Fetal-maternal interactions during the establishment of pregnancy in ruminants

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This review integrates established information with new insights into molecular and physiological mechanisms responsible for events leading to pregnancy recognition, endometrial receptivity, and implantation with emphasis on sheep. After formation of the corpus luteum, progesterone acts on the endometrium and stimulates blastocyst growth and elongation to form a filamentous conceptus (embryo/fetus and associated extraembryonic membranes). Recurrent early pregnancy loss in the uterine gland knockout ewe model indicates that endometrial epithelial secretions are essential for peri-implantation blastocyst survival and growth. The elongating sheep conceptus secretes interferon tau (IFNT) that acts on the endometrium to inhibit development of the luteolytic mechanism by inhibiting transcription of the estrogen receptor alpha (ESR1) gene in the luminal (LE) and superficial ductal glandular (sGE) epithelia, which prevents estrogen-induction of oxytocin receptors (OXTR) and production of luteolytic prostaglandin F2-alpha pulses. Progesterone downregulates its receptors (PGR) in LE and then GE, correlating with a reduction of anti-adhesive MUC1 (mucin glycoprotein one) and induction of secreted LGALS15 (galectin 15) and SPP1 (secreted phosphoprotein one), that are proposed to regulate trophectoderm growth and adhesion. IFNT acts on the LE to induce WNT7A (wingless-type MMTV integration site family member 7A) and to stimulate LGALS15, CTSL (cathepsin L), and CST3 (cystatin C), which may regulate conceptus development and implantation. During the peri-implantation period, trophoblast giant binucleate cells (BNC) begin to differentiate from mononuclear trophectoderm cells, migrate and then fuse with the uterine LE as well as each other to form multinucleated syncytial plaques. Trophoblast giant BNC secrete chorionic somatomammotropin (CSH1 or placental lactogen) that acts on the endometrial glands to stimulate their morphogenesis and differentiated function. The interactive, coordinated and stage-specific effects of ovarian and placental hormones regulate endometrial events necessary for fetal-maternal interactions and successful establishment of pregnancy.

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Introduction

Establishment of pregnancy includes pregnancy recognition signaling for maintenance of a functional corpus luteum, and endometrial differentiation and function for uterine receptivity to implantation of the conceptus (embryo/fetus and associated extraembryonic membranes). The coordinated actions of progesterone and placental hormones regulate fetal-maternal interactions required for establishment and maintenance of pregnancy. This review integrates established information with new insights into molecular and physiological mechanisms responsible for events leading to pregnancy recognition, endometrial receptivity, and implantation with emphasis on sheep.

Overview of pregnancy establishment and conceptus implantation

Establishment of pregnancy in domestic ruminants (sheep, cattle, goats) begins at the blastocyst stage and involves coordinate pregnancy recognition signaling and conceptus implantation (Fig. 1). According to Guillomot (1995), the phases of implantation are designated as: (1) shedding of the zona pellucida; (2) pre-contact and blastocyst orientiation; (3) apposition; (4) adhesion; and (5) endometrial invasion. All of these phases occur in domestic ruminants, but endometrial invasion is very limited (see Figs. 1 and 2).

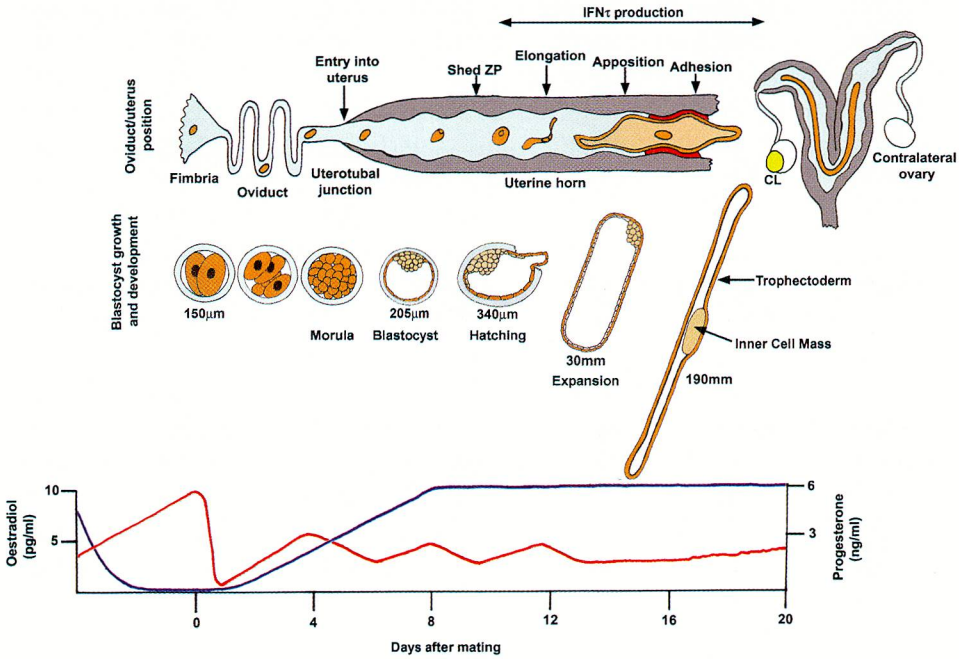

Fig. 1. Early pregnancy events in sheep. This schematic illustrates changes in embryo/blastocyst/conceptus development after fertilization in relation to position in the female reproductive tract and circulating levels of ovarian steroid hormones. Fertilization occurs in the oviduct and morula stage embryos enter into the uterus on Day 4-5. Blastocysts form by Day 6, hatch from the zona pellucida on Day 8, transition from spherical to tubular forms by Day 11 and then elongate to filamentous conceptuses between Days 12 and 16. Elongation of conceptuses marks the beginning of implantation which involves apposition and transient attachment (Days 12 to 15) and firm adhesion by Day 16. By Day 17, the filamentous conceptus occupies the entire ipsilateral horn and has elongated through the uterine body into the contralateral horn.

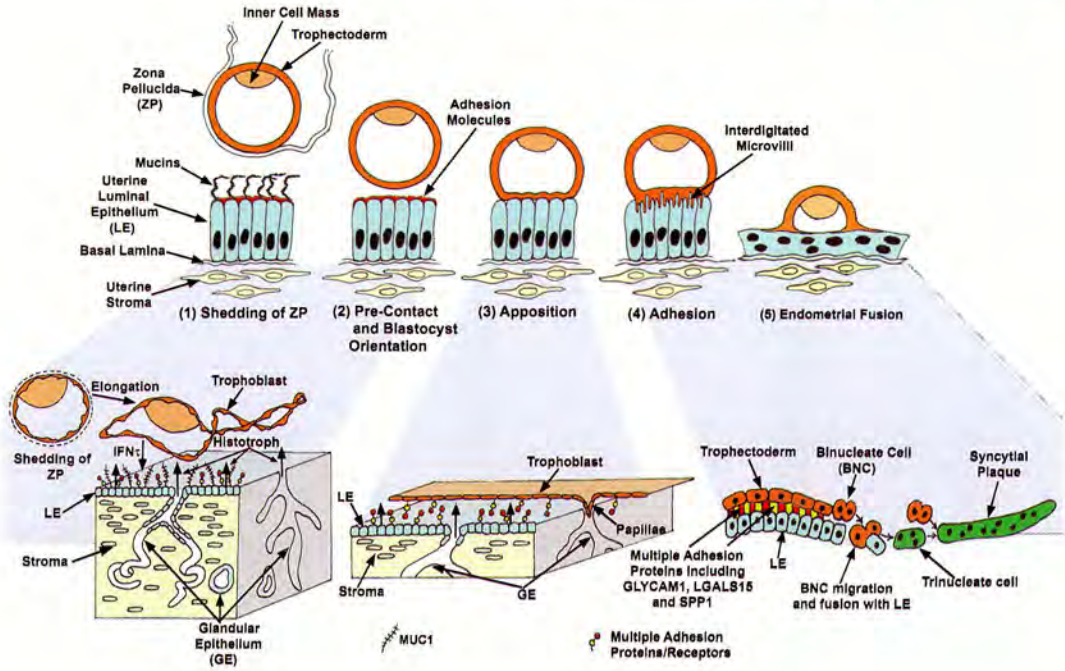

Fig. 2. Phases of blastocyst implantation in sheep. Pre-attachment blastocysts undergo shedding of the zona pellucida (Phase 1) and pre-contact blastocyst orientation (Phase 2). The first phase of implantation is shedding of the zona pellucida on Day 8 that exposes the trophectoderm to begin the second pre-implantation phase wherein blastocyst orientation occurs on Days 9 to 11 as it transitions from a spherical to a tubular conceptus and migrates to the middle region of the uterine horn ipsilateral to the corpus luteum. The anti-adhesive MUC1 present on endometrial LE prevents contact of trophectoderm and adhesive receptors such as integrins. Histotroph, secreted from endometrial LE and GE under the influence of progesterone, nourishes the developing blastocysts. Phase 3 includes apposition and transient attachment. After Day 11, the tubular blastocyst elongates to form a filamentous conceptus. During this period, expression of MUC1 declines on LE to expose constitutively expressed integrins on LE and trophectoderm. Apposition between trophectoderm and endometrial LE occurs followed by formation of trophoblast papillae that extend into superficial ducts of uterine glands. Elongation of conceptuses likely requires apposition and transient attachment of trophectoderm to endometrial LE. Firm adhesion of trophectoderm to endometrial LE occurs during Phase 4 between Days 15 and 16. Available evidence indicates that several molecules (GLYCAM1, LGALS15, SPP1) secreted by the endometrial epithelia interact with receptors (integrins and glycoconjugates) on the apical surface of trophectoderm and LE to facilitate adhesion. Invasion of the endometrium during Phase 5 involves formation of binucleate cells (BNC) that begin differentiate from mononuclear trophoblast cells between Days 14 and 16 and then migrate to and fuse with LE to form multinucleated syncytial plaques. Although the BNC are inherently invasive, they do not cross the basal lamina between the LE and stratum compactum stroma.

In sheep (Fig. 1), morula-stage embryos enter the uterus on Day 4-5 and the blastocyst, formed by Day 6, contains an inner cell mass and a blastocoele or central cavity surrounded by a monolayer of trophectoderm (Guillomot 1995; Spencer *et al.* 2004a). After hatching from the zona pellucida on Day 8, blastocysts develop into a tubular form by Day 11, and then elongate

on Day 12 to 10 cm or more in length by Day 14 and achieve a length of 25 cm or more by Day 17. The elongated or filamentous conceptus is composed mainly of extraembryonic trophectoderm. Hatched blastocysts and trophoblastic vesicles do not elongate *in vitro,* but do so when transferred into the uterus of either sheep or cows (Heyman *et al.* 1984; Flechon *et al.* 1986). Elongation of the blastocyst is critical for developmentally regulated production of interferon tau (IFNT), the pregnancy recognition signal, and for implantation (Farin *et al.* 1989; Guillomot *et al.* 1990; Gray *et al.* 2002). Between Days 9 and 14, close association between conceptus trophectoderm and endometrial luminal epithelium (LE) is achieved and followed first by adhesion and then interdigitation of cytoplasmic projections of trophectoderm cells and microvilli of the LE assures firm attachment in both the caruncular and intercaruncular areas by Day 16 of pregnancy (Fig. 2).

In the sheep conceptus, trophoblast giant binucleate cells (BNC) first appear on Day 14 (Wooding 1984) and are thought to arise from mononuclear trophectoderm cells by consecutive nuclear divisions without cytokinesis (Wooding 1992) also termed mitotic polyploidy. By Day 16, BNC represent 15-20% of trophectoderm cells. Between Days 16 and 24, the LE transforms to syncytial plaques which appear to result from migration of BNC to the microvillar junction and then fusion with individual LE cells to produce trinucleate fetomaternal hybrid cells (Wooding 1984). Continued BNC migration and fusion with trinucleate cells, together with displacement and/or death of the remaining uterine LE, apparently produces the multinucleated syncytial plaques that cover the caruncles by Day 24. The syncytial plaques appear to be linked by tight junctions and limited in size to 20-25 nuclei. This caruncular syncytium, in which no nuclear division has been reported, expands in area during formation and maintenance of cotyledons, presumably deriving nuclei from continued BNC migration and fusion. The BNC have at least two main functions: (1) formation of a hybrid fetomaternal syncytium for successful implantation and subsequent cotyledonary growth in the placentome; and (2) synthesis and secretion of protein and steroid hormones such as CSH1 (chorionic somatomammotropin hormone 1; alias placental lactogen)), PAGs (pregnancy associated glycoproteins), and progesterone (Wooding 1992). As will be discussed later, placental CSH1 binds to prolactin receptors (PRLR) unique to uterine GE and stimulates their growth and differentiated functions during pregnancy (Spencer *et al.* 2004c).

Pregnancy recognition signaling by IFNT

Maternal recognition of pregnancy is the physiological process whereby the conceptus signals its presence to the maternal system and prolongs the lifespan of the corpus luteum (CL) (Bazer *et al.* 1991). In ruminants, IFNT is the pregnancy recognition signal secreted by the elongating conceptus (Roberts *et al.* 1999). In addition to its antiluteolytic actions, IFNT acts on the endometrium to induce or enhance expression of a number of genes (IFNT-stimulated genes or ISGs) that are hypothesized to regulate uterine receptivity and conceptus development during implantation (Hansen *et al.* 1999; Spencer *et al.* 2004a).

Inhibition of the endometrial luteolytic mechanism

Sheep experience uterine-dependent oestrous cycles until establishment of pregnancy (Spencer & Bazer 2004). The oestrous cycle is dependent on the uterus, because it releases prostaglandin F2 alpha (PGF) in a pulsatile manner to induce luteolysis during late diestrus. As illustrated in Fig. 3, the luteolytic pulses of PGF are produced by the endometrial LE and superficial ductal glandular epithelium (sGE) in response to oxytocin binding to oxytocin receptors (OXTR)

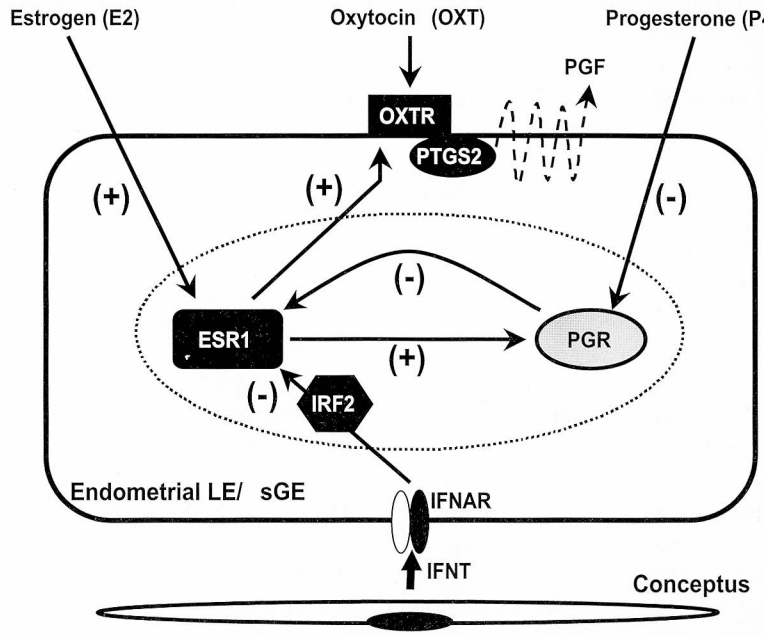

Fig. 3. Schematic illustrating hormonal regulation of the endometrial luteolytic mechanism and antiluteolytic effects of the conceptus on the endometrium in the ovine uterus. During estrus and metestrus, OXTR are present on LE/sGE because circulating estrogens increase expression of ESR1 and OXTR. The PGR are present, but circulating levels of progesterone are inadequate to activate PGR and suppress expression of ESR1 and OXTR. During early diestrus, endometrial ESR1 and estrogen are low and circulating progesterone increases with maturation of the CL so that to activate, via PGR, suppression of expression of ESR1 and OXTR for 8 to 10 days. Continuous exposure of the endometrium to progesterone eventually results in down-regulation of *PGR* in endometrial LE/sGE by Days 11 to 12 of the estrous cycle to end the progesterone block to *ESR1* and *OXTR* expression. Thus, *ESR1* expression increases on Days 11 and 12 post-estrus followed by estrogen induction of OXTR on LE/sGE on Days 13 and 14. The increase in OXTR is facilitated by increasing secretion of estrogen by ovarian follicles. In both cyclic and pregnant sheep, OXT is released from the posterior pituitary and CL beginning on Day 9. In cyclic ewes, OXT binds to OXTR on LE/sGE to induce release of luteolytic pulses of PGF that leads to CL regression through a PTGS-dependent pathway. In pregnant sheep, IFNT is synthesized and secreted by the elongating conceptus between Days 11 to 12 and 21 to 25 of pregnancy. IFNT binds to Type I IFN receptors (IFNAR) on endometrial LE/sGE to inhibit transcription of the *ESR1* gene through a signaling pathway involving IRF2. These antiluteolytic actions of IFNT on the *ESR1* gene prevent ESR1 expression and, therefore, the ability of estrogen to induce expression of OXTR required for pulsatile release of luteolytic PGF. E2, Estrogen; ESR1, estrogen receptor; IFNAR, Type I IFN receptor; IFNT, interferon tau; IRF2, interferon regulatory factor two; OXT, oxytocin; OXTR, oxytocin receptor; P4, progesterone; PGF, prostaglandin F2α; PGR, progesterone receptor; PTGS2, prostaglandin-endoperoxide synthase 2 (prostaglandin G/H synthase and cyclooxygenase)

on those epithelia (McCracken *et al.* 1999). Expression of the *OXTR* is regulated by progesterone and estrogen. Progesterone acts via progesterone receptors (PGR) to "block" expression of

oestrogen receptor alpha (ESR1) and OXTR in LE/sGE between Days 5 and 11 after which time progesterone downregulates PGR in LE/sGE after Day 11 and GE after Day 13 in cyclic ewes which allows rapid increases in expression of *ESR1* on Days 12 to 13 and then *OXTR* on Day 14 (see (Spencer & Bazer 2004)). ESR1, presumably activated by oestrogen from ovarian follicles or possibly growth factors from the stroma, stimulates transcription of the *OXTR* gene. Two GC-rich SP1 binding sites, located within 140 bp of the translational start site, regulate basal activity of the ovine *OXTR* promoter as well as liganded ESR1 induction of promoter activity (Fleming *et al.* 2006). Oxytocin, secreted from as early as Day 9 from posterior pituitary and/or the CL, binds to OXTR to induce pulsatile release of PGF between Days 14 and 16, which elicits structural and functional regression of the CL.

IFNT is synthesized and secreted by conceptus trophectoderm between Days 10 and 20 or so of gestation in sheep (Roberts *et al.* 1999), acts on endometrial LE/sGE to inhibit transcription of the *ESR1* gene and, therefore, *OXTR* gene, which abrogates the luteolytic mechanism required for uterine production of luteolytic pulses of PGF (Wathes & Lamming 1995). IFNT does not inhibit either basal production of PGF or expression of *PTGS2* (prostaglandin-endoperoxide synthase 2; alias COX-2), a rate-limiting enzyme in prostaglandin production, in endometrial LE/sGE of pregnant ewes (Charpigny *et al.* 1997; Kim *et al.* 2003b). Rather, IFNT indirectly inhibits *OXTR* gene transcription by silencing *ESR1* gene transcription in the endometrial LE/sGE. The promoter/enhancer region of the ovine *ESR1* gene contains four IFN regulatory factor elements (IRFEs) and one IFN-stimulated response element (ISRE) that bind IFN regulatory factor two (IRF2) (Fleming *et al.* 2001), a transcriptional repressor expressed specifically in LE/sGE of the ovine uterus that increases in abundance from Day 10 to Day 16 in pregnant, but not cyclic ewes (Choi *et al.* 2001). IFNT and IRF2 inhibit transcriptional activity of the ovine *ESR1* promoter *in vitro*, but the precise cellular and molecular mechanism remains to be determined (Fleming *et al.* 2001). However, IFNT does not affect activity of the ovine *OXTR* gene promoter nor inhibit liganded ESR1 induction of *OXTR* promoter activity (Fleming *et al.* 2006). Thus, available results from sheep support the hypothesis that the antiluteolytic effects of IFNT are to silence *ESR1* gene transcription in LE/sGE subsequent to PGR down-regulation, which precludes induction of *OXTR* gene expression and uterine release of luteolytic pulses of PGF. In cows, the conceptus, presumably via IFNT, inhibits expression of endometrial OXTR before suppressing ESR1, suggesting mechanistic differences between cattle and sheep in terms of the endometrial luteolytic mechanism and pregnancy recognition signaling (Robinson *et al.* 2001).

IFNT-stimulated genes (ISGs) in the endometrium

IFNT induces or increases expression of several ISGs in the endometrium that are hypothesized to be important for conceptus implantation (Hansen *et al.* 1999; Spencer *et al.* 2004a). Since expression of ISGs increases in a stage-specific manner within the endometria of diverse species, including domestic animals, laboratory rodents, primates and humans during early pregnancy, they may be universally important in establishment of uterine receptivity to conceptus implantation.

Bovine endometrial, ovine endometrial and human 2fTGH fibroblast cells have been used to determine that IFNT activates the classical JAK-STAT-IRF (janus kinase-signal transducer and activator of transcription-interferon regulatory factor) signaling pathway used by other Type I IFNs (see (Stark *et al.* 1998) and Fig. 4). Many classical ISGs, such as *ISG15* (ISG15 ubiquitin-like modifier; alias IFI15 or UCRP), are expressed in LE/sGE of the ovine uterus on Days 10 or 11 of the cycle and pregnancy, but are undetectable in LE/sGE by Days 12 to 13 (Johnson *et al.*

1999c) (see Table 1 and Fig. 4). In response to IFNT from elongating conceptuses, *ISG15* is induced in the stratum compactum stroma and GE by Days 13 to 14, and expression extends to the stratum spongiosum stroma, deep glands, and myometrium of the ovine uterus by Days 15 to 16 of pregnancy (Johnson *et al.* 1999c; Johnson *et al.* 2000a). As IFNT production by the conceptus declines, expression of ISGs also declines, but some remain abundant in endometrial stroma and GE on Days 18 to 20 of pregnancy. Similar temporal and spatial alterations in *ISG15* expression occurs in the bovine uterus during early pregnancy (Johnson *et al.* 1999a). Indeed, numerous ISGs are induced or stimulated in the endometrium during conceptus elongation in both cattle and sheep (Klein *et al.* 2005; Gray *et al.* 2006).

Curiously, *in vivo* studies revealed that many classical ISGs *(STAT1, STAT2, ISGF3G (IFN-stimulated gene factor 3 gamma), B2M (beta-2-microglobulin), GBP2 (guanylate binding protein 2), IFI27 (interferon, alpha-inducible protein 27), IFIT1 (interferon-induced protein with tetratricopeptide repeats 1), ISG15, MIC (MHC class I polypeptide),* and *OAS (2',5'-oligoadenylate synthetases 1, 2 and 3)* are not induced or increased by IFNT in LE/sGE of the sheep uterus (Johnson *et al.* 1999c; Choi *et al.* 2001; Johnson *et al.* 2001b; Choi *et al.* 2003; Kim *et al.* 2003a). This finding was initially surprising, because all endometrial cell types express IFNAR1 (interferon (alpha, beta and omega) receptor 1) and IFNAR2 subunits of the common Type I IFN receptor (Rosenfeld *et al.* 2002). Available results indicate that IRF2, a potent transcriptional repressor of ISGs, is expressed specifically in LE/sGE and represses transcriptional activity of ISRE- and IRFE-containing promoters (Choi *et al.* 2001). Thus, IRF2 in LE/sGE is proposed to restrict IFNT induction of many ISGs to stroma and GE of the ovine uterus (see Fig. 4 and Table 1). In fact, all components of ISGF3 (*STAT1, STAT2, ISGF3G*) and the other studied ISGs (*B2M, GBP2, IFI27, IFIT1, ISG15, MIC, OAS*) contain ISREs in their promoters. The silencing of *MIC* and *B2M* genes in endometrial LE/sGE during pregnancy recognition and establishment may be a critical mechanism preventing immune rejection of the conceptus allograft (Choi *et al.* 2003). Given that the critical signaling components of the JAK-STAT signaling system (STAT1, STAT2, ISGF3G) are not expressed in the endometrial LE/sGE, IFNT must utilize a non-classical STAT1-independent cell signaling pathway to regulate expression of genes in endometrial LE (Fig. 4). Transcriptional profiling of human U3A (STAT1 null) cells and ovine endometrium treated with IFNT were used to discover novel ISGs in the endometrial LE/sGE during pregnancy establishment including *WNT7A* (wingless-type MMTV integration site family, member 7A), LGALS15 (lectin, galactoside-binding, soluble, 15), CTSL (cathepsin L) and CST3 (cystatin C) (Kim *et al.* 2003a; Song *et al.* 2005; Gray *et al.* 2006; Song *et al.* 2006b). The expression patterns of classical and novel ISGs are summarized in Table 1, and proposed biological roles of novel epithelial ISGs in conceptus implantation will now be discussed.

WNT7A. During early pregnancy, *WNT7A* mRNA is detected on Day 10, undetectable on Day 12, and is the only gene found to be induced by IFNT between Days 12 and 14 of pregnancy specifically in LE/sGE (Kim *et al.* 2003a). The WNT family (19 genes in human) includes many highly conserved and secreted glycoproteins that regulate cell and tissue growth and differentiation during embryonic development and play a central role in coordinating uterine-conceptus interactions required for implantation in mice and perhaps humans (Mohamed *et al.* 2005). WNT7A from the endometrial LE may activate canonical signaling pathways in the trophectoderm to stimulate proliferation and differentiation. Further, WNT7A may have autocrine actions on LE/sGE to regulate expression of target genes important for uterine receptivity and conceptus implantation, such as *LGALS15, CST3* and *CTSL*.

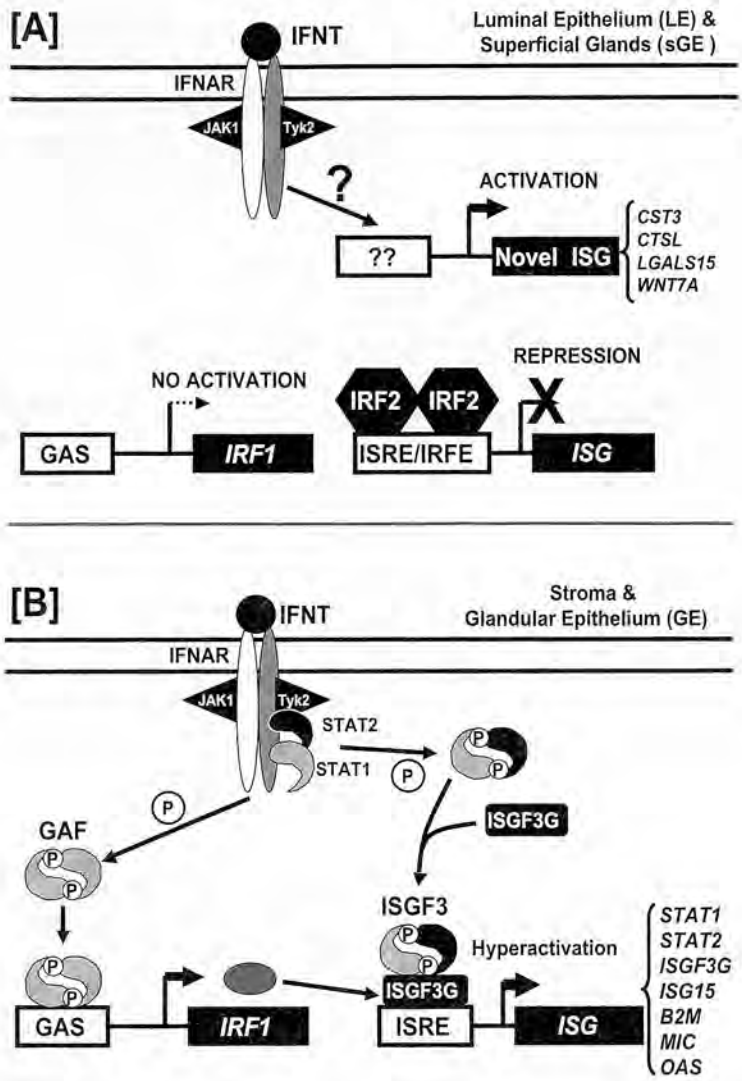

Fig. 4. Schematic illustrating current working hypothesis on IFNT signaling in the endometrium of the ovine uterus. IFNT, produced by developing conceptuses of ruminants, binds to IFNAR present on cells of the ovine endometrium. In LE/sGE, IFNT is prevented from activating ISGs by IRF2 (see upper panel [A]). IRF2, a potent and stable transcriptional repressor present in the nucleus, increases during early pregnancy in LE/sGE. The continual presence of IRF2 inhibits ISRE- and IRFE-containing target genes (*STAT1, STAT2, ISGF3G, BMG, ISG15, MHC, OAS*) through direct ISRE and IRFE binding and coactivator repulsion. Furthermore, critical factors in the JAK-STAT-IRF pathway (STAT1, STAT2, and ISGF3G) are not present, resulting in the absence of ISGF3 or IRF1 transcription factors necessary to stimulate ISGs. However, IFNT does activate an unknown cell signaling pathway that results in induction of *WNT7A* and stimulation of *CST3, CTSL* and *LGALS15* specifically in LE/sGE. In cells of the stroma and middle to deep GE (see lower panel [B]), IFNT-mediated association of IFNAR subunits facilitates cross-phosphorylation and activation of JAK, which in turn phosphorylates the receptor and creates a docking site for STAT2. STAT2 is then phosphorylated, thus creating a docking site for STAT1 which is

then phosphorylated. STAT1 and STAT2 are then released from the receptor and can form two transcription factor complexes. ISGF3 is formed by association of a STAT1-2 heterodimer and ISGF3G in the cytoplasm, translocates to the nucleus, and transactivates genes containing an ISRE(s), such as *STAT1, STAT2, ISGF3G, BMG, ISG15, MHC* and *OAS*. GAF is formed by STAT1 homodimers, which translocates to the nucleus and transactivates genes containing a GAS element(s), such as *IRF1*. IRF1 can also bind and transactivate ISRE-containing genes as well an IRFE-containing genes. The simultaneous induction of *STAT2* and *ISGF3G* by IFNT appears to shift transcription factor formation from GAF towards predominantly ISGF3. Therefore, IFNT activation of the JAK-STAT-IRF signal transduction pathway allows for constant formation of ISGF3 and GAF transcription factor complexes and hyperactivation of ISG expression. B2M, beta-2-microglobulin; CST3, cystatin C; CTSL, cathepsin L; GAF, gamma activated factor; GAS, gamma activation sequence; IFNAR, Type I IFN receptor; IFNT, interferon tau; IRF1, interferon regulatory factor one; IRF2, interferon regulatory factor two; IRFE, IRF-response element; ISGF3G, interferon-stimulated transcription factor 3, gamma 48kDa; ISG15, (ISG15 ubiquitin-like modifier; alias IFI15 or UCRP); ISRE, IFN-stimulated response element; JAK, janus kinase; LGALS15, galectin-15; MIC, MHC class I polypeptide-related sequence; OAS, 2',5'-oligoadenylate synthetases; STAT1, signal transducer and activator of transcription 1, 91kDa; STAT2, signal transducer and activator of transcription 2, 113kDa; WNT7A, wingless-type MMTV integration site family, member 7A.

CTSL and CST3. *CTSL* and *CST3* are induced by progesterone in LE/sGE between Days 10 and 12 and are further increased by IFNT (Song *et al.* 2005; 2006b). Cathepsins are peptidases that can degrade extracellular matrix, catabolize intracellular proteins, process prohormones, and regulate uterine receptivity for implantation and trophoblast invasion in several mammals (Salamonsen 1999). CST3 is an inhibitor of CTSL. A balance of proteases and their inhibitors is likely required to modify the glycocalyx on endometrial LE and trophoblast during apposition and adhesion phases of implantation (Carson *et al.* 2000).

LGALS15. Similar to *CTSL* and *CST3, LGALS15* is induced by progesterone in LE/sGE between Days 10 and 14 and is further increased by IFNT (Gray *et al.* 2004). Galectins are proteins with a conserved carbohydrate recognition domain that bind beta-galactosides, thereby cross-linking glycoproteins as well as glycolipid receptors on the surface of cells, such as integrins, and initiating biological responses (Yang & Liu 2003). LGALS15, originally termed ovgal11, was originally identified in ovine intestinal epithelium as being induced in response to infection by the nematode parasite *Haemonchus contortus* (Dunphy *et al.* 2000). Interestingly, LGALS15 is the 14K protein from sheep endometrium initially characterized as a progesterone-modulated protein associated with crystalline inclusion bodies in uterine epithelia and conceptus trophectoderm (Kazemi *et al.* 1990). LGALS15 is implicated in conceptus implantation (Spencer *et al.* 2004a), because functional studies of other galectins have implicated these proteins in cell growth, differentiation and apoptosis as well as in cell adhesion, chemoattraction and migration (Yang & Liu 2003). Indeed, some galectin family members are involved in both innate and adaptive immune responses and participate in the activation or differentiation of immune cells.

CXCL10. The only ISG with a reported biological effect on trophectoderm growth and adhesion is chemokine (C-X-C motif) ligand 10 (CXCL10; alias IP-10) (Nagaoka *et al.* 2003a; Nagaoka *et al.* 2003b). CXCL10 is a member of the C-X-C chemokine family that regulates multiple aspects of inflammatory and immune responses primarily through chemotactic activity toward subsets of leukocytes. *CXCL10* mRNA was localized to monocytes in the subepithelial stroma of pregnant, but not cyclic uteri of sheep. Whether IFNT directly regulates *CXCL10* in the monocytes or simply attract the monocytes to the endometrium remains to be determined. In the ovine uterus, CXCL10 appeared on Day 17 in the uterine lumen, and the CXCR3 recep-

Table 1. Temporal roadmap of progesterone and IFNT regulated genes during establishment of pregnancy in sheep[1]

	Day of pregnancy				
	10/11	**12/13**	**14/15**	**16/17**	**18/20**
Conceptus	IFNT	IFNT	**IFNT**	**IFNT**	IFNT
				CSH1	CSH1
Uterine	GLYCAM1	GLYCAM1	**GLYCAM1**	**GLYCAM1**	
Lumen	LGALS15	LGALS15	**LGALS15**	**LGALS15**	
Protein			SPP1	**SPP1**	n.d.[2]
		CTSL	**CTSL**	**CTSL**	
		CST3	**CST3**	**CST3**	
			CXCL10	CXCL10	
LE	PGR				
	MUC1	**MUC1**	MUC1	MUC1	
		GLYCAM1	**GLYCAM1**	**GLYCAM1**	**GLYCAM1**
		LGALS15	***LGALS15***	***LGALS15***	***LGALS15***
		CTSL	***CTSL***	***CTSL***	***CTSL***
		CST3	***CST3***	***CST3***	***CST3***
	WNT7A		*WNT7A*	*WNT7A*	*WNT7A*
		PTGS2	*PTGS2*	*PTGS2*	*PTGS2*
	IRF2	IRF2	**IRF2**	**IRF2**	**IRF2**
				G1P3	*G1P3*
	ISG15	*ISG15*			
	MIC	*MIC*			
	B2M	*B2M*			
GE	PGR	PGR			
	PRLR	*PRLR*	*PRLR*	*PRLR*	*PRLR*
		SPP1	*SPP1*	***SPP1***	***SPP1***
			CTSL	***CTSL***	***CTSL***
			CST3	***CST3***	***CST3***
					SERPIN
					STC1
				GRP	*GRP*
			STAT1	***STAT1***	***STAT1***
			STAT2	***STAT2***	***STAT2***
			ISGF3G	***ISGF3G***	***ISGF3G***
			IRF1	***IRF1***	***IRF1***
		GBP2	*GBP2*	***GBP2***	***GBP2***
		IFIT1	*IFIT1*	***IFIT1***	***IFIT1***
			IFI27	***IFI27***	***IFI27***
			ISG15	***ISG15***	***ISG15***
			MIC	***MIC***	***MIC***
			B2M	***B2M***	***B2M***
ST	PGR	PGR	PGR	**PGR**	**PGR**
			STAT1	***STAT1***	***STAT1***
			STAT2	***STAT2***	***STAT2***
			ISGF3G	***ISGF3G***	***ISGF3G***
			IRF1	***IRF1***	***IRF1***
		GBP2	***GBP2***	***GBP2***	***GBP2***
		IFIT1	*IFIT1*	***IFIT1***	***IFIT1***
			ISG15	***ISG15***	***ISG15***
			MIC	***MIC***	***MIC***
			B2M	***B2M***	***B2M***

[1]Relative abundance of mRNAs across days of pregnancy are indicated: gray = low; normal = moderate; and **bold** = high).

[2]Not determined (n.d.) due to inability to flush intact conceptuses from the uterine lumen on Days 18-20 of pregnancy.

tor was localized to trophoblast cells. Subsequently, recombinant CXCL10 was shown to stimulate migration of trophoblast cells and promote their adhesion to fibronectin, as well as increase expression of integrins α5, αV, and ß3 subunit mRNAs in trophoblast cells (Nagaoka *et al.* 2003b; Imakawa *et al.* 2006). Integrins are essential for conceptus implantation (Burghardt *et al.* 2002) and will be discussed later.

Functional role of the endometrial epithelia in blastocyst growth and elongation

All mammalian uteri contain endometrial epithelia that synthesize and secrete or transport a complex mixture of amino acids, ions, glucose, enzymes, growth factors, hormones, transport proteins and other substances termed histotroph (Bazer 1975). During the pre-attachment period, nutrition of the conceptus depends on uterine secretions. The epithelial cells of the uterine lumen are highly secretory during implantation, and the trophectoderm exhibits intense pinocytotic activity which increases as the conceptus develops (Guillomot 1995). Therefore, factors supporting growth of pre- and peri-implantation blastocysts and elongating conceptuses are thought to be obtained primarily from uterine histotroph. This hypothesis is supported by results from studies of asynchronous uterine transfer of embryos and trophoblast vesicles (Lawson *et al.* 1983; Flechon et al. 1986) and from studies of uterine gland knockout (UGKO) ewes (Gray *et al.* 2001b; Gray *et al.* 2002).

The UGKO ewe model is produced by continuous administration of a synthetic, non-metabolizable progestin to neonatal ewes from birth to postnatal day 56 (Gray *et al.* 2000a). This inappropriate exposure to a progestin permanently ablates differentiation and development of the endometrial glands from LE and produces an UGKO phenotype without apparent alterations in development of myometrium, or other Müllerian duct-derived female reproductive tract structures, or function of the hypothalamic-pituitary-ovarian axis (Gray *et al.* 2000b; Gray *et al.* 2001a). The UGKO endometrium is devoid of glands with markedly reduced LE surface area. UGKO ewes exhibit recurrent early pregnancy loss as blastocyst fails to elongate, and transfer of blastocysts from uteri of control ewes into uteri of timed recipient UGKO ewes does not ameliorate this defect (Gray *et al.* 2001b). Morphologically normal blastocysts are present in uterine flushes of bred UGKO ewes on Days 6 and 9 post-mating, but not on Day 14 (Gray *et al.* 2001b; Gray *et al.* 2002) when uteri contain either no conceptus or a severely growth-retarded tubular conceptus. These results demonstrate that histotroph from endometrial epithelia are required for peri-implantation blastocyst survival and elongation in sheep.

Defects in blastocyst survival and elongation in UGKO ewes are not due to alterations in expression of steroid receptors, anti-adhesive mucin glycoprotein one (MUC1), adhesive integrins on the endometrial LE, or responsiveness of the endometrium to IFNT (Gray *et al.* 2000a; Gray *et al.* 2002). However, uterine flushes from Day 14 bred UGKO ewes contain either very low amounts or undetectable amounts of secreted phosphoprotein one (SPP1 or osteopontin) and glycosylated cell adhesion molecule one (GLYCAM1) proteins which are adhesion proteins secreted by the LE and GE that are relatively abundant in uterine histotroph (Gray *et al.* 2002). Therefore, the reduction or absence of adhesion proteins from endometrial GE is a likely cause of recurrent pregnancy loss in UGKO ewes. Other essential, but as yet undefined, components of histotroph are undoubtedly absent or reduced in the uteri of infertile UGKO ewes.

Progesterone, adhesion molecules and implantation

The cellular and molecular mechanism(s) regulating trophectoderm outgrowth during blastocyst elongation, although not well understood, are hypothesized to require progesterone-de-

pendent uterine secretions as well as apposition and transient attachment of the trophoblast to the LE that is mediated by factors of endometrial origin (see Fig. 2).

Progesterone regulation of pre-implantation blastocyst growth and elongation

As the hormone of pregnancy, progesterone stimulates and maintains endometrial functions necessary for conceptus growth, implantation, placentation and development to term (Bazer *et al.* 1979; Geisert *et al.* 1992; Spencer & Bazer 2002; Spencer *et al.* 2004b). Circulating concentrations of progesterone in early pregnancy affect blastocyst survival and growth during early pregnancy (Mann & Lamming 1999). In dairy cattle, successful establishment of pregnancy and rapid blastocyst development occurs in cows with an early rise in progesterone after ovulation and mating (Mann & Lamming 2001). Exogenous progesterone from Days 2 to 5 and Days 5 to 9 enhanced conceptus development and size in heifers on Day 14 and cows on Day 16, respectively (Garrett *et al.* 1988; Mann *et al.* 2006); however, exogenous progesterone from Days 12 to 16 did not affect conceptus development in cows on Day 16 (Mann *et al.* 2006). Heifers and ewes with lower concentrations of progesterone in the early luteal phase had retarded conceptuses that secreted less IFNT (Nephew *et al.* 1991; Mann & Lamming 2001). Indeed, advancement of conceptus development by administration of progesterone during metestrus and early diestrus has been described also in sheep (Kleemann *et al.* 1994).

The mechanisms whereby progesterone stimulates blastocyst survival and growth are not known, but presumed to be mediated by histotroph (Geisert *et al.* 1992). Uterine-derived growth factors, including CSF2 (colony stimulating factor 2 (granulocyte-macrophage)), FGF2 (fibroblast growth factor 2 (basic)), IGF1 (insulin-like growth factor one), and IGF2, stimulate IFNT production by cultured conceptuses and isolated mononuclear trophectoderm cells (Ko *et al.* 1991; Imakawa *et al.* 1993; Michael *et al.* 2006); however, these effects could be either direct or indirect due to effects on cell proliferation. As noted previously, progesterone acts on the endometrium to induce a number of epithelial genes (*CST3, CTSL, GLYCAM1, LGALS15, SPP1*) in a stage-specific manner that are hypothesized to regulate conceptus development during the peri-implantation period of pregnancy. Paradoxically, progesterone induction of those genes appears to be via the loss of PGR in the endometrial epithelia as discussed next.

PGR regulation and endometrial gene expression

In most mammalian uteri, PGR are expressed in endometrial epithelia and stroma during the early to mid-luteal phase, allowing direct regulation (induction or repression) of genes by progesterone. However, continuous exposure of the endometrium to progesterone negatively regulates PGR expression in LE and GE (see Table 1). In the ovine uterus, PGR protein is not detectable in LE and GE after Days 11 and 13 of pregnancy, respectively, but can be detected in the uterine stroma and myometrium throughout gestation (Spencer *et al.* 2004). The paradigm of loss of PGR in uterine epithelia immediately prior to implantation is common in domestic ruminants and across mammals (Carson *et al.* 2000), strongly suggesting that the loss of PGR alters the program of gene expression in the endometrial LE and then GE (see Table 1). PGR loss in those epithelia is determined by timing of the post-ovulatory rise in progesterone and requires continuous exposure to progesterone, which in sheep is at least 8 days. Thus, an earlier increase in circulating progesterone advances the timing of PGR loss from uterine epithelia. Indeed, PGR loss in the endometrial LE is strongly associated with a reduction in expression of the anti-adhesive MUC1 and induction of *LGALS15*, GLYCAM1, *CST3* and *CTSL*.

In GE, PGR loss is associated with induction of *SPP1*, *STC1* (stanniocalcin) and SERPIN (ovine uterine serpin peptidase inhibitor; alias uterine milk proteins). Indeed, inhibition of progesterone action by an anti-progestin prevents PGR down-regulation and, in turn, progesterone induction of GLYCAM1, *LGALS15*, *CTSL*, *CST3*, *SPP1*, *STC1* and *SERPINs*. Several candidate adhesion factors that mediate blastocyst implantation under the influence of progesterone and, for some IFNT, will now be discussed briefly except for LGALS15 that was discussed previously (see (Burghardt *et al.* 2002; Johnson *et al.* 2003; Spencer *et al.* 2004a)).

Mucin glycoprotein one (MUC1)

As the blastocyst approaches the endometrial LE, it encounters the glycocalyx which includes MUC1, a large transmembrane mucin glycoprotein expressed at the apical surface of epithelia in reproductive tracts of several species (Brayman *et al.* 2004). Expression of the glycoproteins MUC1 and MUC4 on uterine LE may block accessibility of trophoblast integrin receptors to their ligands for cell-cell and cell-extracellular matrix (ECM) adhesion necessary for initial stages of implantation (Carson *et al.* 2000; Burghardt *et al.* 2002). Removal of MUC1 from LE after Day 15 of pregnancy in sheep, which correlates to loss of PGR, may be necessary to expose other glycoproteins involved in adhesion between trophoblast and LE (Table 1). Given that the mucins contain large amounts of glycans that may be potentially recognized by blastocysts or secreted animal lectins such as LGALS15, they may be involved in the apposition phase of implantation.

Integrins

Integrins are a family of heterodimeric intrinsic transmembrane glycoprotein receptors that mediate cellular differentiation, motility and adhesion (Giancotti & Ruoslahti 1999). They play a dominant role in interactions with ECM to transduce cellular signals in uterine epithelial cells and conceptus trophoblast (Burghardt *et al.* 2002; Johnson *et al.* 2003). The central role of integrins in the adhesion cascade leading to implantation is binding ECM ligand(s) to induce cytoskeletal reorganization, stabilize adhesion, and mediate cell migration, proliferation and differentiation through numerous cell signaling pathways (Aplin 1997; Burghardt *et al.* 2002). During the peri-implantation period of pregnancy in ewes, integrin subunits α (v, 4 and 5) and ß (1, 3 and 5) are constitutively expressed on the apical surfaces of both conceptus trophectoderm and endometrial LE (Johnson *et al.* 2001a). This suggests that receptivity to implantation does not involve changes in either temporal or spatial patterns of integrin expression, but may require expression of other glycoproteins and ECM proteins such as LGALS15, SPP1 and oncofetal fibronectin which are ligands for heterodimers of these integrins (Johnson *et al.* 2003). More detail on integrins and implantation can be found elsewhere (Aplin 1997; Burghardt *et al.* 2002; Johnson *et al.* 2003; Spencer *et al.* 2004a).

Glycosylated cell adhesion molecule one (GLYCAM1)

GLYCAM1, a sulfated glycoprotein secreted by the endothelium, mediates leukocyte-endothelial cell adhesion (Lasky *et al.* 1992) by functioning as a carbohydrate ligand for the lectin domain of leukocyte cell surface selectin (SELL or L-selectin) in the lymphoid system (Rosen 1993). Ligation of SELL by GLYCAM1 activates ß1 and ß2 integrins and promotes firm adhesion to ECM components, such as fibronectin (Hwang *et al.* 1996). In humans, trophoblast SELL

appears to be responsible for interactions with the uterine epithelium that are considered critical for implantation and establishing pregnancy (Genbacev *et al.* 2003). The temporal and spatial patterns of expression of GLYCAM1 in cyclic and pregnant ovine uteri implicates it as a potential regulator of implantation (Spencer *et al.* 1999a) (see Table 1). In pregnant ewes, the relative amount of immunoreactive GLYCAM1 in uterine flushings is low on Days 11 and 13, but abundant on Days 15 and 17. Thus, a GLYCAM1-like protein may be a secretory product of the endometrial epithelium and/or conceptus trophoblast. Patterns of distribution observed for immunoreactive GLYCAM1 in the endometrial epithelium, combined with proposed functions for lymphoid GLYCAM1, suggest that this mucin glycoprotein may be involved in conceptus-maternal interactions during the peri-implantation period of pregnancy in sheep (Spencer *et al.* 2004a).

Secreted phosphoprotein one (SPP1)

SPP1 is a member of the Small Integrin-Binding Ligand, N-Linked Glycoprotein (SIBLING) family of related ECM proteins recognized as key players in a number of diverse processes such as bone mineralization, cancer metastasis, cell-mediated immune responses, inflammation, angiogenesis, and cell survival (Sodek *et al.* 2000; Johnson *et al.* 2003). During the peri-implantation period of pregnancy in sheep, *SPP1* mRNA is first detected in the endometrial glands of some ewes by Day 13 and is present in all glands by Day 19 (Johnson *et al.* 1999b). In the uterine lumen, SPP1 protein appears on Day 15 and is found at the trophectoderm-LE interface throughout gestation, suggesting that it plays a key role in adhesion of the trophectoderm to LE via integrin receptors (Johnson *et al.* 2001a). Ovine trophectoderm and LE cells show evidence of integrin receptor activation and cytoskeletal reorganization in response to SPP1 binding *in vitro* (Johnson *et al.* 2001a). Progesterone induces expression of *SPP1* in endometrial glands, and this requires loss of PGR (Spencer *et al.* 1999b; Johnson *et al.* 2000b). SPP1 is hypothesized to serve as a bifunctional bridging ligand that mediates adhesion between LE and trophectoderm essential for implantation and placentation in sheep (Johnson *et al.* 2003).

Progesterone and placental hormone (IFNT, CSH1) regulation of uterine gland morphogenesis and secretory function

During early pregnancy, the ovine uterus is exposed sequentially to oestrogen, progesterone, IFNT, and CSH1 which is proposed to initiate and maintain endometrial gland morphogenesis and differentiated secretory functions (see (Spencer *et al.* 1999b; Spencer & Bazer 2002; Spencer *et al.* 2004b) for review). The placentae of a number of species, including rodents, humans, nonhuman primates and sheep, secrete hormones structurally related to pituitary PRL (prolactin) and GH (growth hormone) that are termed CSH1 (alias placental lactogen) (Soares 2004). Ovine CSH1 is produced by trophoblast giant BNC from Days 15 to 16 of pregnancy which is coordinate with onset of expression of *SERPIN*, *SPP1*, *GRP* (gastrin-releasing peptide), and *STC1* (Ing *et al.* 1989; Whitley *et al.* 1998; Stewart *et al.* 2000; Song *et al.* 2006a), which are excellent markers for GE differentiation and secretory function during pregnancy in sheep. A homodimer of the PRLR, as well as a heterodimer of PRLR and GHR (growth hormone receptor), transduce signals by ovine CSH1 (Gertler & Djiane 2002). In the ovine uterus, *PRLR* gene expression is unique to GE (Cassy *et al.* 1999; Stewart *et al.* 2000). Temporal changes in circulating levels of CSH1 are correlated with endometrial gland hyperplasia and hypertrophy and increased production of SERPIN and SPP1 during pregnancy. The sequential exposure of

the pregnant ovine endometrium to estrogen, progesterone, IFNT, and CSH1 appears to be required to activate and maintain endometrial remodeling, secretory function of GE and uterine growth during gestation. Chronic treatment of ovariectomized ewes with progesterone induces expression of *SPP1*, *SERPIN* and *STC1* by GE (Moffatt *et al.* 1987; Spencer *et al.* 1999b; Johnson *et al.* 2000b; Song *et al.* 2006a). However, intrauterine infusions of CSH1 further increases endometrial *SPP1*, *SERPIN* and *STC1* mRNA, but only when ewes receive progesterone and intrauterine infusions of IFNT between Days 11 and 20 (Spencer *et al.* 1999b). The effects of IFNT may be attributed, in part, to stimulation of PRLR in GE (Martin *et al.* 2004). These results indicate that placental hormones play key roles in stimulating endometrial gland morphogenesis and differentiated functions during pregnancy that are required for conceptus growth and development.

Conclusions

During the past decade, knowledge of mechanisms and factors regulating fetal-maternal interactions during establishment of pregnancy has increased in sheep and cattle. Transcriptional profiling studies are now accelerating the pace of discovery; however, our knowledge of cellular and molecular mechanisms governing fetal-maternal interactions and, in particular, progesterone actions and trophectoderm growth and differentiation remain very limited. Results from studies of rodents strongly suggests that implantation involves a multiplicity of receptor-ligand interactions that are organized into a combinatorial cascade . Therefore, individual and integrative roles of adhesion factors must be mechanistically determined using *in vivo*, *ex vivo* and *in vitro* experimental models. Pregnancy loss in ruminants is greatest during the period of pregnancy recognition and establishment prior to placentation (Mann & Lamming 2001). Therefore, a more complete understanding of key molecules and signal transduction pathways that regulate fetal-maternal interactions during establishment and maintenance of pregnancy can be used to diagnose and identify the cause(s) of recurrent pregnancy loss and improve pregnancy rates and reproductive efficiency in domestic animals and humans.

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