus survival and growth in sheep (Gray et al. 2002; Gray et al. 2001c). Indeed, histotroph is absorbed by the placenta, transported into the fetal circulation, and cleared by the kidney into the allantois via the urachus (Bazer 1975; Roberts and Bazer 1988). Although the allantois was traditionally considered to be a reservoir for fetal wastes (Alexander and Williams 1968), studies in the pig and sheep support the idea that components of allantoic fluid have an important role in fetal nutrition (Bazer 1989).

Recently, a new galectin family member and component of uterine histotroph, galectin-15, was discovered in the endometrium of sheep (CHAPTER IV). Galectin-15 was originally identified in ovine intestinal epithelium as being upregulated in response to parasite infestation (Dunphy 2000). However, uterine expression of galectin-15 was detected only after Day 10 of pregnancy in the endometrial LE and sGE and was induced by progesterone and further stimulated by IFNτ, the pregnancy recognition signal produced by the conceptus. In the endometrium, galectin-15 protein had a

nucleocytoplasmic distribution within the LE and sGE and was also concentrated near and on the apical surface. Further, galectin-15 protein was abundant in the histotroph recovered from the uterine lumen and was identified as the previously unknown 14K protein that forms crystals in the endometrial epithelium and conceptus trophectoderm (CHAPTER IV). Galectins bind β-galactosides and functionally cross-link glycoproteins, as well as glycolipid receptors on the surface of cells and initiate biological responses that include cell proliferation, differentiation, motility, adhesion, and apoptosis (Cooper 2002; Yang and Liu 2003). The temporal and spatial alterations in galectin-15 mRNA and protein in the ovine endometrial epithelia and uterine lumen during early pregnancy, combined with the known biological activities of other galectins, make it a strong candidate mediator of conceptus-endometrial interactions during implantation (CHAPTER IV). It is proposed that galectin-15 is critical for successful conceptus implantation. Galectin-15 in the uterine lumen may bind and crosslink β-galactosides on glycoproteins and glycolipids expressed at the surfaces of endometrial LE and conceptus trophectoderm where it functions as a heterophilic cell adhesion molecule bridging the conceptus to the endometrium and stimulates the trophectoderm cells to migrate, proliferate and differentiate. Although the biological role(s) of galectin-15 in the conceptus is not known, the intracellular role of other galectins include modulation of cell growth, differentiation and apoptosis (Hernandez and Baum 2002; Liu et al. 2002).

The biological functions of endometrial galectin-15 on the conceptus are hypothesized to be important throughout pregnancy, but endometrial expression of galectin-15 beyond the peri-implantation period has not been determined. Indeed, many aspects of galectin-15 function are largely unknown, including 1) identity of crystals in the LE and conceptus; 2) ability to form multimers; and 3) functionality to bind to β-galactoside sugars. Our working hypothesis is that galectin-15 is a component of histotroph that is secreted by the endometrial epithelia throughout pregnancy in sheep. Therefore, the present study investigated galectin-15 mRNA, protein and potential roles

in uteroplacental tissues between Days 10 and 120 of pregnancy and in the uterus during the postpartum period.

Materials and Methods

Animals and Tissue Collection

Experimental and surgical procedures on crossbred Suffolk sheep complied with the Guide for Care and Use of Agriculture Animals in Teaching and Research were approved by the Institutional Animal Care and Use Committees of Texas A&M University.

Study One

Cyclic ewes were mated at estrus to either a vasectomized or an intact ram and were hysterectomized (n=5 sheep/day) on Days 12, 14 or 16 of the estrous cycle or on Days 12, 14, 16 of pregnancy (gestation period is 147 days). For all surgeries performed, ewes were administered isofluorane (5%) via an inhalation mask to induce anesthesia, which was maintained with isofluorane (1%–5%). A midventral laparotomy was performed to expose the reproductive tract. On Days 12 to 16, the uterine lumen was flushed with saline and examined for the presence of a morphologically normal conceptus to confirm pregnancy. Uterine flushes were clarified by centrifugation (3000 x *g* for 30 min at 4°C) and frozen at -80°C. Cross-sections of the uterine horn ipsilateral to the ovary bearing the corpus luteum and conceptuses from Day 16 pregnant ewes were fixed for transmission electron microscopy, as described below.

Study Two

As described previously (Kwon et al. 2004b), ewes were mated to rams of proven fertility at estrus and 12 and 24 h later. Ewes were assigned randomly to be hysterectomized (n = 4 per day) on either Day 20, 40, 60, 80, 100, or 120 of gestation to allow collection of uteroplacental tissues and fetal fluids. At hysterectomy, samples of the amniotic and allantoic fluids were collected, snap frozen in liquid nitrogen, and stored at -80^{o}C. Total endometrium was collected on Days 16 and 20 of pregnancy,

whereas caruncular and intercaruncular endometria were dissected from the myometrium collected between Days 40 through 120 of gestation. Portions of these tissues were snap frozen in liquid nitrogen and stored at -80^{o}C. Sections of the uterine wall with the attached trophectoderm were also fixed in freshly prepared 4% paraformaldehyde (w/v) in PBS (pH 7.2) or embedded in OCT compound, snap frozen in liquid nitrogen, and stored at –80^{o}C.

Study Three

As described previously (Gray et al. 2003), multiparous 5 to 8 year-old ewes were bred to rams of proven fertility, and pregnancy was determined by transabdominal ultrasonography. Ewes were assigned randomly to be ovariohysterectomized on postpartum days (PPD) 1, 7, 14 or 28 (n = 4 or 5 ewes/day). Ewes were checked twice daily for signs of labor or parturition. At hysterectomy, the residual placentomal or caruncular and intercaruncular areas of the uterine wall were dissected from the uteri of ewes on PPDs 1, 7 or 14, and sections (~1 cm) were fixed in 4% paraformaldehyde (w/v). On PPD 28, sections from the mid-portion of each uterine horn were fixed in 4% paraformaldehyde (w/v).

Study Four

Uterine milk was collected from the non-gravid uterine horn of unilateral pregnant sheep (n=4) on Day 80 of pregnancy using methods described initially by Bazer et al. (1979a). Uterine milk was clarified by centrifugation and stored at -80°C.

After 24 h, fixed tissues from all studies were dehydrated through a graded series of ethanol solutions and embedded in Paraplast Plus (Oxford Labware) for histological analyses.

Cloning and Expression of Recombinant Ovine Galectin 15

A 450 bp DNA fragment containing the galectin-15 coding sequence was amplified by PCR from plasmid DNA as the template, using the following oligonucleotide primers:

5’AGATGAAGCATATGGACTCCTTGCCGAACCCCTACC-3’ and
5’AGAGTAAGCTTATAACGTATCCACTGAAGTTCAGCA -3’.

The amplified DNA fragment was digested with Nde1 and Hind111 restriction enzymes, and subcloned into the corresponding restriction sites in the pET28b vector (Novagen, Madison, WI) with a N-terminal 6-His tag. The transformed cells were grown to exponential phase at 37ºC in TB media containing kanamycin. Cultures were then induced with 1 mM isopropyl-1-thio-β-D-galactopyranoside followed by incubation for 12 h at 20°C.

Purification of Galectin-15 Protein

The harvested cells were pelleted and resuspended in buffer A (20mM Tris-HCl, pH 7.5, 50mM imidazole) containing 1 mM PMSF and complete EDTA-free protease inhibitors (Roche, Indianapolis, IN). The cells were lysed using a French press and the cell suspension was centrifuged at 15,000 x g for 1 hour. The clear supernatant was loaded onto a Pharmacia Hi-trap Ni^{2+} chelating column (Amersham Pharmacia Biotech, Piscataway, NJ) and washed with 200 ml buffer A containing 20 nM Tris-HCl, pH 7.5, 500 mM NaCl. The His-tagged galectin-15 was eluted from the nickel affinity column using Buffer B (20 mM Tris-HCl, pH7.5, 500 mM imidazole and 500 mM NaCl). The eluate was concentrated by Centriprep (Amicon) to 7.0 mg/ml and applied onto a Sephadex 200 gel-filtration column (Amersham Biosciences) equilibrated with 20 mM tris-HCl (1 mM EDTA and dithiothreitol (pH 7.5)) as a final step. The protein was more than 95% pure as observed on an SDS-PAGE gel.

Western Blot Analyses

Uterine flushes from Study One were concentrated using Centricon-3 columns (Amicon). Protein content of concentrated flushes (Study One) and uterine milk (Study Four) was determined using a Bradford protein assay (Bio-Rad) with BSA as the standard. Proteins were separated by non-denaturing/non-reducing 12% PAGE, and

Western blot was conducted as described previously (Spencer et al. 1999a) using enhanced chemiluminescence detection. Immunoreactive galectin-15 was detected using primary rabbit anti-ovine galectin-15 antibody (kindly provided by Dr. Els N.T. Meeusen, Monash University, Melbourne, Australia) (Dunphy et al. 2000) at a 1:2500 dilution. Negative control blots were performed in which primary antibody was replaced by rabbit serum at the same concentration.

Protein content of allantoic and amniotic fluids from Study Two were determined by Bradford protein assay (Bio-Rad) with BSA as the standard. Allantoic and amniotic fluid proteins (20 μg) were denatured, separated by SDS-PAGE using 15% acrylamide gels, and transferred to nitrocellulose membranes as described previously (Spencer et al. 1999a). Immunoreactive galectin-15 was detected using the rabbit anti-ovine galectin-15 antibody (1:2500).

Carbohydrate Binding Analysis

This analysis was a modified version of methods for the *in vitro* analysis of saccharide binding by galectin-10 (Dyer and Rosenberg 1996). All binding experiments were performed in Dulbecco's PBS (Sigma, St. Louis, MO). Briefly, roGalectin-15 protein (5 μg) was incubated overnight at 4^{o}C with a 50 μl bed volume of lactose- or fetuin-conjugated agarose (Sigma). For competition, lactose or galactose (Sigma) sugar was added at a final concentration of 0, 0.5, 5, 50, and 500 mM. After incubation, the resin was washed three times for 10 min in 1 ml PBS at 4^{o}C and the bound protein eluted with SDS-PAGE reducing sample buffer. The relative amount of galectin-15 protein bound to lactose was determined by 15% SDS-PAGE and Western blot analysis using the rabbit anti-ovine galectin-15 antibody as described above.

Transmission Electron Microscopy (TEM)

Uterine cross-sections and conceptuses from Day 16 of gestation (Study One) were fixed in a solution of 2% paraformaldehyde, 3% glutaraldehyde in 0.08 M sodium cacodylate (pH 7.22) and dehydrated in series from 10% to 100% ethanol and embedded

in Unicryl resin (SPI Supplies, West Chester, PA). Routine staining of semi-thick sections with 0.1% toludine blue and 1% Na-Borate (Borax: $Na_2B_4O_7$ 10 H_2O) was utilized to isolate areas of interest, primarily LE and conceptus trophectoderm. Trimmed materials were then sectioned using an LKB ultramicrotome (Type 4802A). The 70-90 nm thick sections were stained with 10% uranyl acetate in 30% ethanol for 30 minutes followed by Reynolds' lead citrate for 10 minutes (Reynolds 1963). Sections were examined and photographed using a Zeiss 10CA transmission microscope at 80 kV on Kodak Electron Microscope Film 4489.

Immunogold TEM

Sections of Day 16 pregnant endometrium and conceptuses were fixed in 3% (w/v) paraformaldehyde 0.2% glutaraldehyde in 0.1M cacodylate buffer (pH 7.2) containing 5% (w/v) sucrose, dehydrated through ethanol and embedded in Unicryl (SPI Supplies). Ultrathin sections were picked up on 300 hex nickel grids and floated on 10% heat-inactivated normal goat serum in 0.1% BSA/Tris for 30 min. Grids were transferred to drops of rabbit anti-ovine galectin-15 antibody (1:2000) or rabbit serum for 1 h at room temperature. After washing three times with 50 mM Tris (pH 7.4) in 0.1% BSA for 1 min each, five times in 50 mM Tris (pH 7.4) for 1 min each, and 2 times in PBS for 5 min each, the grids were postfixed in 2% glutaraldehyde in PBS for 5 min and rinsed with water. Grids were then dried at room temperature and stained lightly with uranyl acetate in 50% (v/v) alcohol. TEM images were photographed as described above.

RNA Isolation and Analyses

Total cellular RNA was isolated from frozen intercaruncular and caruncular endometria from Study Two and intercaruncular endometrium from Study Three using the Trizol reagent (Gibco-BRL). For each ewe, denatured total cellular RNA (20 μg) was analyzed by slot blot hybridization using a radiolabeled antisense cRNA probe generated by *in vitro* transcription with [α-^{32}P] UTP (Amersham Pharmacia Biotech) as

described previously (CHAPTER IV). Plasmid templates containing cDNAs for ovine galectin-15 (CHAPTER IV) and 18S rRNA (pT718S; Ambion) were used to produce radiolabeled cRNA probes as described previously (Choi et al. 2001). Hybridization signals were detected by exposing washed slot blots to a phosphorimager screen and visualized using a Typhoon 8600 Variable Mode Imager (Molecular Dynamics).

In Situ *Hybridization*

Ovine galectin-15 mRNA was localized in uterine tissue sections by *in situ* hybridization as described previously (Spencer et al. 1999b). Deparaffinized, rehydrated and deproteinated uterine sections (5 μm) were hybridized with radiolabeled antisense or sense ovine galectin-15 (CHAPTER IV) cRNAs generated from linearized plasmid templates using *in vitro* transcription with [α-^{35}S] UTP. Autoradiographs of slides were prepared using Kodak NTB-2 liquid photographic emulsion. Slides were stored at 4^{o}C for 1 to 2 weeks as judged from autoradiographs, developed in Kodak D-19 developer, counterstained with Gill's hematoxylin (StatLab), dehydrated through a graded series of alcohol to xylene, and coverslipped.

Immunofluorescence Analyses

Proteins were localized in frozen intercaruncular and caruncular endometrial tissue sections (8-10 μm) collected in Study Two by immunofluorescence staining as described previously (Johnson et al. 1999a). Briefly, frozen tissues were sectioned (8 μm) with a Hacker-Bright OTF cryostat (Hacker Instruments, Fairfield, NJ) and mounted on Superfrost/Plus microscope slides (Fisher Scientific, Pittsburgh, PA). Frozen sections were fixed in -20C methanol for 10 min, permeabilized with 0.3% Tween-20 (v/v) in 0.02 M PBS, and then blocked in antibody dilution buffer [two parts 0.02 M PBS, 1.0% BSA (w/v), 0.3% Tween-20 (w/v; pH 8.0) and one part glycerol] containing 5% normal goat serum (v/v) for 1 h at room temperature, and incubated overnight at 4^{o}C with the rabbit anti-ovine galectin-15 antibody (1:1000) or rabbit serum

(1:1000). Immunoreactive protein was then detected using a fluorescein-conjugated secondary antibody for 1 h at room temperature. Slides were overlaid with Prolong antifade mounting reagent and then coverslipped (Molecular Probes, Eugene, OR).

Photomicroscopy

Photomicrographs of *in situ* hybridization slides were taken using a Nikon Eclipse E1000 photomicroscope (Nikon Instruments, Inc.). Digital images were captured using a Nikon DXM 1200 digital camera and assembled using Adobe Photoshop 7.0 (Adobe Systems). For immunoflourescence analyses, representative fluorescence images of cross-sections exhibiting immunocrossreactivity were recorded using a Ziess Axioplan microscope (Carl Zeiss, Thornwood, NY) equipped with a Hamamatsu chilled 3CCD color camera (Hamamatsu, Japan) using Adobe Photoshop 7.0 image capture software. TEM negatives were converted to digital images using an Epson Perfection 3200 Photo scanner (Epson America Inc., Long Beach, CA) and assembled using Adobe Photoshop 7.0.

Statistical Analyses

All quantitative data were subjected to LS-ANOVA using General Linear Models procedures of the Statistical Analysis System. Analyses of steady-state levels of endometrial mRNA determined by slot blot hybridization included the 18S rRNA as a covariate to correct for differences in sample loading. Least-squares regression analyses were used to determine effects of day on endometrial galectin-15 mRNA levels. In all analyses, error terms used in tests of significance were identified according to the expectation of the mean squares for error. Data are presented as LSM with overall SE.

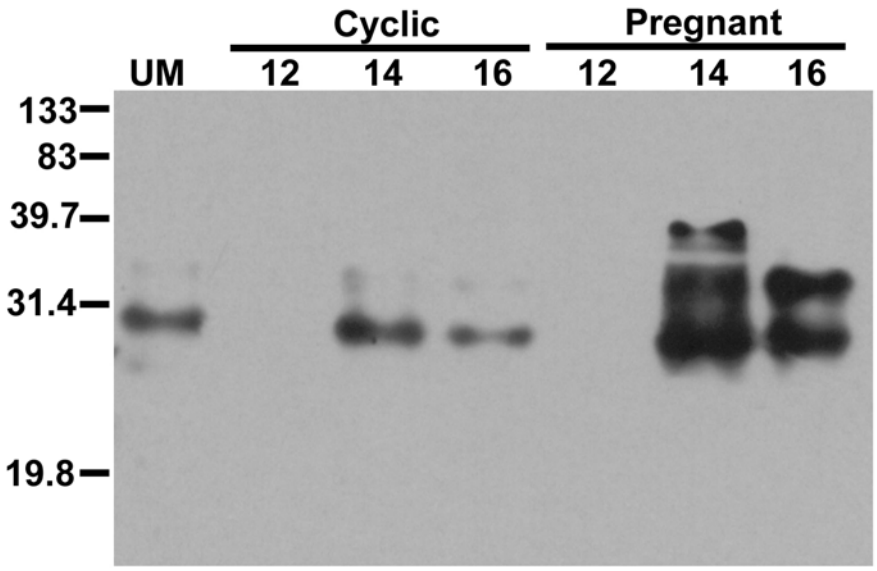

Fig. 5.1. Galectin-15 protein in uterine flushes self-associates to form multimers in the absence of denaturing and reducing agents on Days 14 and 16 of the estrous cycle and early pregnancy. No galectin-15 protein was detectable at 15 kDa, which is the size of the denatured form. Positions of pre-stained molecular weight standards (x 10^{-3}) are indicated.

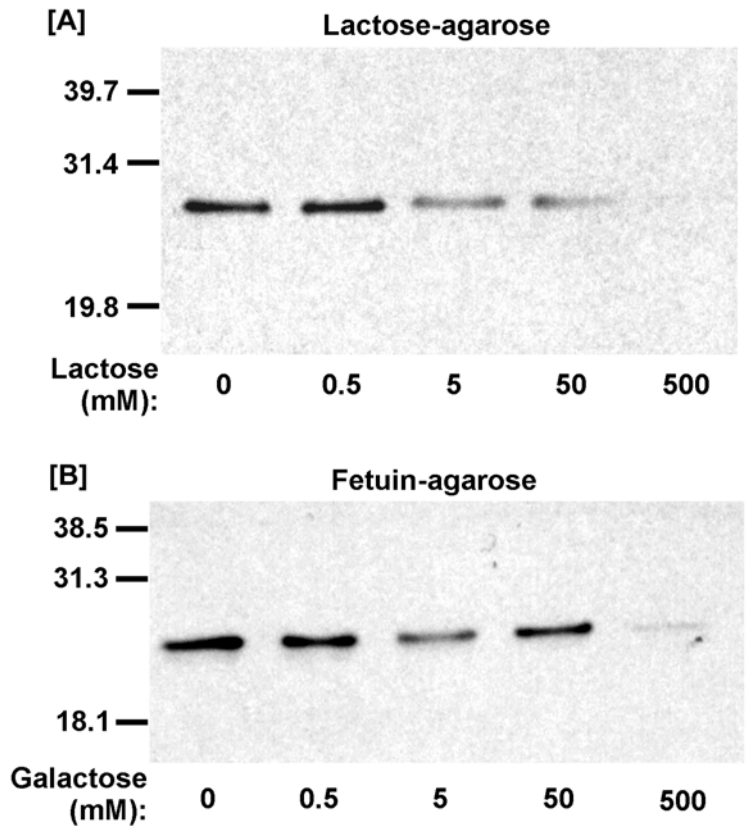

Fig. 5.2. Carbohydrate binding analysis of roGalectin-15 using lactose- or fetuin-agarose. Binding was competed with lactose or galactose sugars at varying concentrations. Galectin-15 was able to bind to both lactose and fetuin and this binding was competed off with lactose and galactose sugars, respectively. Positions of pre-stained molecular weight standards (x 10^{-3}) are indicated.

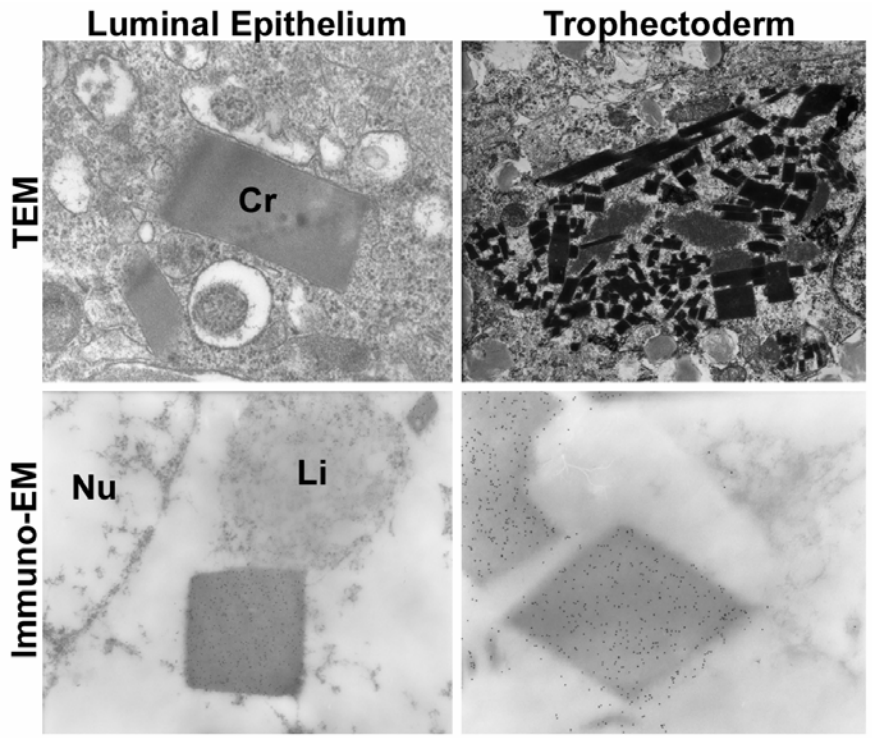

Fig. 5.3. Structural analysis and galectin-15 localization in endometrial LE cells and conceptus trophectoderm. Cross-sections of endometrium and filamentous conceptus collected on gestational day 16 were analyzed via TEM and immunogold-TEM labeling with rabbit anti-ovine galectin-15 antibody. Crystalline structures are present in both LE and trophectoderm and galectin-15 is localized to these crystals. Legend: Nu, nucleus; Cr, crystal protein; Li, lipid. Magnification = 10,000X.

Results

Non-denaturing/Non-reducing Western Analysis

Non-denaturing/non-reducing western blot analyses of uterine flushes from cyclic and pregnant ewes (Study One) revealed multiple immunoreactive galectin-15 protein bands (Fig. 5.1). Galectin-15 appeared as several immunoreactive bands of approximately 60 and 80 kDa on Days 14 and 16 of the estrous cycle and Day 14 of pregnancy. However, on Day 16 of pregnancy galectin-15 appeared as three bands of approximately 60, 80 and 100 kDa. Little or no immunoreactive galectin-15 was detectable on Day 12 of the estrous cycle or pregnancy. No immunoreactive galectin-15 protein was detectable in any of the uterine flushes at 15 kDa, the size of the denatured protein (CHAPTER IV).

Carbohydrate Competition Assay

The carbohydrate binding ability of roGalectin-15 was assessed using lactose-agarose and fetuin-agarose. Binding ability was then competed with various concentrations of lactose and galactose sugars. roGalectin-15 bound to lactose (Fig. 5.2A) and was competed off with free lactose in a dose dependent manner. The addition of 500 mM lactose was sufficient to completely compete off all binding of roGalectin-15 to the lactose agarose. roGalectin-15 was also able to bind to fetuin (Fig. 5.2B) and was competed off with the addition of increasing concentrations of free galactose. However, the addition of 500 mM galactose was not sufficient to compete off all binding of roGalectin-15 to fetuin.

Ultrastructural Analysis of LE and Trophectoderm

Electron Microscopy

Crystalline inclusion bodies are present on Day 16 of gestation in both LE (Fig. 5.3A) and conceptus trophectodermal cells (Fig. 5.3C), as shown previously (Kazemi et al. 1990). These crystal structures are localized in the cytoplasm near the

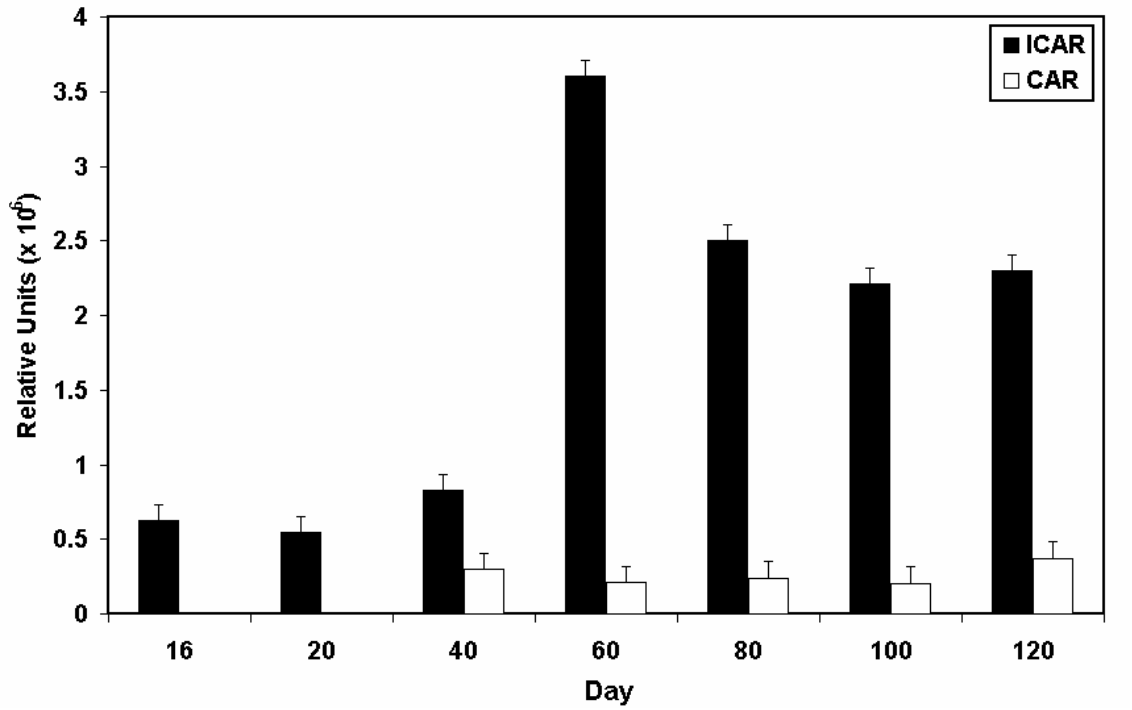

Fig. 5.4. Steady-state levels of galectin-15 mRNA in the intercaruncular (ICAR) and caruncular (CAR) endometrium during pregnancy as determined by slot blot hybridization analysis. Expression of galectin-15 was normalized relative to expression of 18S rRNA mRNA. Data are presented as LSM+SE. Galectin-15 mRNA levels increased after Day 40 of pregnancy ($P<0.001$, quadratic effect of day).

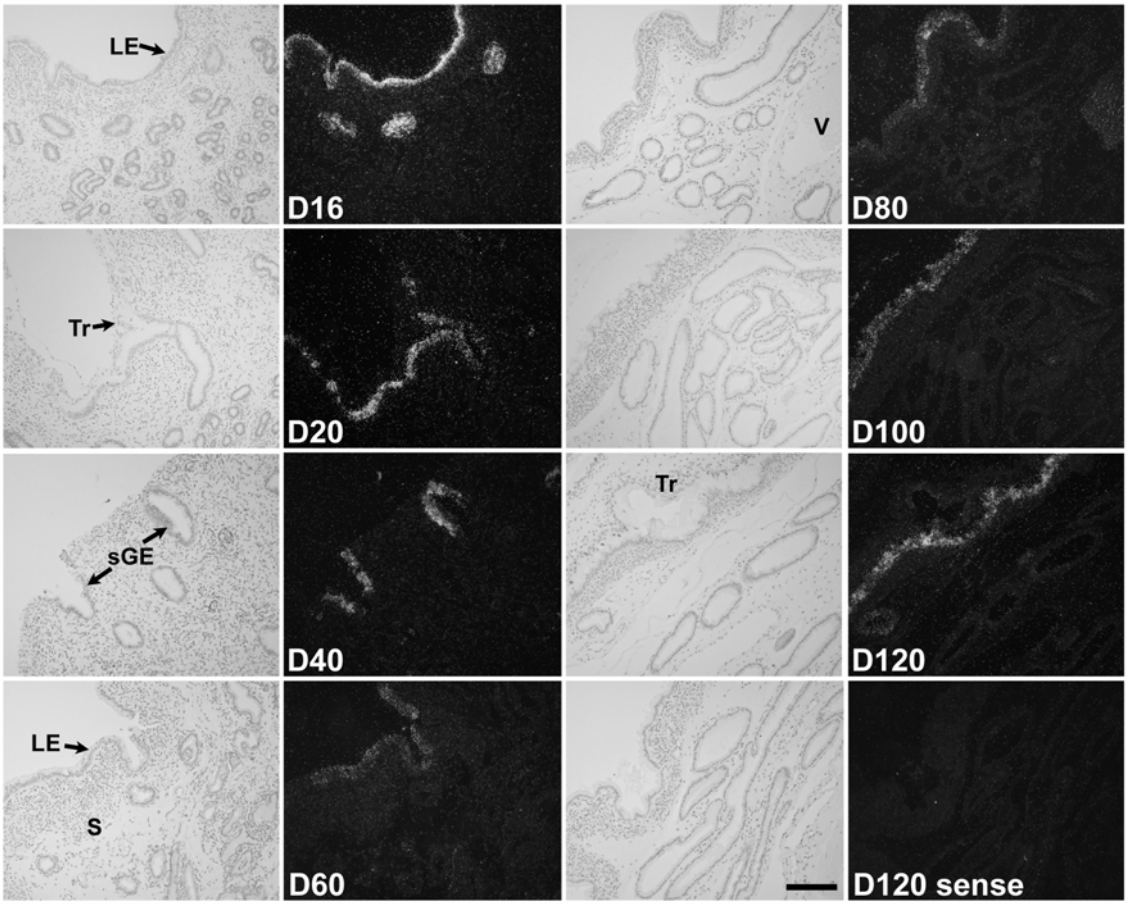

Fig. 5.5. *In situ* hybridization analysis of galectin-15 mRNA expression in the intercaruncular areas of the endometrium from the uterus of pregnant ewes. Cross-sections of the uterine wall were hybridized with radiolabeled antisense or sense cRNA probes generated from linearized plasmid cDNA clones. Hybridized sections were digested with RNase A, and protected transcripts were visualized by liquid emulsion autoradiography. Developed slides were counterstained lightly with hematoxylin, and photomicrographs were taken under brightfield or darkfield illumination. Expression of galectin-15 mRNA was detected only in the endometrial LE and sGE. Legend: LE, luminal epithelium; sGE, superficial ductal glandular epithelium; S, stroma; Tr, trophectoderm; V, blood vessel; Scale bar represents 100 μm.

nucleus in the LE cells and were more abundant in the conceptus trophectodermal cells. The crystals appear to be rhomboidal and are surrounded by a membrane.

Immunolocalization

Immunogold labeling localized galectin-15 protein to the crystalline inclusion bodies in both LE (Fig. 5.3B) and conceptus trophectodermal cells (Fig. 5.3D) on Day 16 of gestation. The crystal structures were the only site of immunoreactive galectin-15 protein in both the LE and conceptus trophectoderm.

Steady-state Levels of Galectin-15 mRNA

In pregnant ewes (Study Two), steady-state levels of galectin-15 mRNA were very low to undetectable in the caruncular endometrium (Fig. 5.4A). Galectin-15 mRNA expression in the intercaruncular endometrium was lowest during early pregnancy from Days 16 to 40, increased 3.5-fold between Days 40 and 60 of pregnancy, and remained elevated to Day 120 (quadratic effect of day, $P<0.001$). In Study Two, steady-state levels of galectin-15 mRNA were highest on PPD 1 and decreased through PPD 28 (linear effect of day, $p=0.08$) (Fig. 5.4B).

Localization of Galectin-15 mRNA

In Study Two, galectin-15 mRNA was expressed specifically by the endometrial LE, if present, and sGE, but not middle to deep GE, stroma, myometrium or placenta (Fig. 5.5). Between Days 20 and 40 of pregnancy, galectin-15 mRNA was only expressed in the sGE of the endometrium, because the LE was absent. However, the LE reappeared between Days 40 and 60. On Day 60 and thereafter, galectin-15 mRNA was detected in both the LE and sGE of the intercaruncular endometrium. Galectin-15 mRNA was not detected in the caruncular or cotyledonary areas of the placentomes (data not shown).

In Study Three, galectin-15 mRNA was localized primarily to the LE during the postpartum period, with low expression also detected in the sGE (Fig. 5.6). On PPD 1,

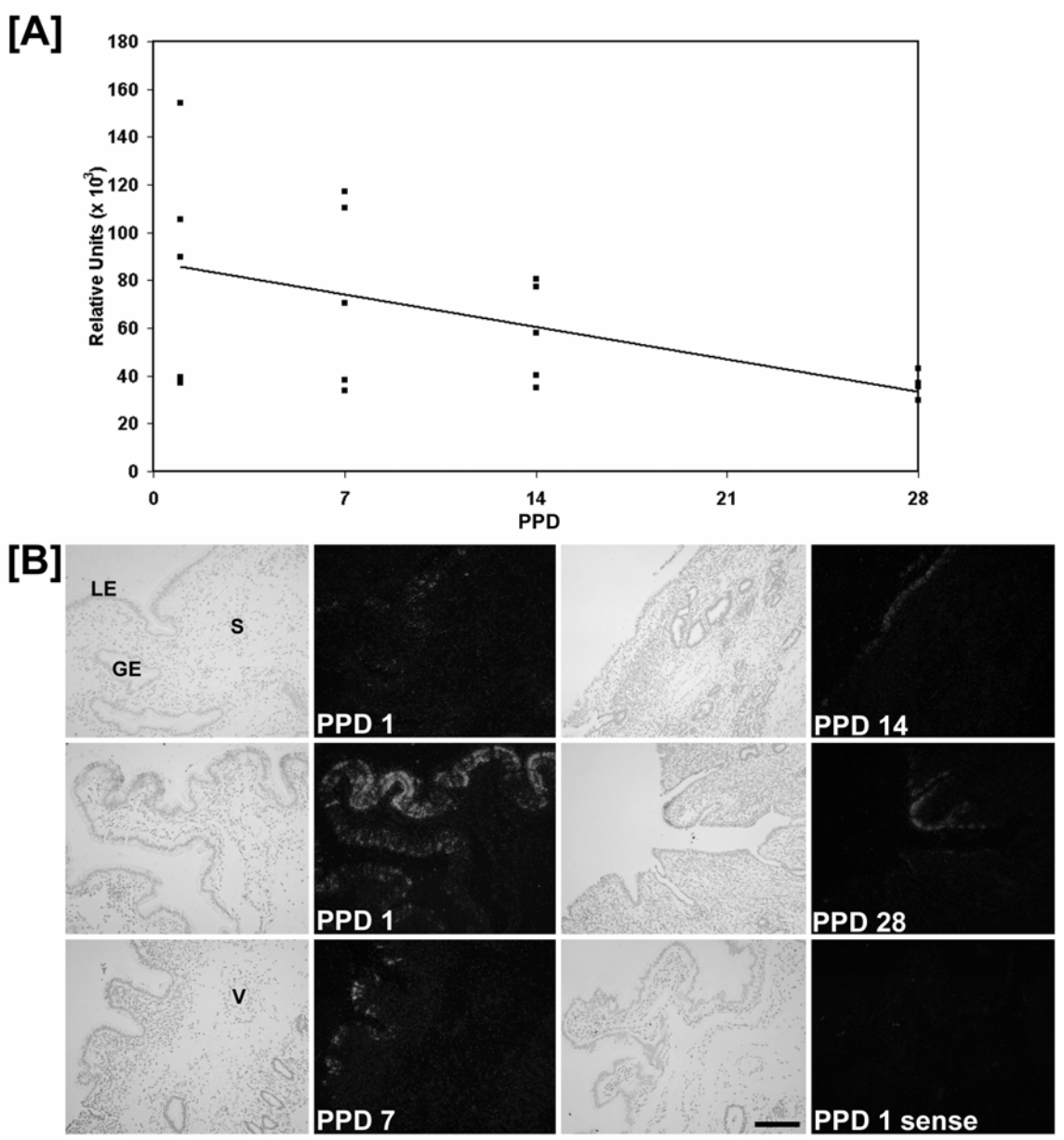

Fig. 5.6. Spatial and temporal patterns of galectin-15 mRNA expression during the postpartum period. Steady-state levels of galectin-15 mRNA in the endometrium during the postpartum period as determined by slot blot hybridization analysis [A]. Data are presented as LSM+SE. Galectin-15 mRNA levels declined after parturition (P=0.08, linear effect of day). *In situ* hybridization analysis of galectin-15 mRNA expression in the intercaruncular areas of the endometrium of the postpartum ovine uterus [B]. Expression of galectin-15 mRNA was detected only in the endometrial LE and sGE. Legend: LE, lumenal epithelium; GE, glandular epithelium; PPD, postpartum day; S, stroma; V, blood vessel. Scale bar represents 100 μm.

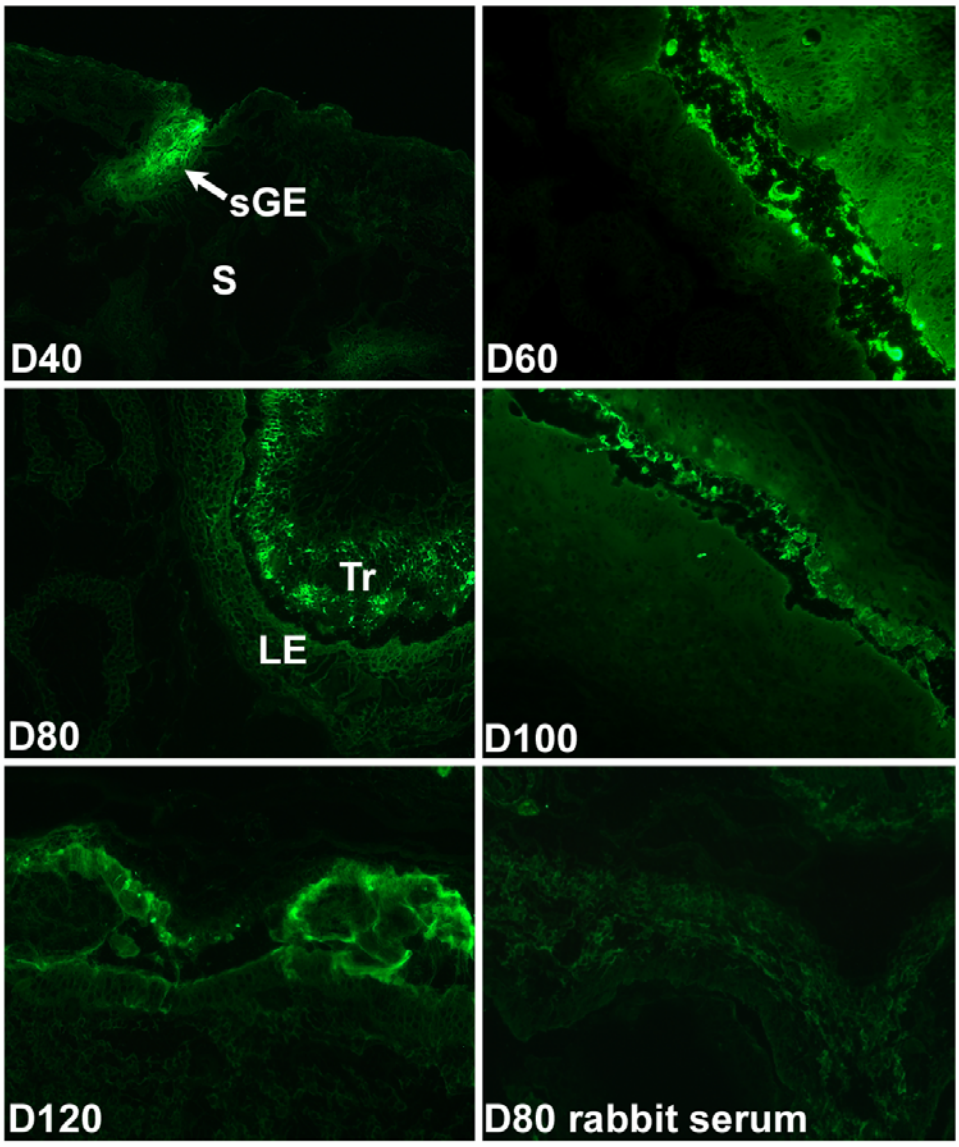

Fig. 5.7. Immunofluorescence localization of galectin-15 protein in the intercaruncular areas of the endometrium from uteri of pregnant ewes on Days 40, 60, 80, 100 and 120. All photomicrographs are shown at the same magnification. Legend: LE, lumenal epithelium; sGE, superficial glandular epithelium; S, stroma; Tr, trophectoderm. The width of the field is 525 μm.

galectin-15 mRNA expression was variable, ranging from high to moderate to low abundance depending on the ewe. The pattern of galectin-15 expression in the LE was variegated, particularly on PPDs 1 and 7. Expression of galectin-15 mRNA was still detected in some areas of the LE on PPD 28.

Localization of Galectin-15 Protein

Immunoreactive galectin-15 was detectable at the chorion-maternal interface throughout gestation (Fig. 5.7). On Day 40 of pregnancy, galectin-15 was observed in at the apical surface of the sGE. By Day 60, galectin-15 protein was observed on the LE as well as within the overlying trophectoderm of the chorion. This spatial pattern of expression was consistent throughout the latter portion of pregnancy. During ovine placentation, the LE fuses with the trophectodermal binucleate cells (BNC) to form a syncytia beginning on Day 16 when the BNC differentiate (Wimsatt 1951; Wooding 1984). The LE reappears in the intercaruncular endometrial areas between Days 40 and 60. Galectin-15 protein was undetectable in the caruncular or cotyledonary areas of the placentomes (data not shown).

Presence of Galectin-15 in Fetal Fluids

Immunoreactive galectin-15 was detectable in allantoic fluid throughout gestation. In the allantoic fluid, galectin-15 appeared as a single immunoreactive protein of approximately 15 kDa (Fig. 5.8). Galectin-15 protein was not detectable in amniotic fluid on any day of gestation (data not shown).

Discussion

Galectin-15 is expressed in LE and sGE of the intercaruncular endometrium of the pregnant ovine uterus from Day 12 to term, encompassing the periods of implantation and placentation, as well as during uterine involution after parturition. In cyclic and early pregnant ewes, galectin-15 mRNA was also confined to the endometrial LE and sGE after Day 10 of the cycle or pregnancy (CHAPTER IV). Further,

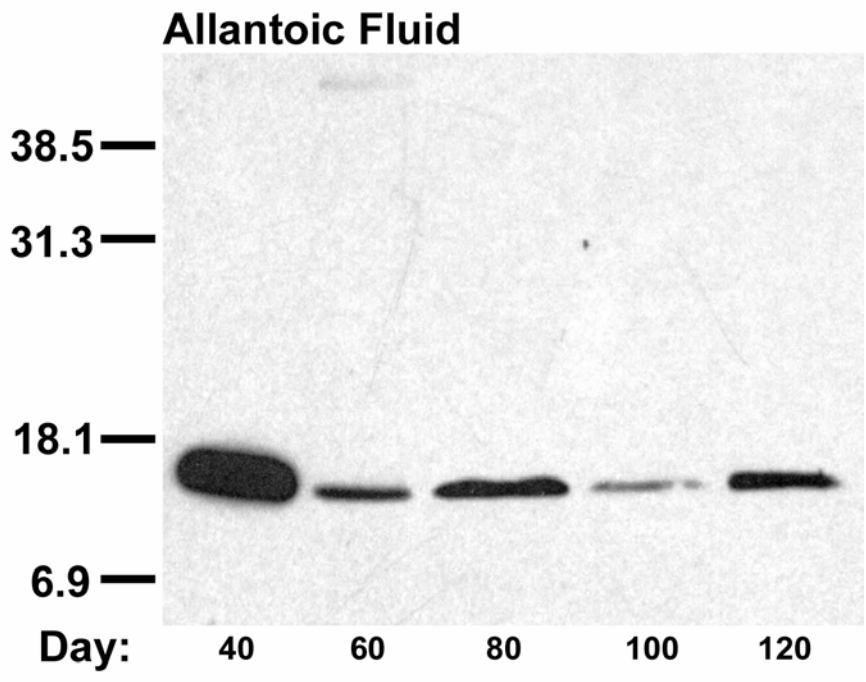

Fig. 5.8. Western blot analysis of galectin-15 protein (~15 kDa) in allantoic fluid obtained from pregnant ewes on Days 40, 60, 80, 100, and 120. Galectin-15 protein was undetectable in amniotic fluid throughout gestation (data not shown). Positions of pre-stained molecular weight standards (x 10^{-3}) are indicated.

progesterone induced and IFNτ stimulated galectin-15 gene expression in the endometrium. Additionally, secreted galectin-15, during early pregnancy, is able to self-aggregate and form multimers, which is a common feature of galectin family members (Cho and Cummings 1996; Dunphy et al. 2002; Yang et al. 1998). The impact that this self-association has on galectin function is not well understood, particularly in regard to ability of galectins to additionally bind to glycoproteins and/or glycolipid receptors. Recombinant galectin-15 is able to bind to sugars (CHAPTER IV) and this binding can be competed off with the addition of sugars. The role galectin-15 may play in binding glycocongugates to promote adhesion during implantation could be critical; however, the importance of galectin-15 binding to carbohydrates during later pregnancy is unknown. Immunogold electron microscopy revealed that within the LE and conceptus trophectoderm, galectin-15 protein was localized to large, membrane-bound rhomboidal crystal structures. Therefore, galectin-15 protein forms crystals within LE cells, is secreted by the endometrial epithelia, taken up by the conceptus from uterine histotroph, and deposited as crystals (Kazemi et al. 1990). These crystals are first observed in the sheep trophoblast on Day 10 and then increase in number and size between Days 10 and 18 of pregnancy (Wintenberger-Torres and Flechon 1974). Indeed, the crystals exhibit a lattice periodicity of about 20 nm in Day 14 blastocysts. The presence of crystals in sheep uterine histotroph (or uterine milk) present between maternal and fetal intercotyledonary membranes is well documented (Hoffman and Olson 1984; Wimsatt 1951). Similar progesterone-induced crystal structures are present in the endometrium and/or conceptus trophectoderm of a number of species, including sheep (Hoffman and Olson 1984; Kazemi et al. 1990; Wintenberger-Torres and Flechon 1974), mouse (Calarco and Szollosi 1973), rabbit (Daniel and Kennedy 1978; Nakoa et al. 1971), and human (Nakoa et al. 1971). Although the biological role(s) of galectin-15 crystals in the uterine epithelia is not known, the intracellular role of other galectins include modulation of cell growth, differentiation and apoptosis through functioning as pre-mRNA splicing factors and interacting with specific intracellular ligands such as Ras and Bcl-2 (Hernandez and Baum 2002; Liu et al. 2002).

During synepitheliochorial placentation in sheep, trophoblast giant BNC begin to differentiate between Days 14 and 16 and then fuse apically with the endometrial LE and form syncytia, thereby assimilating and replacing the endometrial LE (Wimsatt 1951; Wooding 1984). In the intercaruncular endometrium, the LE reappears between Days 40 and 60 of pregnancy, and this was associated with an increase in galectin-15 mRNA in the intercaruncular endometrium in the present study. Galectin-15 mRNA and protein expression was not observed in the placentomes, which are comprised of placental cotyledons and endometrial caruncles devoid of LE. Although the placenta is expelled shortly after parturition, regrowth of the LE in the caruncular endometrium takes at least four weeks during postpartum involution of the uterus (Gray et al. 2003). Galectin-15 mRNA expression pattern in the epithelia is variegated during the postpartum period and steady-state levels decline slowly over the month following parturition.

The temporal and spatial changes in galectin-15 mRNA expression in the endometrial LE and sGE of cyclic and pregnant ewes were inversely correlated to PR protein expression in the same epithelia in cyclic and pregnant ewes (Spencer and Bazer 1995)(CHAPTER IV). Further, Gray and coworkers (CHAPTER IV) determined that progesterone induction of galectin-15 gene expression was likely due to the ability of progesterone to inhibit PR gene expression in the endometrial epithelia. Throughout gestation the ovine epithelia remain PR negative and progesterone action is mediated through stromal receptors (Spencer et al. 2004). However, during the postpartum period, epithelial PR expression is increased in the GE from PPDs 7 to 28, but not in the LE (Gray et al. 2003). Interestingly, PR loss in the epithelia appears to be required for the onset of expression of other secretory proteins during pregnancy such as osteopontin (OPN) and ovine uterine serpins (or uterine milk proteins) (Spencer et al. 2004). With the return of PR in the GE during the postpartum period, GE-specific expression of OPN and uterine serpins declines (Gray et al. 2003). Available results suggest that repression of epithelial galectin-15 gene expression in the endometrium occurs in the presence of functional PR and progesterone (CHAPTER IV). Perhaps the variable decline in galectin-15 mRNA expression during the postpartum period is due to the reappearance

of the PR in only one of the cell types expressing galectin-15. Understanding the transcriptional regulation of the galectin-15 gene is expected to uncover unique cell-specific transcription factors and regulatory networks that regulate activation and repression, given that expression of the galectin-15 gene in the ovine uterus is confined solely to the endometrial LE and sGE.

Although galectin-15 mRNA was found exclusively in the endometrial LE and sGE of the uterus, immunoreactive galectin-15 protein was detected predominantly in the chorion/trophectoderm of the placenta as well as in the allantoic fluid. Galectin-15 protein is present in the uterine lumen of early pregnant ewes as well as in uterine milk recovered from the non-gravid horn of unilaterally pregnant ewes (CHAPTER IV). Other galectin family members also exhibit dual localization, being found in both intracellular (cytoplasm and, in some cases, the nucleus) and extracellular (cell surface and medium) compartments (Hughes 1997). Although galectins are often present on cell surfaces or in the ECM, galectins lack a classical secretion signal sequence and are secreted by a novel apocrine mechanism that does not involve the endoplasmic reticulum-Golgi pathway (Boulianne et al. 2000; Hughes 1999). Similarly, ovine galectin-15 lacks a signal peptide (Dunphy 2000)(CHAPTER IV). After implantation, the chorioallantois develops unique structures, termed areloae, that develop over the mouth of each uterine gland as specialized areas for absorption and transport of uterine histotroph into the conceptus (Bazer 1975). These results support the idea that galectin-15 protein is synthesized by the endometrial LE and sGE and then secreted into the uterine lumen, where it is absorbed by the placenta, transported into the fetal circulation, and cleared by the kidney into the allantois via the urachus (Bazer 1975; Roberts and Bazer 1988). Although the allantois was initially considered a reservoir for waste products of the fetus, it serves to store most secreted proteins from the endometrium (Bazer et al. 1975; Moffatt et al. 1987b). In contrast, amniotic fluid is not in the path for protein clearance by the fetal kidney and, therefore, does not function in this capacity. In the present study, galectin-15 protein was not detected in amniotic fluid, but was in allantoic fluid.

In the allantois, galectin-15 may simply serve as a source of amino acids for fetal and placental growth and function or posess other biological functions. Throughout gestation, amino acids play a vital role in development of the conceptus and are abundant in allantoic fluid (Kwon et al. 2004b). Nitric oxide, synthesized from L-arginine, regulates placental angiogenesis (Reynolds and Redmer 2001) and uterine blood flow during gestation (Sladek et al. 1997). Galectin-1 modulates L-arginine metabolic pathways in rat macrophages (Correa et al. 2003), which are important for placental growth in sheep (Kwon et al. 2004b). Even though galectin family members are traditionally recognized as binding to β-galactoside moieties, galectin-8 is also able to bind to glucose (Nagy et al. 2002). Although the binding affinity of galectin-15 for glucose is unknown, it may bind to glucose or fructose in the allantoic fluid, thus helping maintain high sugar concentrations for fetal nutrition. During human pregnancy, galectin-13 (or placental protein-13) plays a role in calcium-mediated depolarization, liberation of arachidonic acid, and the formation of thromboxane in the placenta (Burger et al. 2004). Indeed, a reduction in galectin-13 is associated with intrauterine growth retardation (Burger et al. 2004). Future experiments will assess the intracellular and extracellular role(s) of galectin-15 in regulation of uterine immune responses, placental morphogenesis and function, and fetal growth.

Another potential biological function of endometrial galectin-15 throughout pregnancy and during involution may be to modulate inflammatory and immune responses. Under the influence of progesterone, uterine lymphocyte dynamics and functions are altered that inhibit maternal responses to the fetal-placental semi-allograft which is critical for pregnancy success (Clark et al. 1999). As in other mammals, the ovine uterus has pregnancy-associated changes in lymphocytes, macrophages and natural killer cells which are differentially regulated by conceptus and maternal factors

(Fox et al. 1998; Gogolin-Ewens et al. 1989; Lee et al. 1992; Tekin and Hansen 2002; Tekin and Hansen 2004). Evidence suggests that progesterone stimulates the endometrium to induce synthesis of regulatory molecules that affect lymphocyte dynamics (Majewski et al. 2001). Indeed, uterine serpins (also termed uterine milk proteins) are a protein family synthesized and secreted by the endometrium that have immunoregulatory functions (Hansen 1998; Leslie and Hansen 1991). Similarly, galectin-15 is a progesterone-induced secretory product of the endometrium (CHAPTER IV). Interestingly, galectin-15 was originally found to be induced in gastrointestinal tissue and secreted into the intestinal lumen in response to inflammation and eosinophil infiltration after infection of sheep with the helminth, *Haemonchus contortus* (Dunphy et al. 2000). Given that several other galectins regulate innate and adaptive immune responses (Rabinovich et al. 2002b; Young and Meeusen 2004), one may speculate that galectin-15 in the ovine endometrium is another progesterone-induced immunoregulatory factor that modulates the maternal intrauterine immune system, thereby protecting the conceptus and promoting placental growth, which is a process strikingly similar to host-parasite interactions (Guimond et al. 1999).

CHAPTER VI
PROTEOMIC ANALYSIS OF UTERINE SECRETIONS FROM DAY 14 PREGNANT AND BRED UTERINE GLAND KNOCK OUT (UGKO) EWES

Introduction

The endometrium of the uterus synthesizes and secretes or selectively transports a variety of substances that are essential for conceptus survival, growth and implantation in mammals (Bazer 1975; Carson et al. 2000; Fazleabas et al. 1997; Kane et al. 1997; Roberts and Bazer 1988). Histotroph is a rather undefined, complex mixture of adhesion proteins, transport proteins, ions, cytokines, growth factors, amino acids, hormones, proteases, protease inhibitors, and other molecules (Bazer 1975; Roberts and Bazer 1988). Uterine secretions are of particular importance for conceptus survival and growth in domestic animals due to their protracted peri-implantation period and non-invasive type of placentation (Bazer 1975; Roberts and Bazer 1988; Roberts et al. 1987). Available evidence from humans, laboratory animals and domestic animals indicate that a reduction in uterine histotroph compromises survival and growth of conceptuses, which can ultimately lead to infertility, recurrent pregnancy loss and intrauterine growth retardation (IUGR) (Burton et al. 2002; Gray et al. 2002; Herrler et al. 2003). The UGKO ewe model was developed to understand the role of uterine histotroph in conceptus survival and development. In this model, postnatal endometrial gland morphogenesis is inhibited by exposing neonatal ewes to a 19-norprogestin from birth to at least 8 weeks of age (Gray et al. 2000a; Spencer et al. 1999c). Progestin exposure specifically ablates development of the endometrial glands in the uterus without altering development of the uterine myometrium or other Müllerian duct-derived female reproductive tract structures (Gray et al. 2001b; Gray et al. 2000b). In addition to the absence of superficial and deep endometrial glands, the endometrium contains decreased surface LE due to the lack of intercaruncular endometrial folds (Gray et al. 2001b). Despite repeated matings to fertile rams, adult UGKO ewes are unable to establish pregnancy (Gray et al. 2000a; Gray et al. 2001b; Gray et al. 2002; Gray et al. 2001c).

Transfer of hatched blastocysts, recovered from superovulated normal donor ewes, into the uteri of timed recipient UGKO ewes failed to ameliorate the pregnancy defect (Gray et al. 2001c). Morphologically normal blastocysts were found in the uterine flushings of bred UGKO ewes on Days 6 and 9 post-mating, but not on Day 14 (Gray et al. 2001b; Gray et al. 2001c). On Day 14, uterine flushings from bred UGKO ewes contained either no conceptus or a severely growth-retarded conceptus that had failed to elongate from a tubular to filamentous form (Gray et al., 2001c). UGKO ewes exhibit a peri-implantation pregnancy defect whose timing correlates with the majority of embryo loss that occurs during pregnancy in livestock as well as humans (Bazer 1975; Kane et al. 1997; Norwitz 2001). Therefore, the UGKO ewe is a useful model to identify critical components of uterine secretions necessary for peri-implantation conceptus survival and growth.

In sheep, the peri-implantation period is marked by rapid elongation of the conceptus from a tubular to filamentous form (Guillomot 1995; Spencer et al. 2004) and production of IFNτ, a Type I IFN that is the antiluteolytic signal for maternal recognition of pregnancy in ruminants (Bazer et al. 1997). Available results support the hypothesis that endometrial insufficiency, due to the absence or reduction in secretion of proteins of endometrial epithelial origin, is the cause of recurrent pregnancy loss in the UGKO ewe model (Gray et al. 2002; Spencer et al. 1999c). These results support the earlier conclusion of Flèchon and coworkers (Flèchon et al. 1986) that histotrophic secretions from the endometrium are required for conceptus elongation in sheep, a developmental event that has not been recapitulated *in vitro*. The objective of this study was to analyze differences in the protein component of endometrial secretions of normal fertile and infertile UGKO ewes using 2D-PAGE combined with MALDI-TOF mass spectrometry.

Materials and Methods

Animals

Experimental and surgical procedures complied with the Guide for Care and Use of Agriculture Animals and were approved by the Institutional Agricultural Animal Care

and Use Committee of the Texas A&M University System Agricultural Experiment Station (Animal Use Protocol 7-286).

UGKO ewes were produced as described previously (Gray et al. 2000a; Spencer et al. 1999c) by implanting crossbred ewes with a single Synchromate B® (Sanofi) implant within 12 h of birth and every two weeks thereafter for a total of eight weeks. Implants were inserted subcutaneously in the periscapular area and released approximately 6 mg of norgestomet, a potent synthetic 19-norprogestin, over a 14 day period (Bartol et al. 1988a). Control ewes did not receive implants.

Study One

In order to synchronize estrus, adult UGKO (n=4) and normal crossbred ewes (n=8) were given two i.m. injections (0700h and 1700h) of 10 mg $PGF_{2\alpha}$ (Lutalyse) nine days apart. Ewes were monitored daily for estrous behavior using vasectomized rams. All UGKO and some normal control ewes (n=4) were bred at estrus and at 12 h and 24 h post-estrus by intact rams of proven fertility. The remaining control ewes (n=4) were assigned to cyclic status, and time of estrus was determined by vasectomized rams. On Day 14 post-estrus/mating, all ewes were subjected to mid-ventral laparotomy, and the uterine lumen flushed with 20 ml sterile saline followed by ovariohysterectomy. Uterine flushes were analyzed using a dissecting microscope to recover conceptuses, if any, and determine their morphology. The flushes were then clarified by centrifugation (3000 x *g* for 15 min at 4^{o}C) and stored in aliquots at -80^{o}C for 2D-PAGE analysis.

Study Two

Cyclic ewes were mated at estrus to either a vasectomized or an intact ram and were hysterectomized (n=5 ewes/day) on Days 10, 12, 14 or 16 of the estrous cycle or on Days 10, 12, 14, 16, 18 or 20 of pregnancy. On Days 10 to 16, the uterine lumen was flushed with saline and examined for the presence of a morphologically normal conceptus to confirm pregnancy.

For both studies, cross-sections of the uterine horn ipsilateral to the ovary bearing the corpus luteum were fixed in 4% paraformaldehyde in phosphate buffered saline (PBS) for 24 h, dehydrated in 70% ethanol, and then embedded in Paraplast-Plus

(Oxford Labware). The remaining endometrial tissues were dissected from myometrium and frozen at -80^{o}C.

Endometrial Explants

Some of the dissected endometrium from Day 14 pregnant and bred UGKO ewes in Study One was placed into warm Dulbecco's Modified Eagle's medium (DMEM)/F-12 culture medium (Sigma) containing penicillin G (100 IU/ml), streptomycin (0.1 mg/ml), and amphotericin B (0.25 µg/ml; Gibco-BRL). The endometrium was then minced with scalpel blades into small pieces (2–3 mm^3). Aliquots of 500 mg minced endometrium were placed into culture dishes (100 x 15 mm) with 5 ml of cysteine-methionine-deficient DMEM culture medium (Sigma) containing 50 µCi/ml of [^{35}S]methionine-cysteine (Promix; Amersham Life Sciences, Arlington Heights, IL) for incorporation into newly synthesized proteins. Cultures were incubated for 24 h with rocking under an atmosphere of 45% nitrogen, 5% carbon dioxide, and 50% oxygen. The culture medium was then harvested, centrifuged (3000 x g for 15 min at 4°C), and stored at -80°C for 2D-PAGE analysis.

2D-PAGE Analysis

Preparation of Protein Samples

Uterine flush and endometrial explant culture media samples (3 ml) from Day 14 pregnant and UGKO ewes were filtered through 0.45 µm cellulose acetate syringe filters and precipitated with 10% volume of trichloroacetic acid (TCA) on ice for 30 min. Precipitated proteins were collected by centrifugation and washed with cold acid acetone (1 part HCL, 37 parts acetone). Samples were then centrifuged again, air dried, and resuspended in 200 µl of solubilization buffer (9.5M deionized urea/4% CHAPS). Protein content was determined using a Bradford protein assay (Bio-Rad) with bovine serum albumin (BSA) as the standard. The amount of radioactivity associated with the precipitated protein was determined using a LS6500 Multi-purpose scintillation counter (Beckman Coulter, Fullerton, CA).

Isoelectric Focusing (IEF)

Proteins were separated by isoelectric point in the first dimension and by molecular weight in the second dimension using facilities in the Protein Chemistry Laboratory at Texas A&M University. Samples (100 μg per ewe) were combined with rehydration buffer [8M deionized urea, 2% CHAPS, 18 mM DTT, 2% IPG buffer (pH 3-10NL) and trace bromophenol blue] for a final volume of 250 μl. The samples were placed in ceramic strip holders and used to rehydrate 13 cm pH 3-10 non-linear immobilized pH gradient gel strips (Amersham Pharmacia Biotech). The samples were subjected to IEF conditions for a total of 50,000 volthours. Subsequently, the focused strips were treated with Laemmli sample buffer that contained 5 mM DTT (reduction) followed by incubation in Laemmli sample buffer that contained 10 mM iodoacetamide (alkylation).

SDS-PAGE

Equilibrated gradient strips were then placed horizontally onto 10% acrylamide SDS slab gels and subjected to electrophoresis in the second dimension. An initial trial run was performed with uterine flush proteins and these gels were silver stained after electrophoresis. For subsequent analysis of proteins by MALDI-TOF mass spectrometry, 2D-PAGE gels of separated uterine flush proteins or radiolabeled endometrial explant proteins were visualized with Coomassie blue, followed by fixation in 50% (v:v) methanol, 5% (v:v) acetic acid. The gels containing radiolabeled proteins were then dried and detected by autoradiography using Bioimax MR film (Kodak). The length of time the gels were exposed to autoradiographs was determined by the counts per minute (CPM) of the endometrial explant cultures run per gel.

Differences in protein spot patterns between pregnant and UGKO samples were identified by visual comparison. Eight protein spots were chosen from the uterine flush samples, and four protein spots were selected from the endometrial explant samples for mass spectrometry. Peptide sequences were obtained from selected protein spots by in gel digestion techniques using trypsin. Peptides from the uterine flush digests were identified using an Axima CFR (Kratos, Japan) MALDI-TOF mass spectrometer at the

University of Massachusetts Medical Center (Worcester, MA). The MS-Tag database (University of California San Francisco Mass Spectrometry Facility) was utilized to fit the fragment-ion tag data of the tandem mass spectrum to a peptide sequence. Peptides from the endometrial explant cultures were identified by ThermoFinnegan (Thermo Electron Corp, San Jose, CA) using a ThermoFinnegan LCQ DecaXP ion trap mass spectrometer equipped with in-line HPLC separation of peptides. Endometrial explant protein tandem mass data was analyzed with Bioworks and TurboSEQUEST software (Thermo Electron Corp).

RNA Isolation and Analyses

RNA Extraction

Total cellular RNA was isolated from endometrial samples using the Trizol reagent (Gibco-BRL).

Cloning of cDNAs

Partial cDNAs for ovine NADP+ dependent isocitrate dehydrogenase (IDH) and CA2 mRNAs were amplified by reverse transcription-PCR of total RNA from Day 14 pregnant ovine endometrium using primers based on the bovine IDH mRNA (GenBank accession no. AF136009; forward, 5'-TTT TCC CTA CGT GGA ACT GG-3'; reverse, 5'-CCA TCG CTT GGT GTG TAG G-3') or human CA2 mRNA (GenBank accession no. BC035424; forward, 5'-CAA TGG TCA TGC TTT CAA CG-3'; reverse, 5'-AAT CCA GGG ATT CAG GAA GG-3'). PCR amplification was conducted as follows using PCR Optimized Buffer (Invitrogen, Carlsbad, CA): 95°C for 5 min, 35 cycles of 95^{o}C for 30 sec, 54^{o}C for 30 sec, and 72^{o}C for 30 sec; and then 72°C for 7 min. The PCR amplified partial IDH cDNA (390 bp) and CA2 cDNA (386 bp) cDNAs were subcloned into pCRII vector using a T/A Cloning Kit (Invitrogen). Partial cDNAs were sequenced in both directions using an ABI PRISM Dye Terminator Cycle Sequencing Kit and ABI PRISM automated DNA sequencer (Perkin-Elmer Applied Biosystems, Foster City, CA) to confirm identity.

Slot Blot Hybridization

For each ewe, denatured total cellular RNA (20 μg) was analyzed by slot blot hybridization using a radiolabeled antisense cRNA probe generated by *in vitro* transcription with [α-^{32}P] UTP (Amersham Pharmacia Biotech) as described previously (Spencer and Bazer 1995). Plasmid templates containing cDNAs for ovine IDH, ovine CA2, and human 18S rRNA (pT718S; Ambion) were used to produce radiolabeled cRNA probes as described previously (Choi et al. 2001). Hybridization signals were detected by exposing washed slot blots to a phosphoimager screen and visualized using a Typhoon 8600 Variable Mode Imager (Molecular Dynamics).

In Situ *Hybridization*

IDH and CA2 mRNAs were localized in uterine tissue sections from both studies by *in situ* hybridization as described previously (Spencer et al. 1999b). Deparaffinized, rehydrated and deproteinated uterine sections (5 μm) were hybridized with radiolabeled antisense or sense IDH or CA2 cRNAs generated from linearized plasmid templates using *in vitro* transcription with [α-^{35}S] UTP. Autoradiographs of slides were prepared using Kodak NTB-2 liquid photographic emulsion. Slides were stored at 4^{o}C for 1 to 2 weeks as judged from autoradiographs, developed in Kodak D-19 developer, counterstained with Gill's hematoxylin (StatLab), dehydrated through a graded series of alcohol to xylene, and coverslipped.

Statistical Analysis

All quantitative data were subjected to LS-ANOVA using the General Linear Models procedures of the Statistical Analysis System. Protein concentrations (micrograms per microliter) from uterine flushes and endometrial explant cultures were analyzed for effect of group (cyclic, pregnant, and UGKO). Amount of radiolabeled protein per microliter was analyzed for effect of group using protein concentration as a covariate. Slot blot hybridization data were corrected for differences in sample loading using the 18S rRNA data as a covariate. Data from Study One were analyzed for effect of group. Data from Study Two were analyzed for effects of day, status (cyclic or

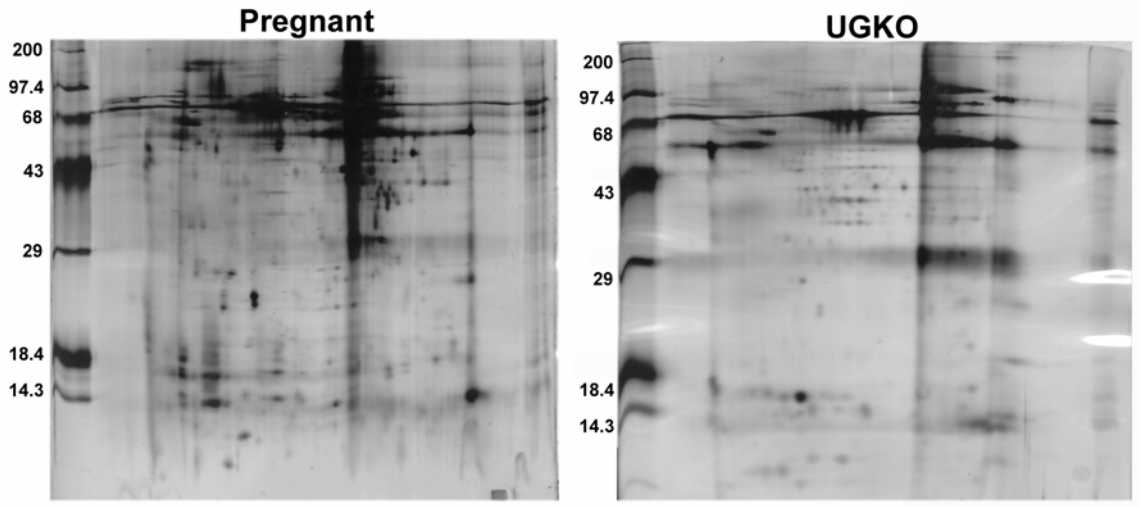

Fig. 6.1. 2D-PAGE analysis of silver stained uterine flush proteins from Day 14 cyclic, pregnant and bred UGKO ewes.

pregnant), and their interaction. If an effect of status was detected, regression analyses were conducted within status to determine effects on day. Values for CA2 were log transformed to alleviate heterogeneity of error variance. A P value of 0.1 or less was accepted to indicate statistical significance. Data are presented as the LSM with overall SE.

Results

Uterine Flushes Contain Secreted and/or Transported Proteins

Pregnancy-associated proteins were identified in uterine flushes by comparing 2D gels of pregnant with cyclic and UGKO samples. Uterine flush samples contain proteins that are synthesized and secreted and/or transported by the endometrial epithelia into the uterine lumen. Total protein content of the uterine flushes was not different (P=0.2) in the uterine flushes from cyclic, pregnant and UGKO ewes in Study One. However, qualitative differences in the distribution and abundance of selected proteins in the uterine flush were observed (Figs. 6.1 and 6.2). As expected, many of the proteins observed in the silver stained gels (Fig. 6.1) were not visible in the Coomassie blue stained gels (Fig. 6.2) due to the relative low abundance of most proteins in the uterine flushes of these Day 14 post-estrus/mating ewes. Eight of the proteins present in the uterine flush of pregnant, but not cyclic or UGKO ewes were excised from the gels (Fig. 6.2) and analyzed by mass spectrometry. The analysis identified only IDH and CA2 (see Table 6.1).

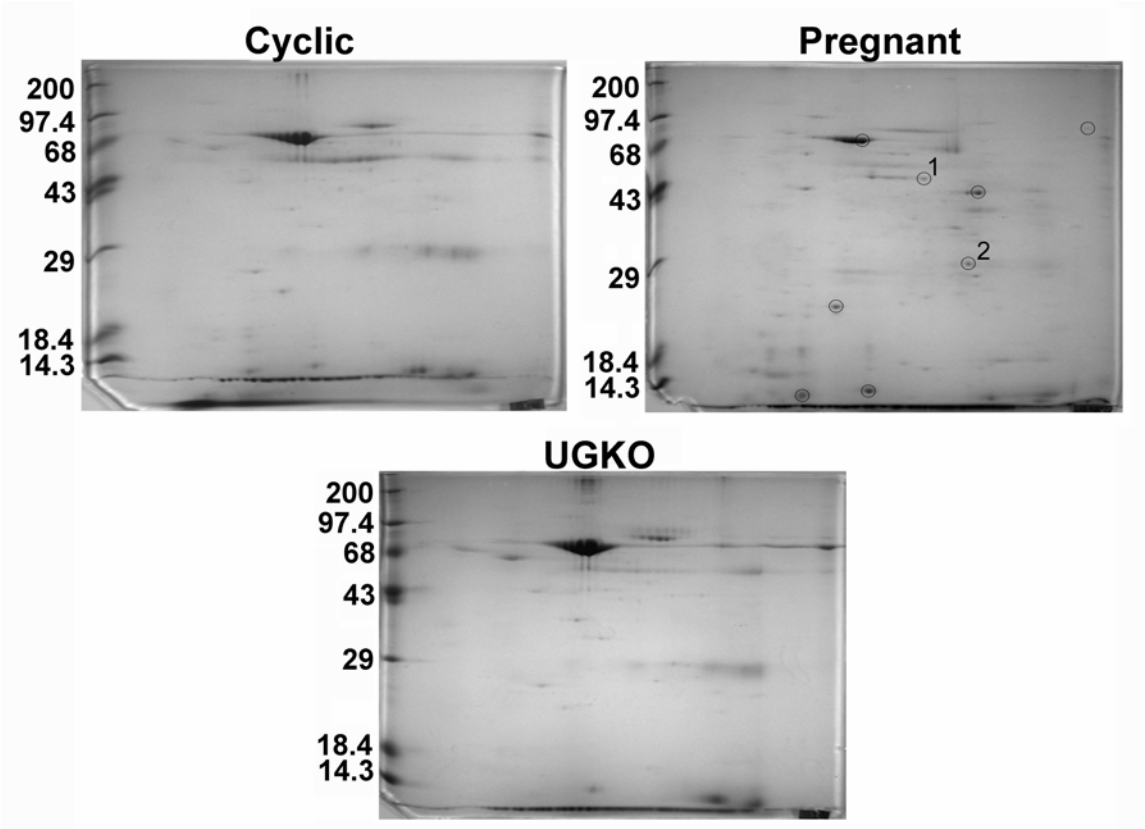

Fig. 6.2. 2-D PAGE analysis of Coomassie blue stained uterine flush proteins from Day 14 cyclic, pregnant and bred UGKO ewes. These proteins were both secreted, as well as and transported by the endometrial epithelia. The circles indicate protein spots that were chosen for mass spectrometry analysis. Protein spots 1 and 2 were identified as IDH and CA2, respectively.

Table 6.1. Proteins identified by 2D-PAGE analysis of uterine flushes and endometrial explant cultures from Day 14 cyclic, pregnant, and UGKO ewes.

Spot ID	Protein ID	Swissprot	kDa[1]	pI[1]	Origin
1	Cytosolic NADP+ dependent IDH	Q9XSG3	46.8	6.13	Uterine Flush
2	CA2	P00921	28.9	6.4	Uterine Flush
3	Aminomethyltransferase	Q9K934	40.3	5.46	Endo Explant
4	Alpha enolase	P19140	47.1	6.36	Endo Explant
5	Cystatin E (Cystatin M)	Q15828	16.5	8.32	Endo Explant

[1]MW and theoretical pI for the complete amino acid sequence were determined with ExPASy

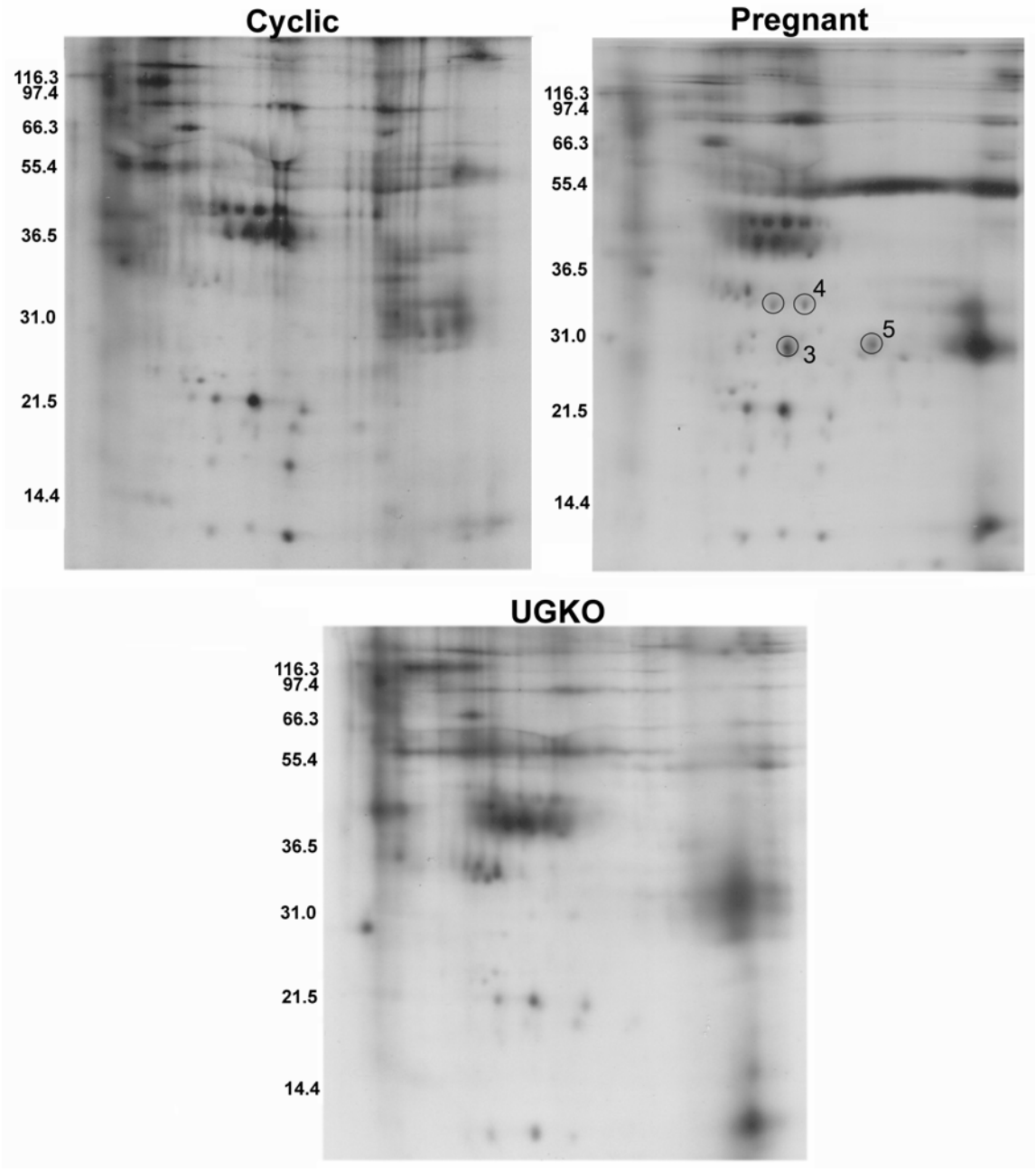

Fig. 6.3. Representative autoradiographs from 2D-PAGE analysis of radiolabeled endometrial explant cultures from Day 14 cyclic, pregnant, and bred UGKO ewes. These proteins were synthesized by the endometrium and secreted into the culture media. The circles indicate protein spots that were excised for mass spectrometry analysis. Protein spots 3,4, and 5 were identified as aminomethyltransferase, alpha enolase, and cystatin E (cystatin M), respectively.

Endometrial Explant Cultures Contain Only Synthesized Proteins

Autoradiographs of proteins present in the medium of endometrial explants from ewes in Study One are illustrated in Fig. 6.3. As found for the amount of protein in the uterine lumen, no differences in total protein or in amount of radiolabeled protein in the media from endometrial explant cultures was detected (P=0.50) in Day 14 cyclic, pregnant or UGKO ewes. However, careful analysis of the fluorographs revealed that at least four proteins were present in the cultures of Day 14 pregnant endometrium as compared to explant cultures of Day 14 cyclic or UGKO ewes. As summarized in Table 6.1, analysis of the mass spectrometry data identified, with high confidence (99-100%), aminomethyltransferase and alpha enolase, whereas another protein may be cystatin M (cystatin E), albeit with low confidence (60%).

IDH

In Study One, there was no effect of treatment on steady-state levels of IDH mRNA (pregnant vs. UGKO, P=0.16; pregnant vs. cyclic, P=0.93; cyclic vs. UGKO, P=0.17; Fig. 6.4A). In Study Two, IDH mRNA was most abundant on Day 10 of the estrous cycle and pregnancy and decreased to Day 16 (day, p>0.002; Fig. 6.4B) and was affected by pregnancy status (day x status, P>0.02). Steady-state levels of IDH mRNA were lowest on Days 18 and 20 of pregnancy.

As illustrated in Fig. 6.5, IDH mRNA was expressed specifically by the LE and sGE. IDH mRNA expression was not observed in the stroma, middle to deep GE, or myometrium. On Days 18 and 20 of pregnancy, IDH mRNA was detected abundantly in the conceptus trophectoderm (Fig. 6.5).

CA2

In Study One, steady-state levels of endometrial CA2 mRNA were not affected by treatment (pregnant vs. cyclic, p=0.24; pregnant vs. UGKO, p=0.98; cyclic vs. UGKO, p=0.21; Fig. 6.6A). In Study Two, endometrial CA2 mRNA levels were

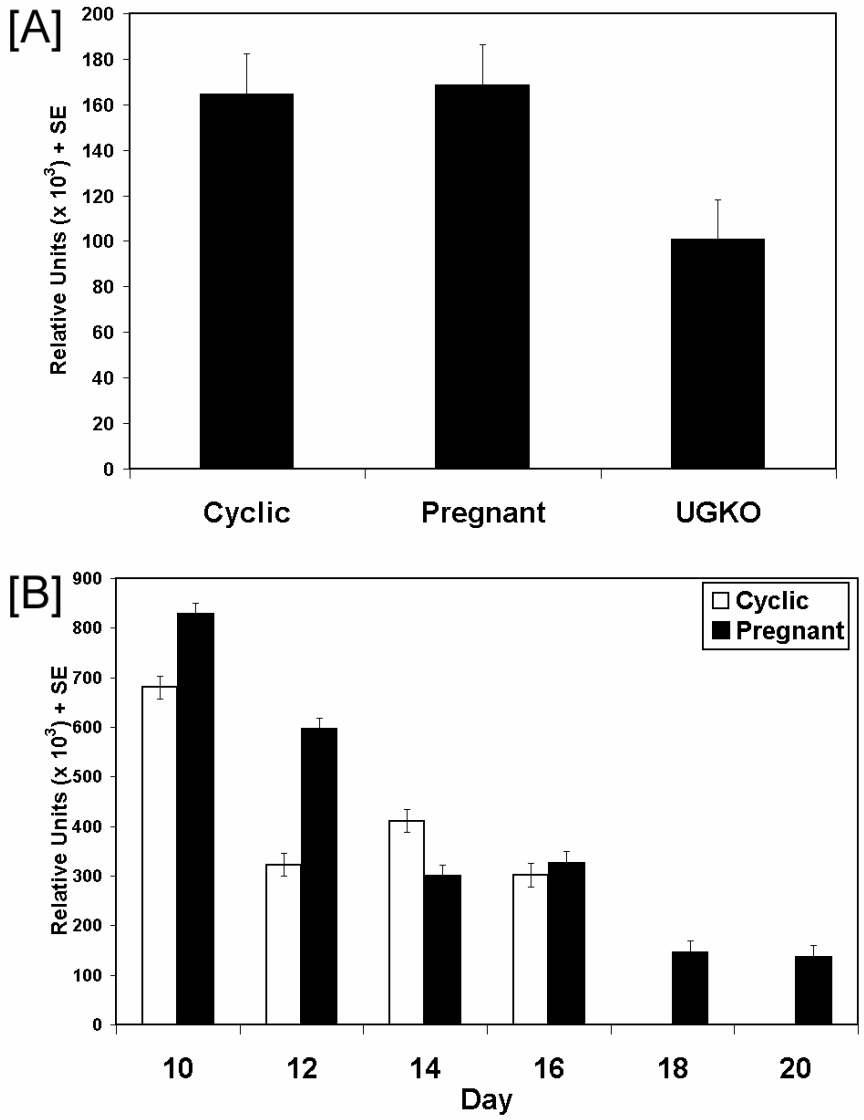

Fig. 6.4. Steady-state levels of IDH mRNA in endometria of Day 14 cyclic, pregnant, and bred UGKO ewes [A] and during the estrous cycle and pregnancy [B], as determined by slot blot hybridization analysis. Expression of IDH was normalized relative to expression of 18S rRNA mRNA. Data are presented as LSM+SE. There was no differences in IDH mRNA levels between Day 14 cyclic, pregnant, and UGKO ewes (pregnant vs. UGKO, P=0.16; pregnant vs. cyclic, P=0.93; cyclic vs. UGKO, P=0.17). IDH mRNA levels were highest on Day 10 and decreased to Day 16 (P>0.02, linear effect of day).

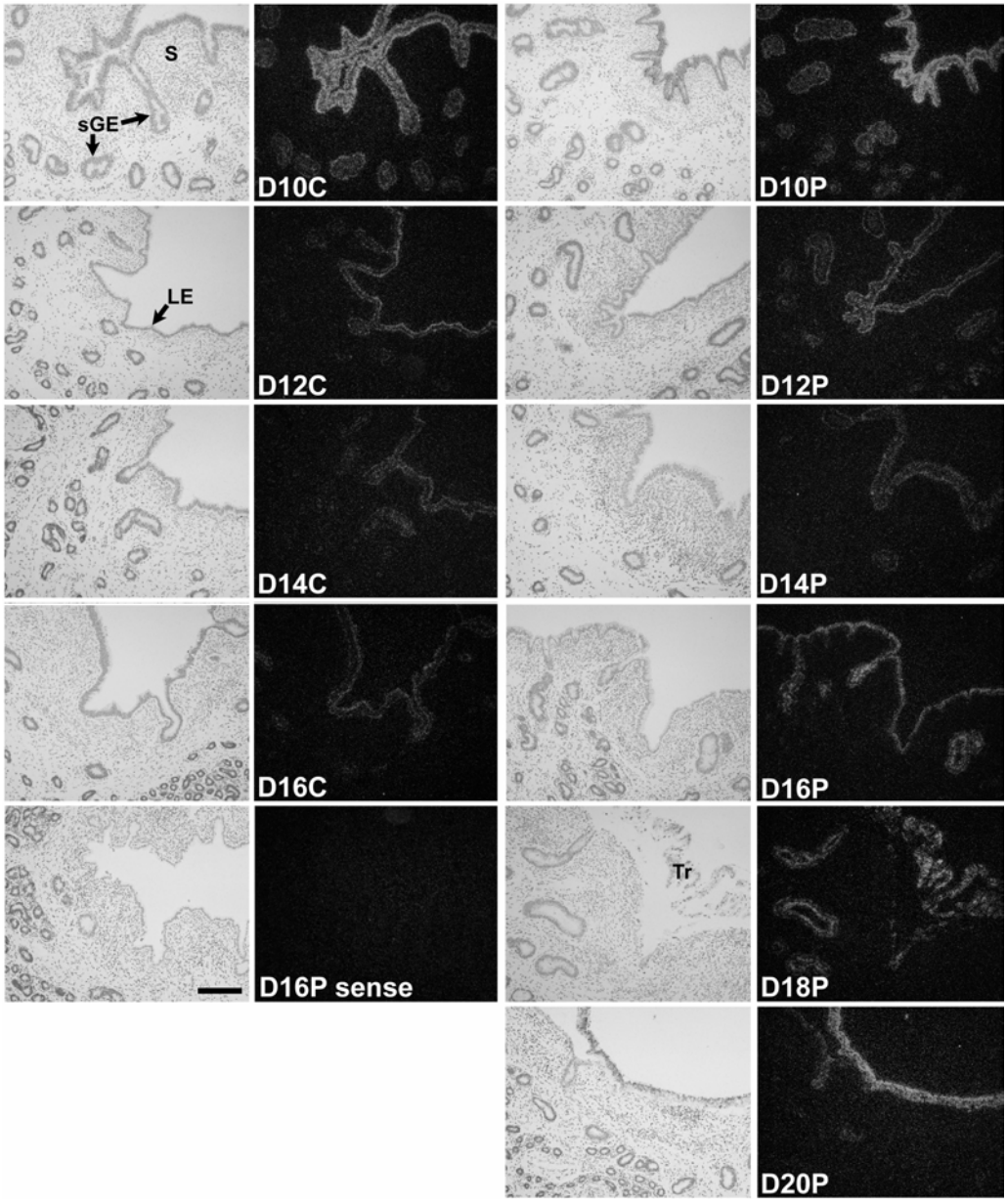

Fig. 6.5. *In situ* hybridization analysis of IDH mRNA expression in the endometrium of cyclic and pregnant ewes. Cross-sections of the uterine wall were hybridized with radiolabeled antisense or sense cRNA probes generated from linearized plasmid cDNA clones. Hybridized sections were digested with RNase A, and protected transcripts were visualized by liquid emulsion autoradiography. Developed slides were counterstained lightly with hematoxylin, and photomicrographs were taken under brightfield or darkfield illumination. Expression of IDH mRNA was detected only in the LE and sGE, as well as the conceptus trophectoderm. Legend: LE, luminal epithelium; sGE, superficial glandular epithelium; S, stroma; Tr, trophectoderm. Scale bar represents 100 μm.

affected by day (P>0.02) and were different in cyclic and pregnant ewes from Days 10 to 16 (day x status, P>0.03). CA2 mRNA levels increased on Days 18 and 20 (Fig. 6.6B).

In Study One, low levels of CA2 mRNA were detectable only in the LE (data not shown). In Study Two, CA2 mRNA was detected at low levels in the LE and superficial GE, but not in the endometrial stroma or myometrium (Fig. 6.7). CA2 was also detectable in the deep GE in some ewes (data not shown). On Day 18 and 20 of pregnancy, CA2 mRNA was observed in the conceptus trophectoderm.

Discussion

The present study used a proteomics approach to identify several components of histotroph present in the uterine lumen that emanate from the endometrial epithelia. IDH and CA2 were the two proteins identified from uterine flushes of Day 14 pregnant ewes. Previous reports of IDH (Elias et al. 1983; Schmidt et al. 1969; Zamiri and Blackshaw 1979) and CA2 (Falk and Hodgen 1972; Friedley and Rosen 1975; Lutwak-Mann 1955) focused on endometrial expression patterns and not whether they were components of uterine secretions in humans, as well as in sheep. Both IDH and CA2 mRNA are expressed by the LE and sGE, but only CA2 was expressed by the GE of the endometrium. Histotroph consists of proteins secreted by both the endometrial LE and GE. Proteins secreted by the LE and sGE appear to be particularly important for peri-implantation conceptus survival and growth, such as galectin-15 (CHAPTER IV), IDH, and CA2. During the process of implantation in sheep, endometrial LE cells are fused apically by trophoblast binucleate cells to form a syncytia that results in the assimilation and replacement of LE (Wimsatt 1951; Wooding 1984). Thus, the LE is not present to produce any secretions until it returns around Day 50 of gestation. Proteins secreted by the middle to deep GE may be more important for post-implantation conceptus survival and placental growth, such as uterine serpins (uterine milk proteins) (Hansen 1998). In this study, many of the proteins were less abundant in the uterine flushes and

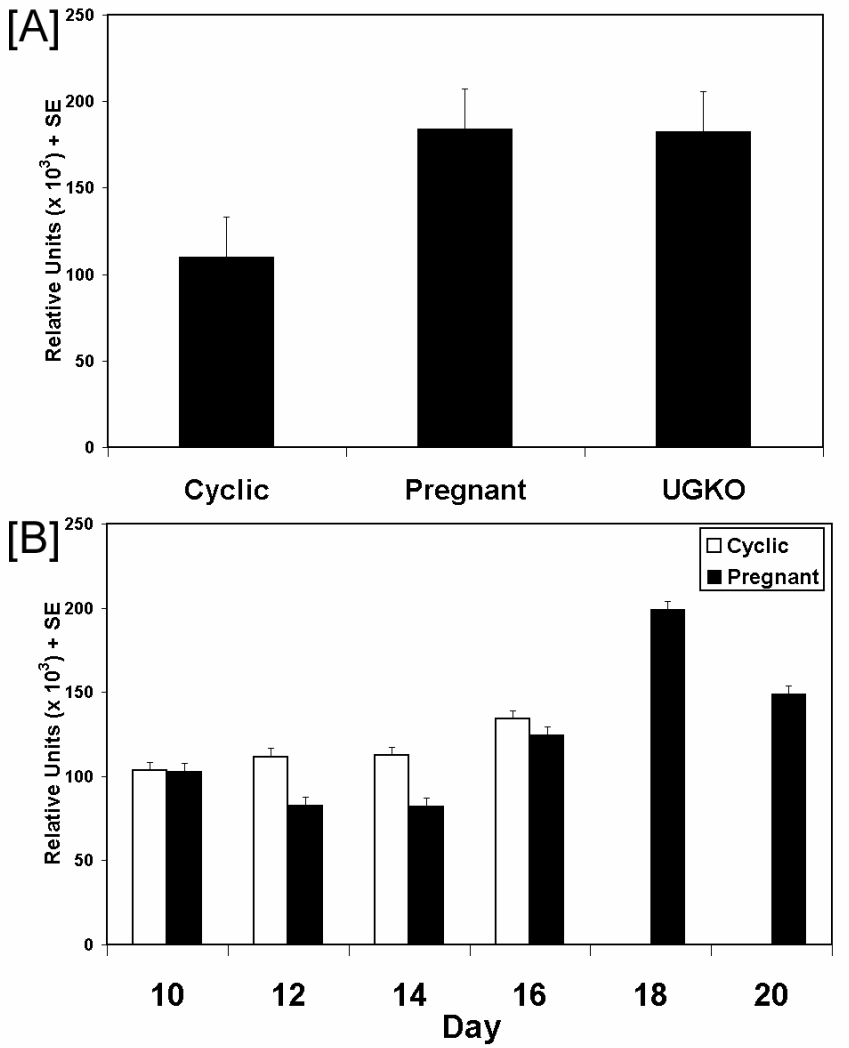

Fig. 6.6. Steady-state levels of CA2 mRNA in the endometria of Day 14 cyclic, pregnant and UGKO ewes [A], as well as in cyclic and early pregnant ewes [B]. Expression of CA2 was normalized relative to expression of 18S rRNA mRNA. Data are presented as LSM+SE. CA2 mRNA levels were not different between cyclic, pregnant, and bred UGKO ewes (pregnant vs. cyclic, P=0.24; pregnant vs. UGKO, P=0.98; cyclic vs. UGKO, P=0.21), but were affected by day (P>0.02) and status (P>0.03).

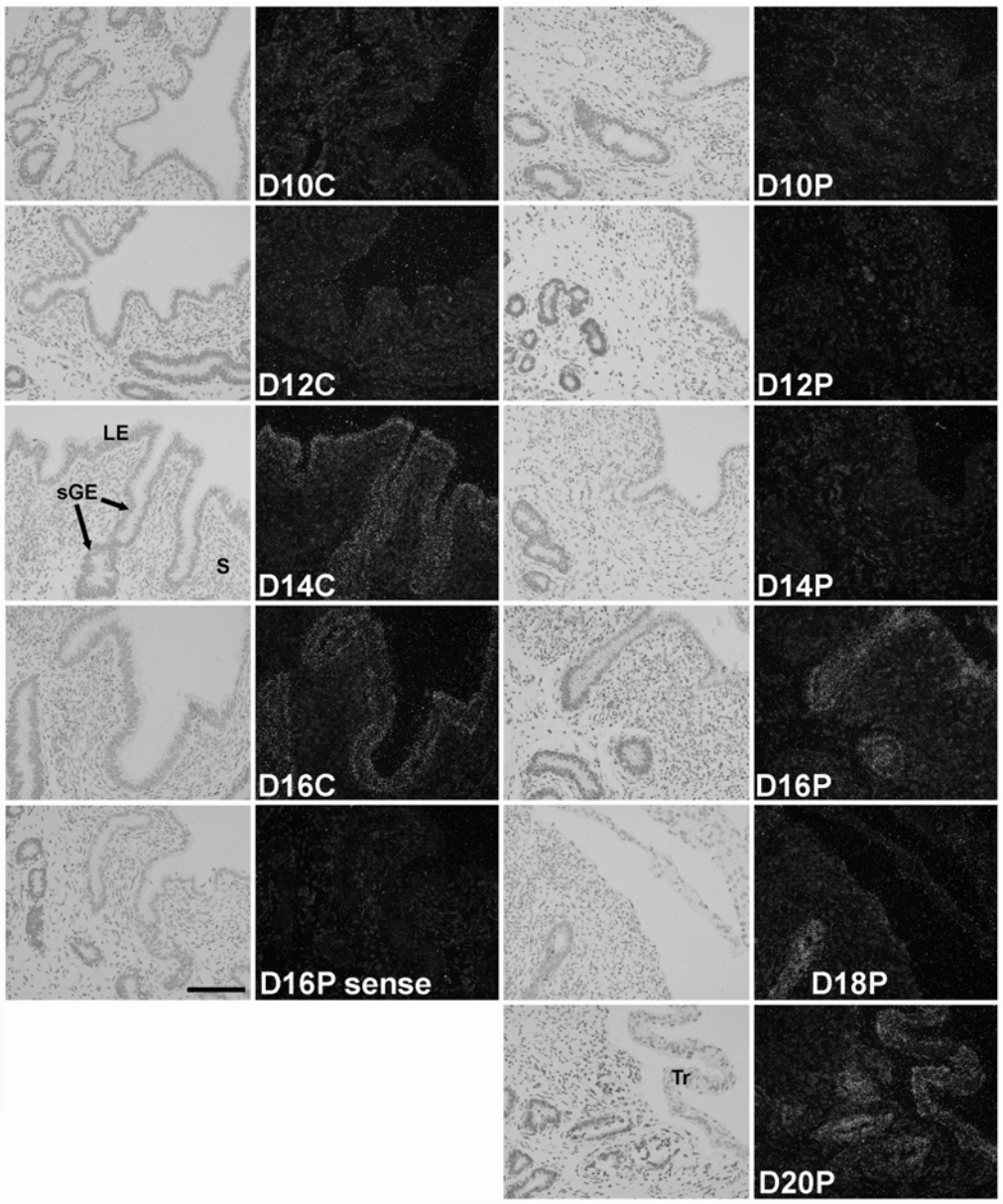

Fig. 6.7. *In situ* hybridization analysis of CA2 mRNA expression in the endometrium of cyclic and pregnant ewes. Cross-sections of the uterine wall were hybridized with radiolabeled antisense or sense cRNA probes generated from linearized plasmid cDNA clones. Hybridized sections were digested with RNase A, and protected transcripts were visualized by liquid emulsion autoradiography. Developed slides were counterstained lightly with hematoxylin, and photomicrographs were taken under brightfield or darkfield illumination. Expression of CA2 mRNA was detectable in the endometrial LE and sGE, as well as the conceptus trophectoderm. Legend: LE, luminal epithelium; sGE, superficial glandular epithelium; S, stroma; Tr, trophectoderm. Scale bar represents 100 μm.

endometrial explant cultures from UGKO or cyclic compared with pregnant ewes; however, most of them were not entirely absent. Therefore, it is possible that the inability for UGKO ewes to maintain pregnancy is due to less abundance of a number of LE-specific proteins, rather than the absence of one or several GE-specific secretions.

There are two forms of IDH, cytosolic and mitochondrial. The sequences from the present study indicate the specific identification of the cytosolic form. It is unknown if there is also a mitochondrial enzyme form expressed in the ovine uterus. Cytosolic IDH catalyzes the reversible reaction:

$$\text{Isocitrate} + \text{NADP} + 2H^+ \leftrightarrow \alpha\text{-ketoglutarate} + \text{NADPH} + H^+ + CO_2$$

IDH is expressed by the human and ovine endometria (Elias et al. 1983; Schmidt et al. 1969; Zamiri and Blackshaw 1979). IDHs protect cells from oxidative damage through the production of NADPH, which is an essential reducing equivalent for the activity of the NADPH-dependent thioredoxin system, as well as for the regeneration of reduced glutathione (Jo et al. 2001; Kwon et al. 1994; Lee et al. 2002). Thioredoxin is also expressed in the ovine uterus by the same cell types that express IDH (CHAPTER III). Recent studies have shown that cytosolic IDH also plays an important role in lipid and cholesterol synthesis (Koh et al. 2004). Thus, IDH could be involved in cholesterol production (Koh et al. 2004), which serves as a precursor for steroid hormone synthesis. Indeed, the sheep conceptus synthesizes progesterone as early as Day 21 (Marcus et al. 1979).

Alternatively, IDH may be involved in prostaglandin production. In the ovine uterus, LE and sGE cells are the predominant endometrial cell type containing a large number of lipid droplets (Brinsfield and Hawk 1973), which corresponds to the same cells which express IDH. Maximum levels of lipid droplets are present on Day 10 of the estrous cycle and pregnancy (Brinsfield and Hawk 1973), which is a similar pattern to that of IDH. These lipid droplets may serve as a source of arachidonic acid for prostaglandin synthesis (Barrau et al. 1975; Boshier et al. 1981; Brinsfield and Hawk 1973; Fetaih et al. 1992; Hall 1975; Paiva et al. 1997). Prostaglandins are produced via

the cyclooxygenase (COX) pathway, in which COX-1 and COX-2 are the rate-limiting enzymes for conversion of arachidonic acid into prostaglandin H_2. COX-2 is also specifically expressed by the LE and sGE cells of the ovine uterus during the estrous cycle and early pregnancy (Kim et al. 2003b), indicating that all of the prostaglandins that are downstream of prostaglandin H_2 (i.e., E_2, $F_{2\alpha}$, etc.), are also produced specifically by these cells. Additionally, both IDH and COX-2 mRNA are both expressed by the conceptus on Day 18 of pregnancy (Kim et al. 2003b). Thus, during early pregnancy the biological role for IDH may be to produce prostaglandins, which mediate uterine vascular permeability and angiogenesis (Lim et al. 1999; Lutwak-Mann 1955; Matsumoto et al. 2002; Yee and Kennedy 1991), as well as blastocyst formation, hatching and elongation (Biggers et al. 1978; Sayre and Lewis 1993).

CAs are zinc metalloproteinases that regulate net acid/base transport, through catalyzing the first part of the following reversible reaction, with the second reaction occurring spontaneously:

$$CO_2 + H_2O \leftrightarrow H_2CO_3 \leftrightarrow HCO_3^- + H^+$$

Thus, CA modify a number of cellular functions, such as ion content, gas transport, calcification, pH, as well as playing a role in intermediary metabolism (Ali Akbar et al. 1998; Maren 1967; Tashian 1989). Intracellular CA2 is located in the cytosol of epithelial cells (Breton 2001) from a number of different tissues, including the ovary, oviduct, and uterine endometrium (Friedley and Rosen 1975). Regulation of pH and ion transport in reproductive tract organs in both the male and female may be crucial for fertilization and pregnancy maintenance (Breton 2001; Ge and Spicer 1988). Indeed, the uterus and oviduct of rabbits have an alkaline pH and a CO_2 and HCO_3 content twice that found in blood (Vishwakarma 1962), likely due to the CA activity throughout the female reproductive tract. Due to the expression of CA2 by a number female reproductive tract structures, inhibitors of enzymatic activity were once considered as contraceptive agents (Friedley and Rosen 1975; Pincus and Bialy 1963). Unfortunately, no data is available regarding the ability of null female mice to establish and/or maintain pregnancy. However, one of the female mice evaluated lacked endometrial glands

(Spicer et al. 1989), and recent studies indicate that CA have a role in development of the endometrial glands in the neonatal mouse (Hu and Spencer 2005).

CA2 has been previously identified in the endometrium of rodents (Ge and Spicer 1988), guinea pig, rabbit, cat, dog, human (Falk and Hodgen 1972; Friedley and Rosen 1975), pig and sheep (Lutwak-Mann 1955; Lutwak-Mann and Averill 1954). In the current study, no differences in steady-state levels of endometrial CA2 mRNA were detectable in pregnant, or UGKO ewes on Day 14 or from ewes on Days 10 to 16 of the estrous cycle or pregnancy. Additionally, previous studies have shown that there are no differences CA activity in estrous, anestrous ewes and early pregnant sheep (Lutwak-Mann 1955; Lutwak-Mann and Averill 1954). This phenomenon is not entirely unexpected, as the uterus prepares for a pregnancy with every estrous cycle and the pH of the female reproductive tract may be critical for sperm motility and subsequent fertilization. However, CA activity is regulated during the menstrual cycle administration in humans (Falk and Hodgen 1972) and by exogenous hormone in rabbits and rodents (Lutwak-Mann 1955). In fact, inhibition of CA activity in the rabbit has a negative impact on implantation (Boving 1960). Thus, CA2 expression by the endometrial epithelia may be important initially for fertilization and subsequently for conceptus survival through mediating uterine pH during the peri-implantation period.

Steady-state levels of CA2 mRNA in the endometrium increased on Days 18 and 20 of pregnancy and remained high throughout gestation (CA Gray and TE Spencer, unpublished observations), as it is expressed by the conceptus trophectoderm in addition to the endometrial epithelia. Lutwak-Mann demonstrated that CA activity was detectable in the placenta of sheep, pig, rabbit, rat, hamster, and guinea pig (Lutwak-Mann 1955). One potential role for CA2 in the placenta is to regulate carbon dioxide-bicarbonate transport from fetal to maternal blood. However, CA2 may also regulate the environmental pH for fetal development, as a decrease in pH resulted in limb malformations during rodent embryonic development (Scott et al. 1990). Thus, placental CA may play a critical role in the environmental regulation of conceptus

survival and development that is either independent of or supplemental to maternal derived CA.

Analyses of endometrial explant cultures identified three proteins, aminomethyltransferase (AMT), alpha enolase, and cystatin M, as being more abundant in the pregnant than cyclic and/or UGKO endometrium on Day 14. AMT catalyzes the degradation of glycine, which is an abundant amino acid in maternal and fetal plasma and in placental fluids during gestation (Kwon et al. 2003). In fact, glycine levels increase in cases of feed restriction-induced IUGR in sheep (Kwon et al. 2004a), which may be related to decreased levels of AMT. However, there are no differences in amino acid levels of uterine flushes in Day 14 cyclic, pregnant, and UGKO ewes (H. Kwon, G. Wu, and T.E. Spencer, unpublished observations). Alpha enolase is a glycolytic enzyme that was identified by 2D-PAGE analysis of human hyperplastic endometrial explant cultures (Byrjalsen et al. 1999). Cystatin M is a cysteine proteinase inhibitor that is expressed at highest levels in the human uterus and liver (Ni et al. 1997). All three of these genes may be important for support of conceptus growth and development and will be investigated in future studies.

The identification of only five uterine secretory proteins by 2D-PAGE analysis is not surprising due to the low total protein content of the uterine flushings on Day 14 of pregnancy. Similar results were seen in previous studies utilizing a proteomics approach to identifying differences in histotroph composition during early pregnancy (Lee et al. 1998). Lee and coworkers did not identify any proteins from their analysis of Day 12 and 14 uterine flushes, but found several proteins that were specific to Days 15 and 17 of pregnancy (Lee et al. 1998). Indeed, the current study was limited by the low abundance of proteins in the uterine flushes. Additionally, some components of uterine histotroph may not be reactive to Coomassie blue stain, such as galectin-15 (Kazemi et al. 1990). An alternative approach to identify differences in endometrial gene expression is to perform transcriptional profiling. In fact, a previous study utilized differential display and PCR-based suppression subtraction hybridization techniques to compare gene expression in fertile ewes and infertile UGKO ewes (Spencer et al. 1999c). The

limitation to this approach was that very few of the epithelial-specific genes that were identified encoded secreted proteins (Spencer et al. 1999c). Therefore, the current approach utilized to identify differences in histotrophic composition revealed few proteins; however, all of the proteins identified demonstrate great potential for regulating the uterine environment to promote conceptus survival and growth.

CHAPTER VII
CONCLUSIONS

Many investigators have hypothesized that endometrial secretions are essential for adult uterine function in mammals. Although some of the components of histotroph have been characterized, little is actually known of their role(s) in supporting conceptus development during gestation. Results presented in this dissertation are the first to combine genomics and proteomics analyses with the unique uterine gland ablation model, the ovine UGKO ewe, to identify genes important for conceptus survival and growth during the peri-implantation period.

Despite repeated matings to fertile rams, adult UGKO ewes are unable to establish pregnancy (Gray et al. 2000a; Gray et al. 2001b; Gray et al. 2001c). Transfer of hatched blastocysts, recovered from superovulated normal donor ewes, into the uteri of UGKO ewes failed to ameliorate the pregnancy defect (Gray et al. 2001c). Normal hatched blastocysts were found in the uterine flushings of bred UGKO ewes on Days 6 and 9 post-mating, but not on Day 14 (Gray et al. 2001b; Gray et al. 2001c). However, on Day 14, uterine flushings from bred UGKO ewes contained either no conceptus or a severely growth-retarded conceptus that had failed to elongate from a tubular to filamentous form (Gray et al. 2001c). Therefore, UGKO ewes exhibit a peri-implantation pregnancy defect whose timing correlates with the majority of embryo loss that occurs during pregnancy in livestock as well as humans (Bazer 1975; Kane et al. 1997). During the peri-implantation period the conceptus is undergoing growth and expansion to filamentous form, producing IFNτ, and preparing for the onset of implantation. In fact, elongation of the conceptus is critical for developmentally regulated production of IFNτ (Farin et al. 1989) and requires the uterus, as hatched blastocyts fail to elongate *in vitro* unless transferred into the uterus (Heyman et al. 1984).

Identification of genes that are expressed by the endometrium of normal Day 14 pregnant ewes and absent in the endometrium of Day 14 bred UGKO ewes is important

for understanding the hormonal, cellular, and molecular mechanisms regulating conceptus elongation and implantation. Transcriptional profiling of the Day 14 UGKO endometrium revealed genes that were both increased and decreased when compared with either cyclic or pregnant ewes (CHAPTER III). These changes in gene expression could be due to differences in endometrial cell-type distribution and/or pregnancy status. The majority of known genes were identified as being higher in pregnant over either cyclic or UGKO endometrium. Additionally, 2D-PAGE analysis of uterine flushes and endometrial explant cultures revealed several proteins that were reduced in Day 14 UGKO compared with pregnant and/or cyclic ewes (CHAPTER VI). Results indicate that analyses of endometrial gene expression and composition of uterine secretions from normal and UGKO ewes are useful approaches to identify candidate factors and pathways regulating conceptus survival, growth and implantation.

Gene expression profiling of ewes treated with progesterone and IFNτ was also performed to identify genes specifically regulated by these two hormones, in contrast to genes upregulated during pregnancy (i.e., those upregulated by other conceptus factors). A number of genes were identified as being induced by progesterone alone and/or by progesterone and IFNτ. In the endometrium, IFNτ signals through the classic Jak-Stat pathway to induce gene expression in the stroma and deep GE (Choi et al. 2001). Many of these ISGs are induced in ewes treated with progesterone and IFNτ combined, regardless of the presence of a PR antagonist (CHAPTER III). In contrast, these studies also identified an ISG, galectin-15, that is increased in the LE and sGE. To date, only one other ISG has been identified to be expressed in these same cell types, Wnt7a (Kim et al. 2003a). The Jak-Stat pathway is not active in LE and sGE cells (Choi et al. 2001), indicating that IFNτ may be signaling through another pathway to induce gene transcription in these cells or acting through a stromal mediator of IFNτ action. Understanding these two conflicting manners of ISG induction may further elucidate critical mechanisms involved in conceptus elongation and survival, as well as implantation. Collectively, genes identified in these studies are expected to reveal novel factors and pathways important in the endometrium for support of conceptus survival,

growth and implantation, as well as provide potential new targets to improve embryo culture and diagnose endometrial dysfunction that leads to infertility.

Galectin-15 is a new member of the galectin family of secreted β-galactoside lectins that was discovered and characterized in the endometrium of sheep (CHAPTERS III and IV). Sequence analysis of galectin-15 identified a conserved CRD, as well as an integrin binding domain at the N-terminus. Galectin-15 mRNA initially increased in the endometrium of both cyclic and pregnant ewes, and then noticeably decreased in cyclic,but not pregnant ewes on Day 16. Both galectin-15 mRNA and immunoreactive protein were detected in the endometrial LE and sGE. During early pregnancy, galectin-15 protein was secreted into the uterine lumen, where it was phagocytosed by the conceptus trophectoderm. Galectin-15 protein forms crystals and was immunologically identical to the 14K protein the forms crystalline inclusion bodies in endometrial epithelia and conceptus trophectoderm on Day 16. Additionally, Fig. 7.1 illustrates the dual localization of galectin-15, both intracellularly as crystals and extracellularly as a component of histotroph, indicating that it may play multiple roles during the peri-implantation period (CHAPTER IV). Further, galectin-15 is also synthesized and secreted by the endometrial epithelium throughout gestation (CHAPTER V). Secreted galectin-15 is phagacytosed by the trophectoderm/chorion at the fetal-maternal interface, transferred into the fetal circulation and cleared through the fetal kidney, to be stored in allantoic fluid, as shown in Fig. 7.2. Thus, secreted galectin-15 may be important for conceptus implantation initially and promotion of angiogenesis during later gestation, while intracellular galectin-15 crystals may be involved in RNA processing or anti-apoptosis. However, galectin-15 was initially identified as playing a role in immune function (Dunphy 2000) and could be involved in regulating this throughout gestation as well.

The CRD of galectin-15 is functional, as the protein is able to bind to sugars and this binding can be competed off (CHAPTER IV). Therefore, secreted galectin-15 may be important for regulation of conceptus implantation by functioning as a heterophilic cell adhesion molecule cross-linking cell-surface glycoprotein(s), glycolipids, or integrin

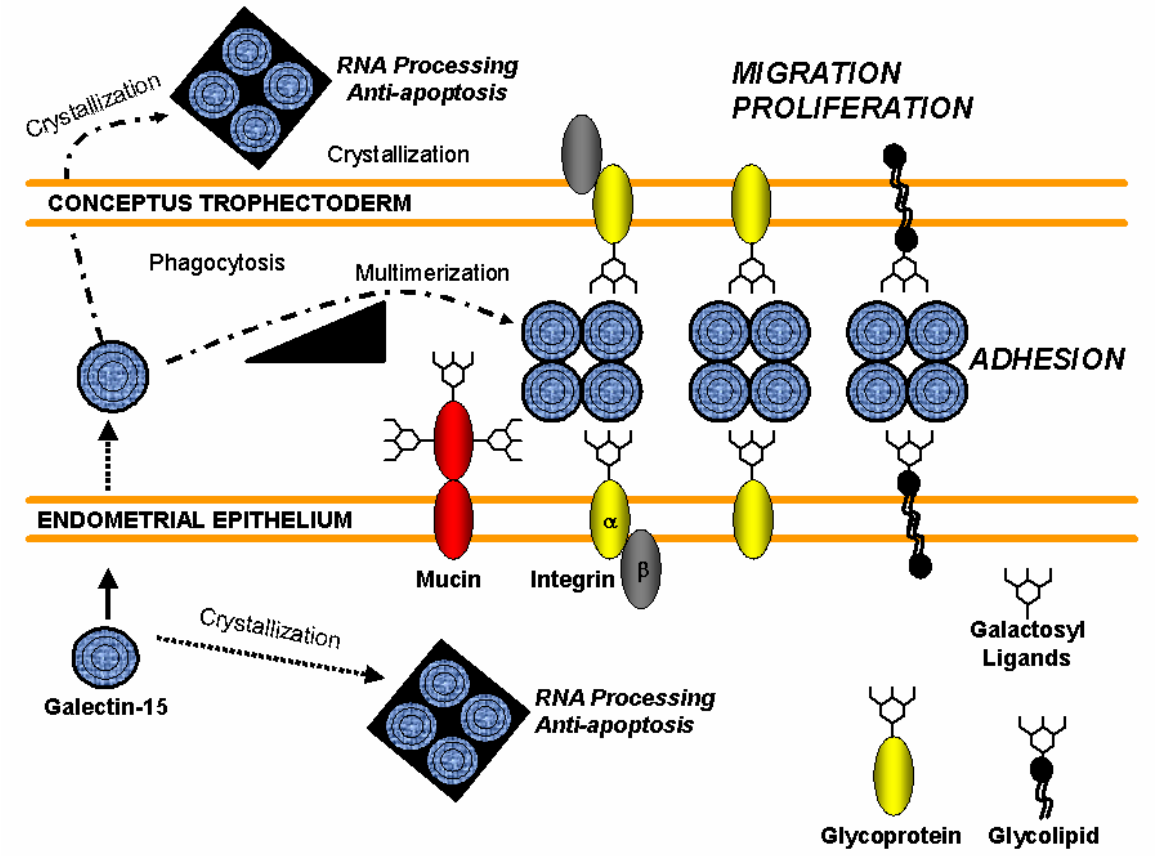

Fig. 7.1. Schematic illustration of the role of galectin-15 during ovine conceptus implantation. Galectin-15 is produced by the endometrial epithelia where it forms intracellular crystals that may play a role in RNA processing or anti-apoptosis. Additionally, galectin-15 is secreted into the uterine lumen where it forms multimers that may bind to glycoprotein or glycolipid receptors on the endometrial epithelia and conceptus trophectoderm to aid in adhesion during implantation. Galectin-15 is also phagocytosed by the trophectoderm where it forms crystals and may promote cellular proliferation and/or migration.

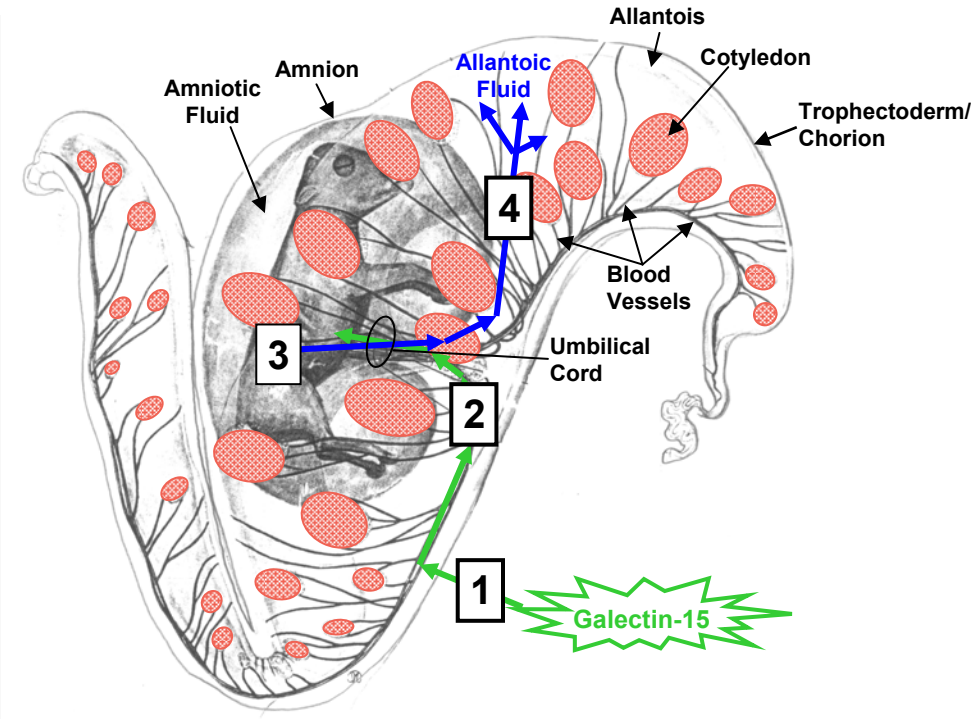

Fig. 7.2. Schematic illustration of the pathway of galectin-15 in ovine uteroplacental tissues during gestion. Galectin-15 is produced by the endometrial epithelia and secreted into the uterine lumen where it is phagocytosed by the trophectoderm/chorion of the conceptus placenta (1). Galectin-15 is then passed into the fetal circulation via the placental vascularization (2) and cleared through the fetal kidney (3) through the urachus and into the allantoic fluid (4). Adapted from (Mullins and Saacke 2003).

subunits on the trophectoderm and endometrial lumenal epithelium. Implantation in ruminants is a highly coordinated process that involves apposition, attachment, and adhesion of the conceptus trophectoderm to the endometrial LE (Guillomot 1995; Guillomot et al. 1981). Integrins are thought to be the dominant glycoproteins that regulate trophectoderm adhesion to endometrial LE during implantation (Johnson et al. 2001a). During the peri-implantation period of pregnancy in sheep, integrin subunits αv, α4, α5, β1, β3 and β5 are constitutively expressed on the conceptus trophectoderm and apical surface of endometrial LE (Johnson et al. 2001a). In sheep, receptivity to implantation does not appear to involve changes in temporal or spatial patterns of integrin expression, but may depend on changes in expression of ECM proteins which are ligands for heterodimers of these integrins (Hughes 2001). As shown in Fig. 7.3, galectin-15 may be similar to GlyCAM-1 and OPN in that it is secreted by the endometrial epithelium and binds to receptors on the conceptus trophoblast and the endometrial LE to serve as a bridging ligand during ovine implantation (Spencer et al. 2004).

Galectin-15 mRNA was induced by progesterone, further stimulated by IFNτ, and expression is inversely correlated to the presence of epithelial PR. The regulation of galectin-15 by progesterone in the absence of epithelial PR is shared with several other secretory proteins, such as UTMP and OPN (Spencer et al. 1999b). This relationship between epithelial PR and gene expression is observed both in the induction of UTMP and galectin-15 during early pregnancy and in the repression of these genes during the postpartum period. However, the pattern of repression is slightly different between UTMP and galectin-15 because of cell-type specificity. UTMP was specifically expressed by the GE and expression is repressed immediately with the return of the PR in the GE within one week after parturition (Gray et al. 2003). Conversely, galectin-15 expression declines in a variable manner during the postpartum period, as the PR does not return to the LE, where galectin-15 was expressed. Therefore, the signal for mRNA downregulation was not as specific or direct for galectin-15 as for UTMP. Gene regulation through the absence of the epithelial PR is particularly effective for

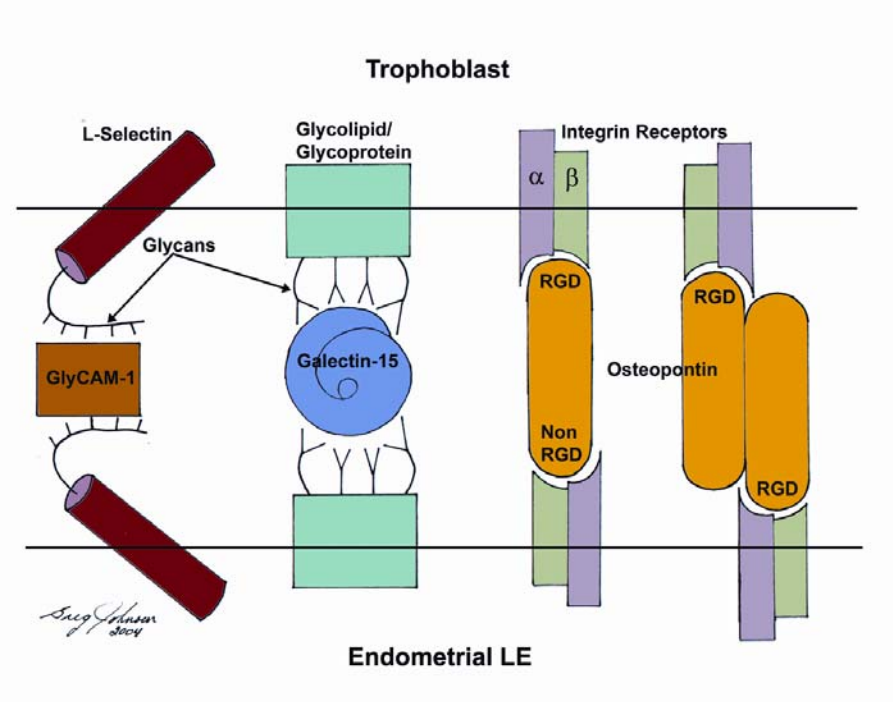

Fig. 7.3. Schematic illustration of adhesive proteins potentially involved in adhesion of the conceptus trophectoderm and the endometrial LE (Spencer et al. 2004). GlyCAM-1 is secreted by LE and GE and binds to L-selectins, galectin-15 is secreted by LE and sGE and may bind to glycolipid/glycoprotein receptors (or integrins), and OPN is secreted by GE and binds to integrin subunits through RGD sequences.

expression of secretory proteins, however the mechanisms of this action are currently unknown.

Experiments in this dissertation have contributed toward the fundamental knowledge of endometrial gene expression, uterine histotroph composition and mechanisms regulating implantation in sheep. These analyses have determined the effectiveness of transcriptional profiling of the UGKO endometrium as a method to identify endometrial genes that are potentially important for conceptus survival, elongation, and development. This technology provides an opportunity for future expression profiling studies in the sheep. Further, these analyses have identified a number of previously unknown genes induced by pregnancy, progesterone, and/or IFNτ, including galectin-15. Additionally, analysis of uterine secretions from cyclic, pregnant, and UGKO ewes on Day 14 revealed several proteins; indicating that this approach is valuable, even in the absence of abundant proteins. Future experiments must be directed toward determining the cellular and molecular mechanisms regulating successful conceptus survival and implantation. These experiments include: (1) characterizing spatial and temporal gene expression for previously unknown ISGs; (2) cloning the promoter of non-classical ISGs, such as galectin-15, Wnt7a and cathepsin L; (3) determining the role of galectin-15 in trophoblast adhesion, migration and cell proliferation; (4) inactivating galectin-15 RNA in the LE and consequences on conceptus development; (5) infusing recombinant galectin-15 into the uterus of UGKO ewes and the effect on conceptus elongation; (6) adding recombinant galectin-15 to embryo culture systems and determine effects on conceptus elongation; (7) investigations into the role of galectin-15 crystals in the LE and conceptus; (8) determining the function of galectin-15 in the allantoic fluid throughout gestation, whether related to prostaglandin production, metabolic regulation, or carbohydrate binding; and (9) characterizing spatial and temporal gene expression for proteins identified in endometrial explant cultures.

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VITA

Catherine Allison Gray was born in Charleston, West Virginia on May 14, 1976 to Scott and Gayle Gray. Due to her father's job, her family lived overseas in Singapore from 1985 to 1990 and in Houston, Texas from 1990 to 1998. While attending high school in Texas, she became very involved with the local FFA chapter. She graduated from Clear Lake High School in May, 1994. She enrolled at Texas A&M University in the fall of 1994 to pursue a degree in Animal Science. At Texas A&M, she was involved with several academic fraternities. In Fall 1996, she began an undergraduate internship in Dr. Fuller Bazer's laboratory during which she was involved in both lab and animal work under the guidance of Dr. Troy Ott and later, Dr. Thomas Spencer. She graduated from Texas A&M University in December, 1997 with a Bachelor of Science degree in animal science. She pursued an interest in reproductive physiology by entering the graduate program of Drs. Thomas Spencer and Fuller Bazer in January, 1998, in the Department of Animal Science at Texas A&M University. She received a Master's of Science degree in reproductive physiology in May, 2000, and continued on with her doctoral research in the same laboratory. While a master's, and later a doctoral candidate she taught undergraduate laboratories in reproductive physiology and conducted research on the role of endometrial glands in adult uterine function using the sheep as an animal model. She was chosen as a Philanthropic and Education Organization (PEO) Scholar and received the Ethel Ashworth-Tsutsui Memorial Research Award, the Dr. A.M. "Tony" Sorenson Achievement Award, and the Vice Chancellors Award for Excellence in Graduate Research. She was elected as the Graduate Representative to the Reproductive Biology Faculty Executive Committee and elected to serve on the Forum Committee. Upon completion of the Doctor of Philosophy degree, she will assume a position as Postdoctoral Associate in the Department of Molecular Genetics at the University of Texas, M. D. Anderson Cancer Center in Houston, Texas to study sexual differentiation and reproductive tract development with Dr. Richard R. Behringer. Allison can be contacted at 7676 Phoenix Drive #1104, Houston, Texas 77030.