IDENTIFICATION OF NOVEL IMPLANTATION-RELATED GENES IN THE OVINE UTERUS

A Dissertation

by

GWON HWA SONG

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 2007

Major Subject: Physiology of Reproduction

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ABSTRACT

Identification of Novel Implantation-Related Genes in the Ovine Uterus. (May 2007)

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The peri-implantation period in mammals is critical with respect to survival of the conceptus and establishment of pregnancy. During this period of pregnancy, reciprocal communication between ovary, conceptus, and endometrium is required for successful implantation and placentation. Therefore, studies were conducted to indentify and characterize novel endometrial genes important for implantation and conceptus development in the ovine uterus.

The first and second studies defined the uterine expression of seven members of the cathepsin (*CTS*) family of lysosomal proteases, and a secreted inhibitor of *CTSL* called cystatin C (*CST3*) during the peri-implantation period. In addition, regulation of *CTS* and *CST3* by progesterone (P4) and interferon tau (IFNT) was evaluated. *CTSL* was the most abundant *CTS* in the ovine ovine uterus and was also coordinately expressed with *CST3* in the endometrial epithelia and conceptus trophectoderm. *CTSL* and *CST3* were found to be novel P4-induced and IFNT-stimulated genes in the luminal epithelial cells of the ovine endometrium.

The third study identified radical S-adenosyl methionine domain containing 2 (*RSAD2*) and interferon-induced with helicase C domain 1 (*IFIH1*) in the ovine uterus. Results of this study indicated that IFNT induces *RSAD2* and *IFIH1* in a P4-independent manner in the stroma, immune cells, and glands of the ovine endometrium. These two genes are proposed to have biological roles in the establishment of uterine receptivity to the conceptus during implantation.

The fourth study characterized endometrial expression of stanniocalcins (*STC*) during pregnancy. *STC1* appeared in the endometrial glands on Day 18 of pregnancy,

increased from Days 18 to 80, and remained abundant through Day 120 of gestation. In addition, this study demonstrated that *STC1* is induced by P4 and increased by placental hormones, such as placental lactogen (*CSH1*) and growth hormone (*GH*), in the ovine endometrial glands.

Collectively, these studies identified genes that are expected to be critical to unraveling the mechanism(s) of reciprocal fetal-maternal interactions required for successful implantation and pregnancy. A more complete understanding of these genes will be important for developing therapeutic strategies to prevent, treat and/or diagnose infertility in domestic animals and humans, because they are biomarkers of P4 and/or IFN effects.

DEDICATION

To my late father,

HAN SOO SONG

ACKNOWLEDGEMENTS

I would like to thank my mentors, Drs. Fuller Bazer and Thomas Spencer, for their guidance and support throughout the course of this research as well as their example of excellence. I would also like to thank my committee members, Drs. Robert Burghardt, Gregory Johnson, and Weston Porter for being a tremendous influence throughout my doctoral research program. Thanks also to the members of the Laboratory for Uterine Biology and Pregnancy for their assistance, patience, and friendship. I particularly appreciate the constant assistance and advice from my friend, Dr. Kathrin Dunlap.

Thank you does not seem a strong enough statement to offer to my family. I am forever indebted to my family for providing me with encouragement and support throughout my life.

TABLE OF CONTENTS

CHAPTER Page

LIST OF FIGURES

FIGURE Page

FIGURE Page

CHAPTER I
INTRODUCTION

In eutherian mammals, including sheep, implantation of the blastocyst is the most important developmental event and an evolutionary advance associated with viviparity (1, 2). During the peri-implantation period in the ovine uterus, the spherical blastocyst elongates to a tubular and then a filamentous form, and develops into a conceptus (embryo/fetus and associated extraembryonic membranes). At this time, interferon tau (IFNT) is synthesized and secreted by the mononuclear trophectodermal cells of the conceptus between Days 10 and 21-25 (maximally on Days 14 to 16) (3-6). In the ovine uterus, IFNT acts directly on the endometrial luminal epithelium (LE) and superficial ductal glandular epithelium (sGE) to suppress transcription of estrogen receptor alpha (*ESR1*) and oxytocin receptor (*OXTR*) genes (7, 8), thereby preventing production of luteolytic pulses of prostaglandin F2α (PGF).

During the estrous cycle, *ESR1* expression increases and progesterone receptor (*PGR*) expression decreases on Days 11 to 13, allowing estrogen (E2) to induce *OXTR* expression on Days 13 to 14 (9, 10). Thus, oxytocin from the posterior pituitary and/or corpus luteum (CL) can then induce release of luteolytic pulses of PGF on Days 15 and 16 (11). During early pregnancy, IFNT produced by the elongating ovine conceptus suppresses *ESR1* expression which then prevents ESR1-induced *OXTR* expression (7, 12-15). Collectively, these results indicate that the antiluteolytic actions of IFNT are to prevent increases in epithelial *ESR1*, *PGR*, and *OXTR* gene expression, which are all E2 responsive genes, by directly inhibiting transcription of the *ESR1* gene and maintaining secretion of progesterone (P4) by the CL (8, 16-18).

In the ovine uterus, establishment and maintenance of pregnancy requires reciprocal communication via endocrine and paracrine signals from the ovary, conceptus,

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This dissertation follows the style of Endocrinology.

and endometrium during implantation and synepitheliochorial placentation (1). Progesterone, the hormone of pregnancy, plays an important role in the establishment and maintenance of a uterine environment that supports conceptus development. Endometrial gland secretions, including growth factors, cytokines, and ions, are predominantly regulated by P4 (19) and are required for peri-implantation conceptus survival, elongation, and development (20-24). Progesterone acts via its cognate receptor, *PGR*. In the ovine endometrium, *PGR* are expressed in epithelia and stroma and allow P4 to directly regulate a variety of genes in the uterus. However, *PGR* expression is down-regulated by continuous exposure to P4 in ovine endometrial LE and GE after Days 11 and 13 of pregnancy, respectively (10). The paradigm of loss of *PGR* in endometrial epithelia immediately before implantation is common to sheep (1, 10), cattle (25), and pigs (26), as well as other mammals studied to date, including humans and mice (see (2)).

During the peri-implantation period, uterine epithelial cell functions might be regulated by interactions between reprogrammed epithelial cells following down-regulation of *PGR* and specific factors produced by *PGR*-positive stromal cells in response to P4, and/or products of the conceptus such as IFNT, placental lactogen (*CSH1*), and placental growth hormone (*GH*) (see (17)). A large number of genes are induced by IFNT throughout the uterine wall. These IFNT-stimulated genes (ISGs) are proposed to have biological roles in pregnancy recognition and uterine receptivity (1). In addition, induction of an antiviral state in the endometrium during early pregnancy may be beneficial by inhibiting sexually transmitted viruses as well as modulating local immune cells to promote tolerance of the allogeneic conceptus and stimulating production of cytokines beneficial for conceptus survival and growth (27-29).

Collectively, knowledge of the complex, precisely orchestrated interaction between P4 and IFNT during the implantation period should provide new insights to improve fertility in humans and domestic animals, and provide key knowledge for interpreting cross-talk mechanisms between maternal endometrium and conceptus. Therefore, these studies were conducted to determine if selected implantation-related

candidate genes are expressed in the ovine uterus and to determine effects of the estrous cycle, pregnancy, P4 and IFNT on expression of these genes in ovine endometria and conceptuses.

CHAPTER II
LITERATURE REVIEW

Early Pregnancy in Sheep

Hormonal Aspects of the Estrous Cycle and Luteolytic Mechanism

Sheep are spontaneous ovulators characterized by recurring estrous cycles with a mean length of 17 days and regulated by the hypothalamic/pituitary/ovarian axis. They are known as short-day breeders because they begin to cycle as day length decreases in late summer or autumn. The estrous cycle can be divided into four stages: (1) proestrus, (2) estrus, (3) metestrus, and (4) diestrus. Estrus lasts about 30 h and at this time, peripheral blood levels of E2 are high and P4 levels are very low. Between proestrus and estrus, follicle-stimulating hormone (FSH) from the anterior pituitary stimulates the growth of Graafian follicles in the ovary and then ovulation occurs in response to an E2-induced surge of lutenizing hormone (LH) from the anterior pituitary. At metestrus, E2 declines and LH stimulates a process termed lutenization that results from differentiation and reorganization of theca and granulosa cells from the ruptured follicle into small and large luteal cells and culminates in formation of a corpus luterum (CL). During diestrus, functional CLs secrete P4 that inhibits estrous behavior and formation of ovulatory follicles by attenuating release of LH and FSH from the anterior pituitary (30). In contrast, the concentrations of E2 in serum remain low. Diestrus lasts until the onset of luteolysis which is the functional and structural regression of the CL (31, 32). However, during proestrus, P4 levels decline and E2 levels increase, as one or more ovulatory Graafian follicles become dominant on the ovary (see Fig. 2.1).

Estrous Cycle and the Luteolytic Mechanism

In sheep, the estrous cycle and luteolysis are dependent on the uterus because the endocrine luteolysin, PGF, from uterus causes the functional and structural regression of the CL (31). Further, hysterectomy in ewes extends the life span of the CL to that characteristic of pregnancy (33). During the estrous cycle, the ovine endometrium

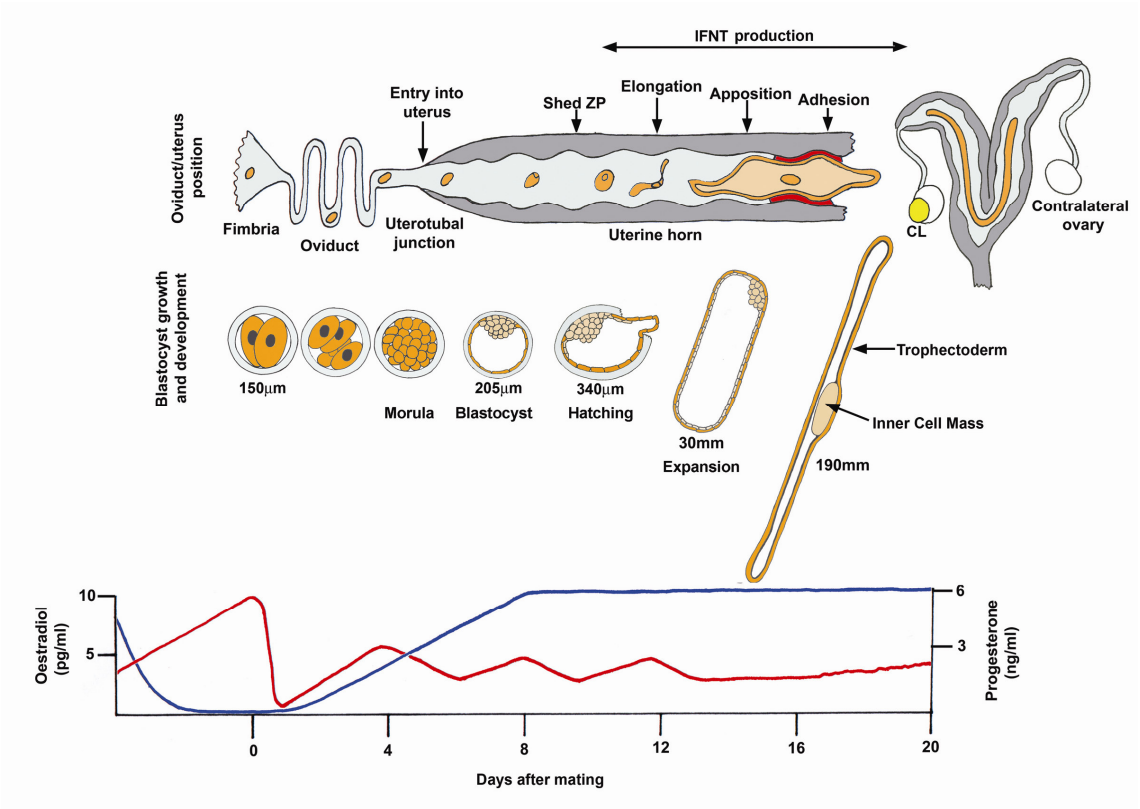

Fig. 2.1. Early pregnancy events in sheep. The embryo enters the uterus on Day 4 after mating (Day 0 = estrus/mating) at the morula stage of development and then develops into a blastocyst on Day 6. Between Days 8 and 9, the blastocyst sheds the zona pellucida by enzyme lysis such as uterine and/or embryonic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

releases luteolytic pulses of PGF from endometrial LE and sGE (11, 21, 34, 35), in response to oxytocin that is synthesized and secreted by large luteal cells (9) as well as from the posterior pituitary (11). In addition, these uterine epithelia express *PTGS2* (prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase); alias COX-2), the rate limiting enzyme in the synthesis of prostaglandins (35), as well as oxytocin receptor (*OXTR*) (34). Development of the endometrial luteolytic mechanism in ovine endometrial epithelia requires sequential effects of P4, E2, and oxytocin, acting through their respective receptors (1, 7, 36). In the ovine uterus, the spatio-temporal expression of *OXTR* is primarily regulated by estrogen receptor alpha (*ESR1*) and progesterone receptor (*PGR*) (7, 9, 10, 36).

During estrus (Day 0 of the estrous cycle) and metestrus, E2 released from ovulatory Graafian follicles stimulates expression of uterine *ESR1*, *PGR*, and *OXTR* in the endometrial LE and sGE (9, 10). During diestrus, increased circulating levels of P4 act through *PGR* to "block" expression of *ESR1* and *OXTR* in the endometrial LE and sGE between Days 3 and 11 of the estrous cycle (36). Therefore, *ESR1* and *OXTR* expression is not detected during most of diestrus. The precise molecular mechanism whereby P4 suppresses *ESR1* gene transcription is unknown. However, after Days 11 to 12 of the estrous cycle, the "P4 block" is removed, because continuous exposure of the uterus to progesterone for 8 to 10 days down-regulates expression of *PGR* in endometrial LE and sGE (9, 15), allowing for rapid increases in expression of *ESR1* on Day 13 followed by *OXTR* on Day 14 in LE and sGE (14, 37). On Days 14 to 16 of the estrous cycle, oxytocin secreted from the posterior pituitary and/or CL binds *OXTR*s on the plasma membrane of endometrial epithelia and activates the protein kinase C (*PKC*) signaling pathway (38) that results in release of luteolytic PGF pulses from the endometrial LE and sGE (11, 34). These 4 to 5 pulses of luteolytic PGF over a 25 h period cause the CL to undergo functional and structural regression that allows the ewe to return to estrus, completing the 17 day estrous cycle.

During the estrous cycle, P4 plays a pivotal role in initiation of endometrial PGF synthesis as it increases phospholipid stores in LE to maximum levels on Days 14 and 15

of the estrous cycle (39), and augments activity of *PTGS2*, the rate limiting enzyme in the biosynthesis of prostaglandins which converts arachidonic acid to *PGG2* (the precursor of various prostaglandins including PGF and PGE (40)). Although *PTGS1* is constitutively expressed, *PTGS2* expression is increased in endometrial LE and sGE on Days 12 to 15 of the estrous cycle (35, 41). Increased expression of *PTGS2* is coincident with *PGR* down-regulation in LE and sGE; therefore *PGR* may be inhibitory to expression of *PTGS2* in the ovine epithelia.

Blastocyst Development and Implatation in Sheep

In sheep, implantation takes place at the blastocyst stage. The timing of implantation differs among species (42, 43) due to variations in the length of the different implantation phases and the degree of endometrial invasion by the trophoblast. In domestic ruminants (sheep, cattle, and goats) and pigs, the spherical blastocyst elongates to tubular and then filamentous forms, and develops into a free-floating conceptus (embryo/fetus and associated extraembryonic membranes) during the latter stages of implantation. This unique developmental event does not occur in humans, primates, rodents, and horses (42, 44, 45). The blastocysts of these species implant rapidly before expansion and the extraembryonic membranes are formed after implantation (42, 46, 47).

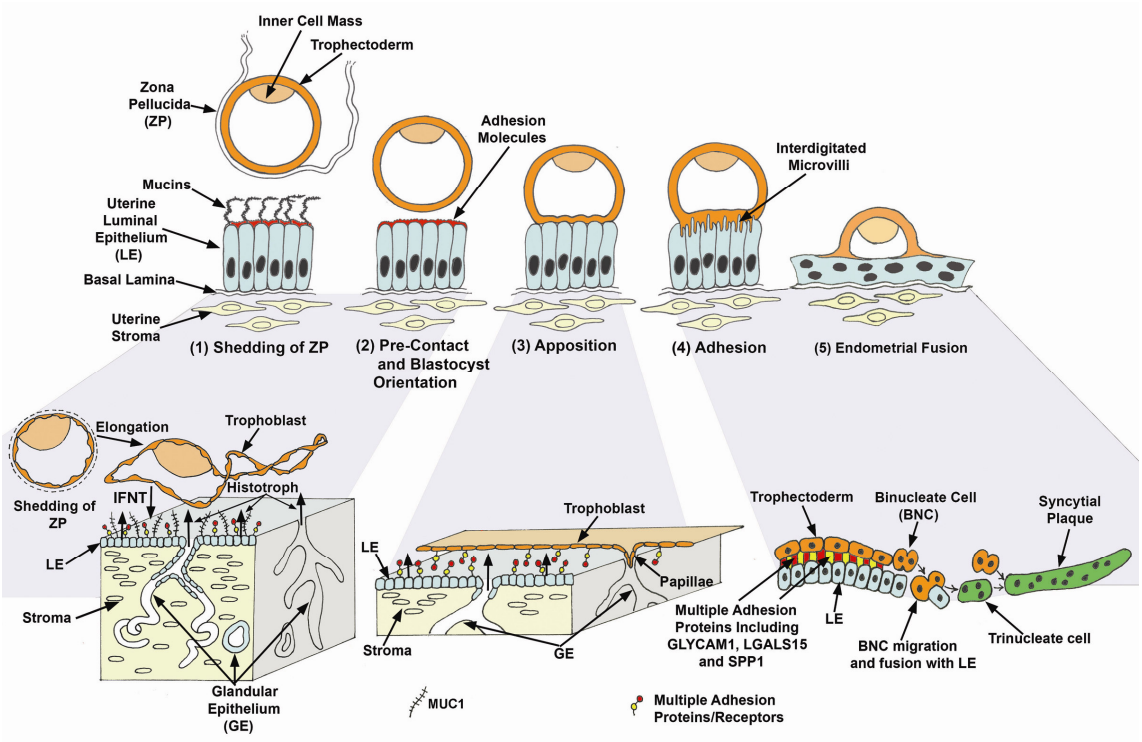

Fig. 2.2. The phases of blastocyst implantation in sheep. Shedding of the zona pellucida (Phase1): The embryo enters the uterus on Day 4. The blastocyst is formed on Day 6 and the zona pellucida is shed on Day 8 or 9 due to blastocyst growth and uterine and/or embryonic proteases. After Day 10, the blastocyst elongates and develops into a tubular and then into a filamentous conceptus. Precontact and blastocyst orientation (Phase 2): Between Days 9 and 14, there is no definitive cellular contact between the conceptus trophectoderm and the endometrial epithelium, but the blastocyst appears to be positioned and immobilized in the uterus. During this time, elongation of the blastocyst plays an important role in production of IFNT in sheep uterus. Apposition (Phase 3): The conceptus trophectoderm associates closely with the endometrial LE followed by unstable adhesion. In ruminants, the trophoblast develops finger-like villi or papillae and thereby penetrates into the superficial ducts of the uterine glands. This event has been hypothesized to anchor the peri-attachment conceptus and allow it to absorb histotroph from glands. Adhesion (Phase 4): On Day 16, the trophoblast begins to adhere firmly to the endometrial LE. The interdigitation of the trophectoderm and endometrial LE occurs in both the caruncular and intercaruncular areas of the endometrium. During this time, the mononuclear trophetoderm cells differentiate into trophoblast giant binucleate cells. Adapted from Spencer et al., 2007 and originally drawn by Dr. Greg A. Johnson.

Collectively, as illustrated in Fig. 2.2, the phases of implantation in sheep include: (1) shedding of the zona pellucida; (2) precontact and blastocyst orientation; (3) apposition; (4) adhesion; and (5) endometrial fusion (2) based on a comparative implantation scheme proposed by Guillomot and colleagues (42-44). The embryo enters the uterus on day 4 after mating (Day 0 = estrus/mating) at the morula stage of development and then develops into a blastocyst by Day 6. Between Days 8 and 9, the blastocyst sheds the zona pellucida by enzyme lysis involving uterine and/or embryonic proteases (Fig. 2.1). After Day 10, the blastocyst elongates into a tubular and then forms a filamentous conceptus (48), which appears to be immobilized in the uterine lumen becoming closely associated with the endometrial LE followed by unstable adhesion.

Between Days 16 and 22, the trophoblast begins to adhere firmly to the LE by interdigitation between uterine epithelial microvilli and projections of the trophectoderm cells (42, 44) and penetrates into the superficial ducts of the uterine glands by using trophoblast papillae (44, 49-51). Beginning on Days 14 to 16, the trophoblast giant binucleate cells begin to differentiate, and then migrate and fuse with the endometrial LE and each other form syncytial plaques (52, 53). As part of synepitheliochorial placentation in sheep, the syncytial plaques cover the caruncular surface of the placentome formed by fusion of placental cotyledons with endometrial caruncles (54).

The Role of Progesterone During Pregnancy Recognition and Maintenance of Pregnancy

In the ovine uterus, establishment and maintenance of pregnancy requires reciprocal communication via endocrine and paracrine signals from the ovary, conceptus, and endometrium during implantation and synepitheliochorial placentation (1). Progesterone, the hormone of pregnancy, plays an important role in the establishment and maintenance of a uterine environment that supports conceptus development. Endometrial gland secretions, including growth factors, cytokines, and ions, are predominantly regulated by P4 (19) and are required for pre-implantation conceptus survival, elongation, and development (20-24). In the ovine endometrium, the *PGR* is expressed in epithelia and stroma and allows P4 to directly regulate a variety of genes. However, *PGR* expression is down-regulated by continuous exposure to P4 in ovine endometrial LE and GE after Days 11 and 13 of pregnancy, respectively (10). The paradigm of loss of *PGR* in endometrial epithelia immediately before implantation is common to sheep (1, 10), cattle (25), and pigs (26), as well as other mammals studied to date, including humans (see (2)).

Therefore, during the peri-implantation period, uterine epithelial cells become reprogrammed following down-regulation of *PGR* and are subject to regulation by specific factors produced by *PGR*-positive stromal cells in response to P4 and/or a product of the conceptus such as interferon tau (IFNT), placental lactogen (*CSH1*), and placental growth hormone (*GH*).

Representative Progesterone-Regulated Genes

Mucin Glycoprotein and Integrins. Down-regulation of mucin glycoproteins (*MUC*) correlates with adhesion and implantation of blastocysts in rodents and sheep, and the decrease of MUC expression in endometrial LE is coincidental with *PGR* loss (55). In uterine LE, *MUC1* is believed to interfere with binding and accessibility between integrins and their cognate receptors on conceptus trophectoderm (47, 56). In addition, ovariectomized gilts treated with P4 decrease MUC1 expression in the apical

surface of endometrial epithelia (57). Integrins are also essential for interactions with ECM to allow communication between uterine epithelia and trophectoderm (56). In the ovine uterus, integrin alpha subunits (v, 4, 5) and beta subunits (1, 3, 5) are expressed on the apical surface of endometrial LE and GE and trophetoderm (58). However, spatio-temporal expression of these integrins is not regulated by pregnancy. Thus, the uterus must secrete ligands that bridge integrin receptors on the conceptus trophectoderm and endometrial LE. In contrast, in the porcine uterus, expression of integrin alpha 4, alpha 5, and beta 1 subunits is regulated by P4 (57).

Uterine Milk Proteins and Osteopontin. In the ovine uterus, continuous exposure to P4 induces production of secretory proteins by endometrial GE that are secreted into the uterine lumen (59-61). Progesterone acts via its cognate receptor, *PGR*, and the *PGR* is expressed in epithelia and stroma and allows P4 to directly and/or indirectly regulate a variety of P4-regulated genes. However, *PGR* expression is down-regulated by continuous exposure to P4 in ovine endometrial LE and GE after Days 11 and 13 of pregnancy, respectively (9, 10). The loss of *PGR* in GE appears to be required for GE remodeling and differentiation (1, 62, 63). Ovine uterine milk proteins, also known as ovine uterine serpins (*SERPIN*; serine protease inhibitors), are members of the serpin family (64). *SERPIN* is an excellent marker for endometrial secretory capacity during pregnancy in sheep, that is expressed only in GE and dramatically increases during gestation (60, 65). Similarly, secreted phosphoprotein-1 (SPP1, also known as osteopontin) is synthesized exclusively in uterine GE in response to P4. However, SPP1 protein is detected at the apical surface of LE, GE, trophectoderm, and at the maternal-fetal interface (66-69), strongly suggesting secretion into the uterine lumen and binding to integrin receptors on LE and trophectoderm. SPP1 is an acidic phosphorylated glycoprotein that binds to integrin heterodimers via its Arg-Gly-Asp sequence and to promote cell adhesion and migration (70). It has been hypothesized that SPP1 binding to integrins stimulates changes in morphology of conceptus extraembryonic membranes and promotes adhesion between LE and trophectoderm for implantation (58). Results strongly support the hypothesis that loss of *PGR* in GE is required for P4-induced

expression of *SERPIN* and *SPP1* in the ovine uterus (65, 69). Administration of P4 with E2 or ZK136,317 (PGR antagonist) ablated effects of P4 alone to induce expression of *SPP1* mRNAs in GE (69).

Maternal Recognition of Pregnancy

History of IFNT in Sheep

The developing conceptus must signal its presence to the mother in order to ensure successful establishment and maintenance of pregnancy, a process termed maternal recognition of pregnancy (71). In the 1960s, Moor and Rowson reported extension of the interestrous interval due to the transfer of Day 13 sheep blastocysts into recipient ewes on Day 12 of the estrous cycle (72), and that removal of blastocysts after Day 13 significantly extended CL life-span (73). In addition, infusion of sheep conceptus homogenates collected between Days 14 and 15 into the uterine lumen of recipient ewes (on or before Day 12 of their cycle) extend CL life-span and the inter-estrous interval of cyclic ewes. However, infusion of pig conceptus homogenates had no effects on estrous cycle length in ewes (74). The transfer of trophoblastic vesicles from blastocysts collected between Days 11 and 13, without the embryonic disc, to recipient ewes on Day 12 of the estrous cycles maintained CL function (75). The first report of secretion of low molecular weight acidic proteins by Day 16 ovine conceptuses was by Wilson et al. (1979) (76). Later, in 1982, Godkin and colleagues characterized secretion of the low molecular weight acid protein by cultured ovine conceptuses collected between Days 13 and 21 of pregnancy and term it Protein X (77). In a later study, Protein X from ovine trophectoderm was termed ovine trophoblast protein 1 (oTP-1) (78). Subsequently, native purified or recombinant oTP-1 was shown to extend the inter-estrous interval of ewes and to attenuate oxytocin-induced PGF release in sheep (79-81).

The primary amino acid sequence of oTP-1 was highly homologous to bovine IFNA (82) and possessed antiviral and antiproliferative properties (83-85). Therefore, oTP-1 was renamed IFNT and was designated as a member of the Type I IFN family

(86-88) by the International Interferon Society. As the most abundant mRNA in the conceptus on Day 14, *IFNT* mRNA increases from Day 12 to 14 and then declines to Day 22 and is localized to mononuclear trophetodermal cells (4, 89). Similar to other Type I IFN family members such as IFNs alpha (IFNA), beta (IFNB), delta (IFND), epsilon (IFNE), kappa (IFNK) and omega (IFNW), IFNT possesses potent antiviral (83-85), antiproliferative (85, 90), and immunomodulatory biological activities and effects (90, 91). IFNT is most closely related to IFNW encoding the 172 amino acid sequence except for six amino acids in the carboxyl terminus that are not responsible for biological activitiy (92). Even though IFNT shares a high-degree of DNA and amino acid sequence identity in ruminants (sheep, cattle, goats) (82, 86, 87), the precise biochemical structure of IFNT is different among species because of different post-translational modifications. Ovine IFNT is not glycosylated, bovine IFNT is glycosylated, and caprine IFNT is found in both glycosylated and nonglycosylated forms (86, 93, 94).

Antiluteolytic Mechanism of Action of Interferon Tau

In ruminants, maternal recognition of pregnancy requires elongation of the blastocyst which produces IFNT (4, 95), a Type I IFN that prevents development of the endometrial luteolytic mechanism through paracrine action on uterine epithelia (1, 5, 96). Thus, the antiluteolytic effect of IFNT maintains a functional CL and production of P4 required for successful pregnancy and development of the conceptus.

During the peri-implantation period, IFNT is synthesized and secreted by the mononuclear trophectodermal cells between Days 10 and 21-25 (maximally on Days 14-16) (3-6). In the ovine uterus, IFNT acts directly on the endometrial LE and sGE to suppress transcription of *ESR1* and *OXTR* genes (7, 8), thereby preventing production of luteolytic pulses of PGF (Fig. 2.3). During the estrous cycle, *ESR1* expression increases and *PGR* expression decreases on Days 11 to 13 and then E2 induces *OXTR* expression on Days 13 to 14 (9, 10), thereby allowing oxytocin from the posterior pituitary and/or CL to induce release of luteolytic pulses of PGF on Days 15 to 16 (11).

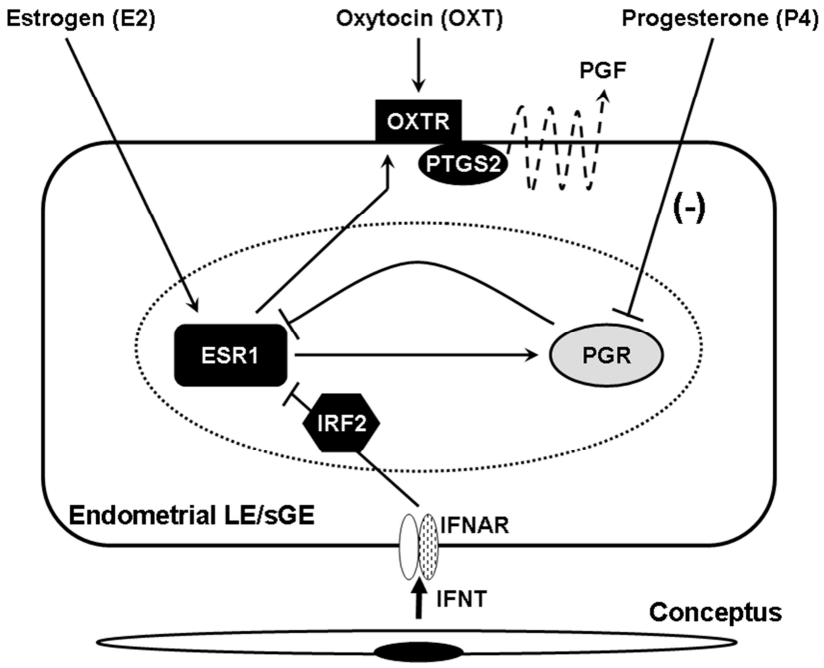

Fig. 2.3. Schematic illustrating the current working hypothesis on hormonal regulation of the endometrial antiluteolytic mechanism and cross-talk between the conceptus and the maternal endometrium. During the peri-implantation period, ovine IFNT synthesized and secreted by the mononuclear trophectodermal cells between Days 10 and 21-25 (maximally on Days 14-16), acts directly on endometrial LE and sGE to suppress transcription of *ESR1* and *OXTR* genes, thereby preventing production of luteolytic pulses of PGF. During the estrous cycle, *ESR1* expression increases and *PGR* expression decreases on Days 11 to 13 and then E2 induces *OXTR* expression on Days 13 to 14, thereby allowing oxytocin from the posterior pituitary and/or CL to induce release of luteolytic pulses of *PGF* on Days 15 to 16. In contrast, during early pregnancy, secreted IFNT from fully elongated conceptus silences *ESR1* expression which prevents E2-induced *OXTR* expression. However, IFNT does not stabilize *PGR* expression in endometrial epithelia during pregnancy. Adapted from Spencer et al., 2007.

In contrast, during early pregnancy, elongating ovine conceptuses secret IFNT to suppress *ESR1* expression which prevents E2-induced *OXTR* expression (7, 12-15). Collectively, these results indicate that the antiluteolytic actions of IFNT are to prevent increases in epithelial *ESR1*, *PGR*, and *OXTR* gene expression, which are all E2 responsive, by directly inhibiting transcription of the *ESR1* gene and maintaining secretion of P4 by the CL (8, 17, 18).

Type I IFN Signal Transduction Pathway

The actions of IFNT to signal pregnancy recognition and induce or increase expression of IFNT-stimulated genes (ISGs) are mediated by the Type I IFN signal transduction pathway. Type I IFNs bind to a common Type I IFN receptor (*IFNAR*), a heterodimer consisting of two subunits, IFNAR1 and IFNAR2, containing tyrosine kinases such as janus kinase 1 (*JAK1*) and tyrosine kinase 2 (*TYK2*) (97, 98). These receptors are present in all endometrial cell types, but are highest in endometrial LE (99). IFNAR classically activate the JAK/STAT (signal transducers and activators of transcription) signaling pathway (100-102). Upon cognate ligand binding, IFNAR1 and IFNAR2 heterodimerize, change their conformation, and activate TYK2 and JAK1 by tyrosine phosphorylation (103, 104). The activated TYK2 phosphorylates STAT2 through the SH2 (src homologous 2) domain and then recruits signal transducers and activators of transcription-1 (STAT1) (105-107). Phosphorylated STAT1 binds phosphorylated STAT2 to form a heterodimer that is released from the receptor complex and translocates to the nucleus after forming a heterotrimeric transcriptional complex by binding with ISGF3G (IFN-stimulated transcription factor 3, gamma 48 kDa), collectively termed ISGF3 (108, 109). In addition to STAT1/2 heterodimerization, Type I IFN also induces formation of phosphorylated STAT1 homodimers, termed GAF (gamma IFN activation factor) (110). In the nucleus, ISGF3 binds to an IFN-stimulated response element (ISRE) in promoter regions of ISGs to activate transcription in cooperation with several coactivators, such as the cAMP response element binding protein (CREB)-binding protein (CBP)/p300 (111). Similarly, GAF enters the nucleus,

binds to GAS (GAF activation sequence) elements to stimulate transcription of ISGs (112).

STATs and IRFs in the Type I IFN Signal Transduction Pathway

The Type I IFN signaling pathway is mediated by two main transcription factor families, STATs and interferon regulatory factors (IRFs).

STATs. The STATs play an essential role in the IFN signaling pathway and seven have been identified (STAT1, -2,-3,-4,-5A, -5B, and 6) in a variety of mammalian species (113-115). All STATs contain a DNA binding domain, except STAT2, a transactivation domain, and a SH2 domain. Usually, STATs are latent in the cellular cytoplasm, but they are immediately activated by receptor-associated tyrosine kinase such as JAKs under cytokine (including IFN) stimulation and then mediate signaling events to downstream transcription factors or DNA sequences in the promoter region of target genes. The function of phosphorylated STAT dimers as activators or repressors of transcription depends on the motif in the promoter of target genes and/or cell types. The most common STAT-binding motif is GAS (TTCNmGAA) (116), and STAT1, -3, and -4 dimers bind to the TTCC(C/G)GGAA motif and STAT5A and -5B bind to the TTC(C/T)N(G/A)GAA, whereas STAT6 binds to TTCN4GAA (117-119). The STATs also interact with coactivators (such as CBP/p300), transcription factor SP1 or c-Jun, and nuclear steroid receptor (such as glucocorticoid receptor) (120-123). This signaling cascade is rapidly down-regulated by dephosphorylation of JAKs and STATs. In the ovine uterus, STAT1 and STAT2 mRNAs and proteins are expressed only in endometrial stroma and GE during early pregnancy (124, 125).

IRFs. The IRFs act as either transcriptional activators or repressors in response to viral infection or IFN and have been identified as IRF1, -2, -3, -4, -5, -6, -7, -8, and -9 (126-128). All IRFs are highly homologous due to their 115 amino acid containing conserved five tryptophan repeats in the amino-termius (129). The IRFs recognize similar DNA sequences in the promoter region of a number of ISGs through their helix-turn-helix motif, such as the IRF element (IRF-E: G/A G/C TTT C G/A G/C TTT (T)C)

and the interferon-stimulated response element (ISRE: AG TTT CNN TTT CN C/T) (101, 130).

IRF1 is significantly induced in response to IFN or viral infection and acts as a transcriptional activator in IFN signaling (124, 131, 132). In contrast, IRF2 acts as a strong transcriptional repressor competing with IRF1 for binding to the same site or by repulsion of coactivators (133, 134). ISGF3G is a component of heterotrimeric ISGF3 and acts as DNA-binding subunits to ISREs in response to both Type I and II IFNS (135-137). In the ovine uterus, IRF1 expression is not detected during the estrous cycle, but is detected on Day 13 and is most abundant on Day 15 of early pregnancy (124, 125). Both IRF6 and ISGF3G are present in endometrial LE/GE or stroma/GE, respectively, in both cyclic and pregnant ewes. IRF2 is detected only in LE and sGE from cyclic and pregnant ewes and proposed to have biological roles in preventing the induction of ISG expression in these cell types in response to IFNT (125).

IFNT-Stimulated Genes (ISGs)

Most ISGs are expressed by endometrial stroma and middle to deep GE of the ovine uterus (Fig. 2.4) (125, 138-141). These ISGs include *STAT1* and *STAT2* (142, 143), *IRF1* (142-144), *IRF9* (124), *ISG15* (138, 140, 145), *Mx* (146), 2',5'-oligoadenylate synthetase (*OAS*) (147, 148), major histocompatibility complex (*MHC*) class I (139), and beta-2-microglobulin (*B2M*) (139, 149). IFNT induces dimerization of Type I IFN receptors in the ovine uterus (150), and hence phosphorylation of receptor-associated STATs (145, 151) that leads to formation of two transcription factor complexes: ISGF3 and GAF (145, 151). For induction and activation of most ISGs, these complexes translocate to the nucleus and bind to specific DNA sequences to activate transcription of target genes (112, 151, 152). For instance, GAF (STAT1 homodimer) regulates transcription of genes containing a GAS element, such as IRF1 (112). ISGF3 is a heterotrimer consisting of STAT1, STAT2, and IRF9 (153) that regulates transcriptional activities of genes containing ISREs, such as STAT1, STAT2, IRF9, and OAS (154, 155).

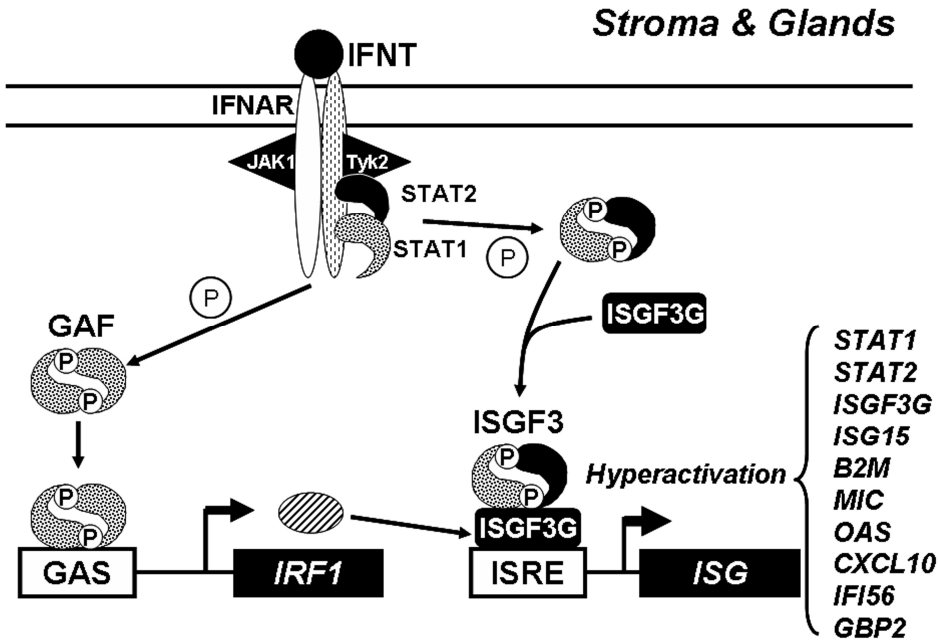

Fig. 2.4. Schematic illustrating the current working hypothesis on IFNT signaling in the ovine endometrial stroma and glandular epithelium. Most IFNT-stimulated genes (ISGs) are expressed by endometrial stroma and middle to deep GE of the ovine uterus because IFNT binds to a common Type I IFN receptor, IFNAR1 and IFNAR2 containing tyrosine kinase such as JAK1 and TYK2, and activates the JAK/STAT signaling pathway. Upon cognate ligand binding, IFNAR1 and IFNAR2 heterodimerize, change their conformation, and activate TYK2 and JAK1 by tyrosine phosphorylation, respectively. The activated TYK2 phosphorylates STAT2 through its SH2 (src homologous 2) domain and then recruits STAT1. Phosphorylated STAT1 binds the phosphorylated STAT2 to form a heterodimer that is released from the receptor complex and translocates to the nucleus after forming a heterotrimeric transcriptional complex by binding with ISGF3G, collectively termed ISGF3. In addition to STAT1/2 heterodimerization, Type I IFN also induces formation of phosphorylated STAT1 homodimers, termed GAF. In the nucleus, ISGF3 binds to the IFN-stimulated response element (ISRE) in promoter regions of ISGs and activates their transcription with the cooperation with several coactivators, such as the cAMP response element binding protein (CREB)-binding protein (CBP)/p300. Similarly, GAF enters the nucleus, binds to GAS elements, and stimulates the transcription of ISGs. Adapted from Spencer et al., 2007.

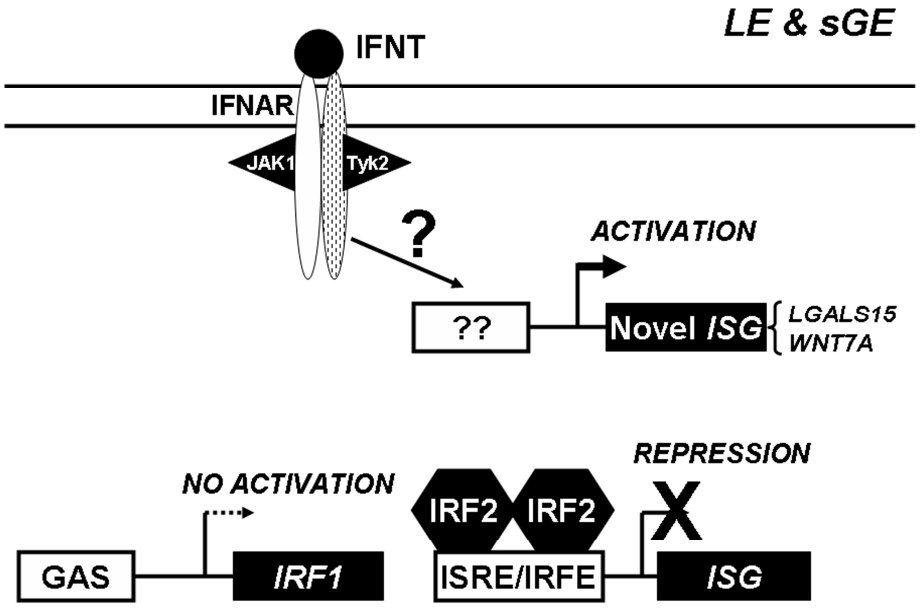

Fig. 2.5. Schematic illustrating the current working hypothesis on IFNT-signaling in the ovine luminal and superficial ductal glandular epithelium. IRF-2, a known transcriptional repressor of Type I ISGs in the ovine uterus constitutively expressed in the endometrial LE and sGE, increases during early pregnancy, and is hypothesized to prevent induction or increases in transcription of ISGs by IFNT. At present, *LGALS15* (also known as galectin-15) and *WNT7A* are the only genes known to be induced in LE and sGE by IFNT utilizing an unknown non-classical signaling pathway that is independent of the classical STAT transcription factors. Adapted from Spencer et al., 2007.

IRF2, a known transcriptional repressor of Type I ISGs in the ovine uterus is constitutively expressed in the endometrial LE and sGE, increases during early pregnancy, and is hypothesized to prevent induction or increases in transcription of ISGs by IFNT (Fig. 2.5) (125, 156). *WNT7A* (156) and *LGALS15* (also known as galectin-15) (157) are the only genes known to be induced in LE and sGE by IFNT utilizing an unknown non-classical signaling pathway that is independent of the classical STAT transcription factors.

Representative STAT1-Dependent ISGs

Ubiquitin Cross-Reactive Protein/IFN-Stimulated Gene 15/17. In humans, a 15 kDa protein called IFN-stimulated gene 15 (*ISG15*) encoding a 15 kDa protein was identified in tumor and lymphoblastoid cells, and was induced by Type I IFNs (IFNA and –B) to greater extent than by Type II IFN (IFNG) (158, 159). However, *ISG15* was renamed ubiquitin cross-reactive protein (*UCRP*) because its sequence is highly homologous to a tandem diubiquitin repeat, and antibodies raised to ISG15 cross-react with ubiquitin (160). In bovine endometrium, the 17 kDa precursor form of UCRP was detected as a 16 kDa form that might have undergone proteolytic cleavage in the endometrium, termed *ISG17* (161, 162). During early pregnancy, in sheep, *ISG15* mRNA increases only in stroma and GE from Days 11 to 15 and then declines thereafter (138). This period is coincident with peak production of IFNT by the ovine conceptus and *ISG15* expression increases in immortalized ovine LE, GE, and stromal cells treated with IFNT (138, 163).

2',5'-Oligoadenylate Synthetase. 2',5'-oligoadenylate synthetase (*OAS*) is induced by Type I and –II IFNs and polymerizes ATP into 2'-5' linked oligomers in order to bind and activate RNase L which can destroy intracellular viral RNAs. Further, *OAS* is involved in antiviral activity, cell growth, differentiation, and apoptosis (164-166). In the ovine uterus, *OAS* is expressed only in the stroma and deep GE in response to IFNT and P4 during early pregnancy (141, 147)

RSAD2. Radical S-adenosyl methionine domain containing 2 (*RSAD2*), also known as viperin, is a cytoplasmic antiviral protein that consists of 361 amino acids, and is encoded for by a gene which contains putative IRF binding sites in the promoter region (167, 168). In humans, stable expression of *RSAD2* in fibroblasts inhibits human cytomegalovirus infection (167), and it is also a potential antiviral effector expressed in patients with atherosclerosis (169) and chronic hepatitis C virus (170). Chin *et al*. reported that *RSAD2* expression is greater in response to Type I than Type II IFN (IFNG) and that it may have an antiviral function (167).

MDA5 (IFIH1). Melanoma differentiation associated gene 5 (*MDA5* also known as *IFIH1*) is a dsRNA-dependent ATPase that responds predominantly to Type I IFNs, similar to *RSAD2*, and is known to be induced during differentiation, cancer reversion, and programmed cell death (171, 172). The *IFIH1* gene contains both CARD and RNA helicase motifs and acts as a positive regulator to sense intracellular viral infection and stimulate innate antiviral responses including the production of Type I IFN (171, 173). The V proteins of a wide variety of paramyxoviruses bind IFIH1 and inhibit its ability to activate the IFNB promoter (174). Further, IFNB promoter stimulator 1, which can induce Type I IFN and IFN-inducible genes through activation of IRF3, IRF7 and NF-κB transcription factors, is known as an adaptor during IFIH1-mediated antiviral immune response (175).

Representative STAT1-Independent ISGs

Wingless-Type Mouse Mammary Tumor Virus Integration Site Family, Member 7A (WNT7A). Most members of the WNT family are involved in embryonic cell growth, development, and differentiation during pregnancy and also in maternal-fetal interactions during implantation (176). In the ovine uterus, Wingless-type mouse mammary tumor virus integration site family, member 7A (*WNT7A*) is the only gene induced by IFNT during early pregnancy and expressed only in LE and sGE (156). Ovine endometrial *WNT7A* may activate the canonical WNT signaling pathway to stimulate proliferation and differentiation of conceptus trophectoderm, and it may also

regulate important genes for uterine receptivity for implantation and conceptus survival (177).

Galectin-15 (LGALS15). Galectins are widely distributed in a variety mammalian species, as well as non-mammalian species including birds, fish, and amphibians (178). They are members of a superfamily of β-galactoside binding lectins that bind β-galactoside via a CRD (carbohydrate recognition domain) (179). In sheep, LGALS15 was identified as the novel 14 kDa form of a P4-modulated protein associated with crystalline inclusion bodies in endometrial LE and conceptus trophectoderm (180). In the ovine uterus, *LGALS15* mRNA is expressed only in endometrial LE and sGE where it was induced by P4 and stimulated by IFNT. In addition, LGALS15 protein had a nucleocytoplasmic distribution within the LE and sGE and was also concentrated near and on the apical surface (157). Therefore, LGALS15 was secreted into the uterine lumen by the LE and sGE, where it may promote adhesion during implantation, as well as was phagocytosed by the trophectoderm and formed intracellular crystals (157, 181).

Cathepsin L (*CTSL*) and Cystatin C (*CST3*)

CTSL

Cathepsins (*CTS*) are a family of lysosomal proteinases that are active in an acidic environment (182). They can degrade extracellular matrix (ECM) molecules, including collagens, laminin, fibronectin and proteoglycans and are also involved in catabolism of intracellular proteins and processing of pro-hormones. Available evidence supports the concept that a variety of proteases, as well as their specific inhibitors regulate trophoblast invasion in many species (e.g. mouse, rat, cat, pig, and human) during conceptus implantation (183-190). CTSL is normally localized in lysosomes where it plays a major role in intracellular protein catabolism. In rodents, interactions between Ctsb, Ctsl, and Cst3 (Ctsb and Ctsl inhibitors) are important for implantation and placentation, because inhibition of endometrial *Ctsb* and *Ctsl* results in abnormal embryonic development and uterine decidualization during the peri-implantation period (183). In cats, CTSL is localized to the GE and can be detected in the uterine lumen

where it is implicated in blastocyst invasion (185). In pigs, *CTSL* is expressed in the endometrial GE and is a P4-regulated component of the uterine lumen during implantation and placentation (188).

CST3

Cystatin C (*CST3*) is a secreted inhibitor of lysosomal cysteine proteases cathepsin B (*CTSB*) and cathepsin L (*CTSL*) (191-194). In mice, *Ctsb* and *Ctsl* are necessary for normal embryonic development and uterine decidualization, and the decidua coordinately expresses *Cst3* to control *Ctsb* and *Ctsl* actions within the implantation site (183). A variety of proteases, as well as their inhibitors, regulate endometrial remodeling and trophoblast invasion in many species (e.g. mouse, rat, cat, sheep, pig, and human) during conceptus implantation and placentation.

Stanniocalcin (*STC*)

Stanniocalcin (*STC*) was originally described as a hormone with calcitonin-like actions in fish (195-198). The hormone was discovered in the corpuscles of Stannius, unique endocrine glands on the kidneys of bony fish (199). Removal of the organ or stanniectomy causes hypercalcemia (200, 201). Fish STC1 was subsequently purified from the corpuscles of Stannius and found to be a homodimeric phosphoglycoprotein that regulates calcium and phosphate homeostasis (202). In fish, STC synthesis and secretion are controlled primarily by serum calcium levels (199) and it acts to restore normocalcemia by acting on the gills to reduce further influx of calcium from the aquatic environment, on the kidneys to promote reabsorption of phosphate and chelate excess calcium, and on the gut to inhibit calcium uptake across the intestinal epithelium (196, 197, 199, 202, 203) .

STC1, a mammalian ortholog of fish STC1, has relatively high amino acid sequence identity (approximately 50%) with fish STC and is expressed in a variety of tissues including brain, kidney, lung, and heart (204). STC2 has lower identity (approximately 35%) with STC1 and fish STC1 (205). Similar to STC1, STC2 is

expressed in a variety of tissues. Research into the functions of STCs in mammals is at an early stage; therefore, its physiological roles have not been established [see for review (195, 206-208)]. Similar to fish STC, mammalian STC1 regulates intracellular calcium and phosphate (Pi) levels in the kidney and intestine (199, 209), but the function of STC2 is unknown. Mammalian STC1 regulates renal transport of phosphate through stimulation of NaPi-2 cotransport activity (196, 210-212). In rodents, Stc1 expression increases in ovarian tissues during gestation and lactation (213), as well as in mesometrial decidua of the uterus during implantation (214). In the rat ovary, STC1 and STC2 are expressed in ovarian theca/interstitial cells and in vitro studies suggest that they act in a paracrine manner to dampen gonadotropin stimulation of granulosa cell differentiation (205, 215). In mice, Stc1 does not appear to be essential for reproduction or growth as null mutatnts have no overt phenotype (216); however, in that study, Stc2 was found in all tissues that normally express Stc1 and may compensate for the lack of Stc1.

CHAPTER III
CATHEPSINS IN THE OVINE UTERUS: REGULATION BY PREGNANCY, PROGESTERONE, AND INTERFERON TAU

Introduction

Cathepsins (CTS) are a family of lysosomal proteinases active in an acidic environment (182). They have the ability to degrade extracellular matrix (ECM) molecules, including collagens, laminin, fibronectin and proteoglycans, and are also involved in the catabolism of intracellular proteins and pro-hormone processing. A member of the cysteine proteinase family, CTSB, can activate matrix metalloproteinases (MMPs) and urokinase type plasminogen activator (uPA) (217) and the closely-related CTSL can cleave pro-uPA into the active form (217). On the other hand, inactive precursors of these CTS can be activated by MMPs (182). In humans, CTSB, CTSH, CTSK, CTSL and CTSS are expressed in the proliferative and secretory phase endometria and appear to be required for normal uterine development and function as well as menstruation (190). Available evidence supports the concept that a variety of proteases as well as their specific inhibitors regulate trophoblast invasion in many species (e.g. mouse, rat, cat, pig, and human) during conceptus implantation (183-190). Specifically, these studies implicate CTS in regulation of uterine receptivity for implantation and trophoblast invasion in a number of mammals (see (218-220) for review).

Regulation of CTS expression in the ovine uterus and conceptus has not been reported. Trophoblast invasion in ruminants (sheep, cattle, goats) is limited to fusion of migrating binucleate cells with uterine epithelium, but considerable tissue remodeling and angiogenesis occurs within the endometrium at implantation which is associated with the cysteine and serine proteases and production of MMPs by the endometrium and conceptus (221, 222). Endometrial function during this period of pregnancy appears to be primarily regulated by progesterone from the corpus luteum and hormones from the conceptus, including interferon tau (IFNT) (223, 224). IFNT is the signal for maternal

recognition of pregnancy in ruminants and is produced between Days 10 and 21 to 25 of pregnancy in sheep by the mononuclear trophoblast cells of the conceptus (1, 225). In sheep, IFNT acts in a paracrine manner on endometrial epithelia to inhibit transcription of the estrogen receptor alpha and oxytocin receptor genes (8, 225), thereby preventing endometrial release of luteolytic pulses of prostaglandin F2α (226). The antiluteolytic actions of IFNT are required for maintenance of a functional corpus luteum and secretion of progesterone, the essential hormone of pregnancy (226). IFNT also induces or stimulates expression of a number of genes in the endometrium that are hypothesized to play important biological roles in conceptus implantation (227). This study determined effects of the estrous cycle, pregnancy, progesterone and IFNT on expression of selected CTS genes in the ovine endometrium. Results indicated that a number of CTS genes are expressed in the endometrium and conceptus during early pregnancy and regulated by progesterone and/or IFNT. In particular, CTSL was found to be novel gene stimulated by progesterone and IFNT only in endometrial luminal (LE) and superficial ductal glandular epithelia (sGE).

Materials and Methods

Animals

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

Experimental Design

Study One. At estrus (Day 0), ewes were mated to either an intact or vasectomized ram as described previously (228) and then hysterectomized (n = 5 ewes/day) on either Day 10, 12, 14 or 16 of the estrous cycle or Day 10, 12, 14, 16, 18 or

20 of pregnancy. Pregnancy was confirmed on Days 10 to 16 post-mating by the presence of a morphologically normal conceptus(es) in the uterus. At hysterectomy, several sections (~0.5 cm) from the mid-portion of each uterine horn ipsilateral to the corpus luteum were fixed in fresh 4% paraformaldehyde in PBS (pH 7.2). After 24 h, fixed tissues were changed to 70% ethanol for 24 h and then dehydrated and embedded in Paraplast-Plus (Oxford Labware, St. Louis, MO). Several sections (1–1.5 cm) from the middle of each uterine horn were embedded in Tissue-Tek OCT compound (Miles, Oneonta, NY), frozen in liquid nitrogen vapor, and stored at -80°C. The remaining endometrium was physically dissected from myometrium, frozen in liquid nitrogen, and stored at -80°C for subsequent RNA or protein extraction. In monovulatory pregnant ewes, uterine tissue samples were marked as either contralateral or ipsilateral to the ovary bearing the corpus luteum. No tissues from the contralateral uterine horn were used for study. Uterine flushes were clarified by centrifugation (3,000 x g for 30 min at 4°C) and frozen at -80°C for Western blot analysis.

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 (229). Ewes were then assigned randomly (n=5 per treatment) to receive daily intramuscular (i.m.) injections of progesterone and/or a progesterone receptor (PGR) antagonist (ZK 136,317; Schering AG, Germany) and intrauterine (i.u.) infusions of control serum proteins and/or recombinant ovine IFNT protein as follows: (1) 50 mg progesterone (P4, Days 5 to 16) and 200 μg control (CX) serum proteins (Days 11 to 16) [P4+CX]; (2) P4 and 75 mg ZK 136,317 (Days 11 to 16) and CX proteins [P4+ZK+CX]; (3) P4 and IFNT (2 x 10^7 antiviral units, Days 11 to 16) [P4+IFN]; or (4) P4 and ZK and IFNT [P4+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 IFNT (5 x 10^6 antiviral units/horn/injection). Recombinant ovine IFNT was produced in *Pichia pastoris* and purified as described previously (230). Proteins were prepared for intrauterine injection as described previously (229). This regimen of progesterone and roIFNT mimics the effects of progesterone and the

conceptus on endometrial expression of hormone receptors and IFNT-stimulated genes during early pregnancy in ewes (148, 156, 231). All ewes were hysterectomized on Day 17, and the uteri and endometria processed as described in Study One.

RNA Isolation

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

Cloning of Partial cDNAs for Ovine CTSB, K, L, H, S, D and Z

Partial cDNAs for ovine *CTSB, CTSD, CTSK, CTSL, CTSH, CTSS*, and *CTSZ* mRNAs were amplified by RT-PCR using total RNA from endometrial tissues from Days 16 to 18 of pregnancy using specific primers. PCR amplification was conducted as follows for ovine CTSB, K, L, H, S, D and Z : a) 95°C for 5 min; b) 95°C for 45 sec, 59.1°C (for CTSB and CTSH) or 56.5°C (for CTSD, CTSK, CTSL, and CTSZ) or 64.5°C (for CTSS) for 1 min, and 72°C for 1 min for 35 cycles; and c) 72°C for 10 min. Partial cDNAs of the correct size were cloned into pCRII using a T/A Cloning Kit (Invitrogen) and their sequences verified by sequencing.

Slot Blot Hybridization Analyses

Steady-state levels of mRNA in ovine endometria were assessed by slot blot hybridization as described previously (125, 232). Radiolabeled antisense and sense cRNA probes were generated by *in vitro* transcription using linearized plasmid template, RNA polymerases, and [α-^{32}P]-UTP. Denatured total endometrial RNA (20 μg) from each ewe in Studies One and Two was hybridized with radiolabeled cRNA probes. To correct for variation in total RNA loading, a duplicate RNA slot membrane was hybridized with radiolabeled antisense 18S cRNA (pT718S; Ambion, Austin, TX). Following washing, the blots were digested with ribonuclease A and radioactivity

associated with slots quantified using a Typhoon 8600 MultiImager (Molecular Dynamics, Piscataway, NJ). Data are expressed as relative units (RU).

In Situ *Hybridization Analyses*

Location of mRNA expression in sections (5 μm) of the ovine uterus was determined by radioactive *in situ* hybridization analysis as described previously (125, 232). Radiolabeled antisense and sense cRNA probes were generated by *in vitro* transcription using linearized plasmid template, RNA polymerases, and [α-^{35}S]-UTP. Deparaffinized, rehydrated and deproteinated uterine tissue sections were hybridized with radiolabeled antisense or sense cRNA probes. After hybridization, washing and ribonuclease A digestion, slides were dipped in NTB-2 liquid photographic emulsion (Kodak, Rochester, NY), and exposed at 4°C for two weeks. Slides were developed in Kodak D-19 developer, counterstained with Gill's hematoxylin (Fisher Scientific, Fairlawn, NJ), and then dehydrated through a graded series of alcohol to xylene. Coverslips were then affixed with Permount (Fisher). Images of representative fields were recorded under brightfield or darkfield illumination using a Nikon Eclipse 1000 photomicroscope (Nikon Instruments Inc., Lewisville, TX) fitted with a Nikon DXM1200 digital camera.

Immunohistochemistry

Immunocytochemical localization of immunoreactive CTSL protein in the ovine uterus was performed as described previously (229) in uterine tissue cross sections from Studies One and Two using rabbit anti-human CTSL polyclonal antibody (Catalog # 3192-100; BioVision, Mountain View, CA) at a final concentration of 1 μg per ml. Antigen retrieval was performed by using boiling citrate buffer as described previously (233). Negative controls included substitution of the primary antibody with non-immune rabbit IgG (Sigma Chemical Co., St. Louis, MO) at the same final concentration.

Western Blot Analyses

Protein concentrations of uterine flushes were determined using the Bradford protein assay (Bio-Rad, Hercules, CA) with bovine serum albumin (BSA) as the standard. Proteins were denatured and separated by 12% SDS-PAGE, and Western blot analysis conducted as described previously (228) by using enhanced chemiluminescence (SuperSignal West Pico, Pierce, Rockford, IL) and X-OMAT AR X-ray film (Kodak, Rochester, NY) according to the manufacturer's recommendations. Immunoreactive CTSL protein was detected using rabbit anti-human CTSL polyclonal antibody (Catalog # 3192-100; BioVision, Mountain View, CA) at 0.5 µg per ml.

Statistical Analyses

Data from slot blot hybridization analyses were subjected to least-squares analysis of variance (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. Data from Study One were analyzed for effects of day, pregnancy status (cyclic or pregnant), and their interaction. Effects of day were determined by least squares regression analysis. Data from Study Two were analyzed using preplanned orthogonal contrasts (P4+CX versus P4+IFN, P4+CX versus P4+ZK+CX, and P4+IFN versus P4+ZK+IFN). Data are presented as least squares means (LSM) with overall standard errors (SE).

Results

Effects of Estrous Cycle and Pregnancy on Expression of CTS mRNAs in Ovine Endometrium (Study One)

Steady-state levels of ovine *CTSB, CTSD, CTSH, CTSK, CTSL, CTSS*, and *CTSZ* mRNAs in endometria from cyclic (C) and pregnant (PX) ewes were determined by slot blot hybridization analyses (Fig. 3.1). Expression of *CTSB* mRNA was lowest on Day 10 and increased to Days 16 or 20 in C and PX ewes, respectively (linear effect of day, $P<0.01$). Endometrial levels of *CTSD* mRNA did not change in C ewes, but increased

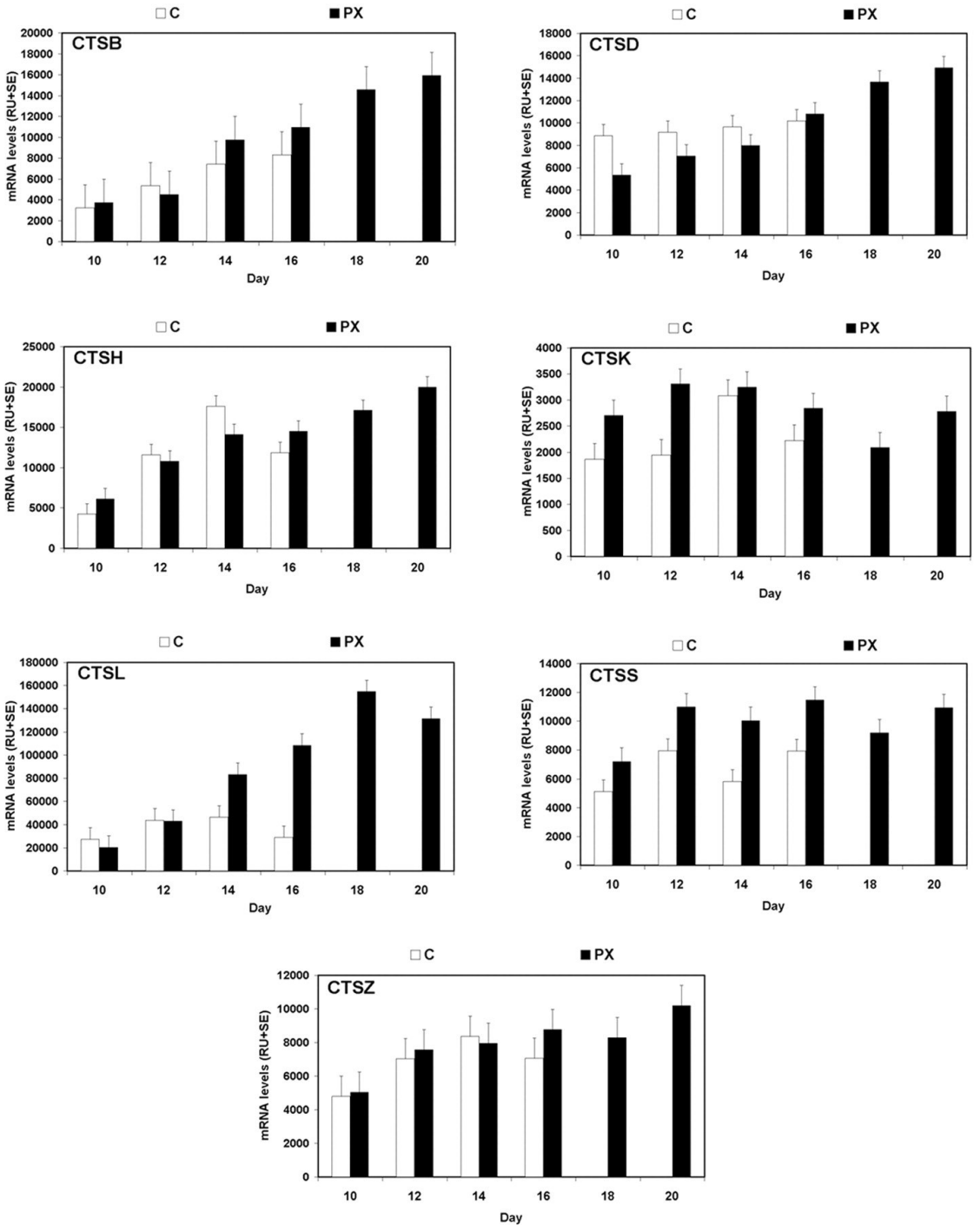

Fig. 3.1. Steady-state levels of CTSB, CTSD, CTSH, CTSK, CTSL, CTSS, and CTSZ mRNAs in endometria from cyclic and pregnant ewes determined by slot blot analysis. See text for description of effects of day of the estrous cycle (C) or pregnancy (PX) on mRNA levels in the endometrium.

from Days 10 to 20 in PX ewes (linear effect of day, P<0.01). *CTSH* mRNA levels increased from Days 10 to 14 in C ewes and from Days 10 to 20 in PX ewes (linear effect of day, P<0.01). In contrast, *CTSK* mRNA did not change (P>0.10) in endometria of C and PX ewes. *CTSL* mRNA was affected (P<0.05) by day, status, and their interaction. In C ewes, *CTSL* mRNA increased from Days 10 to 14 and then decline to Day 16 (quadratic effect of day, P<0.05). In PX ewes, *CTSL* mRNA increased about 8-fold between Days 10 and 18 (linear effect of day, P<0.01). Further, *CTSL* mRNA levels in the endometrium were greater on Days 14 and 16 in PX than C ewes (day x status, P<0.05). Endometrial *CTSS* and *CTSZ* mRNA levels were not affected by pregnancy status or day or their interaction (P>0.10).

In situ hybridization analyses determined the location of *CTS* gene expression in endometria. In C and PX ewes, *CTSB* mRNA was detected in the endometrial luminal epithelium (LE), superficial ductal glandular epithelium (sGE), stratum compactum stroma, and in cells distributed throughout the stroma that appeared to be immune cells based on their morphology (Fig. 3.2). Abundant *CTSB* mRNA was detected in the trophectoderm of the conceptus. *CTSD* mRNA was expressed at low levels in the endometrial LE and sGE, however abundant *CTSD* mRNA was detected in the trophectoderm of the conceptus. *CTSH* mRNA was expressed at moderate levels in the endometrial LE and GE, particularly on Days 18 and 20 in PX ewes. In C and PX ewes, *CTSK* mRNA was expressed at moderate levels in the endometrial LE and stroma as well as in cells within the stroma that appeared to be immune cells based on their morphology and location.

CTSL mRNA was the most abundant CTS genes expressed in the endometrium and it was detected only in endometrial LE and sGE (Fig. 3.3). Further, *CTSL* mRNA was expressed by conceptus trophectoderm on Days 18 and 20 of PX. *CTSS* mRNA was detected at low levels in the endometrial LE and in cells within the stroma that appeared to be immune cells based on their morphology and distribution. The number of *CTSS* mRNA-positive immune-like cells increased between Days 14 and 16 of pregnancy. *CTSZ* mRNA was detected at low levels specifically in the endometrial LE and sGE, as

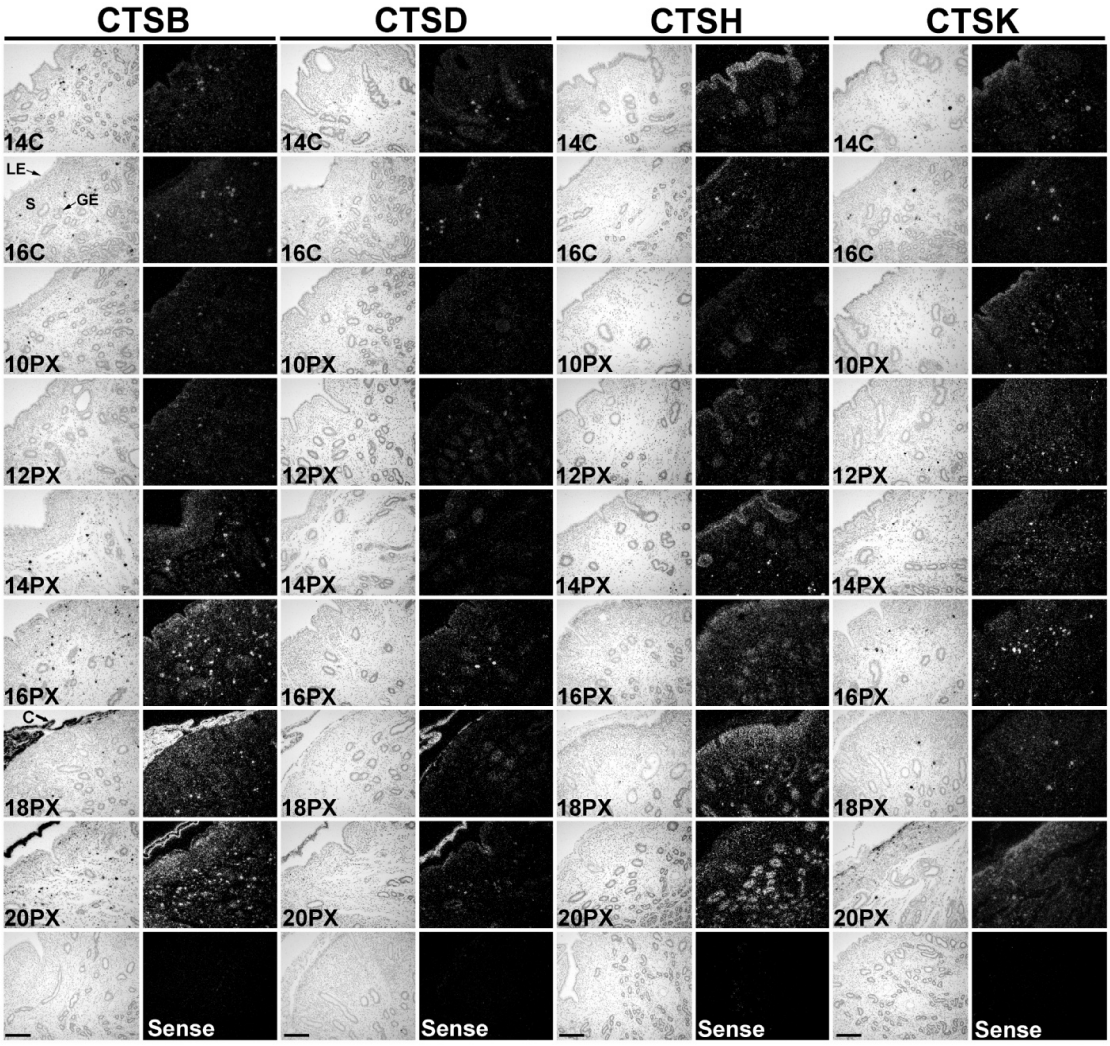

Fig. 3.2. *In situ* hybridization analyses of *CTSB, CTSD, CTSH* and *CTSK* mRNAs in uteri of cyclic and pregnant ewes. Cross-sections of the uterine wall from cyclic (C) and pregnant (PX) ewes were hybridized with radiolabeled antisense or sense ovine *CTS* cRNA probes. Legend: C, conceptus; LE, luminal epithelium; GE, glandular epithelium; S, stroma. Scale bar represents 10 μm.

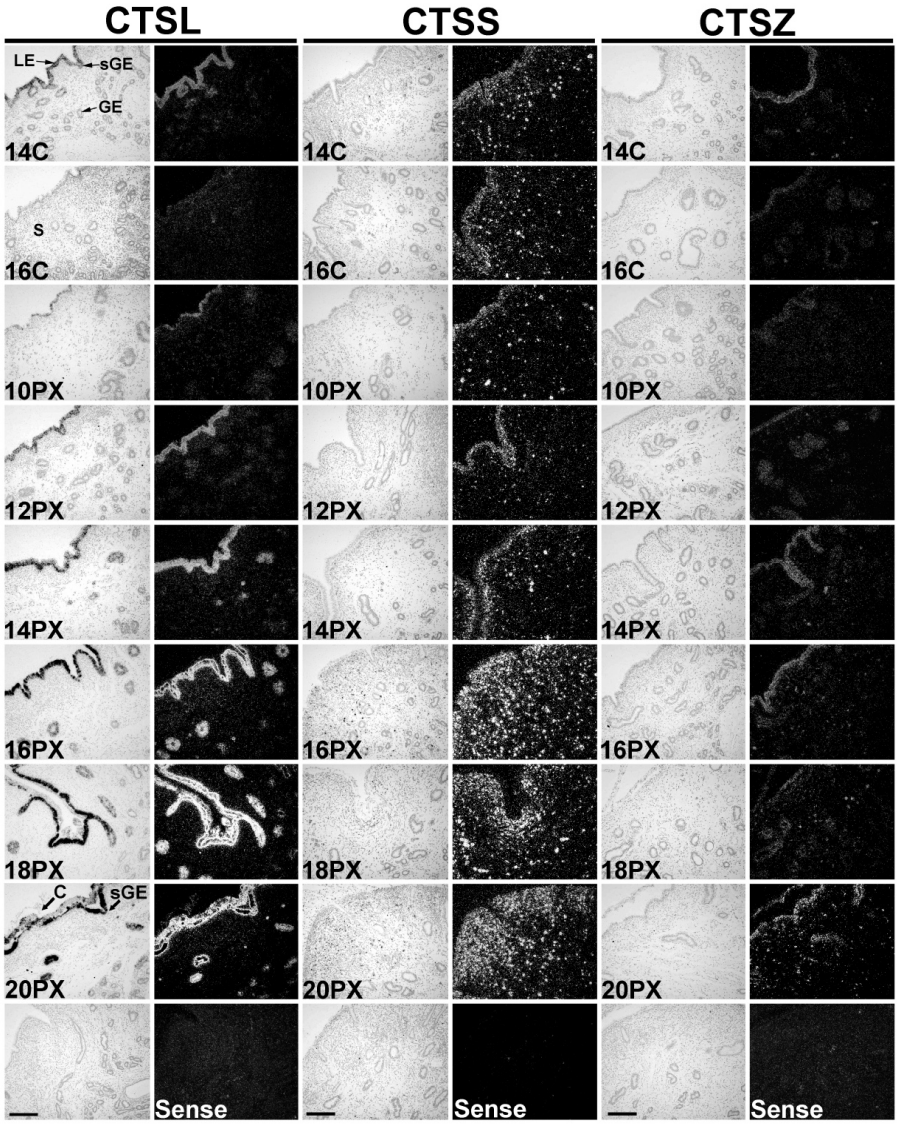

Fig. 3.3. *In situ* hybridization analyses of *CTSL, CTSS*, and *CTSZ* mRNAs in uteri of cyclic and pregnant ewes. Cross-sections of the uterine wall from cyclic (C) and pregnant (PX) ewes were hybridized with radiolabeled antisense or sense ovine *CTS* cRNA probes. Legend: C, conceptus; LE, luminal epithelium; GE, glandular epithelium; S, stroma; sGE, superficial ductal GE. Scale bar represents 10 μm.

well as conceptus trophectoderm on Days 18 and 20 of pregnancy. No differences in expression of *CTS* mRNAs in the LE or stroma of the intercaruncular endometria were found when compared to the caruncular endometria in the uterus of either cyclic or pregnant ewes (data not shown).

Collectively, results of slot blot and *in situ* hybridization analyses indicated that *CTSL* mRNA was the most abundant CTS gene expressed in the endometrium and the only CTS in the endometrium that appeared to be regulated by progesterone and a product of the conceptus. Therefore, CTSL protein was studied in the uterus.

CTSL Protein in the Endometrium and Uterine Lumen (Study One)

Consistent with *in situ* hybridization analyses, immunoreactive CTSL protein was observed predominantly in the LE and sGE in the endometrium of C and PX ewes (Fig. 3.4A). In pregnant ewes, the amount of immunoreactive CTSL protein increased from Days 10 to 16 and was observed predominantly near the apical surface of the LE. Less immunoreactive CTSL protein was detected in the stroma and conceptus trophectoderm.

Western blot analyses detected abundant levels of the 38-40 kDa form of pro-CTSL in the uterine flushings from pregnant, but not cyclic ewes (Fig. 3.4B). Further, the cleaved and active forms of CTSL, made up of 21 and 5 kDa subunits, were also detected at very low abundance in uterine flushings from pregnant ewes.

Effects of Progesterone and IFNT on Endometrial CTS Expression (Study Two)

In order to determine if progesterone (P4) and IFNT regulated *CTS* gene expression in the endometrium, a study was conducted as described in the Materials and Methods (Fig. 3.5A). As illustrated in Fig. 3.5B, treatment with P4 increased *CTSL* mRNA in the endometrium (P4+CX vs P4+ZK+CX, $P<0.001$) which was further stimulated by about 3-fold in ewes receiving intrauterine administration of roIFNT (P4+CX vs P4+IFN, $P<0.01$), but roIFNT did not stimulate *CTSL* mRNA in ewes receiving the ZK anti-progestin (P4+IFN vs P4+ZK+IFN, $P>0.10$).

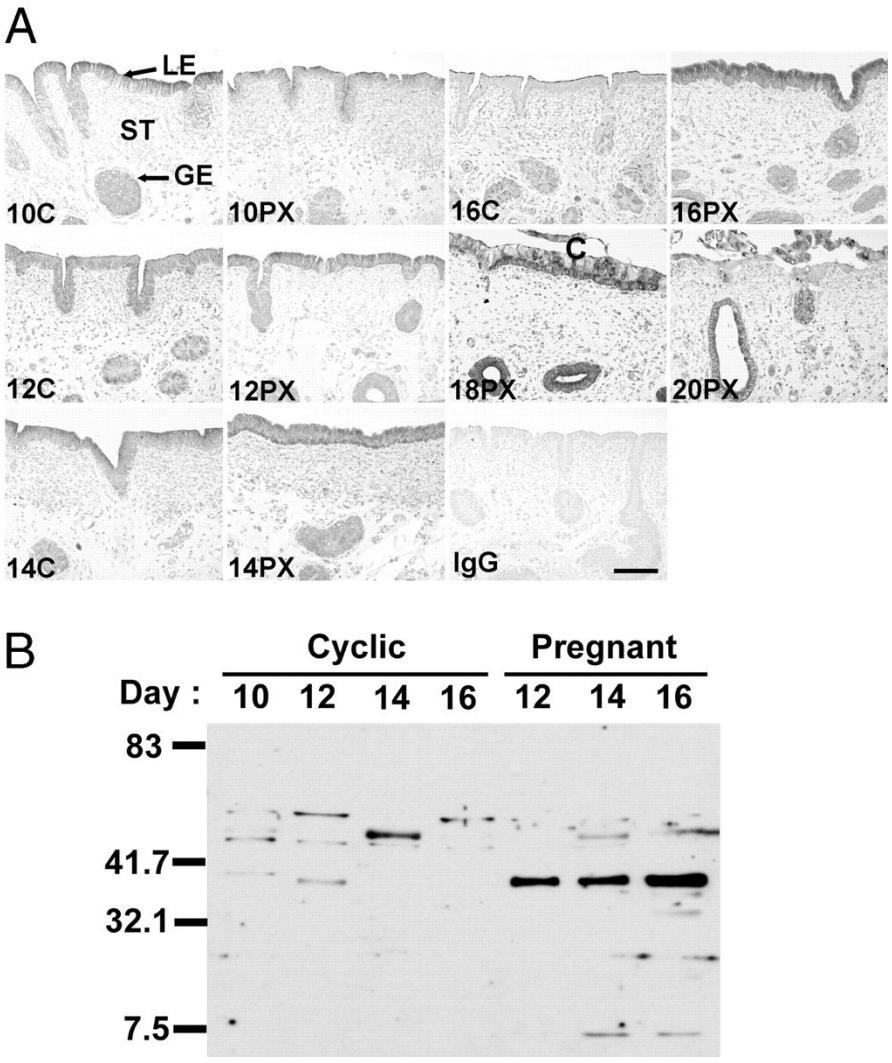

Fig. 3.4. CTSL protein in endometria and uterine flushings from cyclic and pregnant ewes from Study One. (A) Immunoreactive CTSL protein was localized in sections of the uterus using a rabbit anti-human CTSL polyclonal antibody. For the IgG control, normal rabbit IgG was substituted for the primary antibody. Sections were not counterstained. Legend: C, conceptus; LE, luminal epithelium; GE, glandular epithelium; S, stroma. Scale bar represents 10 μm. (B) Representative Western blot analysis of CTSL in uterine flushings. Proteins in uterine flushings were analyzed by 12% SDS-PAGE (10 μg/lane), and immunoreactive protein was detected by Western blot analysis using rabbit anti-human CTSL polyclonal antibody that detects both the proenzyme and the mature forms of CTSL.

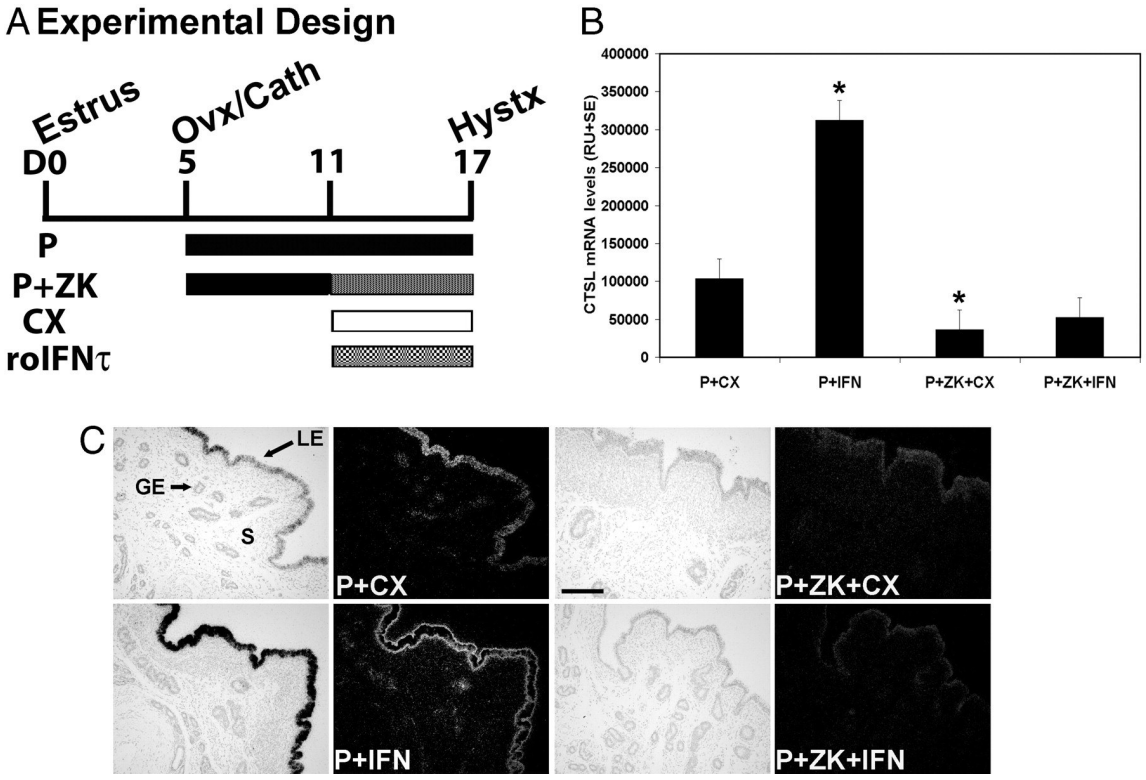

Fig. 3.5. Effects of progesterone and IFNT on *CTSL* mRNA and protein in the uterus (Study Two). (A) Experimental design. See Materials and Methods for complete description. Legend: CX, control serum proteins; Hystx, hysterectomy; Ovx/Cath, ovariectomy and uterine catheterization; P4, progesterone; roIFNT, recombinant ovine interferon tau; ZK, ZK137,316 anti-progestin. (B) Steady-state levels of *CTSL* mRNA in the endometria were determined by slot blot hybridization analysis. Treatment with P4 increased *CTSL* mRNA in the endometrium (P4+CX vs P4+ZK+CX, $P<0.001$) which was further stimulated by about 3-fold in ewes receiving intrauterine administration of roIFNT (P4+CX vs P4+IFN, $P<0.01$), but roIFNT did not stimulate *CTSL* mRNA in ewes receiving the ZK anti-progestin (P4+IFN vs P4+ZK+IFN, $P>0.10$). (C) In situ hybridization analysis of *CTSL* mRNA expression. Cross-sections of the uterine wall from treated-ewes were hybridized with radiolabeled antisense or sense ovine *CTSL* cRNA probes. Legend: LE, luminal epithelium; GE, glandular epithelium; S, stroma; C, conceptus. Bar represents 10 μm.

In situ hybridization analyses revealed that *CTSL* mRNA was only expressed abundantly in the endometrial LE and sGE of ewes treated with P4 (P4+CX and P4+IFN) (Fig. 3.5C).

Endometrial *CTSB* mRNA was stimulated by P4 (P4+CX vs P4+ZK+CX, P<0.02), but decreased by roIFNT in ewes receiving P4 (P4+CX vs P4+IFN, P<0.01), whereas roIFNT increased *CTSB* mRNA in ewes receiving P4 and ZK (P4+ZK+CX vs P4+ZK+IFN, P<0.04) (Fig. 3.6). Expression of *CTSD* mRNA was not affected (P>0.10) by steroid or intrauterine roIFNT treatment. Endometrial *CTSH* mRNA was increased by IFNT (P4+CX vs P4+IFN, P<0.001), but not affected by other treatments (P>0.10). *CTSK* mRNA was decreased by P4 (P4+CX vs P4+ZK+CX, P<0.02), but increased by roIFNT in ewes receiving P4 (P4+CX vs P4+IFN, P<0.01) or P4+ZK (P4+ZK+CX vs P4+ZK+IFN, P<0.001). *CTSS* mRNA was also stimulated by P4 (P4+CX vs P4+ZK+CX, P<0.02). In ewes receiving P4 only, roIFNT decreased *CTSS* mRNA in the endometrium (P4+CX vs P4+IFN, P=0.06). *CTSZ* mRNA was slightly stimulated by P4 (P4+CX vs P4+ZK+CX, P<0.05) and increased by roIFNT in ewes receiving P4 alone (P4+CX vs P4+IFN, P<0.01) or P4+ZK (P4+ZK+CX vs P4+ZK+IFN, P<0.01).

Discussion

Similar to endometria of other mammals, expression of many *CTS* genes was detected in endometria of cyclic and early pregnant ewes. The CTS family of cysteine and aspartyl proteases as well as other proteases, including MMPs and serine proteases, are implicated in the degradation of ECM required for uterine remodeling during decidualization, implantation and placentation (219, 221). In rodents, for example, it has been hypothesized that CTS play a crucial role in digestion of matrix molecules and activation of other pro-enzymes responsible for intracellular breakdown of molecules that are phagocytosed by cells (183). The dynamic and differential expression of CTS genes between cyclic and pregnant ewes suggests functional diversity in mechanisms responsible for expression of CTS genes that may be responsible for optimization of a uterine environment that supports conceptus implantation and placentation during

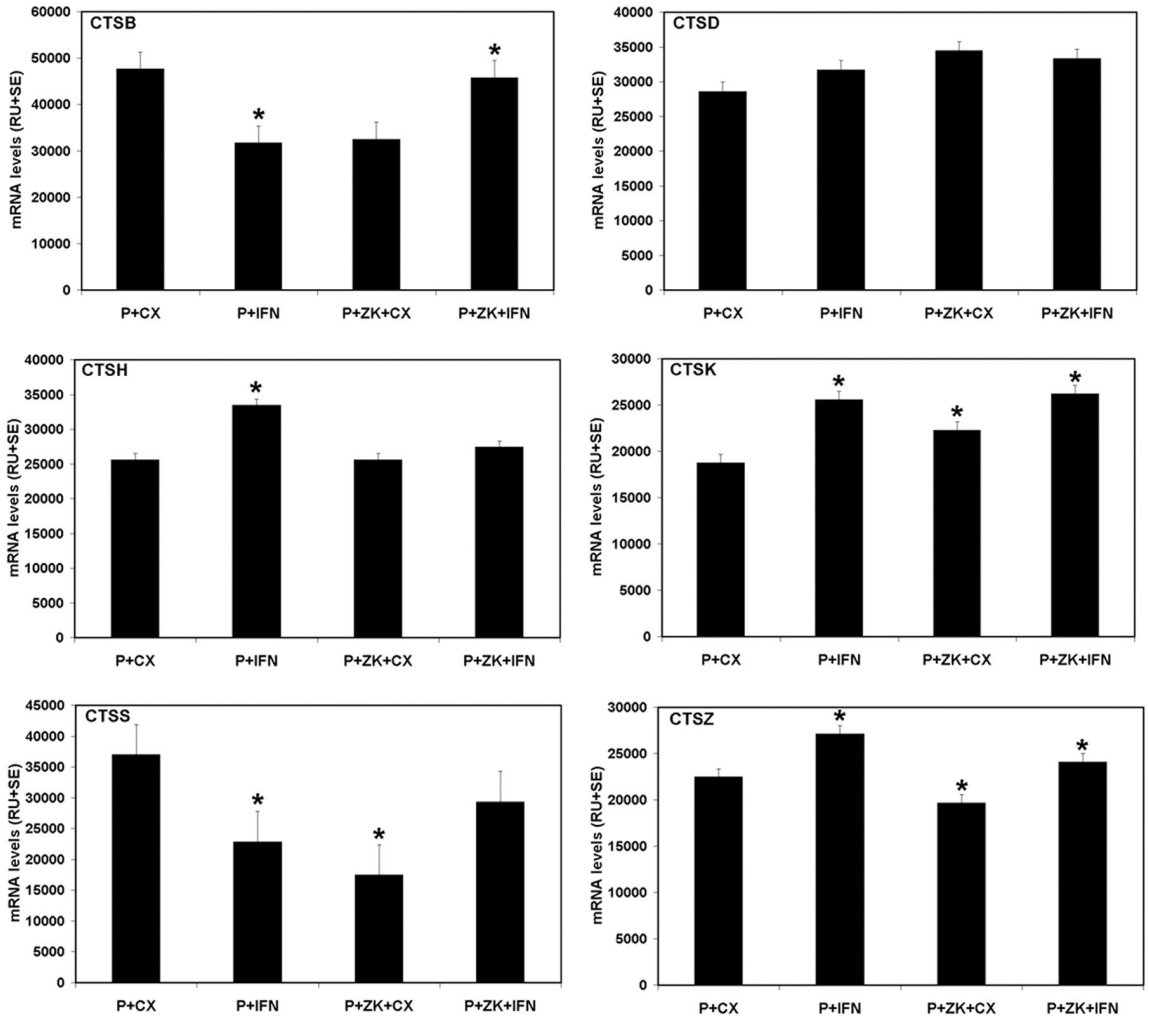

Fig. 3.6. Steady-state levels of *CTSB, CTSD, CTSH, CTSK, CTSS,* and *CTSZ* mRNA in endometria from ewes in Study Two. Steady-state levels of mRNA in endometria from treated ewes were determined by slot blot analysis. See text for description of effects of treatment on mRNA levels in the endometrium. The asterisk (*) denotes an effect of treatment ($P<0.10$).

establishment and maintenance of pregnancy (222). In the present study, cysteine proteases CTSB, CTSH, CTSK, CTSL, CTSS and CTSZ and aspartyl protease CTSD were found to be expressed in the ovine endometrium, and expression of CTSB, CTSD, CTSH, CTSL and CTSZ mRNA increased between Days 10 and 20 of early pregnancy. Consistent with above results, CTSL protein in the porcine uterus was observed in endometrial GE as well as in the uterine lumen and induced by progesterone during the periods of implantation and placentation (188). Interestingly, the ovine placenta expresses large numbers of aspartic proteinase inhibitor genes, termed pregnancy-associated glycoproteins (234), and the endometrial glands express large amounts of serine protease inhibitors, termed serpins or uterine milk proteins (64), that could regulate the activity of endometrial CTS identified in the present study. Therefore, the molecular control of expression of CTS in the ovine endometrium may play an important role in establishing a regulatory network of multiple proteolytic enzymes responsible for ECM remodeling during implantation and placentation. Although decidualization of the endometrial stroma does not occur in sheep, the endometrium undergoes dramatic remodeling after pregnancy recognition and establishment between Days 12 to 20 of early pregnancy. In the intercaruncular endometrium, the endometrial epithelium is removed by the trophoblast giant binucleate cells during synepitheliochorial placentation, the stroma becomes very compact and begins to express new genes such as osteopontin, and the glands undergo hypertrophy followed by hyperplasia (235-238). In the caruncular endometrium, the placental cotyledons attach to the maternal caruncles and develop into placentomes (237). These morphogenetic and differentiation events undoubtedly involve regulation by CTS and extensive remodeling of the ECM.

The present studies found that *CTSL* mRNA was particularly abundant in the endometrial LE and sGE and up-regulated during early pregnancy in association with conceptus elongation and implantation (224). CTSL is normally localized in lysosomes, where it plays a major role in intracellular protein catabolism. In the present studies, the 38-40 kDa latent pro-CTSL form of CTSL protein was abundant in uterine flushings from Day 12, 14 and 16 pregnant ewes. This latent pro-CTSL must be cleaved by

proteases, such as MMPs, to generate the active two-chain form made up of 21 and 5 kDa subunits (182). The presence of the pro-CTSL in uterine flushings from pregnant ewes between Days 12 to 16 of pregnancy suggests that CTSL is secreted by the endometrial LE and/or conceptus. Indeed, the synthesis and secretion of the 39 kDa pro-CTSL has been demonstrated for many tumors, including cancers of the kidney, lung, colon, breast and ovary (239). In rodents, interactions of CTSB, CTSL, and cystatin C, a CTSL inhibitor, are important for implantation and placentation, because inhibition of endometrial CTSL and CTSB results in abnormal embryonic development and uterine decidualization during the peri-implantation period (183). Invasion by the ectoplacental cone of mouse trophoblast was prevented by cysteine proteinase inhibitors *in vitro* (240). Recently, Cheon and coworkers (241) found that cytotoxic T-lymphocyte antigen-2β (CTLA-2β), a cysteine protease inhibitor, was up-regulated by progesterone in the decidua and proposed to regulate blastocyst implantation by neutralizing the activities of one or more proteases, including CTSL, generated by the proliferating trophoblast. CTSL has been studied in uteri of cats (186, 187), pigs (188), and mice (183, 242). In cats, CTSL is localized to the GE and can be detected in the uterine lumen, where it is implicated in blastocyst invasion (185). In pigs, CTSL was also found to be expressed in the endometrial GE and as a progesterone-regulated component of the uterine lumen during implantation and placentation (188). Thus, available results suggest that CTSL may be an essential regulator of endometrial remodeling and conceptus implantation during pregnancy in sheep as well as many other mammals. CTSL is capable of degrading ECM proteins, suggesting a role in conceptus attachment by altering the composition of the ECM present on the apical surfaces of the endometrial LE and/or trophoblast.

In the present study, temporal changes in expression of endometrial *CTSL* mRNA in cyclic and pregnant ewes supported the hypothesis that ovarian progesterone regulates transcription of the *CTSL* gene in the endometrial LE. Similarly, an increase CTSB, CTSD, CTSH, and CTSZ was also observed in the endometrium during early pregnancy. The increase in *CTSL* and *CTSZ* mRNAs in LE and sGE, between Days 10

and 12 post-estrus/mating, and *CTSH* mRNA in LE and GE, between Days 14 and 16 post-mating, is coincident with the disappearance of *PGR* mRNA and protein in these epithelia (10). Similarly, the decrease in *CTSL* and *CTSZ* mRNAs between Days 14 and 16 of the cycle is coincident with the reappearance of PGR protein in endometrial LE. In Study Two, *CTSL* mRNA was detected in endometrial LE and sGE of ovariectomized ewes treated with progesterone for 12 days, but this expression was prevented by administration of the PGR antagonist ZK 136,317. Continuous exposure of the sheep uterus to progesterone for 8 to 10 days down-regulates PGR expression in endometrial LE and sGE, but not stroma or myometrium (231). PGR are present in the endometrial epithelia of P4+ZK-treated sheep (243), because PGR antagonists prevent the inhibitory effects of progesterone on the PGR gene expression. Consequently, progesterone modulation of *CTSL* mRNA may be attributed, at least in part, to down-regulation of PGR by progesterone that occurs in LE and sGE between Days 10 and 12 of the cycle and pregnancy (223). Thus, PGR loss in endometrial epithelia may reprogram these cells, allowing them to increase expression of genes associated with implantation (223, 224). Alternatively, progesterone may act on PGR-positive stromal cells to induce them to express growth factors or changes in the ECM that regulate expression of selected epithelial genes (223).

In addition to regulation by progesterone, the present studies indicate that *CTSH*, *CTSK*, *CTSL* and *CTSZ* are regulated by IFNT. IFNT 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 (223). Of particular note, *CTSL* is a novel gene stimulated by IFNT in endometrial LE and sGE as expression between Days 10 and 18 of early pregnancy parallels the increase in production of IFNT by the elongating conceptus, which is maximal on Day 16 (244). In Study Two, intrauterine administration of roIFNT increased *CTSL* mRNA, but only in progesterone-treated ewes. One hypothesis is that IFNT can only stimulate transcription of the *CTSL* gene in the absence of repression by liganded PGR. Alternatively, the PGR-positive stroma may produce a 'progestamedin' that is also required for LE and

sGE to respond to IFNT (223). The signaling pathway whereby IFNT regulates transcription of the *CTSL* gene is not known, but it clearly does not involve the classical JAK-STAT signaling pathway (1, 125, 156, 223). To date, *WNT7A* and *LGALS15* (galectin-15) are the only other genes identified in endometrial LE and sGE that are induced or stimulated by IFNT, respectively (156, 245). Thus, the diverse actions of IFNT on the endometrium include repression of genes, including ESR1, to abrogate activation of the luteolytic mechanism, as well as stimulation of genes that are critical to implantation, placentation and conceptus growth and development (223). Knowledge of mechanisms whereby IFNT stimulates *CTSL* gene expression in the endometrial LE and sGE is expected to unravel a non-classical signaling pathway for Type I IFNs. Future studies will focus on the role of CTSL, other CTS family members and their inhibitors in endometrial remodeling and conceptus implantation and placentation.

CHAPTER IV
PROGESTERONE AND INTERFERON TAU REGULATE CYSTATIN C IN THE ENDOMETRIUM

Introduction

A variety of proteases, as well as their inhibitors, regulate endometrial remodeling and trophoblast invasion in many species (e.g. mouse, rat, cat, sheep, pig, and human) during conceptus (embryo/fetus and associated extraembryonic membranes) implantation and placentation (219, 220, 246-248). Cathepsins are a family of lysosomal proteinases which can degrade extracellular matrix (ECM) molecules and influence catabolism of intracellular proteins and pro-hormone processing (249). Cystatin C (CST3) is a low molecular weight secretory protein that functions as an inhibitor of lysosomal cysteine proteinases, including cathepsins B (CTSB) and L (CTSL) (191-194). In mice, CTSB and CTSL are necessary for normal embryo development and uterine decidualization, and the decidua coordinately expresses CST3 presumably to control cathepsin actions within the implantation site (183). We recently reported expression of CTSB, CTSD, CTSH, CTSK, CTSL, CTSS, and CTSZ in the endometrium and/or conceptus of sheep during early pregnancy (250). In that study, CTSL was the most abundant cathepsin expressed by the endometrial epithelia and conceptus trophectoderm during early pregnancy, and the *CTSL* gene was induced by progesterone and stimulated by interferon tau (IFNT). However, expression of CST3 has not been investigated in the ovine uterus.

Trophoblast invasion in ruminants (sheep, cattle, goats) is limited to fusion of migrating trophoblast giant binucleate cells with uterine luminal epithelium (54); however, considerable tissue remodeling and angiogenesis occurs within the endometrium at implantation which is associated with the cysteine and serine proteases and production of matrix metalloproteinases (MMPs) by the endometrium and conceptus (251, 252). Endometrial functions during this period of pregnancy are primarily regulated by progesterone from the corpus luteum (CL) and hormones from the

conceptus, including IFNT, placental lactogen and placental growth hormone (2, 17). IFNT is the signal for maternal recognition of pregnancy in ruminants (5) and is produced between Days 10 and 21 to 25 of pregnancy in sheep by the mononuclear trophoblast cells of the conceptus (4). In sheep, IFNT acts in a paracrine manner on endometrial luminal epithelium (LE) and superficial glandular epithelium (sGE) to inhibit transcription of the estrogen receptor alpha gene (8), thereby preventing induction of the oxytocin receptor gene and endometrial release of luteolytic pulses of prostaglandin F2α (5, 16). The antiluteolytic actions of IFNT are required for maintenance of a functional CL and secretion of progesterone, the essential hormone of pregnancy. IFNT also induces or stimulates expression of a number of genes, termed IFNT-stimulated genes or ISGs, in the endometrium that are hypothesized to play important biological roles in uterine receptivity and conceptus implantation (227). In the ovine uterus, most ISGs are induced or increased in the endometrial stroma and middle to deep GE. Indeed, *LGALS15* (galectin-15) (157), *WNT7A* (wingless-type MMTV integration site family, member 7A) (156), and *CTSL* (250) are the only genes known to be induced or increased by IFNT in endometrial LE and sGE.

Therefore, these studies were conducted to determine if the *CST3* gene is expressed in the ovine uterus and to determine effects of the estrous cycle, pregnancy, progesterone and IFNT on *CST3* gene expression in the endometrium and conceptus. The results indicate that *CST3* is expressed coordinately with *CTSL* in the endometrial LE and GE and conceptus during the peri-implantation period of pregnancy. Further, *CST3* is a novel progesterone-induced and IFNT-stimulated gene in the endometrial LE and sGE.

Materials and Methods

Animals

Mature crossbred Suffolk ewes (*Ovis aries*) were observed daily for estrus in the presence of vasectomized rams and used in experiments only after they had exhibited at least two estrous cycles of normal duration (16-18 days). All experimental and surgical

procedures were in compliance with the Guide for the Care and Use of Agriculture Animals and approved by the Institutional Animal Care and Use Committee of Texas A&M University.

Experimental Design

Study One. At estrus (Day 0), ewes were mated to either an intact or vasectomized ram and then hysterectomized (n = 5 ewes/day) on either Day 10, 12, 14 or 16 of the estrous cycle or Day 10, 12, 14, 16, 18 or 20 of pregnancy as described previously (253). At hysterectomy, the uterus was flushed with 20 ml of sterile saline. Pregnancy was confirmed on Days 10 to 16 post-mating by the presence of a morphologically normal conceptus(es) in the uterine flush. It was not possible to obtain uterine flushes on either Day 18 or Day 20 of pregnancy, because the conceptus is firmly adhered to the endometrial luminal epithelium (LE) and basal lamina. At hysterectomy, several sections (~0.5 cm) from the mid-portion of each uterine horn ipsilateral to the CL were fixed in fresh 4% paraformaldehyde in PBS (pH 7.2). After 24 h, fixed tissues were changed to 70% ethanol for 24 h, dehydrated through a graded series of alcohol to xylene, and then embedded in Paraplast-Plus (Oxford Labware, St. Louis, MO). Several sections (1–1.5 cm) from the middle of each uterine horn were embedded in Tissue-Tek OCT compound (Miles, Oneonta, NY), frozen in liquid nitrogen vapor, and stored at -80°C. The remaining endometrium was physically dissected from myometrium, frozen in liquid nitrogen, and stored at -80°C for subsequent RNA or protein extraction. In monovulatory pregnant ewes, uterine tissue samples were marked as either contralateral or ipsilateral to the ovary bearing the CL and only tissues from the ipsilateral uterine horn were used in subsequent analyses. Uterine flushes were clarified by centrifugation (3,000 x *g* for 30 min at 4°C) and frozen at -80°C for western blot analysis.

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 (19). Ewes were then assigned randomly (n=5 per treatment) to receive daily intramuscular (i.m.) injections of progesterone (P4) and/or a progesterone receptor

(PGR) antagonist (ZK 136,317; Schering AG, Germany) and intrauterine (i.u.) infusions of control serum proteins and/or recombinant ovine IFNT protein as follows: (1) 50 mg progesterone (P4, Days 5 to 16) and 200 μg control (CX) serum proteins (Days 11 to 16) [P4+CX]; (2) P4 and 75 mg ZK 136,317 (Days 11 to 16) and CX proteins [P4+ZK+CX]; (3) P4 and IFNT (2 x 10^7 antiviral units, Days 11 to 16) [P4+IFN]; or (4) P4 and ZK and IFNT [P4+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 roIFNT ($5x10^6$ antiviral units/ horn/ injection). The roIFNT was produced in *Pichia pastoris* and purified as described previously (253). Proteins were prepared for intrauterine injection as described previously (19). This regimen of progesterone and roIFNT mimics the effects of progesterone and the conceptus on endometrial expression of hormone receptors and IFNT-stimulated genes during early pregnancy in ewes (14, 141, 156, 254). All ewes were hysterectomized on Day 17, and uteri and endometria processed as described for Study One.

RNA Isolation

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

Cloning of Partial cDNA for Ovine CST3

Partial cDNA for ovine *CST3* mRNA was amplified by RT-PCR using total RNA from Day 18 pregnant ovine endometrial tissues by specific primers based on the bovine *CST3* mRNA (Genbank accession no. NM_174029; forward, 5'-CTG TCC TTT GCG GTC AGC-3'; reverse, 5'-CCT GGC AGC TAA ACT TCA CC-3'). PCR amplification was conducted as follows for ovine *CST3*: 1) 95°C for 5 min; 2) 95°C for 45 sec, 56.5°C for 1 min, and 72°C for 1 min for 35 cycles; and 3) 72°C for 10 min. The partial cDNAs

for *CST3* were cloned into pCRII using a T/A Cloning Kit (Invitrogen) and sequence verified using an ABI PRISM Dye Terminator Cycle Sequencing Kit and ABI PRISM automated DNA sequencer (Perkin-Elmer Applied Biosystems).

Slot Blot Hybridization Analyses

Steady-state levels of mRNA in ovine endometria were assessed by slot blot hybridization as described previously (255). Antisense *CST3* cRNA probes were generated by linearizing the pCR II-CST3 plasmid with *Bam*HI and *in vitro* transcription with T7 RNA polymerase and sense cRNA probes were generated using *Xba*I and SP6 RNA polymerase. And then, radiolabeled antisense and sense cRNA probes were generated by *in vitro* transcription with [α-^{32}P]-UTP. Denatured total endometrial RNA (20 µg) from each ewe was hybridized with radiolabeled cRNA probes. To correct for variation in total RNA loading, a duplicate RNA slot membrane was hybridized with radiolabeled antisense 18S cRNA (pT718S; Ambion, Austin, TX). Following washing, the blots were digested with ribonuclease A and radioactivity associated with slots quantified using a Typhoon 8600 MultiImager (Molecular Dynamics, Piscataway, NJ).

In Situ *Hybridization Analyses*

Location of mRNA expression in sections (5 µm) of the ovine uterine endometrium was determined by radioactive *in situ* hybridization analysis as described previously (255). Briefly, deparaffinized, rehydrated and deproteinated uterine tissue sections were hybridized with radiolabeled antisense or sense cRNA probes generated from linearized ovine *CST3* partial cDNA using *in vitro* transcription with [α-^{35}S]-UTP. After hybridization, washing and ribonuclease A digestion, slides were then dipped in NTB-2 liquid photographic emulsion (Kodak, Rochester, NY), and exposed at 4°C for one week. Slides were developed in Kodak D-19 developer, counterstained with Gill's hematoxylin (Fisher Scientific, Fairlawn, NJ), dehydrated through a graded series of alcohol to xylene, and coverslips affixed with Permount (Fisher). Images of representative fields were recorded under brightfield or darkfield illumination using a

Nikon Eclipse 1000 photomicroscope (Nikon Instruments Inc., Lewisville, TX) fitted with a Nikon DXM1200 digital camera.

Immunohistochemistry

Immunocytochemical localization of CST3 protein in the ovine uterus was performed as described previously (253) using anti-human CST3 polyclonal antibody (Catalog number 06-458; Upstate, Lake Placid, NY) at a 1:2,000 dilution (0.5µg/ml). Antigen retrieval was performed by using Pronase E digestion and 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) and protein content was determined using the Bradford protein assay (Bio-Rad, Hercules, CA) with bovine serum albumin (BSA) as the standard. Proteins were denatured and separated by 15% SDS-PAGE, and Western blot analyses conducted as described previously (253) using enhanced chemiluminescence detection (SuperSignal West Pico, Pierce, Rockford, IL) and X-OMAT AR X-ray film (Kodak, Rochester, NY) according to the manufacturer's recommendations. Immunoreactive CST3 protein was detected by using the rabbit anti-human CST3 polyclonal antibody (Upstate, Lake Placid, NY) at a 1:10,000 (0.1µg/ml) dilution. Negative control blots were performed by replacing the primary antibody with rabbit IgG at the same concentration.

Statistical Analyses

All quantitative data were subjected to least squares analyses of variance (ANOVA) using the General Linear Models (GLM) procedures of the Statistical Analysis System (SAS Institute, Cary, NC). Slot blot hybridization data were corrected for differences in sample loading using the 18S rRNA data as a covariate. Data from Study One were analyzed for effects of day, pregnancy status (cyclic or pregnant), and

their interaction. Next, least squares regression ANOVA was conducted within pregnancy status. Orthogonal contrasts were used to determine effects of treatment in Study Two. 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 0.10 or less was considered significant. Data are presented as least-square means (LSM) with standard errors (SE).

Results

Effects of Estrous Cycle and Early Pregnancy on Expression of CST3 mRNAs in the Ovine Endometrium (Study One)

Steady-state levels of ovine *CST3* mRNAs in endometria from cyclic (C) and pregnant (P) ewes were determined by slot blot hybridization analyses (Fig. 4.1) and found to be affected ($P<0.05$) by day, status, and their interaction. In cyclic ewes, endometrial *CST3* mRNA was low to undetectable on Day 10, increased (quadratic effect of day, $P<0.05$) about 12-fold from Day 10 to Day 12, and then decline to Day 16. In pregnant ewes, *CST3* mRNA levels were also low to undetectable on Day 10, but then increased (linear effect of day, $P<0.01$) about 130-fold between Days 10 and 20.

In situ hybridization analyses to determine the location of *CST3* mRNA in the uterus (Fig. 4.2) revealed that it present only in LE and GE of the endometrium. No hybridization signal was detected in endometrial stroma, myometrium, blood vessels or immune cells. In cyclic ewes, *CST3* mRNA appeared in LE and sGE of the endometrium between Days 10 and 12, but decreased thereafter. In pregnant ewes, *CST3* mRNA was also detected in endometrial LE and sGE between Days 10 and 12. Between Days 12 and 20 of pregnancy, *CST3* mRNA was abundant in the endometrial LE and sGE and also increased in the middle to deep GE by Day 20. In addition, *CST3* mRNA was abundant in the conceptus trophectoderm on Days 18 and 20 of pregnancy.

Consistent with results from *in situ* hybridization analyses, immunoreactive CST3 protein was detected in endometrial LE, sGE and conceptus trophectoderm (Fig. 4.3A). In pregnant ewes, CST3 protein increased after Day 10 and was concentrated

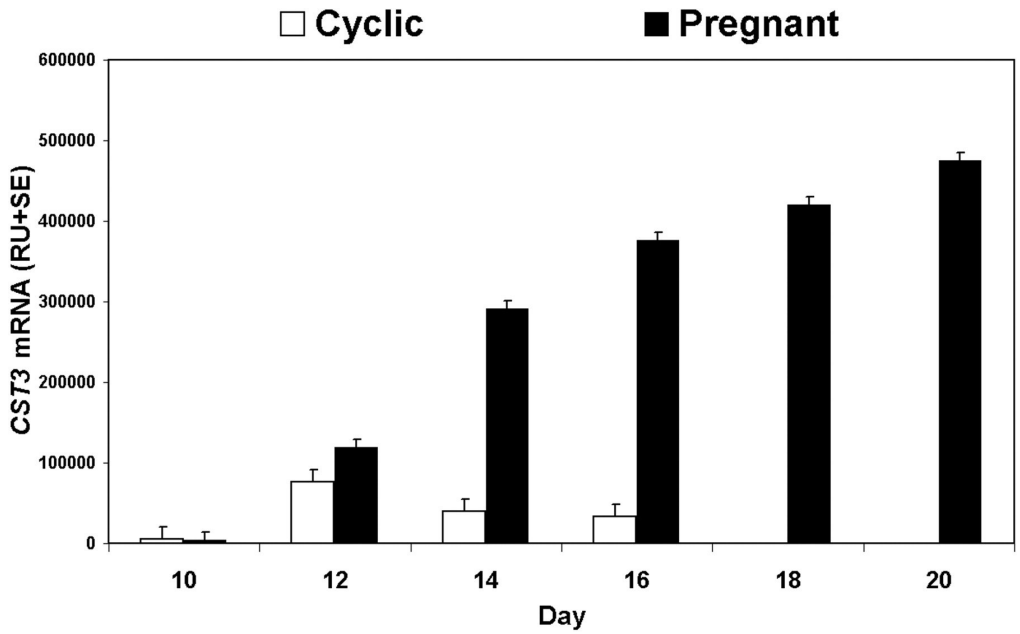

Fig. 4.1. Steady-state levels of *CST3* mRNAs in endometria from cyclic and early pregnant ewes determined by slot blot analysis. In cyclic ewes, *CST3* mRNA was low on Day 10, increased to Day 12 and decreased thereafter (quadratic effect of day, $P<0.05$). In pregnant ewes, *CST3* mRNA was lowest on Day 10 and increased 130-fold between Days 10 and 20 (linear effect of day, $P<0.01$). Data are expressed as LSM relative units (RU) with standard error (SE).

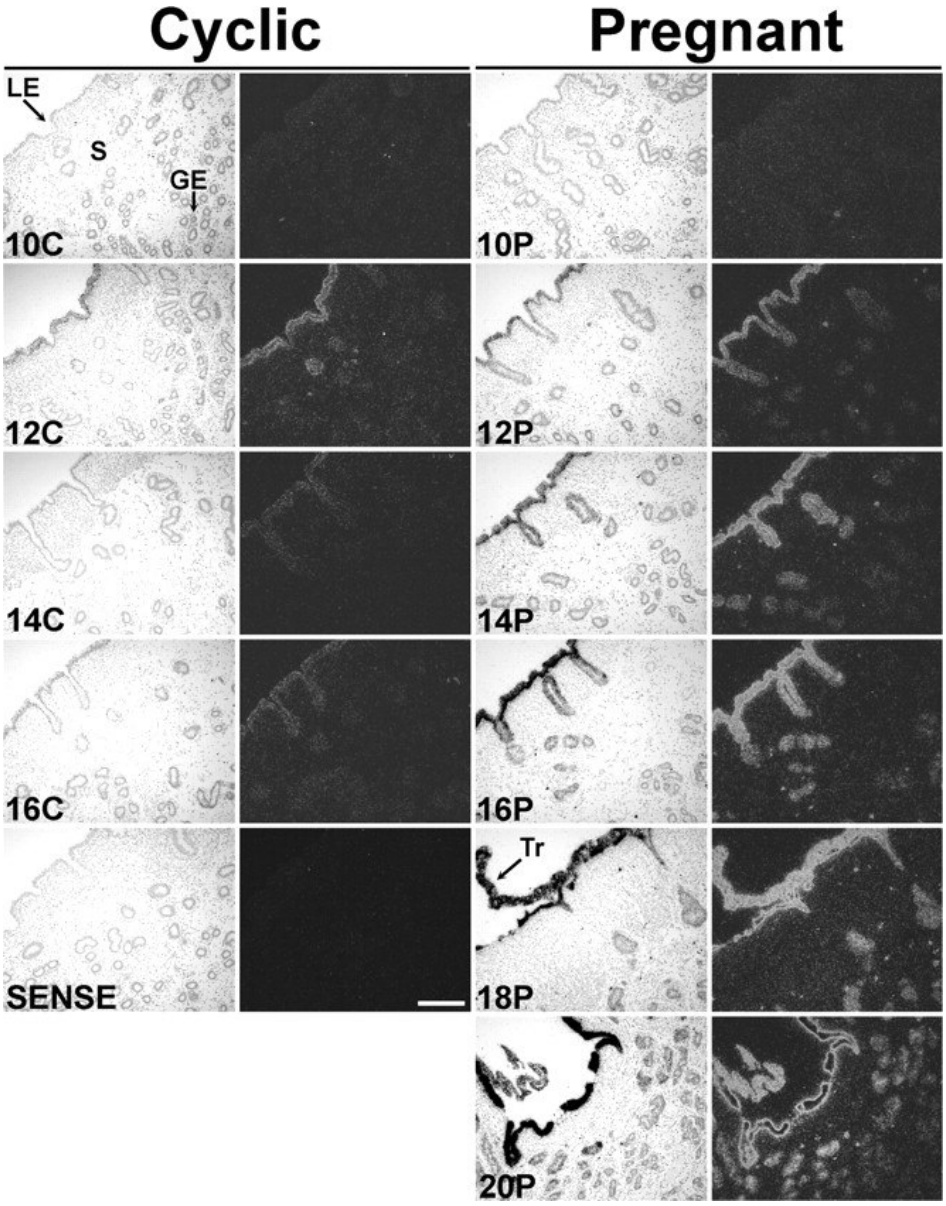

Fig. 4.2. *In situ* hybridization analyses of *CST3* mRNAs in uteri of cyclic and early pregnant ewes. Cross-sections of the uterine wall from cyclic (C) and pregnant (PX) ewes were hybridized with radiolabeled antisense or sense ovine *CST3* cRNA probes. *CST3* mRNA was detected only in endometrial LE and GE, as well as trophectoderm of the conceptus. Legend: LE, luminal epithelium; GE, glandular epithelium; S, stroma; Tr, trophectoderm. Scale bar represents 10 μm.

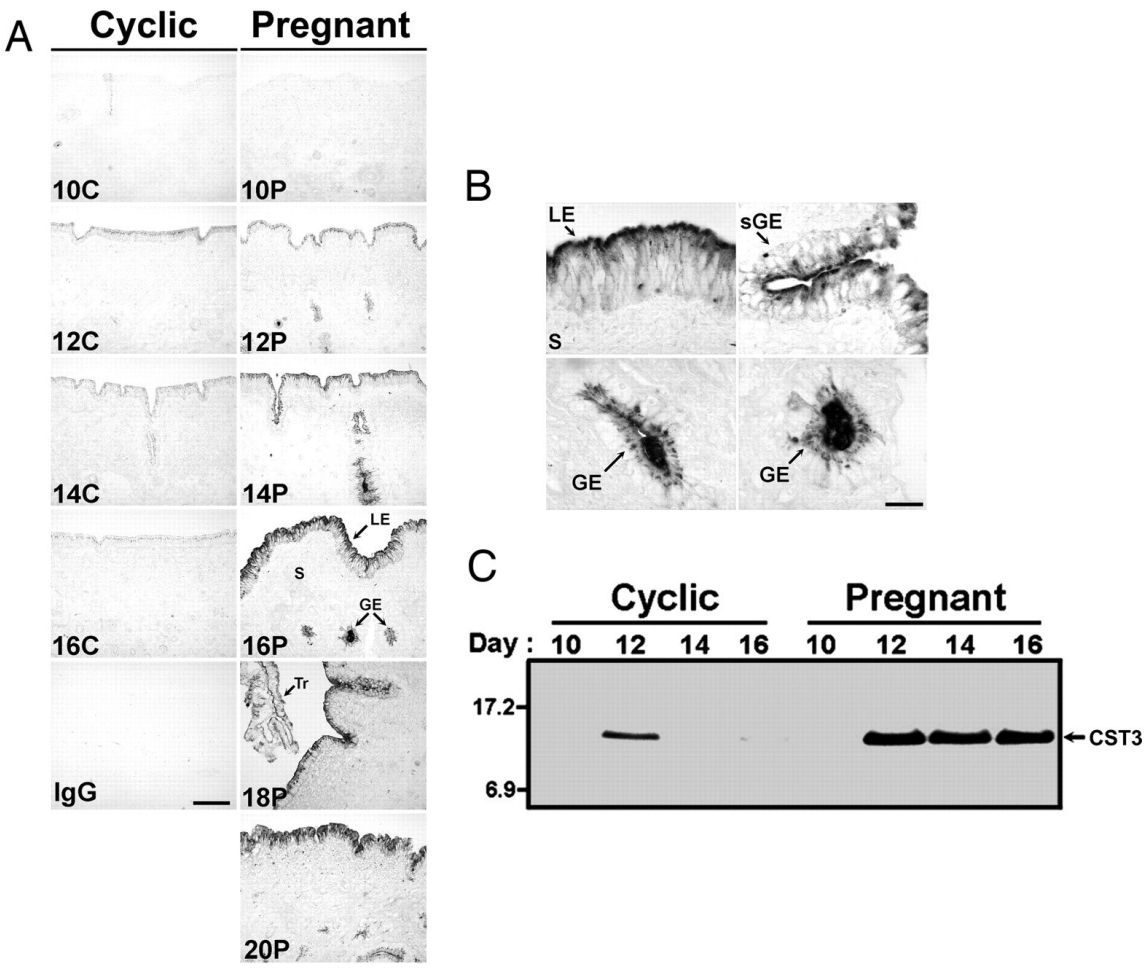

Fig. 4.3. CST3 protein in endometria and uterine flushings from cyclic and pregnant ewes from Study One. (A) Immunoreactive CST3 protein was localized using a rabbit anti-human CST3 polyclonal antibody. For the IgG control, normal rabbit IgG was substituted for the primary antibody. Sections were not counterstained. Legend: LE, luminal epithelium; GE, glandular epithelium; S, stroma; Tr, trophectoderm. Scale bar represents 5 µm. (B) Immunoreactive CST3 protein was localized predominantly near the apical surface of endometrial LE and sGE and was detected in secretions in the lumen of the upper endometrial glands. Sections were not counterstained. Legend: LE, luminal epithelium; GE, glandular epithelium; S, stroma. Scale bar represents 1.25 µm. (C) Representative Western blot analysis of CST3 in uterine flushings. Proteins in uterine flushings were separated by 15% SDS-PAGE (10µg/lane), and immunoreactive CST3 protein detected using rabbit anti-human CST3 polyclonal antibody. Positions of prestained molecular weight standards (x 10-3) are indicated.

near or on the apical surface of LE and sGE as well as in secretions of the uterine glands (Fig. 4.3B). The rabbit anti-human CST3 polyclonal antibody used in these studies detected a single immunoreactive protein of approximately 14 kDa in Western blot analyses of proteins in uterine extracts from cyclic and pregnant ewes (Fig. 4.3C). Consistent with immunohistochemical studies, CST3 protein was detected predominantly in uterine flushes from Day 12 cyclic ewes. In uterine flushes of pregnant ewes, CST3 protein was not detected on Day 10, but was abundant thereafter. Collectively, the temporal and spatial alterations in CST3 mRNA and protein expression during the estrous cycle and early pregnancy are consistent with its regulation by ovarian progesterone and the conceptus.

Progesterone and IFNT Regulate Endometrial CST3 Expression (Study Two)

In order to determine if progesterone (P4) and IFNT regulate endometrial *CST3* gene expression, cyclic ewes were ovariectomized and fitted with indwelling uterine catheters on Day 5 and then treated with progesterone (P4) or P4 and ZK 136,317 anti-progestin (P4+ZK) and infused with control proteins (CX) or roIFNT (see Fig. 4.4A). Slot blot analyses of endometrium found that treatment of ewes with P4 induced a 14-fold increase in *CST3* mRNA (P4+CX vs P4+ZK+CX, $P<0.001$; Fig. 4B). Moreover, intrauterine infusions of roIFNT stimulated a further 2-fold increase in *CST3* mRNA (P4+CX vs P4+IFN, $P<0.01$). However, roIFNT did not stimulate *CST3* mRNA in endometria of ewes receiving the ZK anti-progestin (P4+IFN vs P4+ZK+IFN, $P>0.10$). *In situ* hybridization (Fig. 4C) and immunohistochemistical (Fig. 4D) analyses detected *CST3* mRNA and protein only in endometrial LE and sGE of ewes treated with P4 (P4+CX and P4+IFN).

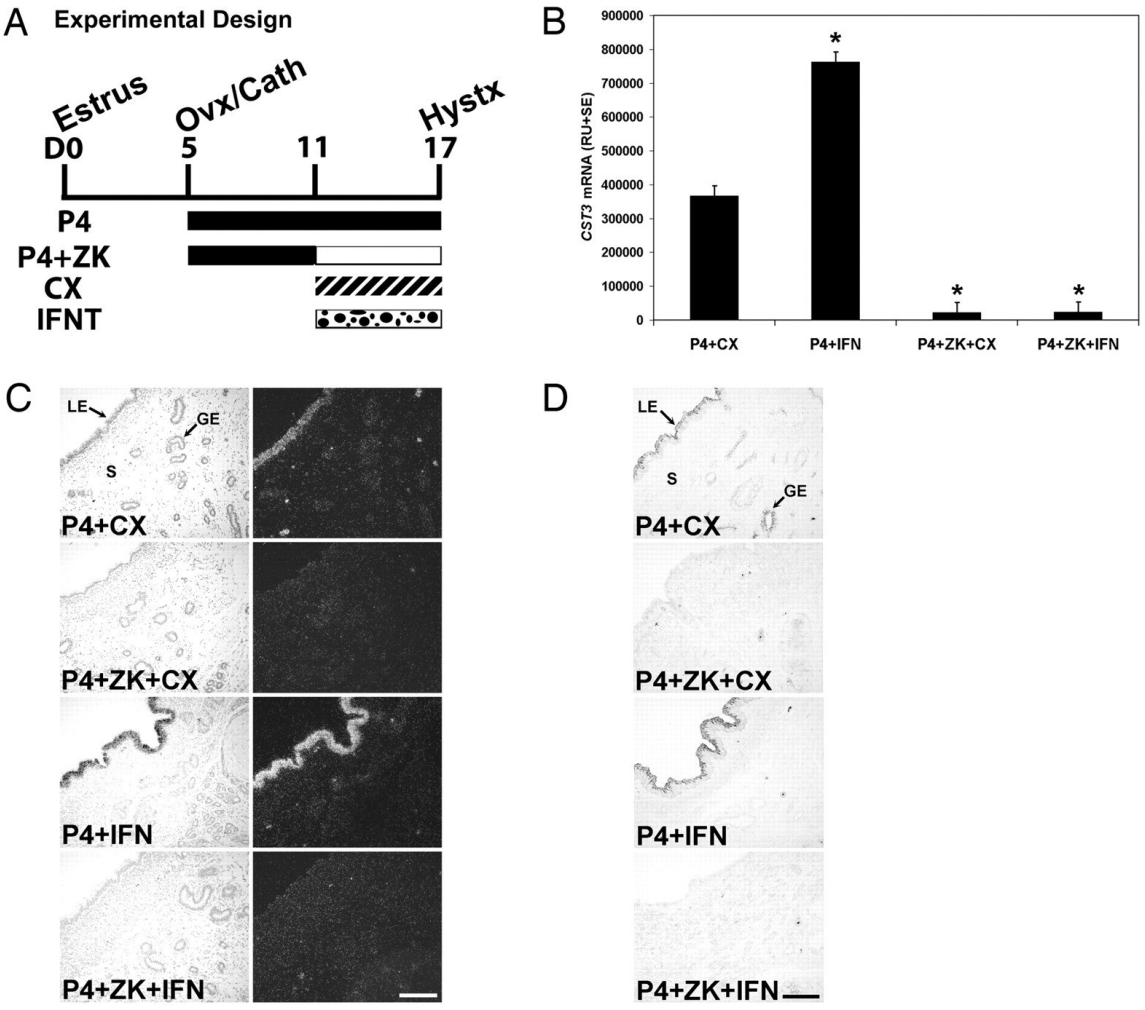

Fig. 4.4. Effects of progesterone and IFNT on *CST3* mRNA and protein in the ovine uterus (Study Two). (A) Experimental design. See Materials and Methods for complete description. Legend: CX, control serum proteins; Hystx, hysterectomy; Ovx/Cath, ovariectomy and uterine catheterization; P4, progesterone; IFNT, recombinant ovine interferon tau; ZK, ZK137,316 anti-progestin. (B) Steady-state levels of *CST3* mRNA in endometria as determined by slot blot hybridization analysis. Treatment of ewes with P4 increased *CST3* mRNA in the endometrium (P4+CX vs P4+ZK+CX, $P<0.001$). Intrauterine infusion of IFNT stimulated *CST3* mRNA in endometria of ewes treated with P4 (P4+CX vs P4+IFN, $P<0.01$), but not in ewes receiving P4 and the ZK anti-progestin (P4+IFN vs P4+ZK+IFN, $P>0.10$). (C) In situ hybridization analysis of *CST3* mRNA expression. Cross-sections of the uterine wall from treated-ewes were hybridized with radiolabeled antisense or sense ovine *CST3* cRNA probes. Scale bar represents 10 μm. (D) Immunoreactive CST3 protein in the uterus. Sections were not counterstained. Legend: LE, luminal epithelium; GE, glandular epithelium; S, stroma. Scale bar represents 5 μm.

Discussion

In the present study, temporal changes in expression of endometrial *CST3* mRNA in cyclic and pregnant ewes supported the hypothesis that ovarian progesterone is required to induce transcription of the *CST3* gene in endometrial LE and sGE. The increase in *CST3* mRNA in LE and sGE, between Days 10 and 12 post-estrus/mating, is coincident with the disappearance of progesterone receptor (*PGR*) mRNA and protein in these epithelia (9, 15). Similarly, the decrease in *CST3* mRNA between Days 14 and 16 of the cycle is coincident with the reappearance of PGR protein in endometrial LE due to regression of the CL and loss of progesterone. In Study Two, *CST3* mRNA was detected in endometrial LE and sGE of ovariectomized ewes treated with progesterone for 12 days, but this expression was prevented by administration of the PGR antagonist ZK 136,317. Continuous exposure of the sheep uterus to progesterone for about 10 days down-regulates PGR expression in endometrial LE and sGE, but not in stroma or myometrium (69). PGR are present in endometrial epithelia of P4+ZK-treated sheep (69), because PGR antagonists prevent the inhibitory effects of progesterone on expression of the *PGR* gene. Consequently, progesterone modulation of *CST3* mRNA may be attributed, at least in part, to down-regulation of PGR by progesterone that occurs in LE and sGE between Days 10 and 12 of the cycle and pregnancy (2, 17). Thus, PGR loss in endometrial epithelia may reprogram these cells, allowing them to increase expression of genes associated with implantation

(17). Alternatively, progesterone may act on PGR-positive stromal cells to induce growth factors or changes in the ECM that regulate expression of selected epithelial genes (17).

In addition to regulation by progesterone, results of the present studies indicate that *CST3* expression is further regulated by IFNT. IFNT is the pregnancy recognition hormone in sheep that acts on the endometrium to prevent development of the luteolytic mechanism, thereby maintaining the CL and its production of progesterone (5, 86). Of particular note, *CST3* is a novel gene stimulated by IFNT in endometrial LE and sGE as expression between Days 10 and 18 of early pregnancy parallels the increase in

production of IFNT by the elongating conceptus, which is maximal on Day 16 (256, 257). In Study Two, intrauterine administration of IFNT increased *CST3* mRNA, but only in progesterone-treated ewes. One hypothesis is that IFNT can only stimulate transcription of the *CST3* gene in the absence of liganded PGR, i.e., after down-regulation of PGR by progesterone. Alternatively, the PGR-positive stroma may produce a 'progestamedin', e.g., fibroblast growth factor 7 (FGF7), FGF10 or hepatocyte growth factor (HGF) , that could be required for LE and sGE to respond to IFNT (1, 17, 156). The signaling pathway whereby IFNT regulates transcription of the *CST3* gene is not known, but it clearly does not involve the classical JAK-STAT-IRF (IFN regulatory factor) signaling pathway (125). The 5' flanking promoter/enhancer region of the bovine *CST3* gene (Genbank NW_928624) does not have any predicted transcription factor binding sites for classical ISGs, such as gamma activation sequence elements for STAT1 binding, IFN-stimulated response elements for ISGF3, or IRF response elements; however, the region does have several predicted PGR response elements (data not shown). To date, *CTSL, WNT7A* and *LGALS15* are the only other genes identified in endometrial LE and sGE that are induced or stimulated by IFNT (156, 157, 250). Thus, the diverse actions of IFNT on the endometrium include repression of genes, including ESR1 (estrogen receptor alpha), to abrogate development of the endometrial luteolytic mechanism, as well as stimulation of genes that are potentially critical to implantation, placentation and conceptus growth and development (17). Knowledge of mechanisms whereby IFNT stimulates *CST3* gene expression in endometrial LE and sGE is expected to help unravel a non-classical signaling pathway for Type I IFNs.

CST3 is an inhibitor of cysteine proteases, e.g. CTSB and CTSL, that have biological roles in the processing and catabolism of proteins (249). Results of the present studies of CST3 in the ovine uterus are similar to those for mice (183), in which expression of CTSL and CTSB by invasive trophoblast giant cells was balanced by coordinated expression of CST3 in the decidualizing stroma at the implantation site. Coordinated increases in CTSL and CTSB with CST3 occur in endometrial LE and sGE as well as in conceptus trophectoderm during early pregnancy (250). Thus, one

biological role of CST3 may be to inhibit the actions of cysteine proteases produced by the conceptus and endometrial epithelia in order to limit the invasive activity of the trophoblast. These results support the general idea that proteases and their inhibitors expressed at the maternal-fetal interface are important for uterine receptivity, endometrial remodeling and conceptus implantation during pregnancy in mammals (219, 220, 246-248). Interestingly, cathepsins and cystatins have recently been implicated in recurrent miscarriage in women (258) who had higher than normal decidual levels of CTSB and CTSH and lower than normal levels of serum CST3. Thus, increased knowledge of uterine proteases and their inhibitors is important for developing therapeutic strategies to prevent, treat and diagnose infertility in humans and domestic animals.

CHAPTER V
PREGNANCY AND INTERFERON TAU REGULATE RSAD2 AND IFIH1 EXPRESSION IN THE OVINE UTERUS

Introduction

Interferon tau (IFNT), the maternal recognition of pregnancy signal in ruminants (sheep, cattle, goats), is secreted by the elongating peri-implantation conceptus (embryo/fetus and associated membranes) and inhibits development of the endometrial luteolytic mechanism (1, 18). IFNT is produced by sheep conceptuses between Days 10 and 21 of gestation with maximal production on Days 14 to 16 (4, 95). During pregnancy recognition, IFNT acts in a paracrine fashion on endometrial luminal epithelium (LE) and superficial ductal glandular epithelium (sGE) of the ovine uterus to repress transcription of the estrogen receptor alpha gene (8, 96), thereby preventing estrogen induction of expression of the oxytocin receptor gene (16) which precludes oxytocin-induced endometrial release of luteolytic pulses of prostaglandin F2 alpha (18). The antiluteolytic actions of IFNT allow maintenance of a functional corpus luteum and secretion of progesterone (P4), which is the hormone of pregnancy necessary for successful implantation and development of the conceptus to term (18). In addition to antiluteolytic effects on the endometrium, IFNT induces a number of IFN-stimulated genes (ISGs) in a cell-specific manner within the endometrium, and ISGs are hypothesized to play important roles in uterine receptivity and conceptus implantation during establishment of pregnancy (18, 227, 259, 260). Several ISGs are first induced by progesterone and stimulated by IFNT, whereas other genes are stimulated by IFNT from the conceptus in a progesterone-independent manner (259).

Recent transcriptional profiling experiments identified *RSAD2* and *IFIH1* as genes induced by IFNT from the conceptus in ovine and bovine endometria during early pregnancy (259, 260). Radical S-adenosyl methionine domain containing 2 (*RSAD2*; alias viperin) is a cytoplasmic antiviral protein induced by Type I IFNs that can inhibit infection of cells with human cytomegalovirus (167). Interferon-induced with helicase

C domain 1 (*IFIH1*; alias *MDA5*) is a RNA helicase that, through its ATP-dependent unwinding of RNA, promotes mRNA degradation by specific RNases and is involved in innate immune defense against viruses as well as cellular growth suppression (171, 172). IFIH1 senses intracellular viral infection and triggers innate antiviral responses including the production of Type I IFNs (173). Both *RSAD2* and *IFIH1* are produced during a viral infection in response to IFNs to limit viral replication and modulate subsequent adaptive immunity (170, 261). Similar to other Type I IFNs, IFNT elicits antiviral, antiproliferative, and immunomodulatory activities in homologous and heterologous cells (66, 68, 84, 138, 161, 163, 262, 263). Induction of an antiviral state in the endometrium during early pregnancy may be beneficial by inhibiting sexually transmitted viruses as well as modulating local immune cells to promote tolerance of the allogeneic conceptus and stimulating production of cytokines beneficial for conceptus survival and growth (27-29).

Although *RSAD2* and *IFIH1* have been identified as pregnancy- and IFNT-stimulated genes in the ovine uterine endometrium, the temporal and spatial alterations in their expression in the endometrium during early pregnancy and in response to progesterone and IFNT have not been investigated. Our working hypothesis that RSAD2 and IFIH1 are induced in the endometrium in a cell type-specific manner by IFNT from the conceptus during early pregnancy and have biological roles in establishing uterine receptivity to implantation by the conceptus. As first step in testing this hypothesis, studies were conducted to determine effects of: (1) stage of the estrous cycle and early pregnancy on *RSAD2* and *IFIH1* expression in the ovine uterus; (2) progesterone and IFNT on *RSAD2* and *IFIH1* expression in the ovine uterus; and (3) IFNT on *RSAD2* and *IFIH1* expression in ruminant endometrial cell lines.

Materials and Methods

Animals

Mature crossbred Suffolk sheep (*Ovis aries*) were observed daily for estrus in the presence of vasectomized rams and used in the experiment after they exhibited at least

two estrous cycles of normal duration (16-18 days). At estrus, ewes were assigned randomly to cyclic or pregnant status. All experimental and surgical procedures were in compliance with the Guide for the Care and Use of Agriculture Animals in Teaching and Research and were approved by the Institutional Animal Care and Use Committee of Texas A&M University.

Experimental Design

Study One. At estrus (Day 0), ewes were mated to either an intact or vasectomized ram as described previously (253) and then hysterectomized (n = 5 ewes/day) on Day 10, 12, 14 or 16 of the estrous cycle or Day 10, 12, 14, 16, 18 or 20 of pregnancy. To confirm pregnancy status, the uterine lumen was flushed with saline on Days 10 to 16 of pregnancy and examined for the presence of a morphologically normal conceptus(es). At hysterectomy, several sections (~0.5 cm) from the mid-portion of each uterine horn ipsilateral to the corpus luteum were fixed in fresh 4% paraformaldehyde in PBS (pH 7.2). After 24 h, fixed tissues were changed to 70% ethanol for 24 h and then dehydrated and embedded in Paraplast-Plus (Oxford Labware, St. Louis, MO). Several sections (1–1.5 cm) from the middle of each uterine horn were embedded in Tissue-Tek OCT compound (Miles, Oneonta, NY), frozen in liquid nitrogen vapor, and stored at -80°C. The remaining endometrium was physically dissected from myometrium, frozen in liquid nitrogen, and stored at -80°C for subsequent RNA extraction. In monovulatory pregnant ewes, uterine tissue samples were marked as either contralateral or ipsilateral to the ovary bearing the corpus luteum; no tissues from the contralateral uterine horn were used for this study.

Study Two. Sixteen cyclic ewes were ovariectomized and fitted with intrauterine catheters on Day 5 post-estrus as described previously (259) and injected daily i.m. with 75 mg progesterone (P4) between Days 5 and 16. Ewes were then assigned randomly (n=5 ewes/treatment) to receive one of the following treatment regimens between Days 11 and 16: (1) P4 and daily intrauterine (IU) infusions of control serum proteins [P4+CX]; (2) P4 and 75 mg of ZK136,317 (Schering), a progesterone receptor (PGR)

antagonist and CX proteins [P4+ZK+CX]; (3) P4 and IU IFNT ($2x10^7$ antiviral units) [P4+IFN]; or (4) P4 and ZK and IU IFNT [P4+ZK+IFN]. The P4 and ZK 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 recombinant ovine IFNT ($5x10^6$ antiviral units/horn/injection with CX proteins). Recombinant ovine IFNT was produced in *Pichia pastoris* and purified as described previously (264). Proteins were prepared for intrauterine injection as described previously . This regimen of P4 and IFNT mimics the effects of P4 and the conceptus on endometrial expression of hormone receptors and IFNT-stimulated genes during early pregnancy in ewes (15, 141, 156). All ewes were hysterectomized on Day 17. The uterus was processed for histology and the endometrium obtained for RNA extraction as described in Study One.

Cell Culture

Immortalized ovine uterine endometrial LE cells were cultured as described previously (163). Bovine endometrial (BEND) cells (161) were kindly provided by Dr. Thomas R. Hansen (Colorado State University, Fort Collins). Ovine LE and BEND cells were maintained in 150 mm culture dishes containing DMEM with F-12 salts (DMEM-F12; Sigma-Aldrich Corp., St. Louis, MO) supplemented with 5% serum and antibiotics. When cells reached 70-80% confluency, they were treated with either IFNT (2×10^7 AVU/ml) or left untreated as a control for 24h in serum-free medium. The experiment was independently repeated three times in each cell type.

RNA Isolation

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

Cloning of Partial cDNAs for Ovine RSAD2 and IFIH1

Partial cDNAs for ovine *RSAD2* and *IFIH1* mRNAs were amplified by RT-PCR using total RNA endometrial tissues from Days 18 of pregnancy using specific primers based on human *RSAD2* mRNA (Genbank NM_080657; forward, 5'-GAG GCC AAG AAA GGT CTG C-3'; reverse, 5'-CCA AGA ACG CTT CAA ACT CC-3') and human *IFIH1* mRNA (Genbank AF095844; forward, 5'-TTC CGC AAA GAG TTC AAA CC-3'; reverse, 5'-AAT GTG TTC TTC GGG TTT GG-3'). The Reverse transcription of cellular total RNA into cDNA was performed as described previously(65). The PCR amplification was conducted as follows for *RSAD2* and *IFIH1*: 1) 95°C for 5 min; 2) 95°C for 30 sec, 56.5°C for 40 sec (for *RSAD2*), 57°C for 40 sec (for *IFIH1*), and 72°C for 1 min for 35 cycles; and 3) 72°C for 10 min. The partial cDNAs for ovine *RSAD2* and *IFIH1* PCR products were cloned into pCRII using a T/A Cloning Kit (Invitrogen) and their sequences verified using an ABI PRISM Dye Terminator Cycle Sequencing Kit and ABI PRISM automated DNA sequencer (Perkin-Elmer Applied Biosystems).

Slot Blot Hybridization Analyses

Steady-state levels of mRNA in ovine endometrium were assessed by slot blot hybridization as described previously (125, 254). For *RSAD2* and *IFIH1* antisense cRNA probes, the plasmids were linearized with *Xba*I and *in vitro* transcription was conducted with SP6 RNA polymerase. Sense cRNA probes were generated using *Bam*HI and T7 RNA polymerase. Radiolabeled antisense and sense cRNA probes were then generated by *in vitro* transcription with [α-^{32}P]-UTP. Denatured total endometrial RNA (20 μg) from each ewe was hybridized with radiolabeled cRNA probes. To correct for variation in total RNA loading, a duplicate RNA slot membrane was hybridized with radiolabeled antisense 18S cRNA (pT718S; Ambion, Austin, TX). Following washing, the blots were digested with ribonuclease A and radioactivity associated with slots quantified using a Typhoon 8600 MultiImager (Molecular Dynamics, Piscataway, NJ).

Semiquantitative RT-PCR Analysis

RSAD2 and *IFIH1* mRNA levels in immortalized ovine endometrial LE and BEND cells were assessed using semi-quantitative RT-PCR as described previously (65). Briefly, isolated total cellular RNA was treated with RQ1 RNase Free-DNase1 (Promega, Madison, WI) and then ethanol-precipitated. The cDNA was synthesized from total cellular RNA (5 µg) isolated from both cell-lines using random and oligo (dT) primers and SuperScript II Reverse Transcriptase (Life Technologies, Gaithersburg, MD). Newly synthesized cDNA was acid-ethanol precipitated, resuspended in 20 µl sterile water, and stored at -20°C. The cDNAs were diluted (1:10) in sterile water before use in PCR. The primers, PCR amplification and verification of their sequences were conducted as described in the section on cloning partial cDNAs. Housekeeping beta-actin (ACTB) primers were forward (5'-ATG AAG ATC CTC ACG GAA CG-3') and reverse (5'-GAA GGT GGT CTC GTG AAT GC-3'), which amplified a 270-base pair product. PCR amplification was conducted as follows for ACTB: 1) 95°C for 5 min; 2) 95°C for 30 sec, 57°C for 30 sec, and 72°C for 1 min for 25 cycles; and 3) 72°C for 10 min. After PCR, equal amounts of reaction product were analyzed using a 1.5% agarose gel, and PCR products were visualized using ethidium bromide staining. The amount of DNA present was quantified by measuring the intensity of light emitted from correctly sized bands under ultraviolet light using a ChemiDoc EQ system and Quantity One software (Bio-Rad, Hercules, CA).

In Situ *Hybridization Analyses*

Location of mRNA expression in uterine sections (5 µm) was determined by radioactive *in situ* hybridization analysis as described previously (125, 254). Briefly, deparaffinized, rehydrated and deproteinated uterine tissue sections were hybridized with radiolabeled antisense or sense cRNA probes generated from linearized *RSAD2* and *IFIH1* partial cDNAs using *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, Rochester, NY), and exposed at 4°C for 1 to 2 weeks. Slides were

developed in Kodak D-19 developer, counterstained with Gill's hematoxylin (Fisher Scientific, Fairlawn, NJ), and then dehydrated through a graded series of alcohol to xylene. Coverslips were then affixed with Permount (Fisher). Images of representative fields were recorded under brightfield or darkfield illumination using a Nikon Eclipse 1000 photomicroscope (Nikon Instruments Inc., Lewisville, TX) fitted with a Nikon DXM1200 digital camera.

Statistical Analyses

All quantitative data were subjected to least-squares analyses of variance (ANOVA) using the Statistical Analysis System (SAS Institute, Cary, NC). Slot blot hybridization data were corrected for differences in sample loading using the 18S rRNA data as a covariate. Data from Study One were analyzed for effects of day, pregnancy status (cyclic or pregnant), and their interaction. Data from Study Two were analyzed using orthogonal contrasts (P4+CX vs P4+IFN; P4+ZK+CX vs P4+ZK+IFN; and P4+CX vs P4+ZK+CX) to elucidate effects of treatment. Semi-quantitative RT-PCR data was analyzed using the ACTB data as a covariate. All tests of significance were performed using the appropriate error terms according to the expectation of the mean squares for error. A P-value of 0.05 or less was considered significant. Data are presented as least-square means (LSM) with standard errors (SE).

Results

RSAD2 and IFIH1 Expression Increases in the Endometrium by a Cell Type-Specific Manner

Expression levels of *RSAD2* and *IFIH1* mRNAs in the endometrium of cyclic ewes were low and not affected (P>0.10) by day (Fig. 5.1). In contrast, *RSAD2* mRNA increased (P<0.01, quadratic) about 6-fold between Days 12 and 16 and was maintained through Day 20 in pregnant ewes. Similarly, *IFIH1* mRNA increased (P<0.01, quadratic) about 2.5-fold between Days 12 and 16. The presence of a conceptus

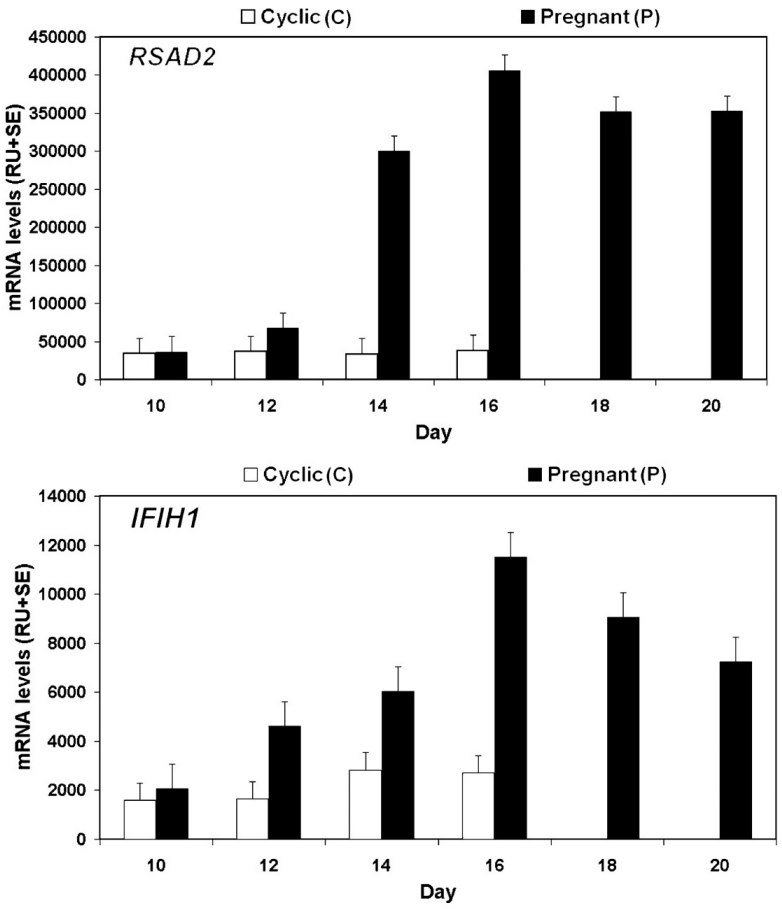

Fig. 5.1. Steady-state levels of *RSAD2* and *IFIH1* mRNAs in endometria from cyclic and early pregnant ewes determined by slot blot hybridization analysis. In cyclic ewes, *RSAD2* mRNA level was low between Days 10 and 16. In contrast, *RSAD2* mRNA increased ($P<0.01$) 6-fold between Days 12 and 16 and was maintained to Day 20. Similarly, *IFIH1* mRNA was very low in the endometria of cyclic ewes and increased ($P<0.01$) about 2.5-fold between Days 12 and 16 of pregnancy. Data are expressed as LSM relative units (RU) with standard error (SE).

increased endometrial *RSAD2* and *IFIH1* mRNA between Days 10 and 16 ($P<0.01$, day x status; Fig. 5.1).

In situ hybridization analyses determined the location of *RSAD2* (Fig 5.2) and *IFIH1* (Fig 5.3) mRNAs in uteri of cyclic and pregnant ewes. *RSAD2* mRNA was low and not different between uteri from Day 10 cyclic and pregnant ewes (Fig. 5.2). Between Days 10 and 12 of pregnancy, *RSAD2* mRNA increased in the middle glands and to a lower extent in the stratum compactum stroma. Between Days 14 and 20 of pregnancy, *RSAD2* mRNA was present predominantly in the endometrial glands, stroma and immune cells, but not in LE, sGE, myometrium or conceptus trophectoderm. Interestingly, *RSAD2* mRNA declined in the endometrial glands after Day 16 of pregnancy. Similar to *RSAD2, IFIH1* mRNA was low and not different between uteri from Day 10 cyclic and pregnant ewes (Fig. 5.3). Between Days 10 and 12 of pregnancy, *IFIH1* mRNA increased slightly in the middle glands and stratum compactum stroma. Between Days 14 and 18 of pregnancy, *IFIH1* mRNA was present predominantly in the stratum compactum stroma of the endometrium, middle glands and immune cells, and not observed in the endometrial LE, sGE, myometrium or conceptus trophectoderm. The melanocytes underneath the LE do not have *IFIH1* mRNA, but rather appear white in darkfield photomicrographs. Between Days 18 and 20, *IFIH1* mRNA abundance declined in the endometrial stroma. The presence of *RSAD2* and *IFIH1* mRNAs in immune cells within the endometrium was based on visual observations of cell morphology.

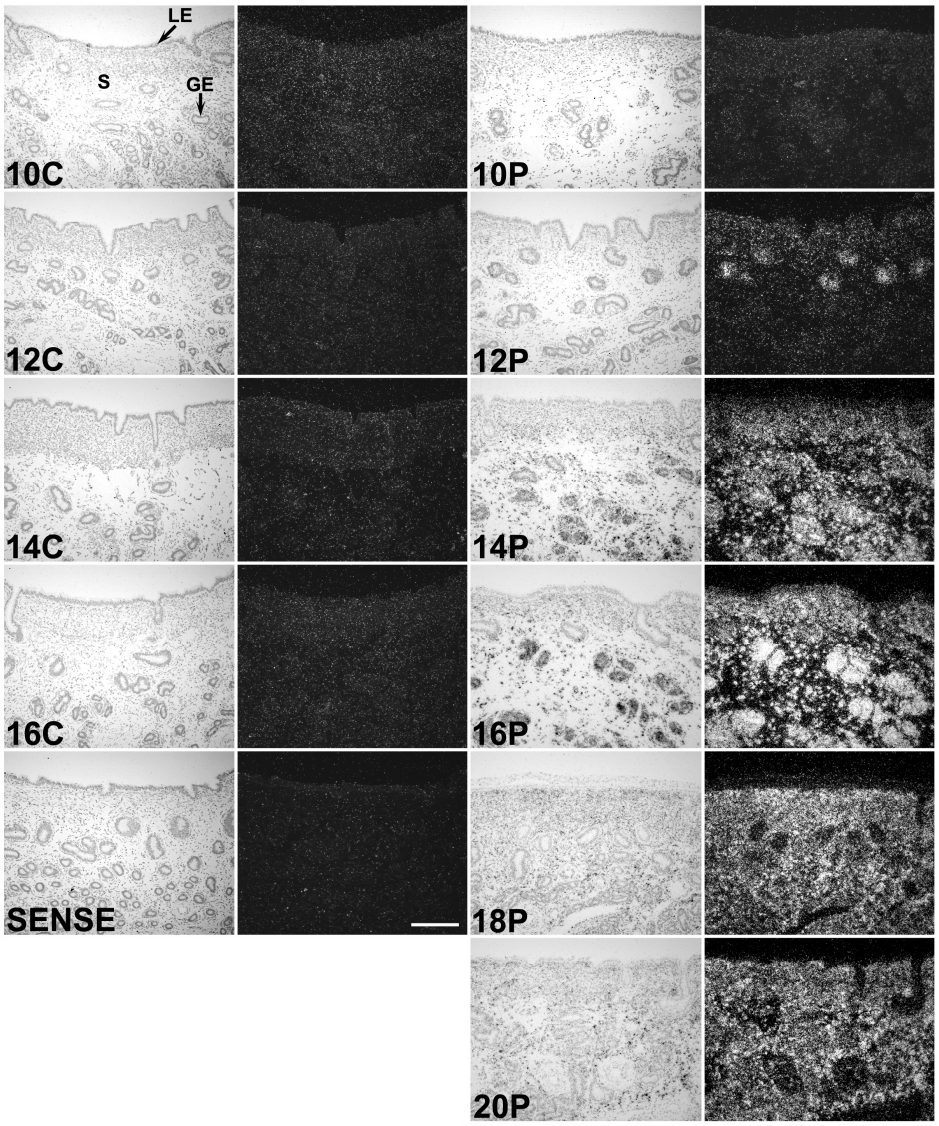

Fig. 5.2. *In situ* hybridization analyses of *RSAD2* mRNA in uteri of cyclic and pregnant ewes. Cross-sections of the uterine wall from cyclic (C) and pregnant (P) ewes were hybridized with radiolabeled antisense or sense ovine *RSAD2* cRNA probes. *RSAD2* mRNA is expressed in endometrial stroma, glands and resident immune cells. Legend: LE, luminal epithelium; GE, glandular epithelium; M, myometrium; S, stroma; Tr, trophectoderm. Scale bar represents 10 μm.

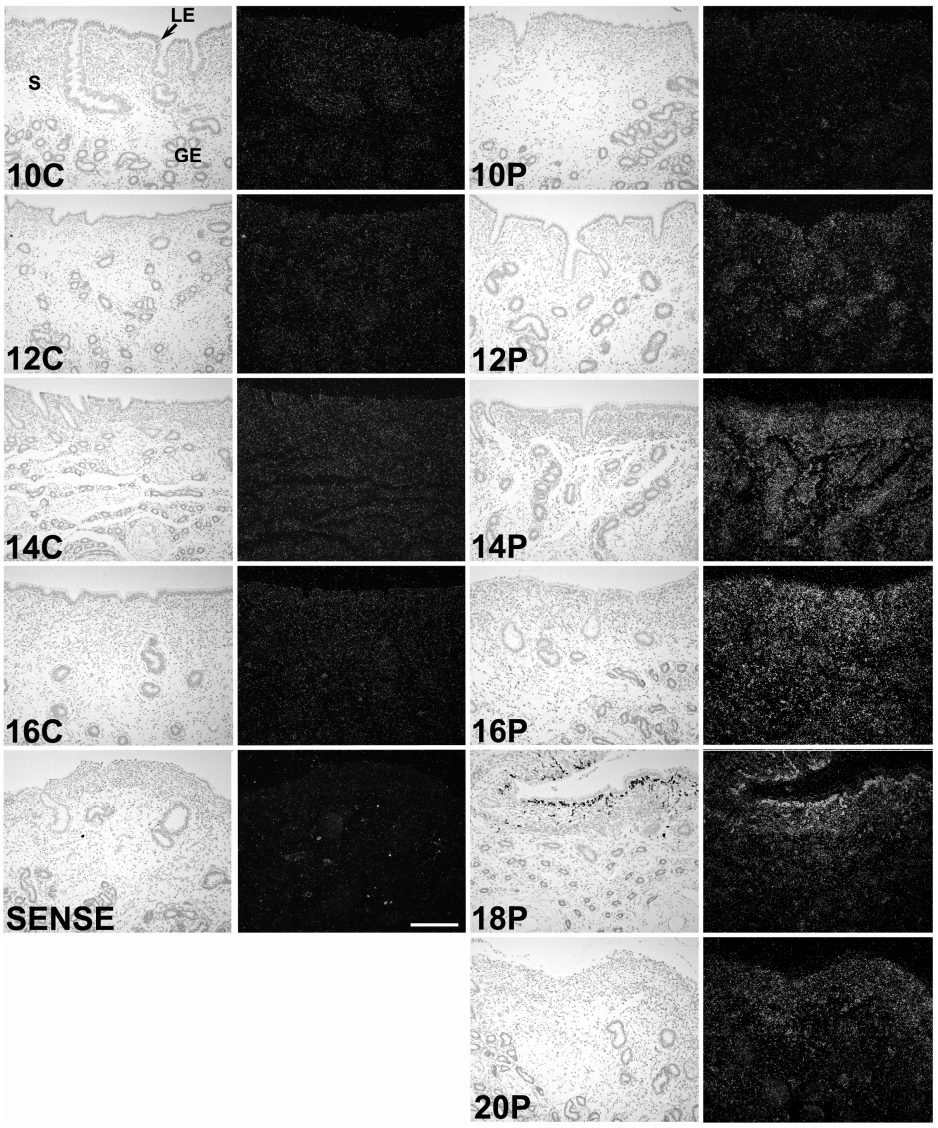

Fig. 5.3. *In situ* hybridization analyses of *IFIH1* mRNA in uteri of cyclic and pregnant ewes. Cross-sections of the uterine wall from cyclic (C) and pregnant (P) ewes were hybridized with radiolabeled antisense or sense ovine *IFIH1* cRNA probes. *IFIH1* mRNA was detected only in endometrial stroma and glands. Legend: LE, luminal epithelium; GE, glandular epithelium; S, stroma; Tr, trophectoderm. Scale bar represents 10 μm.

Intrauterine Administration of Recombinant Ovine IFNT Induces RSAD2 and IFIH1 mRNA in the Ovine Endometrium

In order to determine if differences in expression of the selected genes in endometrium of pregnant compared to cyclic ewes was due to IFNT from the conceptus, cyclic ewes were ovariectomized and fitted with intrauterine (i.u.) catheters on Day 5 and hysterectomized on Day 17 (see Fig. 5.4A). Treatment of ewes with the ZK 136,317 PGR antagonist did not affect ($P>0.10$, P4+CX vs P4+ZK+CX) endometrial *RSAD2* or *IFIH1* mRNA abundance (Fig. 5.4B). For ewes receiving P4 alone, intrauterine recombinant ovine IFNT increased ($P<0.001$) steady-state levels of *RSAD2* and *IFIH1* mRNAs 10-fold and 8.3-fold, respectively, in the endometria ($P<0.001$, P4+CX vs P4+IFN) (Fig. 5.4B and 5.5A). Similarly, for ewes receiving P4+ZK treatment, intrauterine recombinant ovine IFNT increased *RSAD2* and *IFIH1* mRNAs in the endometrium about 9.3-fold and 5.6-fold, respectively ($P<0.001$, P4+ZK+CX vs P4+ZK+IFN).

In situ hybridization analyses verified that IFNT increased *RSAD2* and *IFIH1* mRNA abundance in the endometrium (Figs. 5.4C and 5.5B). Similar to Day 16 to 18 pregnant ewes, *RSAD2* and *IFIH1* mRNA was increased by IFNT primarily in the endometrial stroma and immune cells and, to a lower extent, in the endometrial glands of uteri from ewes receiving P4+IFNT treatment. In P4+ZK ewes, IFNT increased *RSAD2* and *IFIH1* mRNA in the endometrial stroma and immune cells. Further, IFNT increased *IFIH1* mRNA in endometrial LE of ewes receiving P4+ZK treatment (Fig. 5.5B).

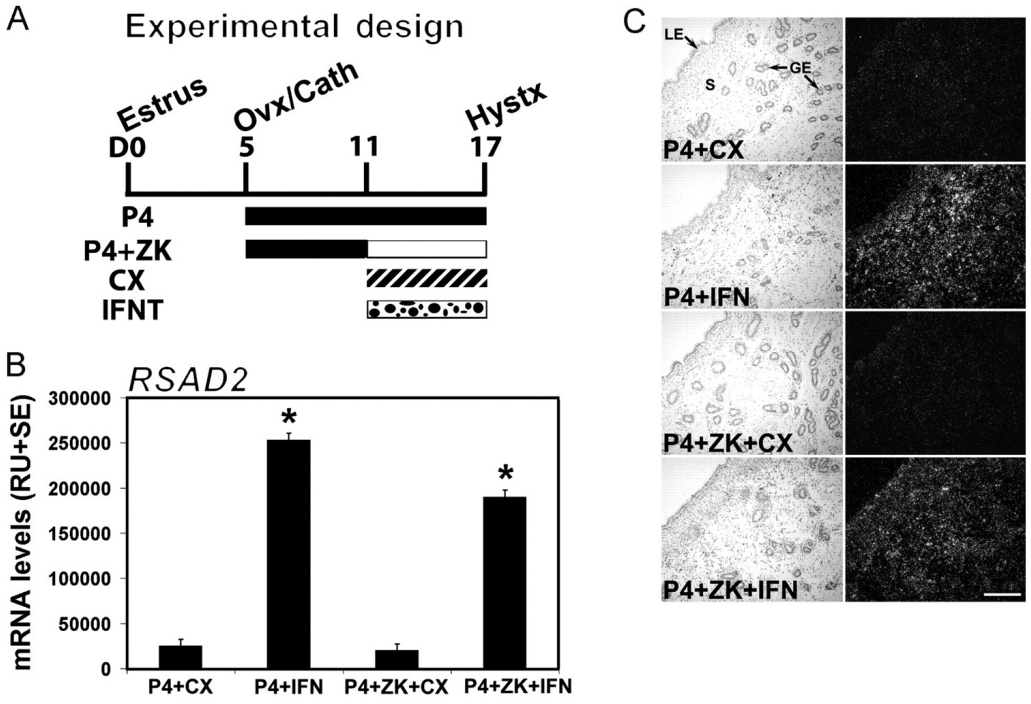

Fig. 5.4. Effects of progesterone and IFNT on *RSAD2* mRNA in the ovine uterus. (A) Experimental design. See Materials and Methods for complete description. Legend: CX, control serum proteins; Hystx, hysterectomy; Ovx/Cath, ovariectomy and uterine catheterization; P4, progesterone; IFNT, recombinant ovine interferon tau; ZK, ZK137,316 anti-progestin. (B) Steady-state levels of *RSAD2* mRNA in endometria as determined by slot blot hybridization analysis. Intrauterine infusion of IFNT increased *RSAD2* mRNA by 10-fold in the endometrium (P4+CX vs P4+IFN, $P<0.001$), but not in ewes receiving the ZK anti-progestin (P4+IFN vs P4+ZK+IFN, $P>0.10$). Similarly, IFNT increased *RSAD2* mRNA 9.3-fold in ewes receiving ZK anti-progestin (P4+ZK+CX vs P4+ZK+IFN, $P<0.001$), but not in ewes receiving the ZK anti-progestin (P4+IFN vs P4+ZK+IFN, $P>0.10$). The asterisk (*) denotes an effect of treatment. (C) In situ hybridization analysis of *RSAD2* mRNA expression. In situ hybridization analyses verified that roIFNT increased *RSAD2* mRNA expression in a cell-type specific manner in P4-treated ewes consistent with increased expression in uteri from Day 16 and 18 pregnant ewes. Intrauterine injections of IFNT increased *RSAD2* mRNAs in endometrial stroma and glands, but not LE, blood vessels or myometrium. Further, IFNT increased *RSAD2* mRNA in endometria of P4+ZK-treated ewes. Scale bar represents 10 μm.

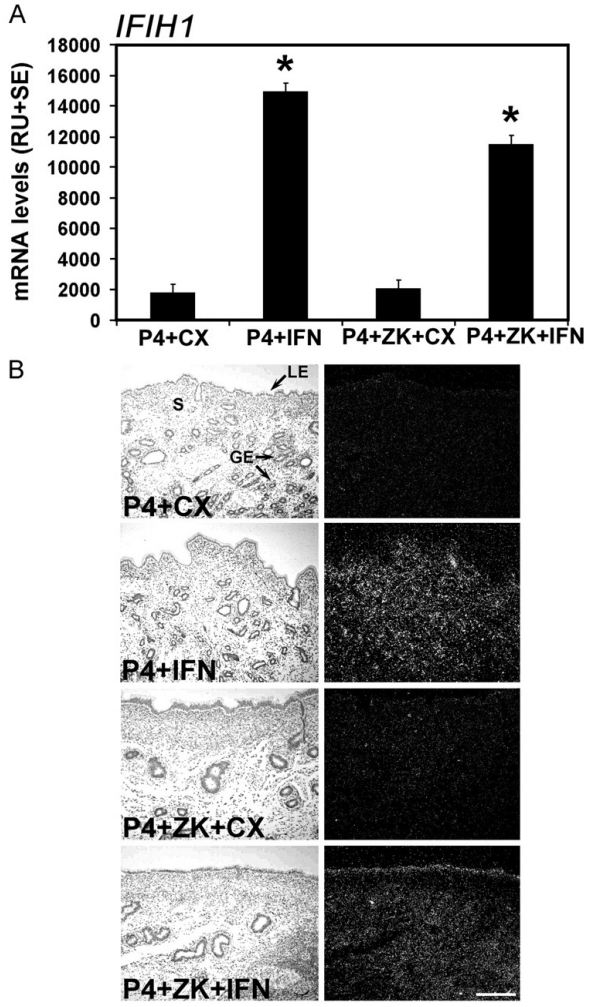

Fig. 5.5. Effects of progesterone and IFNT on *IFIH1* mRNA in the ovine uterus. (A) Steady-state levels of *IFIH1* mRNA in endometria were determined by slot blot hybridization analysis. Intrauterine infusion of IFNT increased *IFIH1* mRNA about 8.3-fold in endometria (P4+CX vs P4+IFN, $P<0.001$), but not in ewes receiving the ZK anti-progestin (P4+IFN vs P4+ZK+IFN, $P>0.10$). The asterisk (*) denotes an effect of treatment. (C) In situ hybridization analysis of *IFIH1* mRNA expression. Intrauterine injections of IFNT increased *IFIH1* mRNA expression in a cell-type specific manner in P4-treated ewes consistent with the increased in expression in uteri from Day 16 and Day 18 pregnant ewes. Infusion of roIFNT increased expression of *IFIH1* mRNA in endometrial stroma and GE, but not LE, blood vessels or myometrium. Further, IFNT increased *IFIH1* mRNA in endometria from ewes treated with P4+ZK. Cross-sections of the uterine wall from treated-ewes were hybridized with radiolabeled antisense or sense ovine *IFIH1* cRNA probes. Scale bar represents 10 μm.

Effects of IFNT on RSAD2 and IFIH1 in Endometrial Cells

In untreated ovine endometrial LE (oLE) and bovine endometrial (BEND) cells maintained in serum-free medium, *IFIH1* but not *RSAD2* mRNA was detected (Fig. 5.6). Treatment of both oLE and BEND cells with recombinant ovine IFNT increased ($P<0.0001$) *RSAD2* and *IFIH1* mRNA levels.

Discussion

During the peri-implantation period of pregnancy, gene expression in endometria of ruminants is programmed primarily by progesterone from the ovarian corpus luteum and IFNT from the conceptus (1, 17). In the present study, we identified two antiviral-related genes, *RSAD2* and *IFIH1*, as being induced in the ovine endometrium in response to IFNT from the conceptus in a progesterone-independent manner. These genes were selected for analysis based on transcriptional profiling studies of ruminant endometria (259, 260) as well as knowledge that both *RSAD2* and *IFIH1* are produced during a viral infection in response to IFNs to limit viral replication and modulate subsequent adaptive immunity (170, 261). In the present study, the ontogeny of *RSAD2* and *IFIH1* in the ovine endometrium correlates directly with increasing amounts of IFNT produced by the rapidly elongating conceptus, which is maximum between Days 14 and 16 and declines thereafter as the trophectoderm begins implantation and trophoblast giant binucleate cells begin to differentiate (95). Clearly, progesterone and IFNT have complex, independent and complementary effects on expression of a number of genes in the ovine endometrium during early pregnancy (see (259, 260)). In the present study, progesterone was found to be not required for IFNT induction of *RSAD2* and *IFIH1* in the endometrium, which is similar to findings for other IFNT-stimulated genes in the ovine endometrium including *CXCL10 (chemokine (C-X-C motif) ligand 10), IFITM3 (interferon induced transmembrane protein 3 (1-8U)), B2M (beta-2-microglobin), MIC (MHC class I polypeptide-related alpha chain)*, and *STAT1* (259).

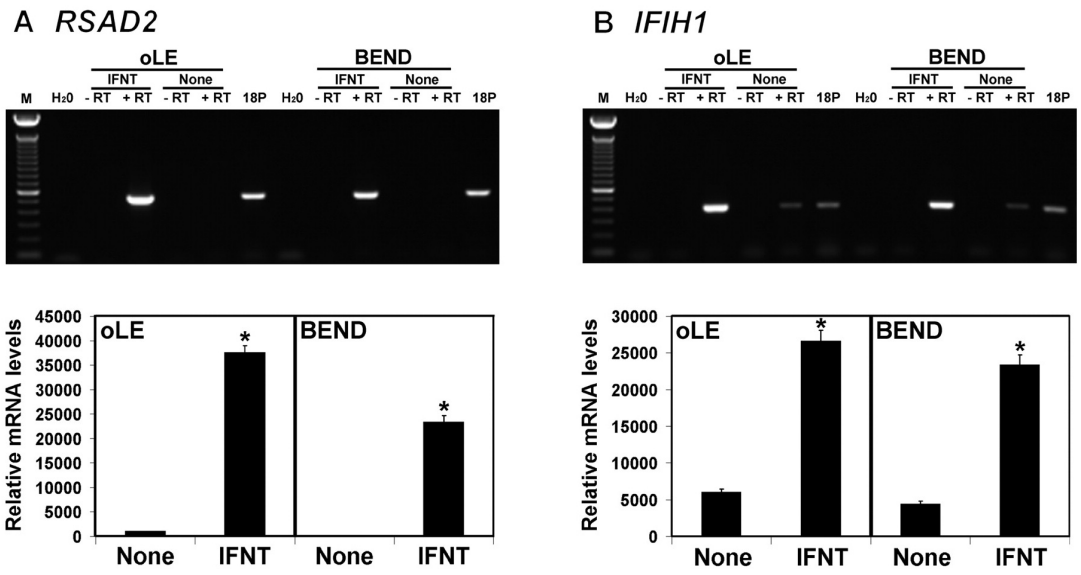

Fig. 5.6. Semi-quantitative RT-PCR analyses of *RSAD2* and *IFIH1* mRNAs in total cellular RNA isolated from immortalized ovine endometrial LE (oLE) and bovine endometrial (BEND) cells. All PCR products were separated in a 1.5% agarose gel and stained with ethidium bromide. Positions of the 100-base pair (bp) DNA marker (M) ladder are shown. Total cellular RNA from endometria of a Day 18 pregnant ewe and sterile water (no template) were positive and negative controls, respectively. A graph illustrating the effect of IFNT on relative mRNA levels for *RSAD2* and *IFIH1* is presented below each gel, and the asterisk (*) denotes a significant (P<0.001) effect of IFNT treatment.

Further, treatment of ovine endometrial LE and BEND cells with recombinant ovine IFNT induced *RSAD2* and *IFIH1* expression without a requirement for serum or progesterone in the medium. In contrast, IFNT induction of several non-classical IFN-stimulated genes, such as *LGALS15 (galectin 15), CTSL (cathespin L)*, and *CST3 (cystatin C)*, in endometrial LE and sGE is dependent on progesterone (157, 250, 265), which is hypothesized to involve progesterone down-regulation of the PGR in those epithelia (17, 259).

In the ovine uterus, induction of *RSAD2* and *IFIH1* mRNA by the presence of the conceptus during pregnancy and by IFNT was limited to endometrial stroma and middle to deep glands as well as resident immune cells based on visual observations of cell morphology. The majority of ISGs induced by IFNT without a requirement for progesterone in the ovine uterus are restricted to endometrial stroma and middle to deep glands as well as immune cells (see (17, 18) for review). A variety of ruminant and human cell lines have been used to determine that IFNT activates the classical JAK-STAT-IRF signaling pathway utilized by other Type I IFNs that involves ISGF3 (STAT1, STAT2, ISGF3G complex), GAF (STAT1 dimer), and IFN regulatory factor one (IRF1) (see (17, 102)) . ISGF3 transactivates genes through binding an IFN-stimulated response element (ISRE), whereas GAF binds to a gamma activation sequence (GAS) element in genes such as *IRF1*. Further, IRF1 transactivates genes through an IRF element (IRFE). Similar to findings for *RSAD2* and *IFIH1* in the present study, results of *in vivo* studies indicate that many classical IFN-stimulated genes (*STAT1, STAT2, IRF1, ISGF3G, GBP2, IFI6, IFI56, ISG15, MIC, B2M, OAS*) are not induced or increased by IFNT in LE and sGE of the sheep uterus (125, 138, 141, 156). This finding was initially surprising because all ovine endometrial cell types express IFNAR1 and IFNAR2 subunits of the common Type I IFN receptor (99). However, available results also indicate that IRF2, a potent transcriptional repressor of IFN-stimulated genes (127), is expressed specifically in endometrial LE and sGE and represses transcriptional activity of promoters containing ISRE or IRFE (125). Thus, IRF2 in LE and sGE is proposed to restrict IFNT induction of many IFN-stimulated

genes to endometrial stroma and glandular epithelium. In fact, all components of ISGF3 (*STAT1, STAT2, ISGF3G*) and other studied IFN-stimulated genes (*B2M, GBP2, G1P2, G1P3, IFI56, MIC*) contain ISREs in their promoters. Further, the promoter regions of the human and fish *RSAD2* genes contain multiple IRFEs (167, 168). Similarly, the promoter region of the human *IFIH1* gene has predicted ISRE and IRFE (unpublished results). Thus, the constitutive presence and pregnancy-specific increase in IRF2 in ovine endometrial LE/sGE *in vivo* is proposed to prevent IFNT induction of *RSAD2* and *IFIH1* in those epithelia. Progesterone appears to be involved in this cell-type specification of IFNT actions, because IFNT induced *IFIH1* mRNA in the LE of the endometrium in P+ZK-treated ewes in the present study. Immortalized ovine endometrial LE and BEND cells lack or have very low levels of *IRF2* mRNA (Song and Spencer, unpublished results); thus, they are fully responsive to IFNT *in vitro* (145, 163, 266). In human 2fTGH cells, Type I IFNB can induce *IFIH1* expression, but this is not the case for STAT1 null U3A cells derived from 2fTGH cells (172). Thus the classical JAK-STAT-IRF signaling pathway active in endometrial stroma and glands, and perhaps resident immune cells, is likely responsible for IFNT induction of *IFIH1* via activation and formation of the ISGF3 complex (172). One interesting finding of the present studies was the loss of *RSAD2* mRNA in the middle to deep endometrial glands between Days 16 and 18 of pregnancy. This loss correlates with a reduction in IFNT production by the conceptus as well as a decline in IRF1 abundance in those glands (125). Available evidence supports the concept that distinct cell-type specific differences exist in the ruminant endometrium with respect to responses to IFNT from the conceptus between the endometrial glands, stroma and resident immune cells.

The IFNT-stimulated genes in endometria of ruminants are hypothesized to be important for conceptus implantation (2, 227, 260). RSAD2 contains a radical S-adenosylmethionine (SAM) domain that catalyzes diverse reactions, including unusual methylations, isomerization, sulphur insertion, ring formation, anaerobic oxidation and protein radical formation. Radical SAM proteins function in DNA precursor, vitamin, cofactor, antibiotic and herbicide biosynthesis, and biodegradation pathways (267),

which could be important in endometrial cells during the peri-implantation period to support conceptus development and implantation. IFIH1 (alias melanoma differentiation associated gene 5) is a RNA helicase induced during differentiation, cancer reversion, and programmed cell death (171, 172). IFIH1 acts to sense intracellular viral infection and mediate a signal for innate antiviral responses including production of Type I IFNs (171, 173). Other Type I IFNs (IFNA and IFNB) are not induced in the endometrium in response to IFNT (Spencer and Bazer, unpublished results).

One biological role of RSAD2 and IFIH1 could be to prevent viral infection of the uterus during the critical peri-implantation period of pregnancy, particularly when the conceptus does not have a developed immune system or antiviral defenses. *RSAD2* and *IFIH1* are implicated in establishing an antiviral state by modulation of innate immune responses. For example, stable expression of *RSAD2* in fibroblasts inhibits human cytomegalovirus infection (167) . Given that IFIH1 also has growth suppressive properties, IFNT induction may suppress the activation of cells within the endometrium, which could be beneficial for pregnancy. In other species such as rodents and humans, resident and recruited immune cells within the endometrium play important roles in placentation and the success of pregnancy (29, 268). Unfortunately, knowledge of which immune cells are present in the ovine uterus during pregnancy and their biological functions is sparse. During the estrous cycle, the density of macrophages and T lymphocytes in the ovine and bovine uteri do not change (269). However, during early pregnancy, the number of $CD45R^+$ lymphocytes increases in both endometrium (270) and uterine and jugular venous blood (271, 272). It has been postulated that these are natural killer cells that produce factors to enhance establishment of pregnancy. In the present study, the number of *IFIH1*- and, in particular, *RSAD2*-positive immune cells markedly increased in the endometria during pregnancy and in response to IFNT, but it is not clear whether these cells were recruited in response to IFNT or already present and stimulated by IFNT. The IFNT stimulated resident immune cells in the endometrium may migrate from the uterus, because IFNT-stimulated genes are higher in the peripheral blood leukocytes isolated from pregnant as compared to non-pregnant ewes and cows

(273). Eosinophils are also present in the endometrium of early pregnant ewes, and their numbers increase between Days 11 and 19 of early pregnancy, perhaps due to actions of both progesterone and perhaps IFNT (274). In fact, IFNT possesses immunoregulatory activity and can inhibit mitogen-induced lymphocyte proliferation (275, 276) as well as modulate activity of natural killer cells (28, 277). These effects of IFNT may prevent immune cell-mediated destruction of the conceptus (27). Finally, some IFNT-stimulated genes, such as CXCL10, from immune cells may have direct effects on conceptus implantation (278, 279). Collectively, available evidence supports the hypothesis that RSAD2 and IFIH1 modulate uterine receptivity to conceptus implantation by induction of an antiviral state and modulation of immune cell functions.

CHAPTER VI
STANNIOCALCIN (STC) IN THE ENDOMETRIAL GLANDS OF THE OVINE UTERUS: REGULATION BY PROGESTERONE AND PLACENTAL HORMONES

Introduction

Stanniocalcin (STC) was originally described as a hormone with calcitonin-like actions in fish (195-198). The hormone was discovered in the corpuscles of Stannius, unique endocrine glands on the kidneys of bony fish (199). Removal of the organ or stanniectomy causes hypercalcemia (200, 201). Fish STC1 was subsequently purified from the corpuscles of Stannius and found to be a homodimeric phosphoglycoprotein that regulates calcium and phosphate homeostasis (202). In fish, STC synthesis and secretion are controlled primarily by serum calcium levels (199) and it acts to restore normocalcemia by acting on the gills to reduce further influx of calcium from the aquatic environment, on the kidneys to promote reabsorption of phosphate and chelate excess calcium, and on the gut to inhibit calcium uptake across the intestinal epithelium (196, 197, 199, 202, 203) .

STC1, a mammalian ortholog of fish STC1, has relatively high amino acid sequence identity (approximately 50%) with fish STC and is expressed in a variety of tissues including brain, kidney, lung, and heart (204). STC2 has lower identity (approximately 35%) with STC1 and fish STC1 (205). Similar to STC1, STC2 is expressed in a variety of tissues. Research into the functions of STCs in mammals is at an early stage; therefore, its physiological roles have not been established [see for review (195, 206-208)]. Similar to fish STC, mammalian STC1 regulates intracellular calcium and phosphate (Pi) levels in the kidney and intestine (199, 209), but the function of STC2 is unknown. Mammalian STC1 regulates renal transport of phosphate through stimulation of NaPi-2 cotransport activity (196, 210-212). In rodents, Stc1 expression increases in ovarian tissues during gestation and lactation (213), as well as in mesometrial decidua of the uterus during implantation (214). In the rat ovary, STC1 and

STC2 are expressed in ovarian theca/interstitial cells and in vitro studies suggest that they act in a paracrine manner to dampen gonadotropin stimulation of granulosa cell differentiation (205, 215). In mice, Stc1 does not appear to be essential for reproduction or growth as null mutatnts have no overt phenotype (216); however, in that study, Stc2 was found in all tissues that normally express Stc1 and may compensate for the lack of Stc1.

The STCs have not been investigated in the reproductive tract of mammals other than mice; therefore, these studies were conducted to determine if the STC genes are expressed in the ovine uterus and to determine the effects of pregnancy, progesterone and placental hormones on *STC1* and *STC2* expression in the endometrium. The results of these studies indicate that STC1 is expressed specifically by the endometrial glands of the pregnant uterus and suggest that it has a biological role(s) in regulating fetal and placental development and physiology.

Materials and Methods

Animals

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

Experimental Designs

Experiment One. At estrus (Day 0), ewes were mated to either an intact or vasectomized ram as described previously (253) and then hysterectomized (n = 5 ewes/day) on either Day 10, 12, 14 or 16 of the estrous cycle or Day 10, 12, 14, 16, 18 or 20 of pregnancy. On Days 10 to 16, the uterine lumen was flushed with 20 ml of sterile saline. Presence of a morphologically normal conceptus(es) confirmed pregnancy in

mated ewes. It was not possible to obtain uterine flushes on either Day 18 or Day 20 of pregnancy, because the conceptus had firmly adhered to the endometrial luminal epithelium (LE) and basal lamina. At hysterectomy, several sections (~0.5 cm) from the mid-portion of each uterine horn ipsilateral to the corpus luteum (CL) were fixed in fresh 4% paraformaldehyde in PBS (pH 7.2). In monovulatory pregnant ewes, uterine tissue samples were marked as either contralateral or ipsilateral to the ovary bearing the CL. No tissues from the contralateral uterine horn were used for this study. After 24 h, fixed tissues were changed to 70% ethanol for 24 h, dehydrated through a graded series of alcohol to xylene, and then embedded in Paraplast-Plus (Oxford Labware, St. Louis, MO). Several sections (1–1.5 cm) from the middle of each uterine horn were embedded in Tissue-Tek OCT compound (Miles, Oneonta, NY), frozen in liquid nitrogen vapor, and stored at -80°C. The remaining endometrium was physically dissected from myometrium, frozen in liquid nitrogen, and stored at -80°C for subsequent RNA extraction. Uterine flushes were clarified by centrifugation (3,000 x g for 30 min at 4°C) and frozen at -80°C for Western blot analysis.

Experiment Two. At estrus (Day 0), ewes were mated to an intact ram as described previously (280). Ewes were then hysterectomized (n = 5 ewes/day) on either Day 40, 60, 80, 100, 120 or 140 of pregnancy (gestation period is 147 days). Allantoic fluid samples were obtained and frozen at -80C. At hysterectomy, the uterus was trimmed free of cervix and oviduct and opened along the mesometrial border. Several sections (~0.5 cm) of both intercarunucular and placentomal uterine wall regions from the midportion of each uterine horn were fixed in fresh 4% paraformaldehyde in PBS (pH 7.2). Placentomes were then removed by physical dissection, and remaining intercaruncular endometrium was dissected from the myometrium. Endometrial samples were frozen in liquid nitrogen and stored at -80°C for RNA extraction.

Experiment Three. Sixteen cyclic ewes were ovariectomized and fitted with intrauterine catheters on Day 5 post-estrus as described previously (69). Ewes were then assigned randomly (n = 4 ewes/treatment) to receive daily i.m. injections of progesterone (Sigma Chemical Co., St. Louis, MO) or progesterone and a progesterone receptor

(PGR) antagonist (ZK 136,317; generously provided by Dr. Kristof Chwalisz, Schering AG, Berlin, Germany) and intrauterine infusions of either control serum proteins or recombinant ovine IFN tau (roIFNT) protein as follows: 1) 50 mg progesterone (P4, Days 5-24) and 200 µg control (CX) serum proteins (Days 11-24) [P4+CX]; 2) P4 and 75 mg of ZK136,317 (Days 11-24) and CX proteins (200 µg) [P4+ZK+CX]; 3) P4 and IFNT ($2X10^7$ antiviral units, Days 11 to 24) [P4+IFN]; or 4) P4 and ZK and IFNT [P4+ZK+IFN]. All ewes were hysterectomized on Day 25 post-estrus. Recombinant ovine IFNT was prepared in a yeast bacterial system and assayed for biological activity using an antiviral assay as described previously (264). Control serum proteins and IFNT were prepared for intrauterine injections as described previously (281).

Experiment Four. Fifteen cyclic ewes were ovariectomized and fitted with intrauterine catheters on Day 5 post-estrus as described previously (19). All ewes received daily i.m. injections of 50 mg P4 (Days 5 to 25) and intrauterine injections of IFNT ($2X10^7$ antiviral units/day) from Days 11 to 20. Ewes (n=5 per treatment group) also received daily intrauterine injections of either control (CX) serum proteins (200 µg) [CX], recombinant ovine placental lactogen (PL; 200 µg) [PL], or recombinant ovine Growth Hormone (GH, 200 µg) [GH] from Day 16 to Day 25 when all ewes were hysterectomized. Recombinant ovine PL and ovine GH were prepared in bacteria and purified as described previously (282).

For both Experiments Three and Four, portions (~0.5 cm) from the middle region of the uterine horn were fixed at hysterectomy in fresh 4% paraformaldehyde in PBS (pH 7.2) for 24 h, washed in 70% ethanol for 24 h, dehydrated through a graded series of alcohol to xylene, and then embedded in Paraplast-Plus (Oxford Labware, St. Louis, MO).

Experiment Five. Ewes (n=4) were made unilaterally pregnant as described previously (283). On Day 80 of pregnancy, uterine secretions, e.g. uterine milk, was collected from the nongravid uterine horn of unilaterally pregnant ewes (n = 4) on Day 80 of pregnancy by flushing the uterine horn with 100 ml of saline. In addition, samples of allantoic fluid and amniotic fluid (50 ml) were obtained using a syringe fitted with a

20-*g* needle from the gravid uterine horn. Uterine milk and allantoic fluids were clarified by centrifugation and stored at –80°C.

RNA Isolation

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

Cloning of Partial cDNAs for Ovine STC1 and STC2

Partial cDNAs for ovine *STC1* and *STC2* mRNAs were amplified by RT-PCR using total RNA from endometrium from ewes on Day 18 of pregnancy. For *STC1*, the sense primer (5'-TGA TCA GTG CTT CTG CAA CC-3') and antisense primer (5'-TCA CAG TCC AGT AGG CTT CG-3') were derived from the bovine *STC1* mRNA coding sequence (GenBank accession no. NM_176669). For *STC2*, the sense primer (5'- AAC GCT GGA AAA TTT GAT GC-3') and antisense primer (5'- CTC TTG CTA CCT CGC TCA CC -3') were derived from the human *STC2* mRNA coding sequence (GenBank accession no. AF055460). PCR amplification was as follows: 1) 95°C for 5 min; 2) 95°C for 45 sec, 59.1°C for 1 min (for *STC1*), 61.8°C for 1 min (for *STC2*), and 72°C for 1 min for 35 cycles; and 3) 72°C for 10 min. Partial ovine *STC1* and *STC2* cDNAs were cloned into pCRII using a T/A Cloning Kit (Invitrogen) and their sequences were verified using an ABI PRISM Dye Terminator Cycle Sequencing Kit and ABI PRISM automated DNA sequencer (Perkin-Elmer Applied Biosystems).

Slot Blot Hybridization Analyses

Steady-state levels of mRNA in ovine endometria from Experiments One and Two were assessed by slot blot hybridization as described previously (125, 254). Antisense cRNA probes were generated by linearizing both pCRII-STC1 and pCRII-

STC2 plasmids with *XbaI* and *in vitro* transcription with SP6 RNA polymerase, whereas sense cRNA probes were generated using *BamHI* and T7 RNA polymerase. Radiolabeled antisense and sense cRNA probes were then generated by *in vitro* transcription with [α-^{32}P]-UTP. Denatured total endometrial RNA (20 µg) from each ewe was hybridized with radiolabeled cRNA probes. To correct for variation in total RNA loading, a duplicate RNA slot membrane was hybridized with radiolabeled antisense 18S cRNA (pT718S; Ambion, Austin, TX). Following washing, the blots were digested with ribonuclease A and radioactivity associated with slots quantified using a Typhoon 8600 MultiImager (Molecular Dynamics, Piscataway, NJ).

In Situ *Hybridization Analyses*

Location of STC mRNA expression in sections (5 µm) of the ovine uterus was determined by radioactive *in situ* hybridization analysis as described previously (125, 254). Briefly, deparaffinized, rehydrated and deproteinated uterine tissue sections were hybridized with radiolabeled antisense or sense cRNA probes generated from linearized ovine *STC1* and *STC2* partial cDNAs using *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, Rochester, NY), and exposed at 4°C for one to two weeks. Slides were developed in Kodak D-19 developer, counterstained with Gill's hematoxylin (Fisher Scientific, Fairlawn, NJ), and then dehydrated through a graded series of alcohol to xylene. Coverslips were then affixed with Permount (Fisher). Images of representative fields were recorded under brightfield and darkfield illumination using a Nikon Eclipse 1000 photomicroscope (Nikon Instruments Inc., Lewisville, TX) fitted with a Nikon DXM1200 digital camera.

In Experiments 3 and 4, the relative abundance of *STC1* mRNA in the endometrial glands was determined with the Scion Image software (Release beta 4.03, Scion Corporation, NIH, USA). Briefly, photomicrographs of at least 10 regions of the uterus from each animal were acquired under darkfield illumination and converted to a TIFF file. Using the Scion Image software, the optical intensity for the mRNA

hybridization signals in the endometrial glands was determined. The inter- and intra-section variation in optical intensity value measurements was less than 5%.

Immunohistochemical Analyses

Immunocytochemical localization of STC1 protein in the ovine uterus was performed as described previously (19) in tissue sections from Experiments One and Two with rabbit anti-human STC1 antiserum (214) at a 1:25,000 dilution. Antigen retrieval was performed by using a boiling citrate buffer and negative controls included substitution of purified rabbit IgG for the primary antibody at the same final concentration.

Western Blot Analyses

Endometrial extracts of uteri from Days 12, 18 and 80 of pregnancy in Experiments One and Two were prepared by homogenizing the uterine tissues in extraction buffer (60 mM Tris pH 7.0, 1 mM Na3VO4, 10% glycerol, 2% SDS and 1X protease inhibitor cocktail (Roche, Indianapolis, IN)). Uterine flushes from Day 16 pregnant ewes in Experiment One were concentrated using Centricon-3 columns (Amicon, Beverly, MA). Protein concentrations of uterine flushes, uterine milk and allantoic fluid were determined using the Bradford protein assay (Bio-Rad, Hercules, CA) with bovine serum albumin (BSA) as the standard. Proteins were denatured and separated by 15% SDS-PAGE, and Western blot analysis was performed as described previously (145) using enhanced chemiluminescence detection (SuperSignal West Pico, Pierce, Rockford, IL) and X-OMAT AR X-ray film (Kodak, Rochester, NY). Immunoreactive STC1 protein was detected using the rabbit anti-human STC1 antiserum (214) at a 1:40,000 final dilution.

Statistical Analyses

All quantitative data were subjected to least-squares regression analyses (ANOVA) using the General Linear Models (GLM) procedures of the Statistical

Analysis System (SAS Institute, Cary, NC). Slot blot hybridization data were corrected for differences in sample loading using the 18S rRNA data as a covariate. Data from Experiments One and Two were analyzed for effects of day, pregnancy status (cyclic or pregnant), tissue (caruncular and intercaruncular endometrium), treatment and their interactions. Within pregnancy status, least squares regression analyses were used to determine effects of day on endometrial mRNA levels. Optical intensity measurements of mRNA abundance in the endometrial glands as determined by *in situ* hybridization analyses of uteri from Experiments Three and Four were analyzed for effects of treatment, animal, and slide. Pre-planned orthogonal contrasts were used to determine main effects of treatment. All tests of significance were performed using the appropriate error terms according to the expectation of the mean squares for error. A P-value of 0.05 or less was considered significant while a P-value of 0.05 to 0.10 was considered a trend toward significance. Data are presented as least-square means (LSM) with standard errors (SE).

Results

Steady-State Levels of STC1 and STC2 mRNA in the Endometrium of the Ovine Uterus

Steady-state levels of *STC1* and *STC2* mRNA in endometria of cyclic and pregnant ewes were determined by slot blot hybridization analysis (Figure 6.1). *STC1* mRNA was detected in the endometrium of pregnant ewes, but not in the endometrium of cyclic ewes. In addition, *STC1* mRNA was not detected in the placentomal tissues of pregnant ewes (data not shown). In pregnant ewes, *STC1* mRNA first appeared on Day 18 of pregnancy, increased ($P<0.01$) ~6-fold to Day 80, and remained abundant thereafter. *STC2* mRNA was found in the endometria of both cyclic and pregnant ewes as well as in the placentomes. Overall, *STC2* mRNA levels were low in the endometrium and not different ($P>0.10$) between cyclic and pregnant ewes on Days 10 to 16. *STC2* mRNA levels increased (linear, $P<0.05$) ~3-fold in the endometrium of

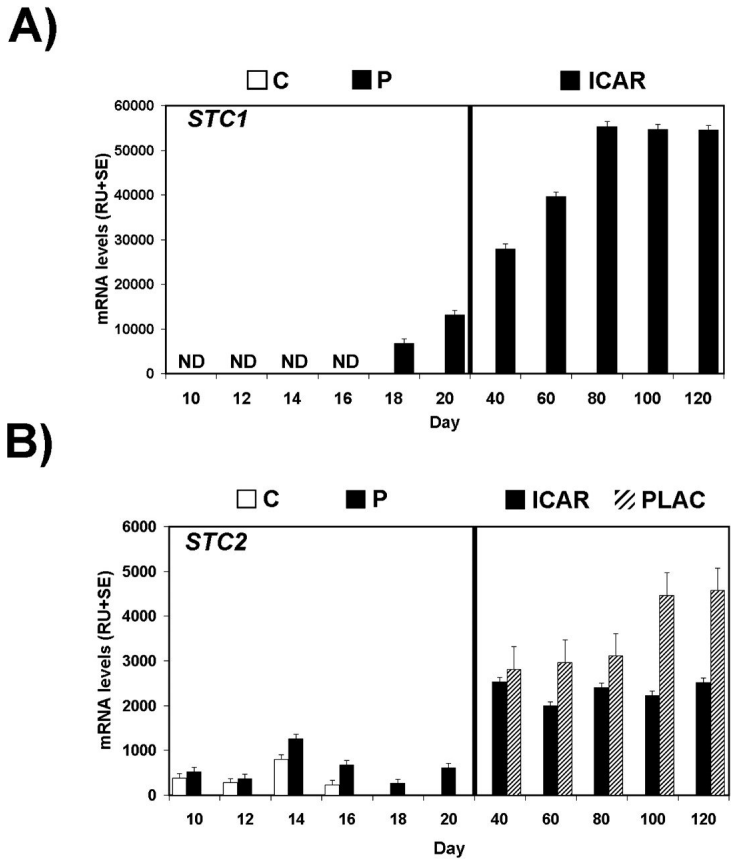

Fig. 6.1. Steady-state levels of *STC1* and *STC2* mRNAs in ovine uterine and placental tissues. (A) *STC1* mRNA in the endometrium of cyclic (C) and early pregnant (P) ewes (Days 10 to 20) and in the intercaruncular (ICAR) endometria of later pregnant ewes (Days 40 to 120). *STC1* mRNA was detected in the endometrium of pregnant ewes, but not in the endometrium of cyclic ewes (ND = not detectable) or in the placentomal tissues of pregnant ewes (data not shown). In pregnant ewes, *STC1* mRNA was first detected on Day 18 of pregnancy, increased ($P<0.01$) ~6-fold to Day 80, and remained abundant thereafter in the intercaruncular (ICAR) endometrium. (B) *STC2* mRNA in the endometrium of cyclic (C) and early pregnant (P) ewes (Days 10 to 20) and in the intercaruncular (ICAR) endometria and placentomes (PLAC) of later pregnant ewes (Days 40 to 120). *STC2* mRNA was detected in the endometria of both cyclic and pregnant ewes as well as in the placentomes. Overall, *STC2* mRNA levels were low in the endometrium and not different ($P>0.10$) between cyclic and pregnant ewes on Days 10 to 16. *STC2* mRNA levels increased ($P<0.05$) ~3-fold in the endometrium of pregnant ewes after Day 20. Low levels of *STC2* mRNA were observed throughout gestation in the placentomal tissues. Data is expressed as LSM relative units (RU) with standard error (SE).

pregnant ewes after Day 20. Low levels of *STC2* mRNA were observed throughout gestation in the placentomal tissues.

Localization of STC1 and STC2 mRNAs in the Ovine Uterus

In situ hybridization analyses determined the location of *STC1* and *STC2* mRNAs in uteri of cyclic and pregnant ewes (Figures 6.2 and 6.3). In cyclic ewes, *STC1* mRNA was not detected between Days 10 and 16 of the estrous cycle or pregnancy (Figure 6.2). Similarly, *STC1* mRNA was not detected in the endometrium of Days 10 to 16 pregnant ewes. On Day 18 of pregnancy, *STC1* mRNA was detected in the endometrial glandular epithelium (GE), but not in any other uterine or placental cell types, including the luminal epithelium (LE), stroma, myometrium, blood vessels, immune cells or conceptus trophectoderm. Throughout pregnancy, *STC1* mRNA was observed only in the endometrial GE. The photomicrographs of Day 60, 80, 100 and 120 placentomes contain red blood cells at the placentome-myometrium interface which diffract light under dark-field, but are not positive for *STC1* mRNA.

In contrast to STC1, *STC2* mRNA was detected at very low levels in the endometrial LE, GE and stroma of cyclic and early pregnant ewes (Fig. 6.3). In later pregnant ewes, *STC2* mRNA was detected predominantly in the endometrial LE and GE as well as conceptus trophectoderm (Tr) with lower levels in the stroma. Given the temporal and spatial alterations in the two *STC* genes in ovine uteroplacental tissues, we focused on STC1 in the remainder of the studies.

Localization of Immunoreactive STC 1 Protein in the Ovine Uterus

Immunohistochemical analysis indicated that STC1 protein was localized predominantly on the apical surface of GE between Days 18 and 140 of gestation (Figure 6.4). Consistent with results from *in situ* hybridization analyses, STC1 protein was predominantly detected in the endometrial glands near the apical surface. In caruncular areas, immunoreactive STC1 was detected only in GE adjacent to the placentome. Areolae are specialized areas of the intercotyledonary placenta that form

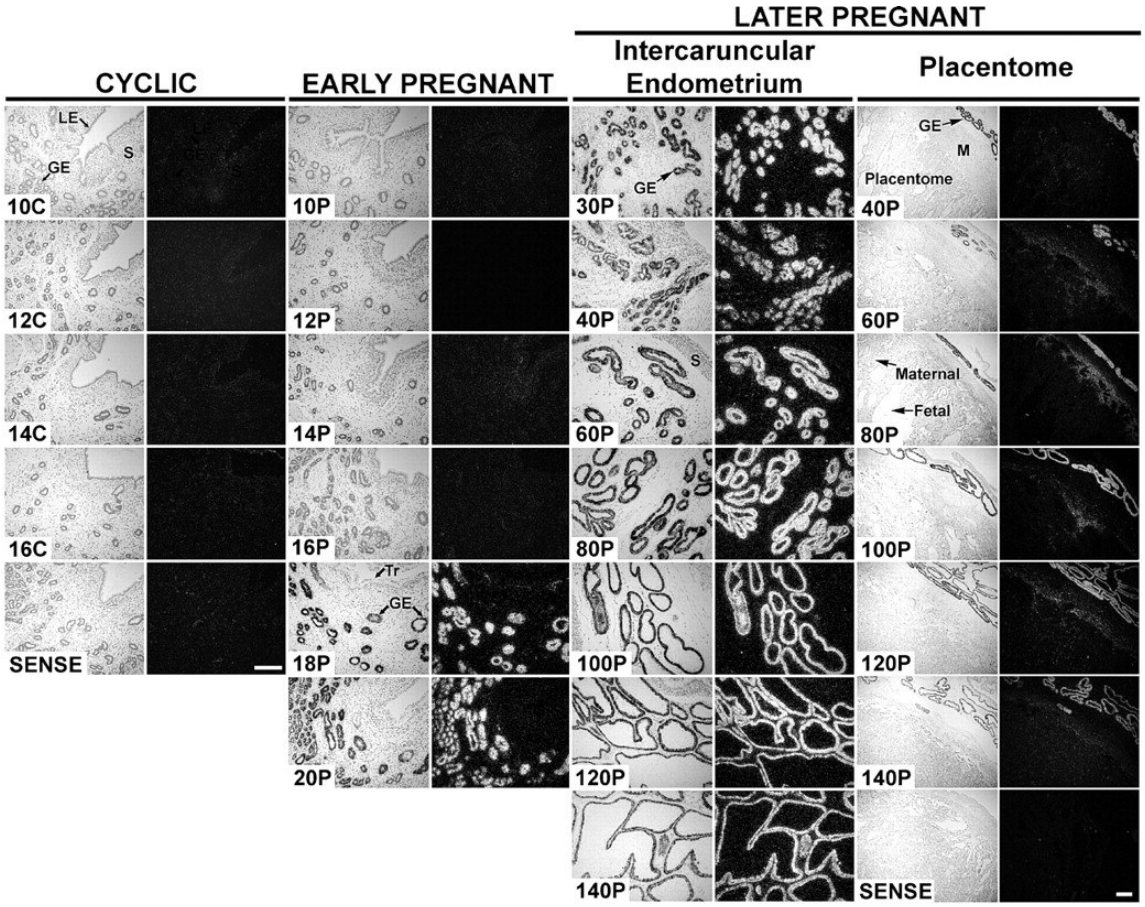

Fig. 6.2. *In situ* hybridization analysis of *STC1* mRNA in the uterus of cyclic and pregnant ewes. Cross-sections of the uterine wall from cyclic (C) and pregnant (P) ewes and placentomes of pregnant ewes were hybridized with radiolabeled antisense or sense ovine *STC1* cRNAs. Note that *STC1* mRNA is expressed only in the glandular epithelia of the endometrium during pregnancy. Legend: GE, glandular epithelium; LE, luminal epithelium; M, myometrium; S, stroma; Tr, trophectoderm. Bar represents 10 μm.

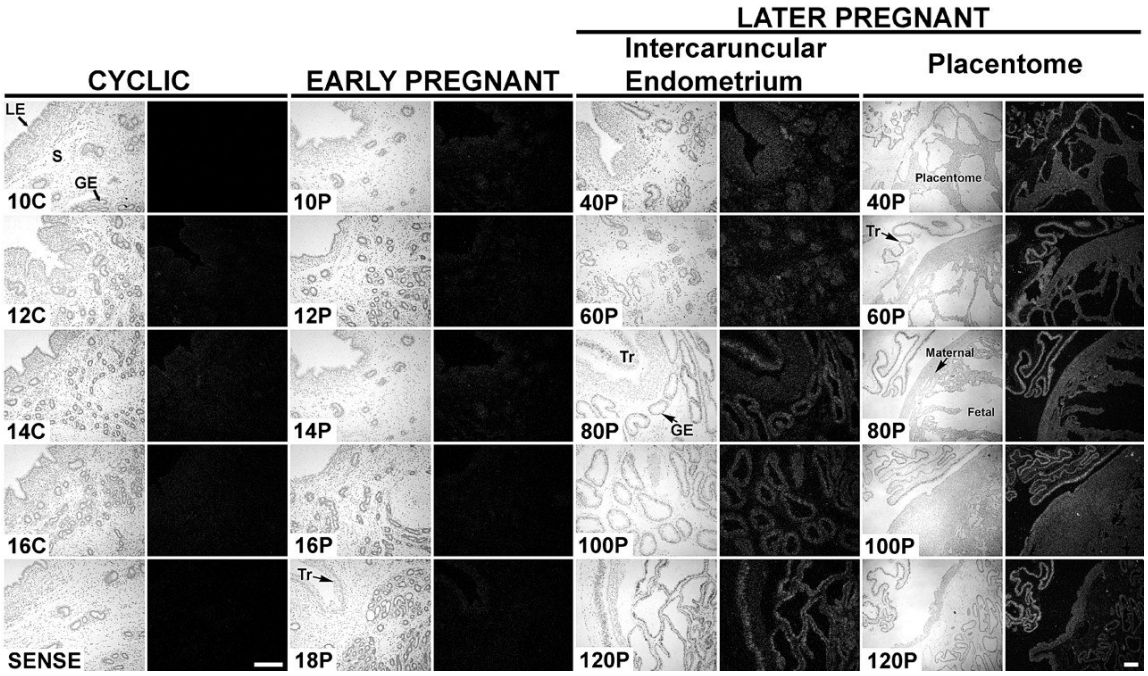

Fig. 6.3. *In situ* hybridization analysis of *STC2* mRNA in endometria of cyclic and pregnant ewes and placentomal tissue of late pregnant ewes. Cross-sections of the uterine wall from cyclic (C) and pregnant (P) ewes were hybridized with radiolabeled antisense or sense ovine *STC2* cRNAs. Very low levels of *STC2* mRNA were detected in the endometrial stroma and glands of the cyclic and early pregnant uterus. In later pregnant ewes, *STC2* mRNA was observed predominantly in the endometrial lumina epithelium (LE), glandular epithelium (GE) and conceptus trophectoderm (Tr) as well as in the maternal caruncular stroma of the placentome. Legend: GE, glandular epithelium; LE, luminal epithelium; S, stroma; Tr, trophectoderm. Bar represents 10 μm.

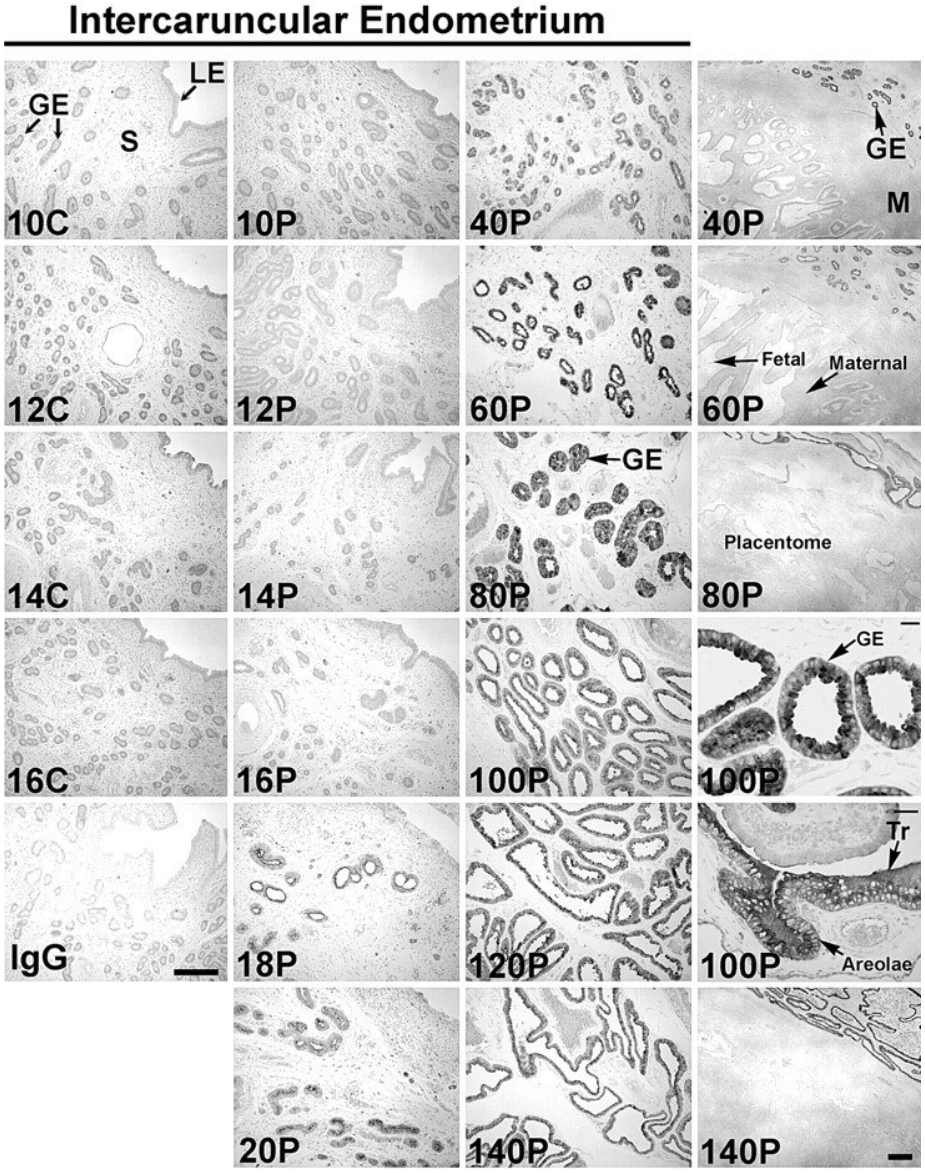

Fig. 6.4. Immunohistochemical localization of STC1 protein in uteri from cyclic and pregnant ewes. In the IgG control, normal rabbit IgG was substituted for rabbit polyclonal antibody to human STC1. Sections were not counterstained. Note the immunoreactive STC1 protein in the endometrial glandular epithelia and accumulation in the placental areolae as shown on Day 100 of pregnancy. Legend: LE, luminal epithelium; GE, glandular epithelium; M, myometrium; S, stroma; Tr, trophectoderm. Solid bar represents 10 μm, except for the bar represents 100 μm in the higher magnifications of the glands and areolae on Day 100 of pregnancy.

over the opening of a gland duct on the endometrial luminal surface (284). STC1 protein was consistently observed in the folded areolae of the intercotyledonary placenta.

Progesterone Induces STC1 mRNA in the Endometrial Glands of the Ovine Uterus

Osteopontin (secreted phosphoprotein one or SPP1 and uterine SERPIN, also known as uterine milk protein or UTMP) is also expressed only the endometrial GE and induced by progesterone (60, 61, 65, 69). Therefore, Experiment Three was conducted to determine if the induction of *STC1* mRNA in the endometrial glands of early pregnant ewes was due to progesterone and/or IFNT from the conceptus (Figure 6.5A). Continuous, long-term progesterone treatment for 20 days alone induced *STC1* mRNA in the endometrium of the ovine uterus (Figure 6.5B). As illustrated in Figure 6.5C, *STC1* mRNA was 11.6-fold higher ($P<0.01$) in the endometrial glands of P4+CX-treated ewes as compared to P4+ZK+CX-treated ewes. Indeed, *STC1* mRNA was not observed in the endometrial glands of uteri from any of the ewes receiving progesterone and the ZK anti-progestin (Figure 6.5B). Intrauterine infusion of IFNT had no effect ($P>0.10$) on *STC1* mRNA abundance in the endometrial glands when P4+IFN-treated ewes were compared to P4+CX-treated ewes. *STC2* mRNA was not detected by *in situ* hybridization analysis in endometria of ewes in any treatment group (data not shown).

Placental Lactogen (PL) and Growth Hormone (GH) Increase STC1 mRNA in the Endometrium

In addition to being progesterone induced genes in the endometrial glands of the ovine uterus, SPP1 and SERPIN are also stimulated by intrauterine administration of ovine PL and ovine GH (19, 285). Therefore, Experiment Four was conducted to determine if ovine PL and(or) ovine GH regulated STC expression in the endometrial glands of the ovine uterus (Figure 6.6A). Intrauterine administration of recombinant ovine PL increased *STC1* mRNA in the endometrial glands of the ovine uterus by 1.8-fold ($P<0.05$, CX vs PL) (Figures 6.6B and 6.6C). Similarly, intrauterine administration of recombinant ovine GH tended to increase *STC1* mRNA in the endometrial glands by

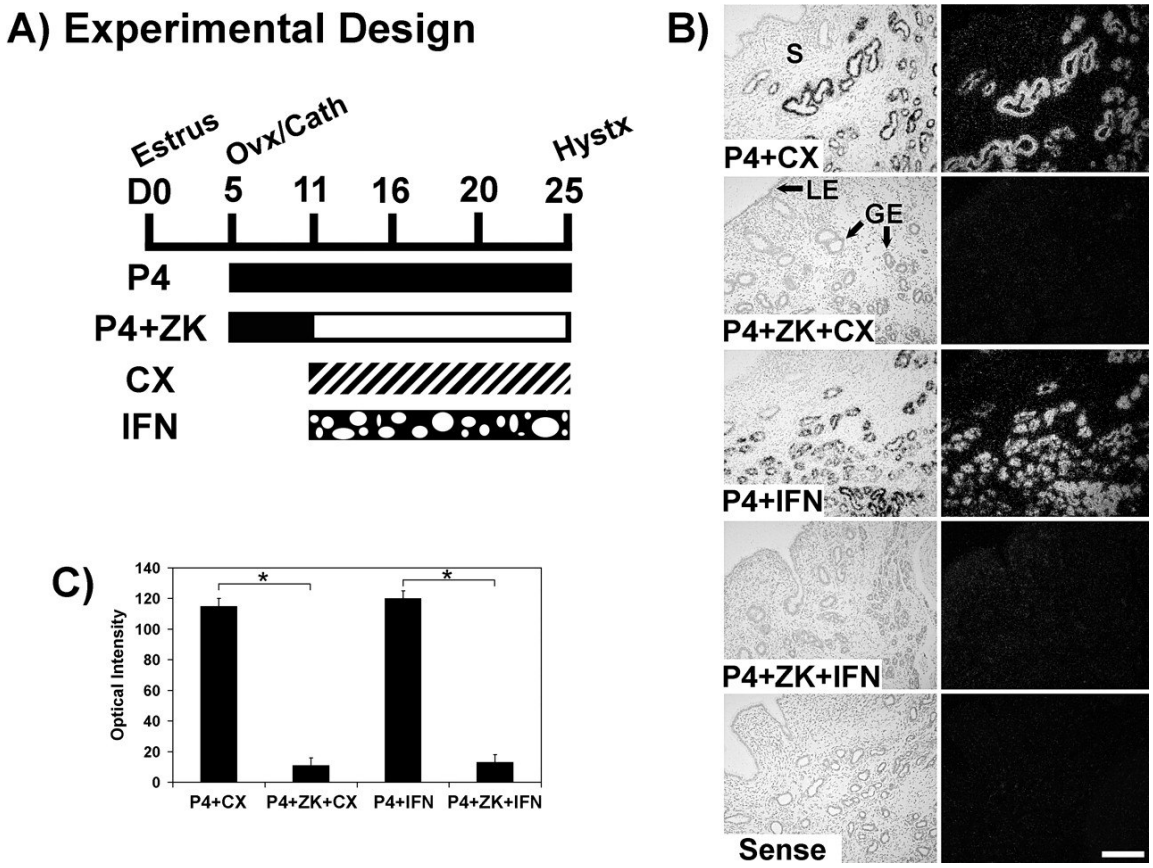

Fig. 6.5. Effects of progesterone and IFNT on endometrial *STC1* mRNA. (A) Experimental design (see Materials and Methods for complete description of experimental design). Legend: CX, control serum proteins; Hystx, hysterectomy; IFN, recombinant ovine interferon tau; Ovx/Cath, ovariectomy and uterine catheterization; P4, progesterone; ZK, ZK137,316; (B) In situ hybridization analysis of *STC1* mRNA in the uterus. Indeed, *STC1* mRNA was not observed in the endometrial glands of uteri from any of the ewes receiving progesterone and the ZK anti-progestin. Legend: LE, luminal epithelium; GE, glandular epithelium; S, stroma. Bar represents 10 μm. (C) Quantification of *STC1* mRNA in the endometrial glands of uteri. *STC1* mRNA was 11.6-fold higher ($P<0.01$) in the endometrial glands of P4+CX-treated ewes as compared to P4+ZK+CX-treated ewes. However, intrauterine infusion of IFNT had no effect ($P>0.10$) on *STC1* mRNA abundance in the endometrial glands when P4+IFN-treated ewes were compared to P4+CX-treated ewes. Data is expressed as LSM optical intensity with SE.

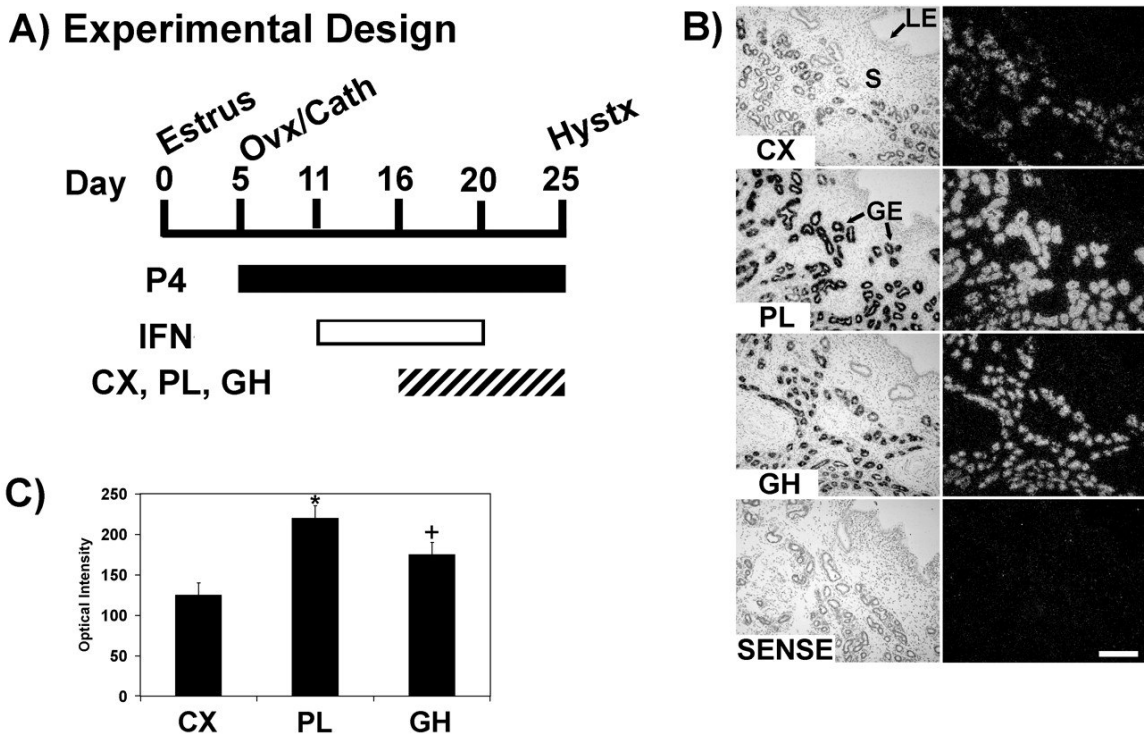

Fig. 6.6. Effects of intrauterine infusion of placental lactogen and growth hormone on endometrial *STC1* mRNA. (A) Experimental design (see Materials and Methods for complete description of experimental design). Legend: CX, control serum proteins; GH, growth hormone; Hystx, hysterectomy; IFN, recombinant ovine interferon tau; Ovx/Cath, ovariectomy and uterine catheterization; P4, progesterone; PL, placental lactogen. (B) In situ hybridization analysis of *STC1* mRNA in the uterus. Legend: LE, luminal epithelium; GE, glandular epithelium; S, stroma. Bar represents 10 μm. (C) Quantification of *STC1* mRNA in the endometrial glands of uteri. Intrauterine administration of recombinant ovine PL increased *STC1* mRNA in the endometrial glands of the ovine uterus by 1.8-fold (* = $P<0.05$, CX vs PL). Intrauterine administration of recombinant ovine GH tended to increase *STC1* mRNA in the endometrial glands by 1.4-fold (+ = $P<0.10$, CX vs GH). Data is expressed as LSM optical intensity with SE.

1.4-fold (P<0.10, CX vs GH). Similar to Experiment Three, *STC2* mRNA was not detected by *in situ* hybridization analysis of the endometria from any of the ewes in the experiment (data not shown).

Western Blot Analysis of STC1 Protein in the Endometrium, Uterine Secretions and Fetal Fluids

Western blot analysis (under reducing conditions) of endometrial extracts, uterine secretions and allantoic fluid from pregnant ewes with rabbit anti-human STC1 antibody detected a single protein of ~25 kDa in size. Immunoreactive STC1 was observed in the uterine luminal fluid, e.g. uterine milk, and allantoic fluid of Day 80 unilateral pregnant ewes, but not in the uterine luminal fluid obtained by flush of Day 16 pregnant ewes (Figure 6.7). In addition, STC1 was not detected in the amniotic fluid from Day 80 pregnant ewes (data not shown). These results support the idea that STC1 is synthesized in GE, secreted by glands into the uterine lumen, transported by the areolae across the placenta into the fetal circulation, cleared by the kidney into the urachus, and then stored in the allantoic fluid during gestation.

Discussion

The results of the present studies demonstrate that STC1 is exclusively expressed in the endometrial glands of the ovine uterus after Day 16 of pregnancy. In sheep, the blastocyst enters the uterus by Day 6, but only begins implantation on Day 16 (2). In rodents, STC1 gene expression was found to shift from the uterine LE to the mesometrial decidua during implantation (214). In contrast, in the endometrium of the ovine uterus, the STC1 gene was uniquely expressed in glandular epithelial cells. Likewise, STC1 protein was present near the apical surface of gland cells and secreted into the uterine lumen, as evidenced by the presence of immunoreactive STC1 protein in uterine secretions, placental areolae, and allantoic fluid. In this context, STC1 would appear to be secreted in an exocrine manner. If STC1 is also secreted in an endocrine direction by the endometrial glands, it may play an additional role in regulating maternal

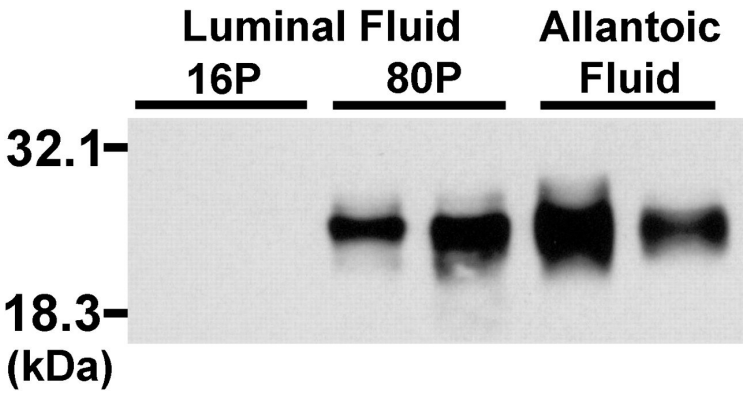

Fig. 6.7. Analysis of immunoreactive STC1 protein in uterine luminal fluid and allantoic fluid of pregnant ewes. Proteins were separated by 15% SDS-PAGE under reducing conditions. Western blot analysis found a single immunoreactive protein of ~25 kDa in uterine luminal fluid and allantoic fluid samples from Day 80 unilaterally pregnant ewes, but not in uterine luminal fluid of Day 16 pregnant ewes or amniotic fluid of Day 80 ewes (data not shown). Positions of prestained molecular weight standards (x 10-3) are indicated.

physiology, and perhaps be useful as an endocrine marker of pregnancy in sheep. STC2, a paralog of STC1, has been identified (205, 286, 287) and detected in various tissues, but its biological roles are not known. In the present study, low levels of STC2 mRNA were detected in the endometrial glands and placenta. Results of the present studies indicate that STC1 is the predominant form of the hormone produced in the ovine uterus and present in uteroplacental tissues of the sheep.

The gland-specific expression of the *STC1* gene in the endometrium of the ovine uterus is similar to *SPP1* and *SERPIN*, which also encode secreted proteins that are present in the uterine lumen and allantoic fluid during pregnancy (67, 68, 288). All three genes are induced in the glands of the endometrium in response to progesterone. Available results indicate that continuous exposure of the uterus to progesterone specifically down-regulates progesterone receptor (PGR) in the endometrial epithelia (1, 17). The disappearance of the PGR from the endometrial GE after Day 13 of pregnancy is associated with subsequent induction of SPP1 after Day 13 followed by SERPIN and STC1 between Days 16 and 18 (10, 65, 66, 69). Indeed, treatment of ewes with an anti-progestin inhibited progesterone-dependent down-regulation of the PGR in the endometrial epithelia of the ovine uterus (69). Furthermore, administration of estrogen with progesterone to ewes up-regulated PGR in the endometrial GE which, in turn, suppressed SPP1 and SERPIN (19). Collectively, available evidence suggests that the STC1 gene is repressed by liganded PGR, and this repression is removed by progesterone down-regulation of the PGR gene that occurs after Day 13 of pregnancy. Thus, progesterone induction of STC1, as well as SPP1 and SERPIN, is not a classical mechanism of gene regulation by progesterone and PGR. Indeed, down-regulation of PGR by progesterone may be requisite for GE remodeling and differentiated function [see (17)]. Given that STC1 gene expression was first observed in the endometrial glands on Day 18 of pregnancy, it is likely that another factor(s) besides progesterone regulates the STC1 gene, because PGR gene expression is lost between Days 13 and 15 of pregnancy (10).

In the present study, ovine PL and GH were found to stimulate STC1 in the endometrial glands. During pregnancy, the uterus is sequentially exposed to progesterone from the ovary and then IFNT, PL and GH from the placenta. IFNT is produced by the mononuclear trophectoderm between Days 11 to 20 of early pregnancy and is the signal for maternal recognition of pregnancy (18). IFNT acts in a paracrine manner on the endometrium to inhibit development of the luteolytic mechanism in the endometrial LE, thereby promoting continued production of progesterone by the corpus luteum. Although IFNT stimulates a large number of genes in the endometrial GE and stroma (18), *STC1* expression in the endometrial glands was not affected by IFNT in the present studies. PL is produced specifically by the trophoblast giant binucleate cells, which first differentiate between Days 14 and 15 in the conceptus (54). Peak concentrations of PL in maternal serum closely parallel dynamic changes in total protein synthesized and secreted by the GE of the ovine endometrium during gestation (61, 289-291). In the current studies, STC1 mRNA and protein was first observed on Day 18 of pregnancy and increased to maximal levels by Day 80 of pregnancy, which is associated with the onset of and increases in PL production by the trophoblast giant binucleate cells. Indeed, SPP1 and SERPIN are also stimulated in the endometrium of ovariectomized ewes treated with progesterone and IFNT (19, 285). Lacroix and coworkers (292, 293) first described the expression of GH in the ovine placenta between Days 35 and 70. Similar to uterine SERPINs, STC1 tended to be stimulated in the endometrial glands by intrauterine infusions of ovine GH. Thus, somatolactogenic hormones from the conceptus act in a paracrine manner on the endometrium to increase *STC1* mRNA in the GE. Future studies will need to focus on the molecular mechanism of PL and GH modulation of *STC1* gene expression. The mechanism likely involves both prolactin receptors (PRLR) and GH receptors (GHR), because ovine PL can signal through a homodimer of the PRLR as well as a heterodimer of the PRLR and GHR, whereas GH signals only via a homodimer of the GHR (294). Indeed, PRLR are expressed exclusively in the endometrial glands of the ovine uterus, and PL binds to those receptors (65, 285, 295). Furthermore, IFNT stimulates PRLR in the endometrial glands

of the ovine uterus (296). Although likely more complicated, available evidence supports the idea that progesterone down-regulates PGR, which is permissive for the onset of STC1, and then IFNT stimulates PRLR that, in turn, respond to PL from the new BNC which further stimulate STC1 gene expression along with GH during later pregnancy.

Although mouse STC1 expression is highly up-regulated in the ovary during lactation (213) and changes dynamically in the uterus during the pre-implantation period (213, 214), its biological and molecular functions in the mammalian uterus during pregnancy are not known. In the present study, we found that STC1 is induced by progesterone and stimulated by PL and GH. The temporal alterations in endometrial STC1 mRNA and protein parallel fetal growth and development. Indeed, our results indicate that STC1 is secreted by the endometrial glands into the uterine lumen, where it is transported into the fetal circulation by the areolae of the intercotyledonary placenta. 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 (59). These results support the idea that STC1 protein is synthesized by the endometrial glands 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 (59, 247). Although the allantois was initially considered a reservoir for waste products of the fetus, it serves to store most secreted proteins from the endometrium, including SERPINs (61, 297). In contrast, amniotic fluid is not in the path for protein clearance by the fetal kidney and, therefore, does not function in this capacity. Alternatively, STC1 may originate from the fetus itself, given that *Stc1* is highly expressed by the mouse fetus, in particular by the kidneys, testes, bone and muscle (298).

Although the functions of uterine STC1 are not known, based on its biological properties in fish and mammals, it may be involved in the regulation of calcium and phosphate transport by placental membranes as well as their homeostasis in the fetus. All of the nutrients and minerals required to provide the anabolic requirements of the

developing ovine fetus must pass from the maternal circulation through either the uterine glands (interplacentomal) or feto-maternal syncytiotrophoblast (placentomal) and then cross the placental trophoblast epithelium (299). Calcium is essential for cellular homeostasis and function. During pregnancy, fetal calcium must cross the placenta and in exponentially increasing amounts during the second half of gestation to support fetal bone growth (300). Calcium transport across the placenta is an active process, because serum calcium in the fetus is higher than the mother (301). Indeed, S100 calcium binding protein G (S100G, also known calbindin-D9K) is present in the maternal endometrial glands and is higher in the trophoblasts of the interplacentomal placenta as compared to that of the placentomes (302, 303). Given the importance of calcium in placental function and fetal growth, STC1 from the endometrial glands may regulate calcium and phosphate homeostasis in the placenta as well as perhaps the fetus.

CHAPTER VII
SUMMARY AND CONCLUSIONS

Summary

Experiments described in this dissertation were conducted to identify and characterize novel implantation-related genes in the ovine uterus during the peri-implantation period. It has been hypothesized that a variety of proteases, as well as their inhibitors, regulate endometrial remodeling and trophoblast invasion in many species (e.g. mouse, rat, cat, sheep, pig, and human) during conceptus implantation and placentation (219). Trophoblast invasion in ruminants is limited to fusion of migrating binucleate cells with uterine epithelium, but considerable tissue remodeling and angiogenesis occurs within the endometrium at implantation which is associated with the cysteine and serine proteases and production of matrix metalloproteinases (MMPs) by the endometrium and conceptus (221, 222).

Results described in Chapters II and III indicate that similar to endometria of other mammals, expression of many cathepsins (*CTS*) and cystatin C (*CST3*; an inhibitor of CTS) were detected in endometria of cyclic and early pregnant ewes. In the present study, cysteine proteases CTSB, CTSH, CTSK, CTSL, CTSS, CTSZ and aspartyl protease CTSD, and CST3 were found to be expressed in the ovine endometrium, and expression of *CTSB, CTSD, CTSH, CTSL, CTSZ,* and *CST3* mRNA increased between Days 10 and 20 of early pregnancy. Results of these studies of CTSL and CST3 in the ovine uterus are very similar to those for mice, in which expression of CTSL and CTSB by invasive trophoblast giant cells was balanced by coordinated expression of CST3 in the decidualizing stroma at the implantation site. Therefore, the dynamic and differential expression of *CTS* and *CST3* genes between cyclic and pregnant ewes suggests functional diversity in mechanisms responsible for expression of *CTS* and *CST3* genes that may be responsible for optimization of a uterine environment that supports conceptus implantation and placentation during establishment and maintenance of pregnancy (222). In Study One, temporal changes in expression of endometrial *CTSL*

and *CST3* mRNA in cyclic and pregnant ewes supported the hypothesis that ovarian P4 regulates transcription of the *CTSL* and *CST3* gene in the endometrial LE. The increase in *CTSL* and *CST3* mRNAs in LE and sGE, between Days 10 and 12 post-estrus/mating, is coincident with the disappearance of PGR mRNA and protein in these epithelia (10). Similarly, the decrease in *CTSL* and *CST3* mRNAs between Days 14 and 16 of the cycle is coincident with the reappearance of PGR protein in endometrial LE due to regression of the CL and loss of P4. In Study Two, *CTSL* and *CST3* mRNA was detected in endometrial LE and sGE of ovariectomized ewes treated with P4 for 12 days, but this expression was prevented by administration of the PGR antagonist ZK 136,317. Continuous exposure of the sheep uterus to P4 for 8 to 10 days down-regulates *PGR* expression in endometrial LE and sGE, but not stroma or myometrium (231). PGR are present in the endometrial epithelia of P4+ZK-treated sheep (243), because PGR antagonists prevent inhibitory effects of P4 on *PGR* gene expression. Consequently, P4 modulation of *CTSL* and *CST3* mRNA may be attributed, at least in part, to down-regulation of *PGR* by P4 that occurs in LE and sGE between Days 10 and 12 of the cycle and pregnancy (223). Thus, PGR loss in endometrial epithelia may reprogram these cells, allowing them to increase expression of genes associated with implantation (223, 224). Alternatively, P4 may act on PGR-positive stromal cells to induce them to express growth factors or changes in the ECM that regulate expression of selected epithelial genes (223).

In addition to regulation by P4, results of these studies indicate that CTSL and CST3 expression is further enhanced by IFNT. In Study Two, intrauterine administration of IFNT increased *CTSL* and *CST3* mRNA, but only in P4-treated ewes. One hypothesis is that IFNT can only stimulate transcription of the *CTSL* and *CST3* gene in the absence of liganded PGR, i.e., after down-regulation of *PGR* by P4. Alternatively, the PGR-positive stroma may produce a 'progestamedin', e.g., FGF7, FGF10 or HGF, that could be required for LE and sGE to respond to IFNT (223). The signaling pathway whereby IFNT regulates transcription of the *CTSL* and *CST3* gene is not known, but it clearly does not involve the classical JAK-STAT-IRF (IFN regulatory factor)

signaling pathway as LE and sGE do not express STAT1, STAT2 or ISGF3G (1, 125, 156, 223).

What are the molecular mechanisms and signal transduction pathways activated by IFNT to regulate transcription of the novel epithelial genes, such as *WNT7A, LGALS15, CTSL,* and *CST3*, only in LE and sGE in the ovine uterus during the peri-implantation period? The current working hypothesis is that IFNT utilizes STAT1-independent signaling pathway(s) to stimulate transcription of those genes in the LE and sGE (Fig. 7.1). In the ovine endometrial LE and sGE, the essential components of the JAK/STAT signal transduction, such as STAT1, -2, and ISGF3G, are not expressed, but IRF2, a potent transcriptional repressor of ISGs, was identified specifically in those cells, where it could repress or suppress the transcriptional acitivity of the promoter regions of ISGs that contain ISREs and IRF-Es (see bottom panel) (124, 125, 145, 151). Further, in our *in silico* study, the enhancer/promoter regions of bovine *WNT7A, LGALS15, CTSL,* and *CST3* genes had conserved transcription factor(s) binding sites for AP-1, CEBPB, CREB, ELK1, GATA, and LEF1/TCF7, but not STATs or IRFs. Are there unknown non-classical JAK/STAT signaling pathways that are independent of STAT1? Recently, Platanias *et al.* reported that the generation of responses to Type I IFN requires the coordination and cooperation of multiple distinct signaling cascades including the mitogen-activated protein (MAP) kinase p38 pathway and the phosphatidylinositol 3-kinase (PI3K) pathway (for review see Platanias 2005). The p38 MAP kinase is phosphorylated and activated in several IFN-sensitive cell lines in response to Type I IFN such as IFNA and its inhibitor (SB203580) blocks IFN-inducible transcription (304, 305). Inhibition of p38 MAP kinase has no effects on the phosphorylation of STAT1 or -2, and formation of the ISGF3 transcriptional complex (304, 306). In addition, Type II IFN (IFNG) did not activate p38 MAP kinase in several cell lines (305, 307). Further, in the bovine uterus, IFNT activates the p38 MAP kinase pathway for induction of PTGS2 in myometrial cells (308). These results indicate that p38 MAP kinase may play a role in Type I IFN-mediated signal transduction that is independent of STATs. Therefore, IFNT activation of p38 MAP kinase may be one signaling pathway whereby IFNT

stimulates transcription of certain genes independent of STAT1 in the ovine uterus. Meanwhile, PI3K is activated in response to Type I or II IFNs. In the case of the Type I IFN signaling pathway, Type I IFNs activate the PI3K-signal pathway downstream of JAKs, in an insulin receptor substrate (IRS)-dependent but STAT-independent manner (306, 309). The proposed model for the IFNT signal transduction cascade that is STAT1-independent in the ovine LE and sGE is illustrated in the upper panel of Fig 7.1 (adapted from Platanias 2005). IFNT-activated JAK1/TYK2 may regulate the phosphorylation of PI3K, resulting in the downstream activation of phosphoinositide-dependent protein kinase 1 (PDK1) and proto oncogenic protein kinase Akt (AKT). The activated AKT translocates into the nucleus and then phosphorylates a variety of target proteins such as CREB (cAMP-response element binding protein)-binding protein (CBP)/p300 or NF-κB. Also, IFNT may activate MAPK kinase kinase (MAPKKK) or Raf which is activated by activated Ras. Activated MAPKKK and/or Raf subsequently regulate activation of downstream effectors including MAPK kinase (MAPKK), p38 MAPK, MEK, or extracellular signal-regulated kinase (ERK). In addition, the mammalian target of rapamycin (mTOR)-p70 ribosomal protein S6 kinase (p70S6K) pathways which are activated by PI3K or AKT, may be involved in mRNA translation of ISGs by phosphorylated ribosomal protein S6 (RPS6) and translational respressor 4EBP1 (eukaryotic translation-initiation factor 4 E (EIF4-E)-binding protein 1). This hypothesis is supported by available results that IFNT and growth factors including insulin-like growth factor 2 (IGF2) stimulate PI3K-AKT and MAPK signal transduction cascades in ovine trophectodermal and LE cells (unpublished observation). Meanwhile, another possible scenario in the ovine uterus during the peri-implantation period is that IFNT may induce WNT7A using the canonical WNT signaling pathway between Days 12 and 16 of pregnancy and then WNT7A acts in an autocrine or paracrine manner to stimulate the *LGALS15, CTSL*, and *CST3* genes in endometrial LE and sGE. Because *WNT7A* is the only gene truly induced by IFNT, its expression is not detected on Day 12 of pregnancy, but is induced by IFNT between Days 14 and 16 (156). In fact, *LGALS15, CTSL,* and *CST3* genes are stimulated by IFNT between Days 14 and 16 of pregnancy,

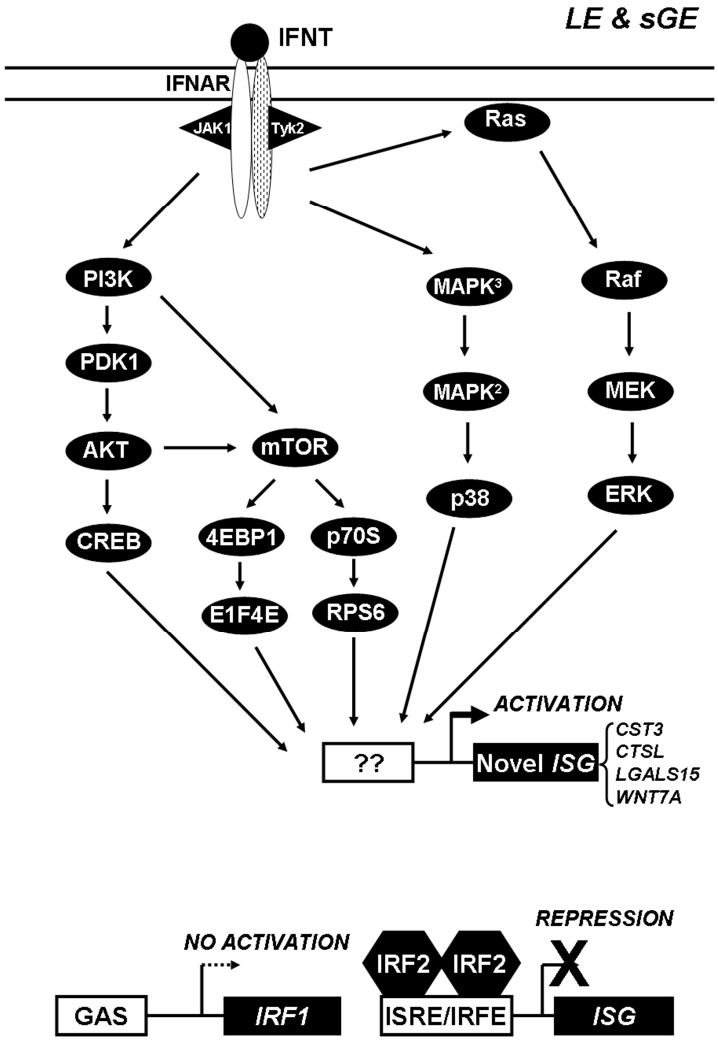

Fig. 7.1. A proposed model of IFNT signal transduction cascades that is independent of STAT1 in the ovine LE and sGE. IFNT-activated JAK1/TYK2 may regulate the phosphorylation of PI3K, resulting in the downstream activation of phosphoinositide-dependent protein kinase 1 (PDK1) and proto oncogenic protein kinase Akt (AKT). The activated AKT translocate into the nucleus and phosphorylate a variety of target proteins such as CREB (cAMP-response element binding protein) binding protein (CBP)/p300 or NF-κB. Also, IFNT may activate MAPK kinase kinase (MAPKKK) or Raf which is activated by activated Ras. Activated MAPKKK and/or Raf subsequently regulate activation of downstream effectors including MAPK kinase (MAPKK), p38 MAPK, or MEK, extracellular signal-regulated kinase (ERK), respectively. In addition, the mammalian target of rapamycin (mTOR)-p70 ribosomal protein S6 kinase (p70S6K) pathways which is activated by PI3K or AKT, may be involved in mRNA translation of ISGs by phosphorylated ribosomal protein S6 (RPS6) and translational respressor 4EBP1 (eukaryotic translation-initiation factor 4 E (EIF4-E)-binding protein 1).

which correlates with *WNT7A* upregulation (157, 250, 265). Therefore, future experiments will be directed toward determining the physiological role of the above epithelial genes and novel STAT1-independent signaling pathways in peri-implantation conceptus development as well as endometrial remodeling in response to IFNT.

Interestingly, the ovine placenta expresses large numbers of aspartic proteinase inhibitor genes, termed pregnancy-associated glycoproteins (234), and the endometrial glands express large amounts of serine protease inhibitors, termed serpins or uterine milk proteins (64), that could regulate the activity of endometrial CTS identified in these studies. Therefore, the molecular control of expression of CTS in the ovine endometrium may play an important role in establishing a regulatory network of multiple proteolytic enzymes responsible for ECM remodeling during implantation and placentation. Futher, coordinated increases in CTSL and CTSB with CST3 occur in endometrial LE and sGE as well as in conceptus trophectoderm during early pregnancy. Thus, one biological role of CST3 may be to inhibit the actions of cysteine proteases produced by the conceptus and endometrial epithelia in order to limit the invasive activity of the trophoblast. These results support the general idea that proteases and their inhibitors expressed at the maternal-fetal interface are important for uterine receptivity, endometrial remodeling and conceptus implantation during pregnancy in mammals.

Experiments described in Chapter IV identified two antiviral-related genes, *RSAD2* and *IFIH1*, as being induced in the ovine endometrium in response to IFNT from the conceptus in a P4-independent manner. Clearly, P4 and IFNT have complex, independent and complementary effects on expression of a number of genes in the ovine endometrium during early pregnancy (259, 260). In this study, P4 was not required for IFNT induction of RSAD2 and IFIH1 in the endometrium. Further, treatment of ovine endometrial LE and BEND cells with recombinant ovine IFNT induced RSAD2 and IFIH1 expression without a requirement for serum or P4 in the medium. In contrast, IFNT induction of several non-classical IFNT-stimulated genes (ISGs), such as *LGALS15* and *WNT7A* in endometrial LE and sGE is dependent on P4 (156, 157, 250,

265), which is hypothesized to involve P4 down-regulation of the PGR in those epithelia (17, 259).

The majority of ISGs induced by IFNT without a requirement for P4 in the ovine uterus are restricted to endometrial stroma and middle to deep glands as well as immune cells (17, 18). This finding was initially surprising because all ovine endometrial cell types express IFNAR1 and IFNAR2 subunits of the common Type I IFN receptor (99). However, available results also indicate that IRF2, a potent transcriptional repressor of ISGs (127), is expressed specifically in endometrial LE and sGE and that it represses transcriptional activity of promoters containing ISRE or IRFE (125). Thus, IRF2 in LE and sGE is proposed to restrict IFNT induction of many ISGs to endometrial stroma and glandular epithelium.

One biological role of RSAD2 and IFIH1 could be to prevent viral infection of the uterus during the critical peri-implantation period of pregnancy, particularly when the conceptus does not have a developed immune system or antiviral defenses. RSAD2 and IFIH1 are implicated in establishing an antiviral state by modulation of innate immune responses. For example, stable expression of RSAD2 in fibroblasts inhibits human cytomegalovirus infection (167). Given that IFIH1 also has growth suppressive properties, IFNT induction may suppress activation of cells within the endometrium, which could be beneficial for pregnancy. In other species such as rodents and humans, resident and recruited immune cells within the endometrium play important roles in placentation and a successful pregnancy (29, 268). Unfortunately, knowledge of which immune cells are present in the ovine uterus during pregnancy and their biological functions is limited. In this study, the number of IFIH1- and, in particular, RSAD2-positive immune cells markedly increased in the endometria during pregnancy and in response to IFNT, but it is not clear whether these cells were recruited in response to IFNT or were already present and stimulated to express RSAD2 by IFNT. The IFNT stimulated resident immune cells in the endometrium may migrate from the uterus, because ISGs are higher in peripheral blood leukocytes isolated from pregnant as compared to non-pregnant ewes and cows (273). In fact, IFNT possesses

immunoregulatory activity and can inhibit mitogen-induced lymphocyte proliferation (275, 276) as well as modulate activity of natural killer cells (28, 277). These effects of IFNT may prevent immune cell-mediated destruction of the conceptus (27). Finally, some ISGs, such as CXCL10, from immune cells may have direct effects on conceptus implantation (278, 279).

Results described in Chapter V demonstrate that *STC1* mRNA is exclusively expressed in the endometrial glands of the ovine uterus after Day 16 of pregnancy. Further, STC1 protein was present near the apical surface of gland cells and secreted into the uterine lumen, as evidenced by the presence of immunoreactive STC1 protein in uterine secretions, placental areolae, and allantoic fluid. The gland-specific expression of the STC1 gene in the endometrium of the ovine uterus is similar to SPP1 and SERPIN, which are also secreted proteins that are present in the uterine lumen and allantoic fluid during pregnancy (67, 68, 288). All three genes are induced in the glands of the endometrium in response to P4. Available results indicate that continuous exposure of the uterus to P4 specifically down-regulates PGR in endometrial epithelia (1, 17) and this is associated with expression of SPP1 after Day 13 followed by SERPIN and STC1 between Days 16 and 18 (10, 65, 66, 69). Indeed, treatment of ewes with an anti-progestin inhibited P4-dependent down-regulation of the PGR in the endometrial epithelia of the ovine uterus (69). Furthermore, administration of E2 with P4 to ewes up-regulated PGR in the endometrial GE which, in turn, suppressed expression of SPP1 and SERPIN (19). Therefore, available evidence suggests that the STC1 gene is repressed by liganded PGR, and this repression is removed by P4 down-regulation of the PGR gene that occurs after Day 13 of pregnancy. Thus, P4 induction of STC1, as well as SPP1 and SERPIN, is not a classical mechanism of gene regulation by P4 and PGR. Indeed, down-regulation of PGR by P4 may be required for GE remodeling and differentiated function (17).

In this study, ovine placental lactogen (CSH1) and growth hormone (GH) were found to stimulate STC1 in the endometrial glands. During pregnancy, the uterus is sequentially exposed to P4 from the ovary and then IFNT, GH and PL from the placenta.

PL is produced specifically by the trophoblast giant binucleate cells, which first differentiate between Days 14 and 15 in the conceptus (54). Peak concentrations of PL in maternal serum closely parallel dynamic changes in total protein synthesized and secreted by GE of the ovine endometrium during gestation (61, 289-291). In this study, STC1 mRNA and protein were first observed on Day 18 of pregnancy and increased to maximal levels by Day 80 of pregnancy, which is associated with the onset of and increases in PL production by the trophoblast giant binucleate cells. Indeed, SPP1 and SERPIN are also stimulated in the endometrium of ovariectomized ewes treated with P4 and IFNT (19, 285). Lacroix and coworkers (292, 293) first described the expression of GH in the ovine placenta between Days 35 and 70 of gestation. Similar to uterine SERPINs, STC1 tended to be stimulated in the endometrial glands by intrauterine infusions of ovine GH. Thus, somatolactogenic hormones from the conceptus act in a paracrine manner on the endometrium to increase *STC1* mRNA in GE.

Conclusions

During the peri-implantation period in sheep, *CTSL* and *CST3* are novel P4-induced and IFNT-stimulated genes in endometrial LE and sGE. The majority of ISGs such as *RSAD2* and *IFIH1* are expressed by endometrial stroma and middle to deep glands as well as immune cells in response to cell signaling involving the classical STAT1-dependent JAK/STAT signal transduction pathway without a requirement for P4 in the ovine uterus. It has been reported and hypothesized that Type I IFNs and many common ISGs are upregulated for the implanting conceptus in the endometrium during pregnancy in humans, rodents, and domestic animals. Recent evidence that ISGs are among the most upregulated genes in human decidualized stromal cells by trophoblast conditioned medium, perhaps due to production of type I IFNs by the trophoblast supports the hypothesis that a lack of ISG expression would compromise pregnancy. In contrast, IFNT induction of several non-classical ISGs, such as *LGALS15*, *WNT7A*, *CTSL*, and *CST3* in endometrial LE and sGE is dependent on P4, which is hypothesized to involve P4-induced down-regulation of PGR in those epithelia, as well as induction of

an unknown STAT1-independent signaling pathway. Thus, knowledge of mechanisms whereby IFNT stimulates *CTSL* and *CST3* gene expression in endometrial LE and sGE is expected to elucidate a non-classical signaling pathway for Type I IFNs. Further, increased knowledge of expression of uterine proteases and their inhibitors is important for developing therapeutic strategies to prevent, treat and diagnose infertility in humans and domestic animals. Meanwhile, biological roles of *RSAD2* and *IFIH1* could be to prevent viral infection of the uterus during the critical peri-implantation period of pregnancy, particularly when the conceptus does not have a developed immune system or antiviral defenses. In the ovine uterus, *STC1* is induced by P4 and stimulated by PL and GH. Indeed, results described in this dissertation support the idea that STC1 protein is synthesized by the endometrial glands, secreted into the uterine lumen, absorbed by placental areolae and transported into the fetal circulation, cleared by the kidney into the allantois via the urachus, and reabsorbed into the fetal circulation to influence conceptus/fetus growth and development.

REFERENCES

1. **Spencer TE, Bazer FW** 2002 Biology of progesterone action during pregnancy recognition and maintenance of pregnancy. Front Biosci 7:d1879-98
2. **Spencer TE, Johnson GA, Bazer FW, Burghardt RC** 2004 Implantation mechanisms: insights from the sheep. Reproduction 128:657-68
3. **Ashworth CJ, Bazer FW** 1989 Changes in ovine conceptus and endometrial function following asynchronous embryo transfer or administration of progesterone. Biol Reprod 40:425-33
4. **Farin CE, Imakawa K, Roberts RM** 1989 In situ localization of mRNA for the interferon, ovine trophoblast protein-1, during early embryonic development of the sheep. Mol Endocrinol 3:1099-107
5. **Bazer FW** 1992 Mediators of maternal recognition of pregnancy in mammals. Proc Soc Exp Biol Med 199:373-84
6. **Roberts RM, Ealy AD, Alexenko AP, Han CS, Ezashi T** 1999 Trophoblast interferons. Placenta 20:259-64
7. **Spencer TE, Bazer FW** 1996 Ovine interferon tau suppresses transcription of the estrogen receptor and oxytocin receptor genes in the ovine endometrium. Endocrinology 137:1144-7
8. **Fleming JA, Choi Y, Johnson GA, Spencer TE, Bazer FW** 2001 Cloning of the ovine estrogen receptor-alpha promoter and functional regulation by ovine interferon-tau. Endocrinology 142:2879-87
9. **Wathes DC, Hamon M** 1993 Localization of oestradiol, progesterone and oxytocin receptors in the uterus during the oestrous cycle and early pregnancy of the ewe. J Endocrinol 138:479-92
10. **Spencer TE, Bazer FW** 1995 Temporal and spatial alterations in uterine estrogen receptor and progesterone receptor gene expression during the estrous cycle and early pregnancy in the ewe. Biol Reprod 53:1527-43

11. **Hooper SB, Watkins WB, Thorburn GD** 1986 Oxytocin, oxytocin-associated neurophysin, and prostaglandin F2 alpha concentrations in the utero-ovarian vein of pregnant and nonpregnant sheep. Endocrinology 119:2590-7
12. **Stevenson KR, Riley PR, Stewart HJ, Flint AP, Wathes DC** 1994 Localization of oxytocin receptor mRNA in the ovine uterus during the oestrous cycle and early pregnancy. J Mol Endocrinol 12:93-105
13. **Lamming GE, Wathes DC, Flint AP, Payne JH, Stevenson KR, Vallet JL** 1995 Local action of trophoblast interferons in suppression of the development of oxytocin and oestradiol receptors in ovine endometrium. J Reprod Fertil 105:165-75
14. **Spencer TE, Becker WC, George P, Mirando MA, Ogle TF, Bazer FW** 1995 Ovine interferon-tau inhibits estrogen receptor up-regulation and estrogen-induced luteolysis in cyclic ewes. Endocrinology 136:4932-44
15. **Spencer TE, Becker WC, George P, Mirando MA, Ogle TF, Bazer FW** 1995 Ovine interferon-tau regulates expression of endometrial receptors for estrogen and oxytocin but not progesterone. Biol Reprod 53:732-45
16. **Fleming JG, Spencer TE, Safe SH, Bazer FW** 2006 Estrogen regulates transcription of the ovine oxytocin receptor gene through GC-rich SP1 promoter elements. Endocrinology 147:899-911
17. **Spencer TE, Johnson GA, Burghardt RC, Bazer FW** 2004 Progesterone and placental hormone actions on the uterus: insights from domestic animals. Biol Reprod 71:2-10
18. **Spencer TE, Burghardt RC, Johnson GA, Bazer FW** 2004 Conceptus signals for establishment and maintenance of pregnancy. Anim Reprod Sci 82-83:537-50
19. **Spencer TE, Gray A, Johnson GA, Taylor KM, Gertler A, et al.** 1999 Effects of recombinant ovine interferon tau, placental lactogen, and growth hormone on the ovine uterus. Biol Reprod 61:1409-18
20. **Gray CA, Bartol FF, Tarleton BJ, Wiley AA, Johnson GA, et al.** 2001 Developmental biology of uterine glands. Biol Reprod 65:1311-23

21. **Gray CA, Bartol FF, Taylor KM, Wiley AA, Ramsey WS, et al.** 2000 Ovine uterine gland knock-out model: effects of gland ablation on the estrous cycle. Biol Reprod 62:448-56

22. **Gray CA, Bazer FW, Spencer TE** 2001 Effects of neonatal progestin exposure on female reproductive tract structure and function in the adult ewe. Biol Reprod 64:797-804

23. **Gray CA, Taylor KM, Bazer FW, Spencer TE** 2000 Mechanisms regulating norgestomet inhibition of endometrial gland morphogenesis in the neonatal ovine uterus. Mol Reprod Dev 57:67-78

24. **Gray CA, Taylor KM, Ramsey WS, Hill JR, Bazer FW, et al.** 2001 Endometrial glands are required for preimplantation conceptus elongation and survival. Biol Reprod 64:1608-13

25. **Kimmins S, MacLaren LA** 2001 Oestrous cycle and pregnancy effects on the distribution of oestrogen and progesterone receptors in bovine endometrium. Placenta 22:742-8

26. **Geisert RD, Pratt TN, Bazer FW, Mayes JS, Watson GH** 1994 Immunocytochemical localization and changes in endometrial progestin receptor protein during the porcine oestrous cycle and early pregnancy. Reprod Fertil Dev 6:749-60

27. **Hansen PJ** 1995 Interactions between the immune system and the ruminant conceptus. J Reprod Fertil Suppl 49:69-82

28. **Tekin S, Hansen PJ** 2002 Natural killer-like cells in the sheep: functional characterization and regulation by pregnancy-associated proteins. Exp Biol Med (Maywood) 227:803-11

29. **Croy BA, Esadeg S, Chantakru S, van den Heuvel M, Paffaro VA, et al.** 2003 Update on pathways regulating the activation of uterine Natural Killer cells, their interactions with decidual spiral arteries and homing of their precursors to the uterus. J Reprod Immunol 59:175-91

30. **Baird DT, Land RB, Scaramuzzi RJ, Wheeler AG** 1976 Endocrine changes associated with luteal regression in the ewe; the secretion of ovarian oestradiol, progesterone and androstenedione and uterine prostaglandin F2alpha throughout the oestrous cycle. J Endocrinol 69:275-86

31. **McCracken JA, Baird DT, Goding JR** 1971 Factors affecting the secretion of steroids from the transplanted ovary in the sheep. Recent Prog Horm Res 27:537-82 passim

32. **McCracken JA, Custer EE, Lamsa JC** 1999 Luteolysis: a neuroendocrine-mediated event. Physiol Rev 79:263-323

33. **Wiltbank J, Casida L** 1956 Alteration of ovarian activity by hysterectomy. J Anim Sci 15:134-140

34. **Wathes DC, Lamming GE** 1995 The oxytocin receptor, luteolysis and the maintenance of pregnancy. J Reprod Fertil Suppl 49:53-67

35. **Charpigny G, Reinaud P, Tamby JP, Creminon C, Guillomot M** 1997 Cyclooxygenase-2 unlike cyclooxygenase-1 is highly expressed in ovine embryos during the implantation period. Biol Reprod 57:1032-40

36. **McCracken JA, Schramm W, Okulicz WC** 1984 Hormone receptor control of pulsatile secretion of PGF-2alpha from the ovine uterus during luteolysis and its abrogation in early pregnancy. Anim Reprod Sci 7:31-55

37. **Hixon JE, Flint AP** 1987 Effects of a luteolytic dose of oestradiol benzoate on uterine oxytocin receptor concentrations, phosphoinositide turnover and prostaglandin F-2 alpha secretion in sheep. J Reprod Fertil 79:457-67

38. **Silvia WJ, Raw RE** 1993 Regulation of pulsatile secretion of prostaglandin F2 alpha from the ovine uterus by ovarian steroids. J Reprod Fertil 98:341-7

39. **Boshier DP, Fairclough RJ, Holloway H** 1987 Assessment of sheep blastocyst effects on neutral lipids in the uterine caruncular epithelium. J Reprod Fertil 79:569-73

40. **Eggleston DL, Wilken C, Van Kirk EA, Slaughter RG, Ji TH, Murdoch WJ** 1990 Progesterone induces expression of endometrial messenger RNA encoding for cyclooxygenase (sheep). Prostaglandins 39:675-83

41. **Kim S, Choi Y, Spencer TE, Bazer FW** 2003 Effects of the estrous cycle, pregnancy and interferon tau on expression of cyclooxygenase two (COX-2) in ovine endometrium. Reprod Biol Endocrinol 1:58

42. **Guillomot M, Flechon JE, Leroy F** 1993 Blastocyst development and implantation. In: C Thibault, MC Levasseur & RHF Hunter (eds.), Reproduction in Mammals and Man, Paris: Ellipses, pp. 387-411.

43. **Guillomot M** 1995 Cellular interactions during implantation in domestic ruminants. Journal of Reproduction and Fertility Supplement:39-51

44. **Guillomot M, Flechon JE, Wintenberger-Torres S** 1981 Conceptus attachment in the ewe: an ultrastructural study. Placenta 2:169-82

45. **Allen WR, Stewart F** 2001 Equine placentation. Reprod Fertil Dev 13:623-34

46. **Renfree M** 1982 Implantation and placentation. In: CR Austin & RV Short (eds.), Reproduction in Mammals and Man, Cambridge: Cambridge University Press, pp. 26-69.

47. **Carson DD, Bagchi I, Dey SK, Enders AC, Fazleabas AT, et al.** 2000 Embryo implantation. Dev Biol 223:217-37

48. **Wintenberger-Torres S, Flechon JE** 1974 Ultrastructural evolution of the trophoblast cells of the pre-implantation sheep blastocyst from day 8 to day 18. J Anat 118:143-53

49. **Boshier DP** 1969 A histological and histochemical examination of implantation and early placentome formation in sheep. J Reprod Fertil 19:51-61

50. **Guillomot M, Guay P** 1982 Ultrastructural features of the cell surfaces of uterine and trophoblastic epithelia during embryo attachment in the cow. Anat Rec 204:315-22

51. **Wooding FB, Staples LD, Peacock MA** 1982 Structure of trophoblast papillae on the sheep conceptus at implantation. J Anat 134 (Pt 3):507-16

52. **Wooding FB** 1984 Role of binucleate cells in fetomaternal cell fusion at implantation in the sheep. Am J Anat 170:233-50

53. **Wimsatt WA** 1951 Observations on the morphogenesis, cytochemistry, and significance of the binocleate giant cells of the placenta of ruminants. Am J Anat 89:233-81

54. **Wooding FB** 1992 Current topic: the synepitheliochorial placenta of ruminants: binucleate cell fusions and hormone production. Placenta 13:101-13

55. **Johnson GA, Bazer FW, Jaeger LA, Ka H, Garlow JE, et al.** 2001 Muc-1, integrin, and osteopontin expression during the implantation cascade in sheep. Biol Reprod 65:820-8

56. **Burghardt RC, Johnson GA, Jaeger LA, Ka H, Garlow JE, et al.** 2002 Integrins and extracellular matrix proteins at the maternal-fetal interface in domestic animals. Cells Tissues Organs 172:202-17

57. **Bowen JA, Bazer FW, Burghardt RC** 1997 Spatial and temporal analyses of integrin and Muc-1 expression in porcine uterine epithelium and trophectoderm in vitro. Biol Reprod 56:409-15

58. **Johnson GA, Burghardt RC, Bazer FW, Spencer TE** 2003 Osteopontin: roles in implantation and placentation. Biol Reprod 69:1458-71

59. **Bazer FW** 1975 Uterine protein secretions: Relationship to development of the conceptus. J Anim Sci 41:1376-82

60. **Moffatt J, Bazer FW, Hansen PJ, Chun PW, Roberts RM** 1987 Purification, secretion and immunocytochemical localization of the uterine milk proteins, major progesterone-induced proteins in uterine secretions of the sheep. Biol Reprod 36:419-30

61. **Moffatt RJ, Bazer FW, Roberts RM, Thatcher WW** 1987 Secretory function of the ovine uterus: effects of gestation and steroid replacement therapy. J Anim Sci 65:1400-10

62. **Tong W, Pollard JW** 1999 Progesterone inhibits estrogen-induced cyclin D1 and cdk4 nuclear translocation, cyclin E- and cyclin A-cdk2 kinase activation, and cell proliferation in uterine epithelial cells in mice. Mol Cell Biol 19:2251-64

63. **Musgrove EA, Swarbrick A, Lee CS, Cornish AL, Sutherland RL** 1998 Mechanisms of cyclin-dependent kinase inactivation by progestins. Mol Cell Biol 18:1812-25

64. **Peltier MR, Hansen PJ** 2001 Immunoregulatory activity, biochemistry, and phylogeny of ovine uterine serpin. Am J Reprod Immunol 45:266-72

65. **Stewart MD, Johnson GA, Gray CA, Burghardt RC, Schuler LA, et al.** 2000 Prolactin receptor and uterine milk protein expression in the ovine endometrium during the estrous cycle and pregnancy. Biol Reprod 62:1779-89

66. **Johnson GA, Spencer TE, Burghardt RC, Bazer FW** 1999 Ovine osteopontin: I. Cloning and expression of messenger ribonucleic acid in the uterus during the periimplantation period. Biol Reprod 61:884-91

67. **Johnson GA, Burghardt RC, Joyce MM, Spencer TE, Bazer FW, et al.** 2003 Osteopontin is synthesized by uterine glands and a 45-kDa cleavage fragment is localized at the uterine-placental interface throughout ovine pregnancy. Biol Reprod 69:92-8

68. **Johnson GA, Burghardt RC, Spencer TE, Newton GR, Ott TL, Bazer FW** 1999 Ovine osteopontin: II. Osteopontin and alpha(v)beta(3) integrin expression in the uterus and conceptus during the periimplantation period. Biol Reprod 61:892-9

69. **Johnson GA, Spencer TE, Burghardt RC, Taylor KM, Gray CA, Bazer FW** 2000 Progesterone modulation of osteopontin gene expression in the ovine uterus. Biol Reprod 62:1315-21

70. **Butler WT** 1989 The nature and significance of osteopontin. Connect Tissue Res 23:123-36

71. **Short RV** 1969 Maternal recognition of pregnancy. In: Wolstenholm GEW, O'Conner M, editors. Foetal Anatomy. Churchill, London: Wiley, pp. 2-26

72. **Moor RM, Rowson LE** 1964 Influence of the Embryo and Uterus on Luteal Function in the Sheep. Nature 201:522-3

73. **Moor RM, Rowson LE** 1966 The corpus luteum of the sheep: effect of the removal of embryos on luteal function. J Endocrinol 34:497-502

74. **Rowson LE, Moor RM** 1967 The influence of embryonic tissue homogenate infused into the uterus, on the life-span of the corpus luteum in the sheep. J Reprod Fertil 13:511-6

75. **Heyman Y, Camous S, Fevre J, Meziou W, Martal J** 1984 Maintenance of the corpus luteum after uterine transfer of trophoblastic vesicles to cyclic cows and ewes. J Reprod Fertil 70:533-40

76. **Wilson ME, Lewis GS, Bazer FW** 1979 Proteins of ovine blastocyst origin. Proc Soc Study Reprod, Quebec, Canada; p. 101A

77. **Godkin JD, Bazer FW, Moffatt J, Sessions F, Roberts RM** 1982 Purification and properties of a major, low molecular weight protein released by the trophoblast of sheep blastocysts at day 13-21. J Reprod Fertil 65:141-50

78. **Godkin JD, Bazer FW, Roberts RM** 1984 Ovine trophoblast protein 1, an early secreted blastocyst protein, binds specifically to uterine endometrium and affects protein synthesis. Endocrinology 114:120-30

79. **Fincher KB, Bazer FW, Hansen PJ, Thatcher WW, Roberts RM** 1986 Proteins secreted by the sheep conceptus suppress induction of uterine prostaglandin F-2 alpha release by oestradiol and oxytocin. J Reprod Fertil 76:425-33

80. **Vallet JL, Bazer FW, Fliss MF, Thatcher WW** 1988 Effect of ovine conceptus secretory proteins and purified ovine trophoblast protein-1 on interoestrous interval and plasma concentrations of prostaglandins F-2 alpha and E and of 13,14-dihydro-15-keto prostaglandin F-2 alpha in cyclic ewes. J Reprod Fertil 84:493-504

81. **Ott TL, Van Heeke G, Hostetler CE, Schaule TK, Olmsted JJ, et al.** 1993 Intrauterine injection of recombinant ovine interferon-tau extends the interestrous interval in sheep. Theriogenology 40:757-69

82. **Imakawa K, Anthony RV, Kazemi M, Marotti KR, Polites HG, Roberts RM** 1987 Interferon-like sequence of ovine trophoblast protein secreted by embryonic trophectoderm. Nature 330:377-9

83. **Pontzer CH, Torres BA, Vallet JL, Bazer FW, Johnson HM** 1988 Antiviral activity of the pregnancy recognition hormone ovine trophoblast protein-1. Biochem Biophys Res Commun 152:801-7

84. **Pontzer CH, Bazer FW, Johnson HM** 1991 Antiproliferative activity of a pregnancy recognition hormone, ovine trophoblast protein-1. Cancer Res 51:5304-7

85. **Roberts RM, Imakawa K, Niwano Y, Kazemi M, Malathy PV, et al.** 1989 Interferon production by the preimplantation sheep embryo. J Interferon Res 9:175-87

86. **Roberts RM, Cross JC, Leaman DW** 1992 Interferons as hormones of pregnancy. Endocr Rev 13:432-52

87. **Roberts RM, Leaman DW, Cross JC** 1992 Role of interferons in maternal recognition of pregnancy in ruminants. Proc Soc Exp Biol Med 200:7-18

88. **Bazer FW, Spencer TE, Ott TL** 1996 Placental interferons. Am J Reprod Immunol 35:297-308

89. **Hansen TR, Imakawa K, Polites HG, Marotti KR, Anthony RV, Roberts RM** 1988 Interferon RNA of embryonic origin is expressed transiently during early pregnancy in the ewe. J Biol Chem 263:12801-4

90. **Fillion C, Chaouat G, Reinaud P, Charpigny JC, Martal J** 1991 Immunoregulatory effects of ovine trophoblastin protein (oTP): all five isoforms suppress PHA-induced lymphocyte proliferation. J Reprod Immunol 19:237-49

91. **Tennakoon DK, Smith R, Stewart MD, Spencer TE, Nayak M, Welsh CJ** 2001 Ovine IFN-tau modulates the expression of MHC antigens on murine

cerebrovascular endothelial cells and inhibits replication of Theiler's virus. J Interferon Cytokine Res 21:785-92

92. **Ealy AD, Alexenko AP, Keisler DH, Roberts RM** 1998 Loss of the signature six carboxyl amino acid tail from ovine interferon-tau does not affect biological activity. Biol Reprod 58:1463-8

93. **Nephew KP, Whaley AE, Christenson RK, Imakawa K** 1993 Differential expression of distinct mRNAs for ovine trophoblast protein-1 and related sheep type I interferons. Biol Reprod 48:768-78

94. **Baumbach GA, Duby RT, Godkin JD** 1990 N-glycosylated and unglycosylated forms of caprine trophoblast protein-1 are secreted by preimplantation goat conceptuses. Biochem Biophys Res Commun 172:16-21

95. **Guillomot M, Michel C, Gaye P, Charlier N, Trojan J, Martal J** 1990 Cellular localization of an embryonic interferon, ovine trophoblastin and its mRNA in sheep embryos during early pregnancy. Biol Cell 68:205-11

96. **Spencer TE, Ott TL, Bazer FW** 1996 tau-Interferon: pregnancy recognition signal in ruminants. Proc Soc Exp Biol Med 213:215-29

97. **Novick D, Cohen B, Rubinstein M** 1994 The human interferon alpha/beta receptor: characterization and molecular cloning. Cell 77:391-400

98. **Domanski P, Yan H, Witte MM, Krolewski J, Colamonici OR** 1995 Homodimerization and intermolecular tyrosine phosphorylation of the Tyk-2 tyrosine kinase. FEBS Lett 374:317-22

99. **Rosenfeld CS, Han CS, Alexenko AP, Spencer TE, Roberts RM** 2002 Expression of interferon receptor subunits, IFNAR1 and IFNAR2, in the ovine uterus. Biol Reprod 67:847-53

100. **Darnell JE, Jr.** 1997 STATs and gene regulation. Science 277:1630-5

101. **Darnell JE, Jr., Kerr IM, Stark GR** 1994 Jak-STAT pathways and transcriptional activation in response to IFNs and other extracellular signaling proteins. Science 264:1415-21

102. **Stark GR, Kerr IM, Williams BR, Silverman RH, Schreiber RD** 1998 How cells respond to interferons. Annu Rev Biochem 67:227-64

103. **Gauzzi MC, Velazquez L, McKendry R, Mogensen KE, Fellous M, Pellegrini S** 1996 Interferon-alpha-dependent activation of Tyk2 requires phosphorylation of positive regulatory tyrosines by another kinase. J Biol Chem 271:20494-500

104. **Muller M, Briscoe J, Laxton C, Guschin D, Ziemiecki A, et al.** 1993 The protein tyrosine kinase JAK1 complements defects in interferon-alpha/beta and -gamma signal transduction. Nature 366:129-35

105. **Colamonici O, Yan H, Domanski P, Handa R, Smalley D, et al.** 1994 Direct binding to and tyrosine phosphorylation of the alpha subunit of the type I interferon receptor by p135tyk2 tyrosine kinase. Mol Cell Biol 14:8133-42

106. **Colamonici OR, Uyttendaele H, Domanski P, Yan H, Krolewski JJ** 1994 p135tyk2, an interferon-alpha-activated tyrosine kinase, is physically associated with an interferon-alpha receptor. J Biol Chem 269:3518-22

107. **Yan H, Krishnan K, Greenlund AC, Gupta S, Lim JT, et al.** 1996 Phosphorylated interferon-alpha receptor 1 subunit (IFNaR1) acts as a docking site for the latent form of the 113 kDa STAT2 protein. Embo J 15:1064-74

108. **Silvennoinen O, Ihle JN, Schlessinger J, Levy DE** 1993 Interferon-induced nuclear signalling by Jak protein tyrosine kinases. Nature 366:583-5

109. **Shuai K, Horvath CM, Huang LH, Qureshi SA, Cowburn D, Darnell JE, Jr.** 1994 Interferon activation of the transcription factor Stat91 involves dimerization through SH2-phosphotyrosyl peptide interactions. Cell 76:821-8

110. **Haque SJ, Williams BR** 1994 Identification and characterization of an interferon (IFN)-stimulated response element-IFN-stimulated gene factor 3-independent signaling pathway for IFN-alpha. J Biol Chem 269:19523-9

111. **Bhattacharya S, Eckner R, Grossman S, Oldread E, Arany Z, et al.** 1996 Cooperation of Stat2 and p300/CBP in signalling induced by interferon-alpha. Nature 383:344-7

112. **Pine R, Canova A, Schindler C** 1994 Tyrosine phosphorylated p91 binds to a single element in the ISGF2/IRF-1 promoter to mediate induction by IFN alpha and IFN gamma, and is likely to autoregulate the p91 gene. Embo J 13:158-67

113. **Fu XY** 1992 A transcription factor with SH2 and SH3 domains is directly activated by an interferon alpha-induced cytoplasmic protein tyrosine kinase(s). Cell 70:323-35

114. **Hou J, Schindler U, Henzel WJ, Ho TC, Brasseur M, McKnight SL** 1994 An interleukin-4-induced transcription factor: IL-4 Stat. Science 265:1701-6

115. **Yamamoto K, Quelle FW, Thierfelder WE, Kreider BL, Gilbert DJ, et al.** 1994 Stat4, a novel gamma interferon activation site-binding protein expressed in early myeloid differentiation. Mol Cell Biol 14:4342-9

116. **Lew DJ, Decker T, Strehlow I, Darnell JE** 1991 Overlapping elements in the guanylate-binding protein gene promoter mediate transcriptional induction by alpha and gamma interferons. Mol Cell Biol 11:182-91

117. **Horvath CM, Stark GR, Kerr IM, Darnell JE, Jr.** 1996 Interactions between STAT and non-STAT proteins in the interferon-stimulated gene factor 3 transcription complex. Mol Cell Biol 16:6957-64

118. **Leonard WJ, O'Shea JJ** 1998 Jaks and STATs: biological implications. Annu Rev Immunol 16:293-322

119. **Soldaini E, John S, Moro S, Bollenbacher J, Schindler U, Leonard WJ** 2000 DNA binding site selection of dimeric and tetrameric Stat5 proteins reveals a large repertoire of divergent tetrameric Stat5a binding sites. Mol Cell Biol 20:389-401

120. **Zhang X, Shu MA, Harvey MB, Schultz GA** 1996 Regulation of urokinase plasminogen activator production in implanting mouse embryo: effect of embryo interaction with extracellular matrix. Biol Reprod 54:1052-8

121. **Look DC, Pelletier MR, Tidwell RM, Roswit WT, Holtzman MJ** 1995 Stat1 depends on transcriptional synergy with Sp1. J Biol Chem 270:30264-7

122. **Schaefer TS, Sanders LK, Nathans D** 1995 Cooperative transcriptional activity of Jun and Stat3 beta, a short form of Stat3. Proc Natl Acad Sci USA 92:9097-101

123. **Zhang Z, Jones S, Hagood JS, Fuentes NL, Fuller GM** 1997 STAT3 acts as a co-activator of glucocorticoid receptor signaling. J Biol Chem 272:30607-10

124. **Stewart MD, Choi Y, Johnson GA, Yu-Lee LY, Bazer FW, Spencer TE** 2002 Roles of Stat1, Stat2, and interferon regulatory factor-9 (IRF-9) in interferon tau regulation of IRF-1. Biol Reprod 66:393-400

125. **Choi Y, Johnson GA, Burghardt RC, Berghman LR, Joyce MM, et al.** 2001 Interferon regulatory factor-two restricts expression of interferon-stimulated genes to the endometrial stroma and glandular epithelium of the ovine uterus. Biol Reprod 65:1038-49

126. **Nguyen H, Hiscott J, Pitha PM** 1997 The growing family of interferon regulatory factors. Cytokine Growth Factor Rev 8:293-312

127. **Mamane Y, Heylbroeck C, Genin P, Algarte M, Servant MJ, et al.** 1999 Interferon regulatory factors: the next generation. Gene 237:1-14

128. **Taniguchi T, Ogasawara K, Takaoka A, Tanaka N** 2001 IRF family of transcription factors as regulators of host defense. Annu Rev Immunol 19:623-55

129. **Miyamoto M, Fujita T, Kimura Y, Maruyama M, Harada H, et al.** 1988 Regulated expression of a gene encoding a nuclear factor, IRF-1, that specifically binds to IFN-beta gene regulatory elements. Cell 54:903-13

130. **Tanaka N, Kawakami T, Taniguchi T** 1993 Recognition DNA sequences of interferon regulatory factor 1 (IRF-1) and IRF-2, regulators of cell growth and the interferon system. Mol Cell Biol 13:4531-8

131. **Fujita T, Kimura Y, Miyamoto M, Barsoumian EL, Taniguchi T** 1989 Induction of endogenous IFN-alpha and IFN-beta genes by a regulatory transcription factor, IRF-1. Nature 337:270-2

132. **Harada H, Takahashi E, Itoh S, Harada K, Hori TA, Taniguchi T** 1994 Structure and regulation of the human interferon regulatory factor 1 (IRF-1) and

IRF-2 genes: implications for a gene network in the interferon system. Mol Cell Biol 14:1500-9

133. **Harada H, Fujita T, Miyamoto M, Kimura Y, Maruyama M, et al.** 1989 Structurally similar but functionally distinct factors, IRF-1 and IRF-2, bind to the same regulatory elements of IFN and IFN-inducible genes. Cell 58:729-39

134. **Senger K, Merika M, Agalioti T, Yie J, Escalante CJ, et al.** 2000 Gene repression by coactivator repulsion. Mol Cell 6:931-7

135. **Levy DE, Kessler DS, Pine R, Darnell JE, Jr.** 1989 Cytoplasmic activation of ISGF3, the positive regulator of interferon-alpha-stimulated transcription, reconstituted in vitro. Genes Dev 3:1362-71

136. **Levy DE, Raz R, Durbin JE, Bluyssen H, Muzaffar R, Pisharody S** 1995 Cytoplasmic transcription factors: mediators of cytokine signaling. Agents Actions Suppl 47:79-85

137. **Veals SA, Schindler C, Leonard D, Fu XY, Aebersold R, et al.** 1992 Subunit of an alpha-interferon-responsive transcription factor is related to interferon regulatory factor and Myb families of DNA-binding proteins. Mol Cell Biol 12:3315-24

138. **Johnson GA, Spencer TE, Hansen TR, Austin KJ, Burghardt RC, Bazer FW** 1999 Expression of the interferon tau inducible ubiquitin cross-reactive protein in the ovine uterus. Biol Reprod 61:312-8

139. **Choi Y, Johnson GA, Spencer TE, Bazer FW** 2003 Pregnancy and interferon tau regulate major histocompatibility complex class I and beta2-microglobulin expression in the ovine uterus. Biol Reprod 68:1703-10

140. **Johnson GA, Spencer TE, Burghardt RC, Joyce MM, Bazer FW** 2000 Interferon-tau and progesterone regulate ubiquitin cross-reactive protein expression in the ovine uterus. Biol Reprod 62:622-7

141. **Johnson GA, Stewart MD, Gray CA, Choi Y, Burghardt RC, et al.** 2001 Effects of the estrous cycle, pregnancy, and interferon tau on 2',5'-oligoadenylate synthetase expression in the ovine uterus. Biol Reprod 64:1392-9

142. **Johnson GA, Burghardt RC, Newton GR, Bazer FW, Spencer TE** 1999 Development and characterization of immortalized ovine endometrial cell lines. Biol Reprod 61:1324-30

143. **Stewart DM, Johnson GA, Vyhlidal CA, Burghardt RC, Safe SH, et al.** 2001 Interferon-tau activates multiple signal transducer and activator of transcription proteins and has complex effects on interferon-responsive gene transcription in ovine endometrial epithelial cells. Endocrinology 142:98-107

144. **Spencer TE, Ott TL, Bazer FW** 1998 Expression of interferon regulatory factors one and two in the ovine endometrium: effects of pregnancy and ovine interferon tau. Biol Reprod 58:1154-62

145. **Stewart DM, Johnson GA, Vyhlidal CA, Burghardt RC, Safe SH, et al.** 2001 Interferon-tau activates multiple signal transducer and activator of transcription proteins and has complex effects on interferon-responsive gene transcription in ovine endometrial epithelial cells. Endocrinology 142:98-107

146. **Ott TL, Yin J, Wiley AA, Kim HT, Gerami-Naini B, et al.** 1998 Effects of the estrous cycle and early pregnancy on uterine expression of Mx protein in sheep (Ovis aries). Biol Reprod 59:784-94

147. **Mirando MA, Short EC, Jr., Geisert RD, Vallet JL, Bazer FW** 1991 Stimulation of 2',5'-oligoadenylate synthetase activity in sheep endometrium during pregnancy, by intrauterine infusion of ovine trophoblast protein-1, and by intramuscular administration of recombinant bovine interferon-alpha I1. J Reprod Fertil 93:599-607

148. **Johnson GA, Stewart MD, Gray CA, Choi Y, Burghardt RC, et al.** 2001 Effects of the estrous cycle, pregnancy, and interferon tau on 2',5'-oligoadenylate synthetase expression in the ovine uterus. Biol Reprod 64:1392-9

149. **Vallet JL, Barker PJ, Lamming GE, Skinner N, Huskisson NS** 1991 A low molecular weight endometrial secretory protein which is increased by ovine trophoblast protein-1 is a beta 2-microglobulin-like protein. J Endocrinol 130:R1-4

150. **Han CS, Mathialagan N, Klemann SW, Roberts RM** 1997 Molecular cloning of ovine and bovine type I interferon receptor subunits from uteri, and endometrial expression of messenger ribonucleic acid for ovine receptors during the estrous cycle and pregnancy. Endocrinology 138:4757-67

151. **Stewart MD, Johnson GA, Bazer FW, Spencer TE** 2001 Interferon-tau (IFNtau) regulation of IFN-stimulated gene expression in cell lines lacking specific IFN-signaling components. Endocrinology 142:1786-94

152. **Decker T, Lew DJ, Mirkovitch J, Darnell JE, Jr.** 1991 Cytoplasmic activation of GAF, an IFN-gamma-regulated DNA-binding factor. Embo J 10:927-32

153. **Schindler C, Shuai K, Prezioso VR, Darnell JE, Jr.** 1992 Interferon-dependent tyrosine phosphorylation of a latent cytoplasmic transcription factor. Science 257:809-13

154. **Levy DE, Kessler DS, Pine R, Reich N, Darnell JE, Jr.** 1988 Interferon-induced nuclear factors that bind a shared promoter element correlate with positive and negative transcriptional control. Genes Dev 2:383-93

155. **Reich N, Evans B, Levy D, Fahey D, Knight E, Jr., Darnell JE, Jr.** 1987 Interferon-induced transcription of a gene encoding a 15-kDa protein depends on an upstream enhancer element. Proc Natl Acad Sci USA 84:6394-8

156. **Kim S, Choi Y, Bazer FW, Spencer TE** 2003 Identification of genes in the ovine endometrium regulated by interferon tau independent of signal transducer and activator of transcription 1. Endocrinology 144:5203-14

157. **Gray CA, Adelson DL, Bazer FW, Burghardt RC, Meeusen EN, Spencer TE** 2004 Discovery and characterization of an epithelial-specific galectin in the endometrium that forms crystals in the trophectoderm. Proc Natl Acad Sci USA 101:7982-7

158. **Farrell PJ, Broeze RJ, Lengyel P** 1979 Accumulation of an mRNA and protein in interferon-treated Ehrlich ascites tumour cells. Nature 279:523-5

159. **Korant BD, Blomstrom DC, Jonak GJ, Knight E, Jr.** 1984 Interferon-induced proteins. Purification and characterization of a 15,000-dalton protein from human and bovine cells induced by interferon. J Biol Chem 259:14835-9

160. **Haas AL, Ahrens P, Bright PM, Ankel H** 1987 Interferon induces a 15-kilodalton protein exhibiting marked homology to ubiquitin. J Biol Chem 262:11315-23

161. **Johnson GA, Austin KJ, Collins AM, Murdoch WJ, Hansen TR** 1999 Endometrial ISG17 mRNA and a related mRNA are induced by interferon-tau and localized to glandular epithelial and stromal cells from pregnant cows. Endocrine 10:243-52

162. **Austin KJ, Ward SK, Teixeira MG, Dean VC, Moore DW, Hansen TR** 1996 Ubiquitin cross-reactive protein is released by the bovine uterus in response to interferon during early pregnancy. Biol Reprod 54:600-6

163. **Johnson GA, Burghardt RC, Newton GR, Bazer FW, Spencer TE** 1999 Development and characterization of immortalized ovine endometrial cell lines. Biol Reprod 61:1324-30

164. **Samuel CE** 1991 Antiviral actions of interferon. Interferon-regulated cellular proteins and their surprisingly selective antiviral activities. Virology 183:1-11

165. **Lengyel P** 1993 Tumor-suppressor genes: news about the interferon connection. Proc Natl Acad Sci USA 90:5893-5

166. **Salzberg S, Hyman T, Turm H, Kinar Y, Schwartz Y, et al.** 1997 Ectopic expression of 2-5A synthetase in myeloid cells induces growth arrest and facilitates the appearance of a myeloid differentiation marker. Cancer Res 57:2732-40

167. **Chin KC, Cresswell P** 2001 Viperin (cig5), an IFN-inducible antiviral protein directly induced by human cytomegalovirus. Proc Natl Acad Sci U S A 98:15125-30

168. **Sun BJ, Nie P** 2004 Molecular cloning of the viperin gene and its promoter region from the mandarin fish Siniperca chuatsi. Vet Immunol Immunopathol 101:161-70

169. **Olofsson PS, Jatta K, Wagsater D, Gredmark S, Hedin U, et al.** 2005 The antiviral cytomegalovirus inducible gene 5/viperin is expressed in atherosclerosis and regulated by proinflammatory agents. Arterioscler Thromb Vasc Biol 25:e113-6

170. **Helbig KJ, Lau DT, Semendric L, Harley HA, Beard MR** 2005 Analysis of ISG expression in chronic hepatitis C identifies viperin as a potential antiviral effector. Hepatology 42:702-10

171. **Kang DC, Gopalkrishnan RV, Wu Q, Jankowsky E, Pyle AM, Fisher PB** 2002 mda-5: An interferon-inducible putative RNA helicase with double-stranded RNA-dependent ATPase activity and melanoma growth-suppressive properties. Proc Natl Acad Sci USA 99:637-42

172. **Kang DC, Gopalkrishnan RV, Lin L, Randolph A, Valerie K, et al.** 2004 Expression analysis and genomic characterization of human melanoma differentiation associated gene-5, mda-5: a novel type I interferon-responsive apoptosis-inducing gene. Oncogene 23:1789-800

173. **Yoneyama M, Kikuchi M, Matsumoto K, Imaizumi T, Miyagishi M, et al.** 2005 Shared and unique functions of the DExD/H-box helicases RIG-I, MDA5, and LGP2 in antiviral innate immunity. J Immunol 175:2851-8

174. **Andrejeva J, Childs KS, Young DF, Carlos TS, Stock N, et al.** 2004 The V proteins of paramyxoviruses bind the IFN-inducible RNA helicase, mda-5, and inhibit its activation of the IFN-beta promoter. Proc Natl Acad Sci U S A 101:17264-9

175. **Kawai T, Takahashi K, Sato S, Coban C, Kumar H, et al.** 2005 IPS-1, an adaptor triggering RIG-I- and Mda5-mediated type I interferon induction. Nat Immunol 6:981-8

176. **Mohamed OA, Jonnaert M, Labelle-Dumais C, Kuroda K, Clarke HJ, Dufort D** 2005 Uterine Wnt/beta-catenin signaling is required for implantation. Proc Natl Acad Sci USA 102:8579-84

177. **Spencer TE, Johnson GA, Bazer FW, Burghardt RC, Palmarini M** 2007 Pregnancy recognition and conceptus implantation in domestic ruminants: roles of progesterone, interferons and endogenous retroviruses. Reprodution, Fertility and Development 19:65-78

178. **Cooper DN, Barondes SH** 1999 God must love galectins; he made so many of them. Glycobiology 9:979-84

179. **Barondes SH, Castronovo V, Cooper DN, Cummings RD, Drickamer K, et al.** 1994 Galectins: a family of animal beta-galactoside-binding lectins. Cell 76:597-8

180. **Kazemi M, Amann JF, Keisler DH, Ing NH, Roberts RM, et al.** 1990 A progesterone-modulated, low-molecular-weight protein from the uterus of the sheep is associated with crystalline inclusion bodies in uterine epithelium and embryonic trophectoderm. Biol Reprod 43:80-96

181. **Gray CA, Dunlap KA, Burghardt RC, Spencer TE** 2005 Galectin-15 in ovine uteroplacental tissues. Reproduction 130:231-40

182. **Kirschke H, Barrett AJ, Rawlings ND** 1998 Lysosomal Cysteine Proteases. Oxford: Oxford University Press

183. **Afonso S, Romagnano L, Babiarz B** 1997 The expression and function of cystatin C and cathepsin B and cathepsin L during mouse embryo implantation and placentation. Development 124:3415-25

184. **Elangovan S, Moulton BC** 1980 Blastocyst implantation in the rat and the immunohistochemical distribution and rate of synthesis of uterine lysosomal cathepsin D. Biol Reprod 23:663-8

185. **Li W, Jaffe RC, Verhage HG** 1992 Immunocytochemical localization and messenger ribonucleic acid levels of a progesterone-dependent endometrial secretory protein (cathepsin L) in the pregnant cat uterus. Biol Reprod 47:21-8

186. **Li WG, Jaffe RC, Fazleabas AT, Verhage HG** 1991 Progesterone-dependent cathepsin L proteolytic activity in cat uterine flushings. Biol Reprod 44:625-31

187. **Verhage HG, Boomsma RA, Mavrogianis PA, Li W, Fazleabas AT, Jaffe RC** 1989 Immunological characterization and immunocytochemical localization of a progesterone-dependent cat endometrial secretory protein. Biol Reprod 41:347-54

188. **Geisert RD, Blair RM, Pratt T, Zavy MT** 1997 Characterization and proteolytic activity of a cathepsin L-like polypeptide in endometrium and uterine flushings of cycling, pregnant and steroid-treated ovariectomized gilts. Reprod Fertil Dev 9:395-402

189. **Roberts RM, Bazer FW, Baldwin N, Pollard WE** 1976 Progesterone induction of lysozyme and peptidase activities in the porcine uterus. Arch Biochem Biophys 177:499-507

190. **Jokimaa V, Oksjoki S, Kujari H, Vuorio E, Anttila L** 2001 Expression patterns of cathepsins B, H, K, L and S in the human endometrium. Mol Hum Reprod 7:73-8

191. **Abrahamson M, Barrett AJ, Salvesen G, Grubb A** 1986 Isolation of six cysteine proteinase inhibitors from human urine. Their physicochemical and enzyme kinetic properties and concentrations in biological fluids. J Biol Chem 261:11282-9

192. **Abrahamson M, Olafsson I, Palsdottir A, Ulvsback M, Lundwall A, et al.** 1990 Structure and expression of the human cystatin C gene. Biochem J 268:287-94

193. **Hall A, Hakansson K, Mason RW, Grubb A, Abrahamson M** 1995 Structural basis for the biological specificity of cystatin C. Identification of leucine 9 in the N-terminal binding region as a selectivity-conferring residue in the inhibition of mammalian cysteine peptidases. J Biol Chem 270:5115-21

194. **Grubb A, Lofberg H** 1982 Human gamma-trace, a basic microprotein: amino acid sequence and presence in the adenohypophysis. Proc Natl Acad Sci U S A 79:3024-7

195. **Gerritsen ME, Wagner GF** 2005 Stanniocalcin: no longer just a fish tale. Vitam Horm 70:105-35

196. **Lu M, Wagner GF, Renfro JL** 1994 Stanniocalcin stimulates phosphate reabsorption by flounder renal proximal tubule in primary culture. Am J Physiol 267:R1356-62

197. **Butkus A, Roche PJ, Fernley RT, Haralambidis J, Penschow JD, et al.** 1987 Purification and cloning of a corpuscles of Stannius protein from Anguilla australis. Mol Cell Endocrinol 54:123-33

198. **Sundell K, Bjornsson BT, Itoh H, Kawauchi H** 1992 Chum salmon (Oncorhynchus keta) stanniocalcin inhibits in vitro intestinal calcium uptake in Atlantic cod (Gadus morhua). J Comp Physiol [B] 162:489-95

199. **Wagner GF** 1994 The molecular biology of the corpuscles of stannius and regulation of stanniolcalcin gene expression. In: Hochachka PW, Mommsen TP (eds.), Fish Physiology, Amsterdam: Academic Press; pp. 273-306.

200. **Fenwick JC, Brasseur JG** 1991 Effects of stanniectomy and experimental hypercalcemia on plasma calcium levels and calcium influx in American eels, Anguilla rostrata, LeSueur. Gen Comp Endocrinol 82:459-65

201. **So YP, Fenwick JC** 1977 Relationship between net 45calcium influx across a perfused isolated eel gill and the development of post-stanniectomy hypercalcemia. J Exp Zool 200:259-64

202. **Wagner GF, Hampong M, Park CM, Copp DH** 1986 Purification, characterization, and bioassay of teleocalcin, a glycoprotein from salmon corpuscles of Stannius. Gen Comp Endocrinol 63:481-91

203. **Wagner GF, Jaworski E** 1994 Calcium regulates stanniocalcin mRNA levels in primary cultured rainbow trout corpuscles of stannius. Mol Cell Endocrinol 99:315-22

204. **Varghese R, Wong CK, Deol H, Wagner GF, DiMattia GE** 1998 Comparative analysis of mammalian stanniocalcin genes. Endocrinology 139:4714-25

205. **Luo CW, Pisarska MD, Hsueh AJ** 2005 Identification of a stanniocalcin paralog, stanniocalcin-2, in fish and the paracrine actions of stanniocalcin-2 in the mammalian ovary. Endocrinology 146:469-76

206. **Ishibashi K, Imai M** 2002 Prospect of a stanniocalcin endocrine/paracrine system in mammals. Am J Physiol Renal Physiol 282:F367-75

207. **Chang AC, Jellinek DA, Reddel RR** 2003 Mammalian stanniocalcins and cancer. Endocr Relat Cancer 10:359-73

208. **Yoshiko Y, Aubin JE** 2004 Stanniocalcin 1 as a pleiotropic factor in mammals. Peptides 25:1663-9

209. **Madsen KL, Tavernini MM, Yachimec C, Mendrick DL, Alfonso PJ, et al.** 1998 Stanniocalcin: a novel protein regulating calcium and phosphate transport across mammalian intestine. Am J Physiol 274:G96-102

210. **Olsen HS, Cepeda MA, Zhang QQ, Rosen CA, Vozzolo BL** 1996 Human stanniocalcin: a possible hormonal regulator of mineral metabolism. Proc Natl Acad Sci USA 93:1792-6

211. **Haddad M, Roder S, Olsen HS, Wagner GF** 1996 Immunocytochemical localization of stanniocalcin cells in the rat kidney. Endocrinology 137:2113-7

212. **Wagner GF, Vozzolo BL, Jaworski E, Haddad M, Kline RL, et al.** 1997 Human stanniocalcin inhibits renal phosphate excretion in the rat. J Bone Miner Res 12:165-71

213. **Deol HK, Varghese R, Wagner GF, Dimattia GE** 2000 Dynamic regulation of mouse ovarian stanniocalcin expression during gestation and lactation. Endocrinology 141:3412-21

214. **Stasko SE, DiMattia GE, Wagner GF** 2001 Dynamic changes in stanniocalcin gene expression in the mouse uterus during early implantation. Mol Cell Endocrinol 174:145-9

215. **Luo CW, Kawamura K, Klein C, Hsueh AJ** 2004 Paracrine regulation of ovarian granulosa cell differentiation by stanniocalcin (STC) 1: mediation through specific STC1 receptors. Mol Endocrinol 18:2085-96

216. **Chang AC, Cha J, Koentgen F, Reddel RR** 2005 The murine stanniocalcin 1 gene is not essential for growth and development. Mol Cell Biol 25:10604-10

217. **Schmitt M, Goretzki L, Janicke F, Calvete J, Eulitz M, et al.** 1991 Biological and clinical relevance of the urokinase-type plasminogen activator (uPA) in breast cancer. Biomed Biochim Acta 50:731-41

218. **Salamonsen LA, Woolley DE** 1996 Matrix metalloproteinases in normal menstruation. Hum Reprod 11 Suppl 2:124-33

219. **Salamonsen LA** 1999 Role of proteases in implantation. Rev Reprod 4:11-22

220. **Curry TE, Jr., Osteen KG** 2003 The matrix metalloproteinase system: changes, regulation, and impact throughout the ovarian and uterine reproductive cycle. Endocr Rev 24:428-65

221. **Salamonsen LA, Nagase H, Woolley DE** 1995 Matrix metalloproteinases and their tissue inhibitors at the ovine trophoblast-uterine interface. J Reprod Fertil 49:29-37

222. **Salamonsen L** 1999 Role of proteases in implantation. Rev Reprod 4:11-22

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

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

225. **Spencer TE, Ott TL, Bazer FW** 1996 tau-Interferon: pregnancy recognition signal in ruminants. Proc Soc Exp Biol Med 213:215-29

226. **Spencer TE, Burghardt RC, Johnson GA, Bazer FW** 2004 Conceptus signals for establishment and maintenance of pregnancy. Anim Reprod Sci 82-83:537-50

227. **Hansen TR, Austin KJ, Perry DJ, Pru JK, Teixeira MG, Johnson GA** 1999 Mechanism of action of interferon-tau in the uterus during early pregnancy. J Reprod Fertil Suppl 54:329-39

228. **Spencer TE, Bartol FF, Bazer FW, Johnson GA, Joyce MM** 1999 Identification and characterization of glycosylation-dependent cell adhesion molecule 1-like protein expression in the ovine uterus. Biol Reprod 60:241-50

229. **Spencer TE, Gray A, Johnson GA, Taylor KM, Gertler A, et al.** 1999 Effects of recombinant ovine interferon tau, placental lactogen, and growth hormone on the ovine uterus. Biol Reprod 61:1409-18.

230. **Van Heeke G, Ott TL, Strauss A, Ammaturo D, Bazer FW** 1996 High yield expression and secretion of the ovine pregnancy recognition hormone interferon-tau by Pichia pastoris. J Interferon Cytokine Res 16:119-26.

231. **Spencer TE, Becker WC, George P, Mirando MA, Ogle TF, Bazer FW** 1995 Ovine interferon-tau regulates expression of endometrial receptors for estrogen and oxytocin but not progesterone. Biol Reprod 53:732-45.

232. **Spencer TE, Stagg AG, Ott TL, Johnson GA, Ramsey WS, Bazer FW** 1999 Differential effects of intrauterine and subcutaneous administration of recombinant ovine interferon tau on the endometrium of cyclic ewes. Biol Reprod 61:464-70

233. **Taylor KM, Gray CA, Joyce MM, Stewart MD, Bazer FW, Spencer TE** 2000 Neonatal ovine uterine development involves alterations in expression of receptors for estrogen, progesterone, and prolactin. Biol Reprod 63:1192-204.

234. **Green JA, Xie S, Quan X, Bao B, Gan X, et al.** 2000 Pregnancy-Associated Bovine and Ovine Glycoproteins Exhibit Spatially and Temporally Distinct Expression Patterns During Pregnancy. Biol Reprod 62:1624-1631

235. **Johnson GA, Burghardt RC, Bazer FW, Spencer TE** 2003 Osteopontin: roles in implantation and placentation. Biol Reprod 69:1458-71

236. **Johnson GA, Burghardt RC, Joyce MM, Spencer TE, Bazer FW, et al.** 2003 Osteopontin is synthesized by uterine glands and a 45-kDa cleavage fragment is

localized at the uterine-placental interface throughout ovine pregnancy. Biol Reprod 69:92-8

237. **Wimsatt WA** 1950 Hew Histological Observations on the Placenta of the Sheep. Am J Anat 87:391-436

238. **Wimsatt WA** 1951 Observations of the morphogenesis, cytochemistry and significance of the binucleate giant cells of the placenta of ruminants. J Anat 89:233-282

239. **Nomura T, Katunuma N** 2005 Involvement of cathepsins in the invasion, metastasis and proliferation of cancer cells. J Med Invest 52:1-9

240. **Babiarz BS, Romagnano LC, Kurilla GM** 1992 Interaction of mouse ectoplacental cone trophoblast and uterine decidua in vitro. In Vitro Cell Dev Biol 28A:500-8

241. **Cheon Y-P, DeMayo FJ, Bagchi MK, Bagchi IC** 2004 Induction of cytotoxic T-lymphocyte antigen-2β, a cysteine protease inhibitor in decidua: A Potential Regulator of Embryo Implantation. J. Biol. Chem. 279:10357-10363

242. **Hamilton RT, Bruns KA, Delgado MA, Shim JK, Fang Y, et al.** 1991 Developmental expression of cathepsin L and c-rasHa in the mouse placenta. Mol Reprod Dev 30:285-92

243. **Johnson GA, Spencer TE, Burghardt RC, Taylor KM, Gray CA, Bazer FW** 2000 Progesterone modulation of osteopontin gene expression in the ovine uterus. Biol Reprod 62:1315-21.

244. **Farin CE, Imakawa K, Roberts RM** 1989 In situ localization of mRNA for the interferon, ovine trophoblast protein-1, during early embryonic development of the sheep. Mol Endocrinol 3:1099-107.

245. **Gray CA, Adelson DL, Bazer FW, Burghardt RC, Meeusen EN, Spencer TE** 2004 Discovery and characterization of an epithelial-specific galectin in the endometrium that forms crystals in the trophectoderm. Proc Natl Acad Sci USA 101:7982-7987

246. **Bazer FW, Roberts RM** 1983 Biochemical aspects of conceptus--endometrial interactions. J Exp Zool 228:373-83

247. **Roberts RM, Bazer FW** 1988 The functions of uterine secretions. J Reprod Fertil 82:875-92

248. **Roberts RM, Murray MK, Burke MG, Ketcham CM, Bazer FW** 1987 Hormonal control and function of secretory proteins. Adv Exp Med Biol 230:137-50

249. **Kirschke H, Barrett AJ, Salvesen G, Grubb A** 1998 Lysosomal cysteine proteases. Oxford, UK: Oxford University Press.

250. **Song G, Spencer TE, Bazer FW** 2005 Cathepsins in the ovine uterus: regulation by pregnancy, progesterone, and interferon tau. Endocrinology 146:4825-33

251. **Salamonsen LA** 1992 Local regulators and the establishment of pregnancy: a review. Reprod Fertil Dev 4:125-34

252. **Salamonsen LA, Nagase H, Woolley DE** 1995 Matrix metalloproteinases and their tissue inhibitors at the ovine trophoblast-uterine interface. J Reprod Fertil Suppl 49:29-37

253. **Spencer TE, Bartol FF, Bazer FW, Johnson GA, Joyce MM** 1999 Identification and characterization of glycosylation-dependent cell adhesion molecule 1-like protein expression in the ovine uterus. Biol Reprod 60:241-50

254. **Spencer TE, Stagg AG, Ott TL, Johnson GA, Ramsey WS, Bazer FW** 1999 Differential effects of intrauterine and subcutaneous administration of recombinant ovine interferon tau on the endometrium of cyclic ewes. Biol Reprod 61:464-70

255. **Spencer TE, Stagg AG, Joyce MM, Jenster G, Wood CG, et al.** 1999 Discovery and characterization of endometrial epithelial messenger ribonucleic acids using the ovine uterine gland knockout model. Endocrinology 140:4070-80

256. **Chen C, Spencer TE, Bazer FW** 2000 Fibroblast growth factor-10: a stromal mediator of epithelial function in the ovine uterus. Biol Reprod 63:959-66

257. **Chen C, Spencer TE, Bazer FW** 2000 Expression of hepatocyte growth factor and its receptor c-met in the ovine uterus. Biol Reprod 62:1844-50

258. **Nakanishi T, Ozaki Y, Blomgren K, Tateyama H, Sugiura-Ogasawara M, Suzumori K** 2005 Role of cathepsins and cystatins in patients with recurrent miscarriage. Mol Hum Reprod 11:351-5

259. **Gray CA, Abbey CA, Beremand PD, Choi Y, Farmer JL, et al.** 2006 Identification of endometrial genes regulated by early pregnancy, progesterone, and interferon tau in the ovine uterus. Biol Reprod 74:383-94

260. **Klein C, Bauersachs S, Ulbrich SE, Einspanier R, Meyer HH, et al.** 2006 Monozygotic twin model reveals novel embryo-induced transcriptome changes of bovine endometrium in the preattachment period. Biol Reprod 74:253-64

261. **Katze MG, He Y, Gale M, Jr.** 2002 Viruses and interferon: a fight for supremacy. Nat Rev Immunol 2:675-87

262. **Alexenko AP, Leaman DW, Li J, Roberts RM** 1997 The antiproliferative and antiviral activities of IFN-tau variants in human cells. J Interferon Cytokine Res 17:769-79

263. **Khan OA, Jiang H, Subramaniam PS, Johnson HM, Dhib-Jalbut SS** 1998 Immunomodulating functions of recombinant ovine interferon tau: potential for therapy in multiple sclerosis and autoimmune disorders. Mult Scler 4:63-9

264. **Van Heeke G, Ott TL, Strauss A, Ammaturo D, Bazer FW** 1996 High yield expression and secretion of the ovine pregnancy recognition hormone interferon-tau by Pichia pastoris. J Interferon Cytokine Res 16:119-26

265. **Song G, Spencer TE, Bazer FW** 2006 Progesterone and interferon-tau regulate cystatin C in the endometrium. Endocrinology 147:3478-83

266. **Perry DJ, Austin KJ, Hansen TR** 1999 Cloning of interferon-stimulated gene 17: the promoter and nuclear proteins that regulate transcription. Mol Endocrinol 13:1197-206

267. **Sofia HJ, Chen G, Hetzler BG, Reyes-Spindola JF, Miller NE** 2001 Radical SAM, a novel protein superfamily linking unresolved steps in familiar

biosynthetic pathways with radical mechanisms: functional characterization using new analysis and information visualization methods. Nucleic Acids Res 29:1097-106

268. **Croy BA, He H, Esadeg S, Wei Q, McCartney D, et al.** 2003 Uterine natural killer cells: insights into their cellular and molecular biology from mouse modelling. Reproduction 126:149-60
269. **Hansen PJ** 1998 Regulation of uterine immune function by progesterone--lessons from the sheep. J Reprod Immunol 40:63-79
270. **Segerson EC, Matterson PM, Gunsett FC** 1991 Endometrial T-lymphocyte subset infiltration during the ovine estrous cycle and early pregnancy. J Reprod Immunol 20:221-36
271. **Lee CS, Gogolin-Ewens K, Brandon MR** 1988 Identification of a unique lymphocyte subpopulation in the sheep uterus. Immunology 63:157-64
272. **Alders RG, Shelton JN** 1990 Lymphocyte subpopulations in lymph and blood draining from the uterus and ovary in sheep. J Reprod Immunol 17:27-40
273. **Yankey SJ, Hicks BA, Carnahan KG, Assiri AM, Sinor SJ, et al.** 2001 Expression of the antiviral protein Mx in peripheral blood mononuclear cells of pregnant and bred, non-pregnant ewes. J Endocrinol 170:R7-11
274. **Asselin E, Johnson GA, Spencer TE, Bazer FW** 2001 Monocyte chemotactic protein-1 and -2 messenger ribonucleic acids in the ovine uterus: regulation by pregnancy, progesterone, and interferon-tau. Biol Reprod 64:992-1000
275. **Tekin S, Ealy AD, Wang SZ, Hansen PJ** 2000 Differences in lymphocyte-regulatory activity among variants of ovine IFN-tau. J Interferon Cytokine Res 20:1001-5
276. **Newton GR, Vallet JL, Hansen PJ, Bazer FW** 1989 Inhibition of lymphocyte proliferation by ovine trophoblast protein-1 and a high molecular weight glycoprotein produced by the peri-implantation sheep conceptus. Am J Reprod Immunol 19:99-107

277. **Tuo W, Ott TL, Bazer FW** 1993 Natural killer cell activity of lymphocytes exposed to ovine, type I, trophoblast interferon. Am J Reprod Immunol 29:26-34

278. **Nagaoka K, Sakai A, Nojima H, Suda Y, Yokomizo Y, et al.** 2003 A chemokine, interferon (IFN)-gamma-inducible protein 10 kDa, is stimulated by IFN-tau and recruits immune cells in the ovine endometrium. Biol Reprod 68:1413-21

279. **Imakawa K, Imai M, Sakai A, Suzuki M, Nagaoka K, et al.** 2006 Regulation of conceptus adhesion by endometrial CXC chemokines during the implantation period in sheep. Mol Reprod Dev 73:850-8

280. **Kwon H, Spencer TE, Bazer FW, Wu G** 2003 Developmental changes of amino acids in ovine fetal fluids. Biol Reprod 68:1813-20

281. **Spencer TE, Ing NH, Ott TL, Mayes JS, Becker WC, et al.** 1995 Intrauterine injection of ovine interferon-tau alters oestrogen receptor and oxytocin receptor expression in the endometrium of cyclic ewes. J Mol Endocrinol 15:203-20

282. **Sakal E, Bignon C, Grosclaude J, Kantor A, Shapira R, et al.** 1997 Large-scale preparation and characterization of recombinant ovine placental lactogen. J Endocrinol 152:317-27

283. **Bazer FW, Roberts RM, Basha SM, Zavy MT, Caton D, Barron DH** 1979 Method for obtaining ovine uterine secretions from unilaterally pregnant ewes. J Anim Sci 49:1522-7

284. **Wimsatt WA** 1950 New histological observations on the placenta of the sheep. Am J Anat 87:391-457

285. **Noel S, Herman A, Johnson GA, Gray CA, Stewart MD, et al.** 2003 Ovine placental lactogen specifically binds to endometrial glands of the ovine uterus. Biol Reprod 68:772-80

286. **Chang AC, Reddel RR** 1998 Identification of a second stanniocalcin cDNA in mouse and human: stanniocalcin 2. Mol Cell Endocrinol 141:95-9

287. **DiMattia GE, Varghese R, Wagner GF** 1998 Molecular cloning and characterization of stanniocalcin-related protein. Mol Cell Endocrinol 146:137-40

288. **Ing NH, Roberts RM** 1989 The major progesterone-modulated proteins secreted into the sheep uterus are members of the serpin superfamily of serine protease inhibitors. J Biol Chem 264:3372-9

289. **Chan JS, Robertson HA, Friesen HG** 1978 Maternal and fetal concentrations of ovine placental lactogen measured by radioimmunoassay. Endocrinology 102:1606-13

290. **Handwerger S, Crenshaw C, Jr., Maurer WF, Barrett J, Hurley TW, et al.** 1977 Studies on ovine placental lactogen secretion by homologous radioimmunoassay. J Endocrinol 72:27-34

291. **Kelly PA, Robertson HA, Friesen HG** 1974 Temporal pattern of placental lactogen and progesterone secretion in sheep. Nature 248:435-7

292. **Lacroix MC, Devinoy E, Cassy S, Servely JL, Vidaud M, Kann G** 1999 Expression of growth hormone and its receptor in the placental and feto-maternal environment during early pregnancy in sheep. Endocrinology 140:5587-97

293. **Lacroix MC, Devinoy E, Servely JL, Puissant C, Kann G** 1996 Expression of the growth hormone gene in ovine placenta: detection and cellular localization of the protein. Endocrinology 137:4886-92

294. **Gertler A, Djiane J** 2002 Mechanism of ruminant placental lactogen action: molecular and in vivo studies. Mol Genet Metab 75:189-201

295. **Cassy S, Charlier M, Guillomot M, Pessemesse L, Djiane J** 1999 Cellular localization and evolution of prolactin receptor mRNA in ovine endometrium during pregnancy. FEBS Lett 445:207-11

296. **Martin C, Pessemesse L, De La Llosa-Hermier MP, Martal J, Djiane J, Charlier M** 2004 Interferon-tau upregulates prolactin receptor mRNA in the ovine endometrium during the peri-implantation period. Reproduction 128:99-105

297. **Bazer FW, Chen TT, Knight JW, Schlosnagle D, Baldwin NJ, Roberts RM** 1975 Presence of a progesterone-induced, uterine specific, acid phosphatase in allantoic fluid of gilts. J Anim Sci 41:1112-9

298. **Jiang WQ, Chang AC, Satoh M, Furuichi Y, Tam PP, Reddel RR** 2000 The distribution of stanniocalcin 1 protein in fetal mouse tissues suggests a role in bone and muscle development. J Endocrinol 165:457-66

299. **Wooding FB, Flint AP** 1994 Placentation. In: Lamming GE (ed.), Marshall's Physiology of Reproduction, 4th ed. New York: Chapman and Hall, pp. 233-460

300. **Grace ND, Watkinson JH, Martinson PL** 1986 Accumulation of minerals by the fetus and conceptus of single and twin bearing ewes. N Z J Agric Res 29:207-222

301. **Care AD, Abbas SK, Pickard DW, et al.** 1990 Stimulation of ovine placental transport of calcium and magnesium by mid-molecule fragments of human parathyroid hormone-related protein. Exp Physiol 75:605-8

302. **Morgan G, Wooding FB, Care AD, Jones GV** 1997 Genetic regulation of placental function: a quantitative in situ hybridization study of calcium binding protein (calbindin-D9k) and calcium ATPase mRNAs in sheep placenta. Placenta 18:211-8

303. **Wooding FB, Morgan G, Jones GV, Care AD** 1996 Calcium transport and the localisation of calbindin-D9k in the ruminant placenta during the second half of pregnancy. Cell Tissue Res 285:477-89

304. **Uddin S, Majchrzak B, Woodson J, Arunkumar P, Alsayed Y, et al.** 1999 Activation of the p38 mitogen-activated protein kinase by type I interferons. J Biol Chem 274:30127-31

305. **Katsoulidis E, Li Y, Mears H, Platanias LC** 2005 The p38 mitogen-activated protein kinase pathway in interferon signal transduction. J Interferon Cytokine Res 25:749-56

306. **Platanias LC** 2005 Mechanisms of type-I- and type-II-interferon-mediated signalling. Nat Rev Immunol 5:375-86

307. **Uddin S, Lekmine F, Sharma N, Majchrzak B, Mayer I, et al.** 2000 The Rac1/p38 mitogen-activated protein kinase pathway is required for interferon alpha-dependent transcriptional activation but not serine phosphorylation of Stat proteins. J Biol Chem 275:27634-40

308. **Doualla-Bell F, Koromilas AE** 2001 Induction of PG G/H synthase-2 in bovine myometrial cells by interferon-tau requires the activation of the p38 MAPK pathway. Endocrinology 142:5107-15

309. **Kaur S, Uddin S, Platanias LC** 2005 The PI3' kinase pathway in interferon signaling. J Interferon Cytokine Res 25:780-7

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