

## Focus on Implantation

# Implantation mechanisms: insights from the sheep

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### Abstract

Implantation in all mammals involves shedding of the zona pellucida, followed by orientation, apposition, attachment and adhesion of the blastocyst to the endometrium. Endometrial invasion does not occur in domestic ruminants; thus, definitive implantation is achieved by adhesion of the mononuclear trophoblast cells to the endometrial luminal epithelium (LE) and formation of syncytia by the fusion of trophoblast binucleate cells with the LE. This review highlights new information on mechanisms regulating the implantation cascade in sheep. The embryo enters the uterus on day 4 at the morula stage of development and then develops into a blastocyst by day 6. The blastocyst sheds the zona pellucida (day 8), elongates to a filamentous form (days 11–16), and adheres to the endometrial LE (day 16). Between days 14 and 16, the binucleate cells begin to differentiate in the trophoblast and subsequently migrate and fuse with the endometrial LE to form syncytia. Continuous exposure of the endometrium to progesterone in early pregnancy downregulates the progesterone receptors in the epithelia, a process which is associated with loss of the cell-surface mucin MUC1 and induction of several secreted adhesion proteins. Recurrent early pregnancy loss in the uterine gland knockout ewe model indicates that secretions of the endometrial epithelia have a physiologic role in blastocyst elongation and implantation. A number of endometrial proteins have been identified as potential regulators of blastocyst development and implantation in sheep, including glycosylated cell adhesion molecule 1 (GlyCAM-1), galectin-15, integrins and osteopontin. The epithelial derived secreted adhesion proteins (GlyCAM-1, galectin-15 and osteopontin) are expressed in a dynamic temporal and spatial manner and regulated by progesterone and/or interferon tau, which is the pregnancy recognition signal produced by the trophoblast during blastocyst elongation. The noninvasive and protracted nature of implantation in domestic animals provides valuable opportunities to investigate fundamental processes of implantation that are shared among all mammals. Understanding of the cellular and molecular signals that regulate uterine receptivity and implantation can be used to diagnose and identify causes of recurrent pregnancy loss and to improve pregnancy outcome in domestic animals and humans.

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### Introduction

Implantation of the blastocyst in the uterus is an evolutionary advance associated with viviparity and is required for sustained efficient nutrition and protection of the conceptus (embryo/fetus and associated extraembryonic membranes) during gestation. Because of its recent evolution, there is considerable diversity in types of implantation among eutherian mammals. Despite the diversity of implantation and placentation strategies, the initial events that occur between the trophoblast and maternal uterine endometrial luminal epithelium (LE) are shared among species. Consequently, the comparative biology of implantation is useful to discover and understand the physiologic, hormonal, cellular and molecular mechanisms regulating implantation in mammals. By the blastocyst stage, the trophoblast has acquired competence to attach and adhere to the

endometrial LE, which appears to be the primary site of hormonally regulated uterine receptivity. If blastocyst development is synchronous with uterine receptivity, an adhesion cascade initiated at the apical surfaces of trophoblast and LE results in definitive implantation. This review highlights new information on the mechanisms and factors regulating implantation that is focused primarily on the sheep.

### Anatomic and cellular aspects of conceptus implantation in sheep

Implantation in domestic ruminants (sheep, cattle and goats) takes place at the blastocyst stage. The blastocyst develops from the preceding morula stage embryo as the result of compaction and contains a blastocoele or central cavity surrounded by a cell monolayer or trophoblast.

The trophoblast is involved in adhesive interactions with the endometrial epithelia that results in implantation. The structure of the endometrium has common features in all species. The endometrial mucosa is formed by a mono- or pseudostratified epithelium which is separated from the conjunctive stroma by a basal lamina. The stroma is highly vascularized and contains coiled and branched glands whose ducts open into the uterine lumen. The endometrial surface epithelium is composed of secretory cells with microvilli and ciliated cells, the latter being concentrated around the openings of the endometrial glands.

The timing of implantation differs among species and is not particularly related to the length of gestation (Guillomot *et al.* 1993). Differences among species arise from the length of the different implantation phases (hours in rodents to days in humans and domestic animals), the evolution of the cell–cell contacts, and the degree of endometrial invasion by the trophoblast.

In domestic ruminants and pigs, the blastocyst elongates during the latter stages of implantation, but this unique developmental event does not occur in laboratory rodents, horses, primates or humans (Guillomot *et al.* 1993, Allen & Stewart 2001). The polar trophoblast over the inner cell mass (Raubert's layer) is removed before elongation of the blastocyst. Before and perhaps during elongation of the blastocyst, the extraembryonic endoderm originates from the inner cell mass and migrates under the trophoblast as the blastocoele expands. The mesoderm then originates from the inner cell mass and migrates between the endoderm and trophoblast. This interposed mesoderm then cavitates; the outer layer forms the chorion with the trophoblast, whereas the inner layer forms the yolk sac wall with endoderm. Therefore, the extraembryonic membranes form before implantation in domestic ruminants and pigs. It is tempting to speculate that the extraembryonic membranes are important for trophoblast elongation. In summary, the spherical blastocyst becomes tubular and then filamentous as it elongates and becomes a conceptus (embryo/fetus and associated extraembryonic membranes). In contrast, the blastocyst of laboratory rodents, primates and humans implants rapidly before expansion, and the extraembryonic membranes are formed after implantation (Renfree 1982, Guillomot *et al.* 1993, Carson *et al.* 2000). Furthermore, the polar trophoblast does not disappear, but rather proliferates and gives rise to peripheral polyploid cells. However, the initial early stages of implantation are common to all species.

Based on a comparative implantation scheme proposed by Guillomot and colleagues (Guillomot *et al.* 1981, 1993, Guillomot 1995), the phases of implantation include: 1. shedding of the zona pellucida; 2. precontact and blastocyst orientation; 3. apposition; 4. adhesion; 5. endometrial invasion. In contrast to rodents and humans, true endometrial invasion does not occur in ruminants. Following is an anatomic and cellular description of the phases of implantation in sheep, which are illustrated in Figs 1 and 2.

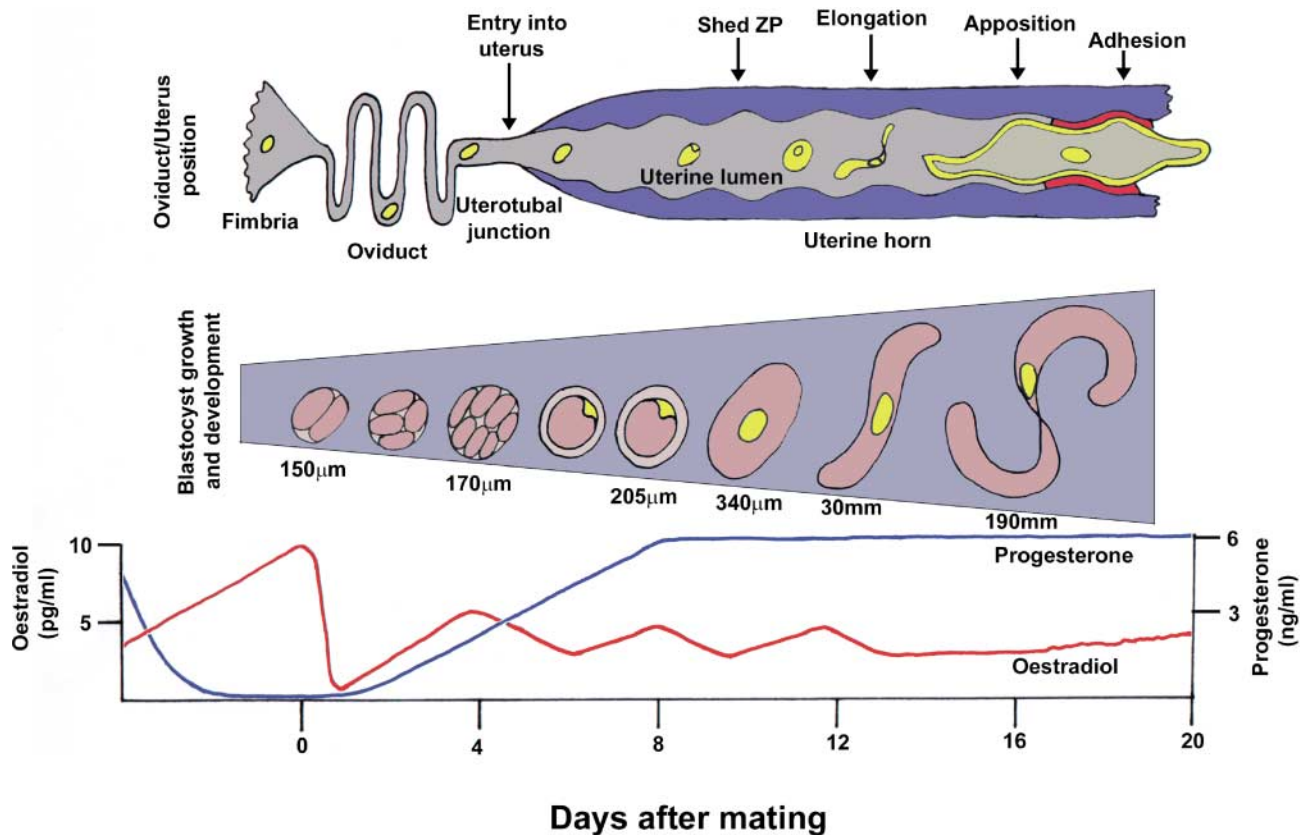
### ***Shedding of the zona pellucida (phase 1)***

The morula (16–32 cells) stage embryo enters the uterus from the oviduct on day 4 after mating (day 0 = estrus/mating) (Fig. 1). The blastocyst is formed on day 6, and the zona pellucida is shed between days 8 and 9. Loss of the zona pellucida appears to be achieved by rupture and hatching after blastocyst growth or after enzymatic lysis by uterine and/or embryonic proteases. This stage can occur in blastocysts derived by *in vitro* maturation, fertilization and culture. In general, the zona pellucida is thought to prevent the trophoblast from contacting and attaching to the endometrial LE. The blastocyst is spherical on day 8, measures 200 µm in diameter and contains approximately 300 cells. By day 10, it measures 400–900 µm in diameter and contains approximately 3000 cells. After day 10, elongation of the blastocyst occurs, and it develops first into a tubular and then into a filamentous conceptus (Wintenberger-Torres & Flechon 1974).

### ***Precontact and blastocyst orientation (phase 2)***

Between days 9 and 14, no definitive cellular contacts are observed between the trophoblast and the endometrial epithelium. The blastocyst appears to be positioned and immobilized in the uterus after loss of the zona pellucida. However, the blastocyst can be easily recovered from the uterus by lavage without causing structural damage. The nonrandom orientation of the blastocyst represents a biologic constant of a given species, and the blastocyst position in the uterine horn is central in species characterized by large expansion of the blastocyst, as in domestic animals.

Starting on day 11, the spherical or slightly tubular blastocyst begins to elongate until it reaches a length of 25 cm or more by day 17 and resembles a long filament composed mainly of extraembryonic trophoblast. By day 12, it has elongated markedly, reaching a length of 10–22 mm. At day 14, the filamentous conceptus is about 10 cm long. The primitive streak appears at this stage and somites soon thereafter. The conceptus, first located in the uterine horn ipsilateral to the corpus luteum, elongates into the contralateral horn on day 13 and may fill more than half of its length on day 17 when only one ovulation has occurred (Rowson & Moor 1966). Hatched blastocysts and trophoblastic vesicles are not able to elongate *in vitro* unless transferred into the uterus (Heyman *et al.* 1984, Flechon *et al.* 1986). Elongation of the blastocyst is critical for developmentally regulated production of interferon tau (IFN $\tau$ ) (Farin *et al.* 1989, Guillomot *et al.* 1990, Gray *et al.* 2002), a type I IFN that is the signal for maternal recognition of pregnancy and acts in a paracrine manner on the endometrial epithelia to inhibit development of the luteolytic mechanism (Bazer 1992). The cellular and molecular mechanisms regulating blastocyst elongation are not well understood, but are hypothesized to require apposition and transient attachment of the trophoblast to the LE.



**Figure 1** Early pregnancy events in sheep. This schematic summarizes the relative changes in embryo/blastocyst 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 the morula stage embryo enters the uterus on day 4. The blastocyst is formed by day 6 and hatches from the zona pellucida on days 8–9. The blastocyst develops from a spherical to a tubular form by day 11 and then elongates to a filamentous conceptus between days 12 and 16. The elongation of the blastocyst marks the beginning of implantation, which involves apposition and transient attachment (days 12–15) and firm adhesion by day 16.

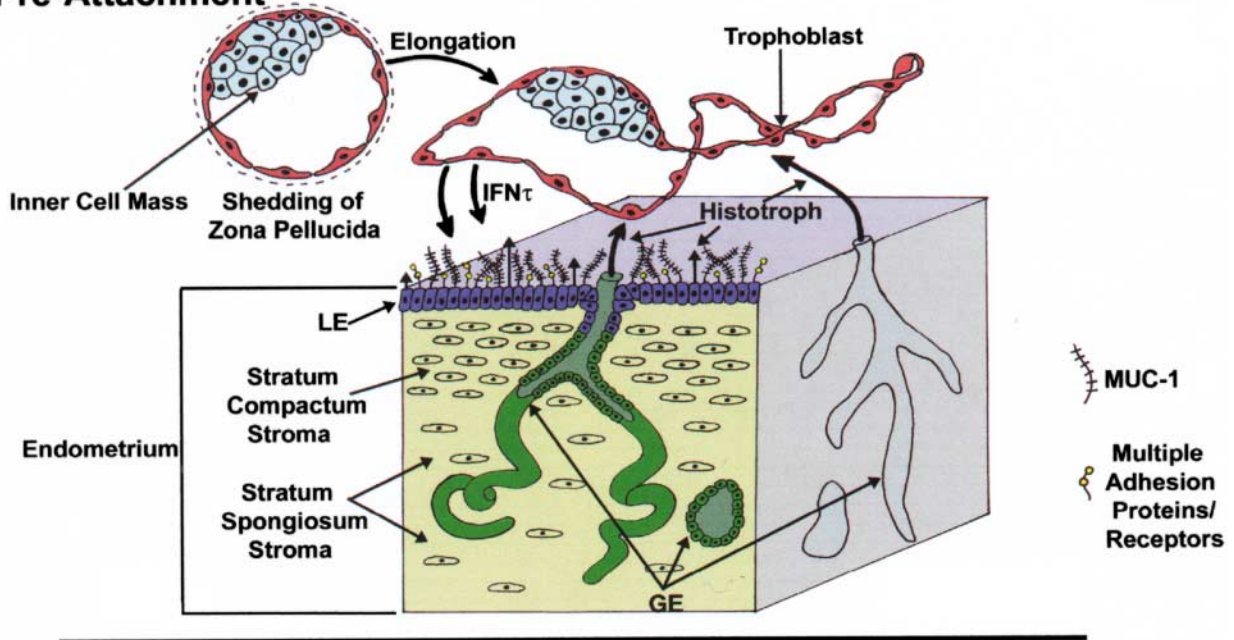
### Apposition (phase 3)

Apposition of the conceptus involves the trophoblast becoming closely associated with the endometrial LE followed by unstable adhesion. After day 14, the filamentous conceptus appears to be immobilized in the uterine lumen. The elongating blastocyst maintains close contact with the endometrial LE, which appears to imprint its rounded shape on the trophoblast in fixed specimens (Guillomot *et al.* 1993). A close association of the apical membranes of both cell types is observed, although the conceptus can still be recovered intact from the uterus by lavage. In most species, the onset of apposition is accompanied by a reduction of the apical microvilli covering the trophoblast, a reduction which occurs between days 13 and 15 on the sheep conceptus (Guillomot *et al.* 1981, 1993). In rodents, the endometrial epithelium undergoes the same modification, allowing a closer association with the trophoblast (Enders & Schlafke 1969); however, loss of apical microvilli on the uterine LE does not appear to occur in sheep (Guillomot *et al.* 1981, 1982). The permeability of uterine capillaries increases for pontamine blue at the same time (Boshier 1970). The

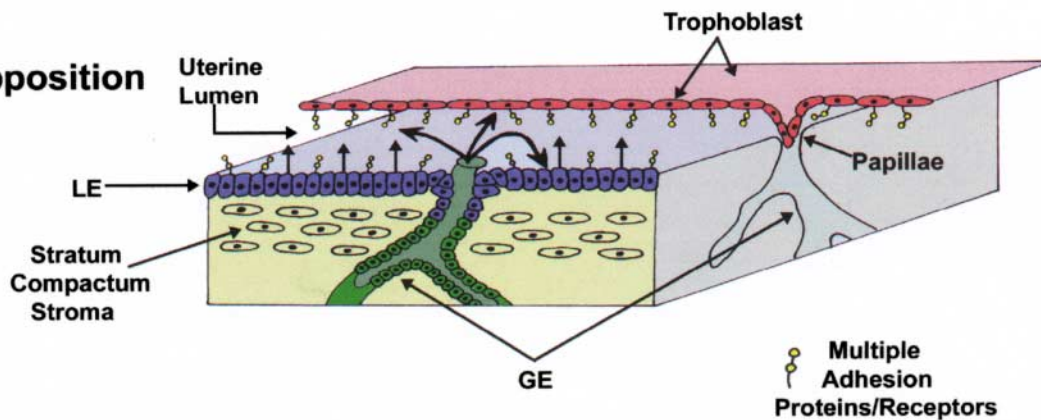
apposition of the blastocyst is ensured by interdigitation of cytoplasmic projections of the trophoblast cells and uterine epithelial microvilli (Guillomot *et al.* 1981, 1993). In sheep, apposition occurs first in the vicinity of the inner cell mass, that is, the embryo, and spreads toward the extremity of the elongated conceptus.

In ruminants, the uterine glands are also sites of apposition (Guillomot *et al.* 1981, Guillomot & Guay 1982). Between the caruncles, the trophoblast develops finger-like villi or papillae, which penetrate into the mouths of the superficial ducts of the uterine glands at days 15–18 (Guillomot *et al.* 1981, Wooding *et al.* 1982). During their short life (they vanish at day 20), these trophoblastic differentiations are hypothesized to anchor the periattachment conceptus and absorb the histotrophic secretions of the glands (Guillomot *et al.* 1981). Furthermore, the trophoblast papillae are hypothesized to facilitate the formation of more robust adhesive interactions between the trophoblast and endometrial LE (Wooding *et al.* 1982). Similar features were described in the cow conceptus from day 15 of pregnancy, but, curiously, the goat conceptus lacked trophoblast papillae.

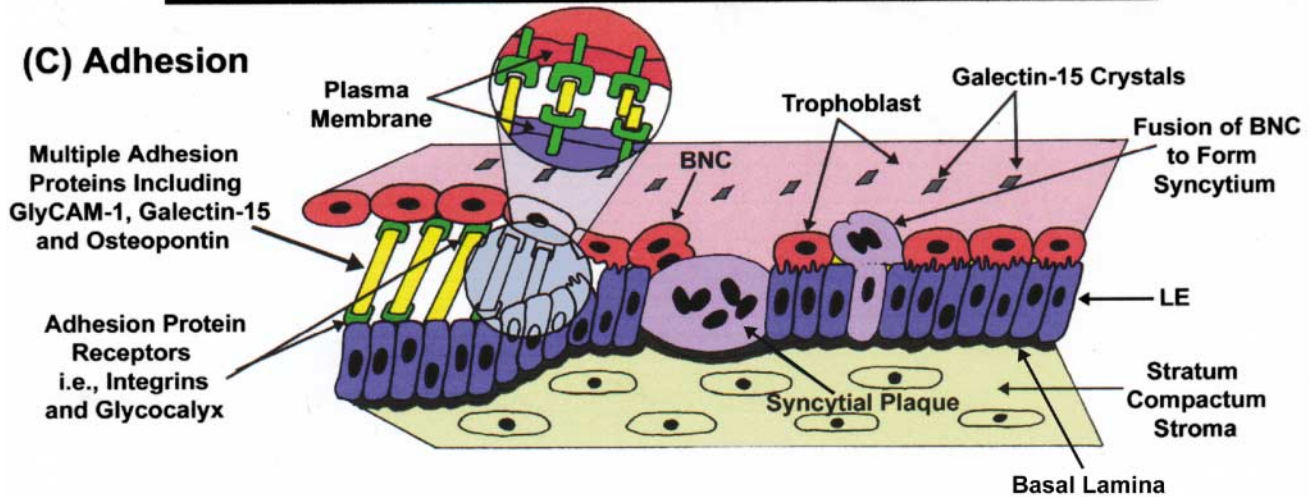
**(A) Pre-Attachment**



**(B) Apposition**



**(C) Adhesion**



The ovine uterine wall can be functionally divided into the endometrium and the myometrium. The normal adult ovine endometrium consists of LE, glandular epithelium (GE), several types of stroma (stratum compactum and stratum spongiosum), blood vessels and immune cells. In sheep, the endometrium has two distinct areas – aglandular caruncular and glandular intercaruncular. The caruncular areas have LE and compact stroma and are the sites of superficial implantation and placentation (Wimsatt 1950, Amoroso 1951). Synepitheliochorial placentation in sheep involves the fusion of placental cotyledons with endometrial caruncles to form placentomes, which serve a primary role in fetal–maternal gas exchange and derivation of nutrients by the placenta. The first changes in the endometrial LE begin on day 14 in both uterine horns (Guillomot *et al.* 1981). The caruncles become edematous with a folded and depressed surface. These modifications are progressive and do not occur simultaneously on all caruncles. Caruncular folding is perhaps the first step in the formation of crypts that constitute the maternal side of the future placentomes, which are structures that form with placental cotyledons (Wimsatt 1950). Dome-like cytoplasmic protrusions also appear on the caruncular epithelial cells, which have a convex apex. Similar protrusions, which are sites of endocytosis, are termed pinopods and have also been described on the uterine epithelium at the time of implantation in the mouse, rat, human and rabbit (Guillomot *et al.* 1981).

#### Adhesion (phase 4)

On day 16, the trophoblast begins to adhere firmly to the endometrial LE. Uterine lavage to recover the conceptus causes superficial structural damage at this time. The interdigitation of the trophoblast and endometrial LE occurs in both the caruncular and intercaruncular areas of the endometrium. Adhesion of the trophoblast to the endometrial LE progresses along the uterine horn and appears to be completed around day 22 (Boshier 1969, Guillomot *et al.* 1981). Interestingly, the arrest of IFN $\gamma$  gene expression occurs in regions of the mononuclear

trophoblast which have established cellular contacts with the LE during the implantation process (Guillomot *et al.* 1990).

The trophoblast giant binucleate cells (BNC) have differentiated from the mononuclear trophoblast by day 16, but only mononuclear trophoblast cells are thought to adhere to the endometrial LE. The BNC of the ruminant placenta may be analogous in many respects to the trophoblast giant cells of the syncytiotrophoblast in humans (Hoffman & Wooding 1993). The BNC have at least two main functions: 1. to form a hybrid fetomaternal syncytium essential for successful implantation and subsequent placentomal growth; 2. to synthesize and secrete protein and steroid hormones, such as placental lactogen and progesterone, that regulate maternal physiology (Wooding 1992, Hoffman & Wooding 1993, Spencer *et al.* 2004). Trophoblast BNC are thought to arise from the mononuclear trophoblast stem cells by consecutive nuclear divisions without cytokinesis, migrate through the apical trophoblast tight junctions of the chorion, and flatten as they become apposed to the apical surface of the endometrial LE (Wimsatt 1951, Wooding 1984). The BNC then fuse apically with the endometrial LE and form syncytia of trinucleate cells, thereby assimilating and replacing the endometrial epithelium. Subsequently, the trinucleate cells enlarge by continued BNC migration and fusion to form syncytial plaques linked by tight junctions that appear to be limited in size in the ewe to 20–25 nuclei (Wooding 1984). The syncytial plaques eventually cover the caruncular surface and aid in formation of the placentome. Indeed, BNC migrate and fuse with the uterine epithelial cells or their derivatives throughout most of pregnancy. The uterine LE persists but is modified to a variable degree, depending on species, into a hybrid fetomaternal syncytium formed by the migration and fusion of the fetal BNC with those of the endometrial epithelium (Wooding 1992). The mature sheep placenta is defined as synepitheliochorial, being neither entirely syndesmochorial without uterine epithelium, nor completely epitheliochorial with two

**Figure 2** Apposition and adhesion phases of blastocyst implantation in sheep. (A) Preattachment involving shedding of the zona pellucida (phase 1) and precontact and blastocyst orientation (phase 2). The first phase of implantation is the shedding of the zona pellucida on day 8 that exposes the trophoblast. The second phase of implantation is before contact and involves the blastocyst orientation which occurs on days 9–11 as the blastocyst grows from a spherical to a tubular conceptus and migrates to the middle region of the uterine horn ipsilateral to the corpus luteum. The antiadhesive mucin MUC-1 is present on the endometrial LE, thereby preventing contact of the trophoblast with adhesive receptors such as integrins. Histotroph, secreted from the endometrial LE and GE, nourishes the developing blastocyst. (B) Apposition and transient attachment (phase 3). After day 11, the tubular blastocyst elongates to form a filamentous conceptus. During this period, expression of MUC-1 declines on the LE, which exposes constitutively expressed integrins on the LE as well as trophoblast. Apposition occurs between the trophoblast and endometrial LE and between the trophoblast papillae and GE ducts. Elongation of the blastocyst probably requires apposition and transient attachment to the endometrial LE. (C) Adhesion (phase 4). Firm adhesion of the mononuclear cells of the trophoblast to the LE occurs between days 15 and 16. Available evidence indicates that several molecules (GlyCAM-1, galectin-15 and osteopontin) interact with receptors (integrins and glycoconjugates) on the apical surfaces of the trophoblast and LE to facilitate adhesion. The binucleate cells (BNC) differentiate from the mononuclear trophoblast and then migrate to and fuse with the LE to form a multinucleated syncytial plaque. Although the BNC are inherently invasive, they do not cross the basal lamina that supports the LE.



apposed cell layers whose only anatomic interaction is interdigitated microvilli, as in the pig.

### Functional role of the endometrial epithelium and secretions in blastocyst survival and elongation

All mammalian uteri contain endometrial epithelia that synthesize and secrete or transport a complex array of proteins and related substances termed 'histotroph' (Wimsatt 1950, Amoroso 1952, Bazer 1975); that is, a complex mixture of enzymes, growth factors, cytokines, lymphokines, hormones, transport proteins and other substances. Evidence from human, primate and subprimate species during the last century supports an unequivocal role for secretions of endometrium as primary regulators of conceptus survival, development, production of pregnancy recognition signals, implantation and placentation (reviewed in (Bazer *et al.* 1979, Roberts & Bazer 1988, Carson *et al.* 2000, Gray *et al.* 2001a, Burton *et al.* 2002). The microvillous epithelial cells of the uterine lumen present a high secretory activity during the luteal phase of the cycle and at the beginning of implantation (Guillomot *et al.* 1981). The sheep trophoblast appears to be the site of intense pinocytotic activity that increases as the blastocyst develops (Wintenberger-Torres & Flechon 1974). Therefore, it has been hypothesized that metabolites necessary for growth of the elongating conceptus are obtained from uterine histotroph. This hypothesis is supported by studies on the asynchronous transfer of embryos and trophoblast vesicles (Lawson *et al.* 1983, Flechon *et al.* 1986), but especially by results from studies of the uterine gland knockout (UGKO) ewe (Gray *et al.* 2001c, 2002).

The UGKO ewe model is produced by continuous administration of a synthetic, nonmetabolizable progestin to neonatal ewes from birth to at least postnatal day 56 (Gray *et al.* 2000a). This inappropriate exposure to a progestin permanently ablates differentiation and development of the glandular epithelia (GE) from LE in the endometrium and produces an UGKO phenotype without altering development of myometrium or other Müllerian duct-derived female reproductive tract structures or the hypothalamic–pituitary–ovarian axis (Gray *et al.* 2000a,b, 2001b). The endometrium is devoid of middle to deep endometrial glands and the LE surface area is markedly reduced. UGKO ewes exhibit recurrent early pregnancy loss in which the blastocyst fails to elongate. Transfer of blastocysts from normal fertile ewes into the uteri of timed recipient UGKO ewes did not ameliorate this defect (Gray *et al.* 2001c). Morphologically normal blastocysts are present in uterine flushes of bred UGKO ewes on days 6 and 9 after mating, but not on day 14 (Gray *et al.* 2001c, 2002). On day 14, uterine flushes of mated UGKO ewes contain either no conceptus or a severely growth-retarded tubular conceptus. Therefore, histotrophic secretions from the endometrial epithelia are required for peri-implantation blastocyst survival and elongation in sheep.

Available results indicate that the defects in blastocyst survival and elongation in UGKO ewes are not due to alterations in expression of steroid receptors, mucin glycoprotein 1 (MUC1) or adhesive integrins on the endometrial LE, or to the responsiveness of the endometrium to the conceptus pregnancy recognition signal IFN $\tau$  (Gray *et al.* 2000a, 2002). However, when uterine flushes of day 14 bred UGKO ewes were analyzed for the presence of osteopontin (OPN) and glycosylated cell adhesion molecule 1 (GlyCAM-1) proteins, which are adhesion proteins secreted primarily by GE (Johnson *et al.* 1999a,b, Spencer *et al.* 1999a), very low levels of OPN and GlyCAM-1 were found compared to control day 14 pregnant ewes (Gray *et al.* 2002). Therefore, the reduction or absence in adhesion proteins of endometrial epithelial origin is proposed as the cause of recurrent pregnancy loss in the UGKO ewe.

### Adhesion molecules and implantation

The external surface of the trophoblast and endometrial LE cells is composed of a glycoprotein coat, or glycocalyx. Biochemical changes occur in the composition or distribution of the trophoblastic glycocalyx during the process of blastocyst attachment in the ewe (Guillomot *et al.* 1982), but little information is available on the relative glycoprotein composition of the apical membrane of either the trophectoderm or endometrial LE. Recently, several candidate adhesion factors that mediate blastocyst implantation under the influence of progesterone have emerged in the sheep. As the hormone of pregnancy, progesterone plays a pivotal and indisputable role in the establishment and maintenance of pregnancy in mammals. In a number of mammalian uteri, progesterone receptors (PR) are expressed in the endometrial epithelia and stroma during the early to midluteal phase, allowing direct regulation of a number of genes by progesterone via activation of the PR. However, continuous exposure of the endometrium to progesterone negatively regulates PR expression in the endometrial epithelium. Expression of PR protein is not detectable in endometrial LE and GE in sheep after days 11 and 13 of pregnancy respectively (Spencer & Bazer 1995). Furthermore, PR expression is detected only in the endometrial stroma and myometrium throughout most of gestation in the ovine uterus (Spencer *et al.* 2004). The paradigm of loss of PR in uterine epithelia immediately before implantation is common across mammals (Carson *et al.* 2000, Spencer *et al.* 2004). Thus, regulation of endometrial epithelial function during the peri-implantation period may be dependent on loss of epithelial cell PR and/or be directed by specific factors produced by PR-positive stromal cells (Spencer & Bazer 2002, Spencer *et al.* 2004). The loss of the PR by the endometrial epithelium can be directly correlated with reduced expression of certain genes, such as the antiadhesive protein MUC1. Furthermore, PR loss in the endometrial GE appears to be required for the onset of

expression of other genes during pregnancy, such as galectin-15, osteopontin and ovine uterine serpins (or uterine milk proteins) (Spencer *et al.* 2004), several of which are discussed further below.

### **MUC1**

As the blastocyst approaches the endometrial LE, it encounters the glycocalyx. One component of the glycocalyx is MUC1, a large, transmembrane mucin glycoprotein expressed at the apical surface of a variety of reproductive tract epithelia (Brayman *et al.* 2004). MUC1 is particularly abundant on the microvilli and cilia that extend from the apical cell surface of the endometrial LE. The extracellular domain of MUC1 contains a very large amount of glycans (Aplin & Hey 1995). In fact, the core protein is 120–220 kDa, but with glycosylation it can be over 400 kDa. In both humans and rodents, the expression pattern of the glycoproteins MUC1 and MUC4 on uterine LE may control the accessibility of trophoblast integrin receptors to their ligands by sterically blocking cell–cell and cell–extracellular matrix (ECM) adhesion and access of conceptus trophoblast to uterine LE, due to their extensive glycosylation and extended extracellular structure (Carson *et al.* 2000, Burghardt *et al.* 2002). The implantation adhesion cascade in sheep is initiated after down-regulation of MUC1, and this is coincident with loss of PR from uterine epithelium (Johnson *et al.* 2001). Immunoreactive MUC1 expression by LE decreases at days 9–17 of early pregnancy in normal (Johnson *et al.* 2001) and UGKO (Gray *et al.* 2002) ewes. This pattern of MUC1 expression contrasts with that in rabbits and humans, in which there is an overall increase in MUC1 expression during the receptive phase under the influence of progesterone; however, MUC1 is locally reduced at implantation sites, via the activity of cell-surface proteases that are triggered by the blastocyst or mediated by paracrine signals from blastocysts (Carson *et al.* 2000, Brayman *et al.* 2004). Regardless of the mechanisms by which MUC1 is downregulated, removal of this antiadhesive barrier is hypothesized to be necessary to expose other glycoproteins involved in the adhesion between trophoblast and LE. Given that the mucins contain a large number of glycans that can be potentially recognized by the blastocyst or secreted animal lectins, they may also be involved in the apposition phase of implantation (Aplin & Hey 1995, Brayman *et al.* 2004).

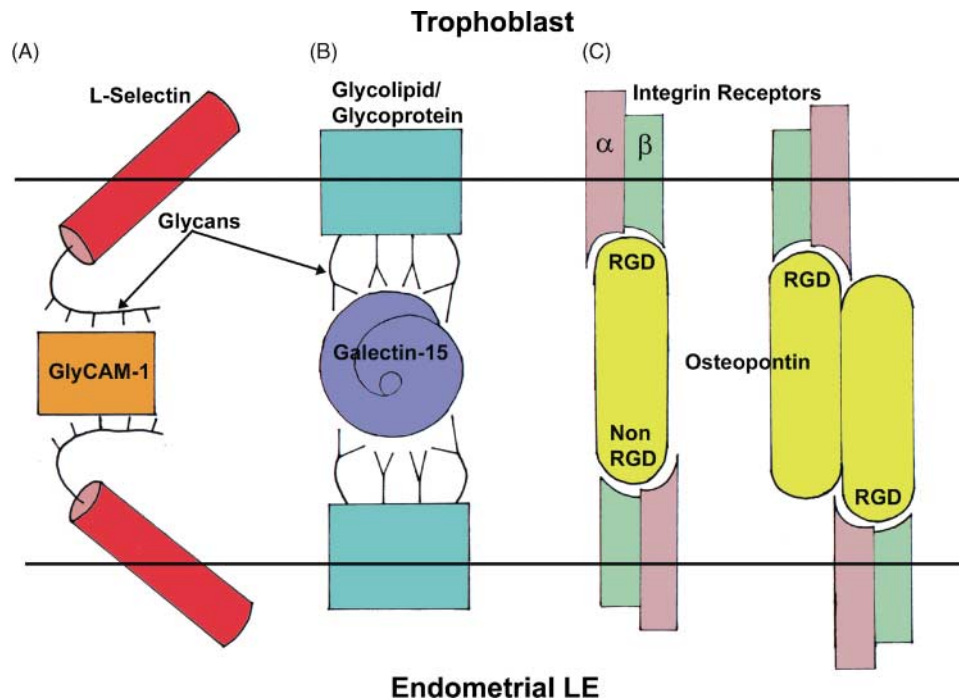
### **Glycosylated cell adhesion molecule 1 (GlyCAM-1)**

GlyCAM-1 is a sulfated glycoprotein secreted by the endothelium that mediates leukocyte–endothelial cell adhesion (Lasky *et al.* 1992). GlyCAM-1 is a member of the mucin family of glycoproteins, with approximately 70% of the native molecular mass composed of O-linked carbohydrates found in two serine/threonine-rich domains (Rosen 1993). This mucin glycoprotein is expressed predominantly at the luminal surface of high endothelial

venules of peripheral and mesenteric lymph nodes. As illustrated in Fig. 3, GlyCAM-1 functions as a carbohydrate ligand for the lectin domain of leukocyte cell-surface selectin (L-selectin) in the lymphoid system (Rosen 1993). Ligation of L-selectin by GlyCAM-1 activates  $\beta$ 1 and  $\beta$ 2 integrins and promotes firm adhesion to fibronectin (Hwang *et al.* 1996, Giblin *et al.* 1997). In humans, trophoblast L-selectin appears to mediate interactions with the uterine epithelium that may be critical to establishing human pregnancy (Genbacev *et al.* 2003). The temporal and spatial patterns of GlyCAM-1 in the uterus of cyclic and pregnant ewes implicate GlyCAM-1 as a potential regulator of implantation (Spencer *et al.* 1999a). In cyclic ewes, GlyCAM-1 expression increases in the endometrial LE and superficial GE between days 1 and 5 and then decreases between days 11 and 15. In pregnant ewes, GlyCAM-1 in the LE and superficial ductal GE is low on days 11 and 13, increases on day 15 and is abundant on days 17 and 19. Immunoreactive GlyCAM-1 is also detected in the conceptus trophoblast on days 13–19. In pregnant ewes, the relative amount of immunoreactive GlyCAM-1 in uterine flushings is low on days 11 and 13, but abundant on days 15 and 17. Thus, a GlyCAM-1-like protein may be a secretory product of the endometrial epithelium and/or conceptus trophoblast. Patterns of distribution observed for immunoreactive GlyCAM-1-like protein in the endometrial epithelium, combined with proposed functions for lymphoid GlyCAM-1, suggest that this mucin glycoprotein may be involved in conceptus–maternal interactions during the peri-implantation period of pregnancy in sheep.

### **Galectin-15**

Galectins are proteins with a conserved carbohydrate recognition domain (CRD) that bind  $\beta$ -galactosides, thereby cross-linking glycoproteins as well as glycolipid receptors on the surface of cells and initiating biologic responses (Cooper 2002, Yang & Liu 2003) (Fig. 3). Recently, a new galectin family member, galectin-15, was discovered in the endometrium of sheep by gene expression profiling to investigate the peri-implantation pregnancy defect in UGKO ewes (Gray *et al.* 2004). Ovine endometrial galectin-15 contains a conserved carbohydrate recognition domain (CRD) that binds  $\beta$ -galactosides, but the carbohydrate-binding specificity for each galectin appears to be different (Cho & Cummings 1995). In addition to a conserved CRD, galectin-15 also contains predicted cell attachment sequences (LDV and RGD) that could mediate binding to integrins in ECM proteins (Kimber & Spanswick 2000, Wang & Armant 2002). Other galectins bind integrins as well as fibronectin, laminin and MUC16/CA-125, because these proteins are modified with  $\beta$ -galactoside sugars (Cooper 2002, Seelenmeyer *et al.* 2003, Yang & Liu 2003). Functional studies of other galectins have implicated these proteins in cell growth, differentiation and apoptosis as well as in cell adhesion, chemoattraction



**Figure 3** Possible interactions between trophoblast and endometrial epithelial cells regulating adhesion during implantation. Evidence for these interactions are from *in vivo* and *in vitro* studies in sheep, mice and humans. (A) Homotypic binding of GlyCAM-1 by L-selectin. GlyCAM-1 functions as a carbohydrate ligand for the lectin domain of L-selectin. (B) Homotypic and heterotypic binding of glycans on proteins and/or lipids via the multifunctional, secreted bridging ligand galectin-15. There is evidence that galectin-15 can bind to a large number of proteins and lipids, including mucins and integrins, which contain  $\beta$ -galactosides, using a carbohydrate recognition domain. (C) Homotypic and heterotypic integrin-mediated adhesion via the bifunctional, secreted bridging ligand OPN. There is evidence that OPN can homodimerize and bind integrins in a RGD-dependent and RGD-independent manner.

and migration (Barondes *et al.* 1994, Hughes 2001, Yang & Liu 2003).

In the ovine uterus, galectin-15 mRNA was detected only in the endometrial LE and superficial ductal GE (Gray *et al.* 2004), which are the primary sites of blastocyst apposition and adhesion. In endometria of cyclic and pregnant ewes, galectin-15 mRNA was not detected before day 10, appeared and then increased 13-fold between days 10 and 14, and then noticeably decreased between days 14 and 16 in cyclic, but not pregnant, ewes. Immunoreactive galectin-15 protein was concentrated near and on the apical surface of the luminal and superficial ductal epithelia and localized within discrete cytoplasmic structures of conceptus trophoderm. In uterine flushings, galectin-15 was present at low levels on days 10 and 12, but was abundant on days 14 and 16 of pregnancy. Progesterone induced and  $\text{IFN}\gamma$  increased galectin-15 mRNA in the endometrium. Thus, galectin-15 and *Wnt7a* are the only genes currently known to be increased by  $\text{IFN}\gamma$  in the endometrial LE of the ovine uterus (Choi *et al.* 2001, Kim *et al.* 2003, Gray *et al.* 2004).

The temporal and spatial alterations in galectin-15 mRNA and protein in endometrial LE and lumen of the ovine uterus during pregnancy, combined with the functional aspects of galectin-15 and its family members, make it a strong candidate for a mediator of conceptus–endometrial interactions during implantation. Therefore,

the proposed extracellular role of galectin-15 in the uterine lumen is functionally to bind and cross-link  $\beta$ -galactosides on glycoproteins, such as mucins, integrins, fibronectin, laminin and other glycoproteins and glycolipids, thereby allowing it to function as a heterophilic cell adhesion molecule bridging the blastocyst and the endometrial LE. The biologic responses of the trophoblast to galectin-15 may also include migration, proliferation and differentiation, which are critical for successful conceptus implantation.

Interestingly, galectin-15 appears to be the 14K protein from sheep endometrium initially characterized as a progesterone-modulated protein associated with crystalline inclusion bodies in uterine epithelia and conceptus trophoblast (Kazemi *et al.* 1990). The 14K protein was originally identified as a component of conceptus-conditioned culture medium and uterine flushes (Salamonsen *et al.* 1984). Release of the 14K protein was attributed to the cellular breakdown of conceptuses in culture (Kazemi *et al.* 1990). Immunogold electron microscopy revealed that within trophoblast, the 14K protein was localized to large, membrane-bound rhomboidal or needle-shaped crystal structures. Thus, it was suggested that the protein was secreted by the endometrial epithelia, taken up by the conceptus from uterine histotroph, and deposited as crystals (Kazemi *et al.* 1990). These crystals are first



observed in the sheep trophoblast on day 10 and then increase in number and size between days 10 and 18 of pregnancy (Wintenberger-Torres & Flechon 1974). Indeed, the crystals exhibit a lattice periodicity of about 20 nm in day-14 blastocysts. Similar progesterone-induced crystal proteins are present in endometrium and conceptus trophoblast of many mammals, including rabbit, mouse, pig and human (Nakao *et al.* 1971, Calarco & Szollosi 1973, Daniel & Chilton 1978, Daniel & Kennedy 1978, Hoffman & Olson 1984, Hernandez & Baum 2002). However, the crystals are generally absent in blastocysts derived *in vitro* or in parthenotes (Daniel & Kennedy 1978, Talbot *et al.* 2000). Accordingly, galectin family members are likely to be expressed in the endometrium of many mammals to facilitate conceptus–endometrial interactions. Although the biologic role of galectin-15 crystals in the conceptus is not known, the intracellular roles of other galectins include modulation of cell growth, differentiation and apoptosis through functioning as pre-mRNA splicing factors and interacting with specific intracellular ligands such as Ras and Bcl-2 (Hernandez & Baum 2002, Liu *et al.* 2002).

### Integrins

Integrins comprise 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 (Johnson *et al.* 2001, Burghardt *et al.* 2002, Johnson *et al.* 2003a). The central role of integrins in the implantation adhesion cascade is to bind ECM ligand(s) to cause cytoskeletal reorganization, stabilize adhesion, and mediate cell migration, proliferation and differentiation through numerous signaling intermediates (Giancotti & Ruoslahti 1999). Altered expression of integrins is correlated with several causes of infertility (Lessey 1998), null mutations of several integrins leads to peri-implantation lethality (Hynes 1996), and functional blockade of selected integrins reduces the number of implantation sites (Illera *et al.* 2000). During the peri-implantation period of pregnancy in ewes, integrin subunits  $\alpha$  (v, 4, 5) and  $\beta$  (1, 3 and 5) are constitutively expressed on the apical surfaces of both conceptus trophoblast and endometrial LE (Johnson *et al.* 2001). These integrin subunits are detected at the apical surfaces of the LE and GE and on conceptus trophoblast; expression of these integrins is constitutive and not influenced by pregnancy or presence of the conceptus. In the sheep, receptivity to implantation does not appear to involve changes in either temporal or spatial patterns of integrin expression, but may depend on expression of other glycoproteins and ECM proteins, such as galectin-15, OPN and fibronectin, which are ligands for heterodimers of these integrins (Johnson *et al.* 2003a, Gray *et al.* 2004). In species such as pig, mouse and humans, interactions

between specific integrins and ECM proteins frame the putative window of implantation (Carson *et al.* 2000, Burghardt *et al.* 2002, Lessey 2002).

### Osteopontin (OPN)

OPN is a member of the small integrin-binding ligand, N-linked glycoprotein (SIBLING) family of genetically 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.* 2003a). OPN has also been linked to pregnancy (Johnson *et al.* 2003a). Microarray profiling identified OPN as the most highly upregulated ECM adhesion molecule in human endometrium that is receptive to implantation (Carson *et al.* 2002, Kao *et al.* 2002). Multiple integrin receptors for OPN are present on trophoblasts and LE of humans and domestic animals, some of which increase during the peri-implantation period (Lessey *et al.* 1994, Bowen *et al.* 1997, Johnson *et al.* 2001). Ovine and porcine trophoblast and LE cells show evidence of integrin receptor activation and cytoskeletal reorganization in response to OPN binding *in vitro* (Johnson *et al.* 2001, Garlow *et al.* 2002), and polymerized OPN has high tensile strength when simultaneously binding receptors on different cells during adhesion and matrix assembly (Goldsmith *et al.* 2002). Finally, disruption of the OPN gene in OPN-null and OPN heterozygote mice decreases reproductive success at midgestation, and OPN-null embryos are significantly smaller than wild-type counterparts at term (Weintraub *et al.* 2004).

OPN has been detected in epithelia and in secretions of many tissues, including the uterus (Johnson *et al.* 2003a). OPN binds to integrin heterodimers ( $\alpha\beta$ 1,  $\alpha\beta$ 3,  $\alpha\beta$ 5,  $\alpha\beta$ 6,  $\alpha\beta$ 8,  $\alpha$ 4 $\beta$ 1,  $\alpha$ 5 $\beta$ 1 and  $\alpha$ 8 $\beta$ 1) via its Arg-Gly-Asp (RGD) sequence, and to  $\alpha$ 4 $\beta$ 1 and  $\alpha$ 9 $\beta$ 1 by other sequences to promote cell adhesion, spreading and migration (Fig. 3). In sheep, OPN is also a component of histotroph secreted from endometrial GE into the uterine lumen during pregnancy. During the peri-implantation period of pregnancy in sheep, OPN mRNA is expressed only by the endometrial glands, is first detected in some glands of some ewes by day 13, and is present in all glands by day 19 (Johnson *et al.* 1999b). Progesterone induces expression of OPN in the endometrial glands, and this induction is associated with a loss of PR in the GE (Spencer *et al.* 1999b, Johnson *et al.* 2000). The 45 kDa form of OPN is present in greater amounts in uterine flushings from pregnant than cyclic ewes (Johnson *et al.* 1999a, 2001). The 45 kDa fragment of OPN has greater binding affinity for  $\alpha\beta$ 3 integrin than the native 70 kDa form (Senger *et al.* 1996). Evidence suggests that secreted OPN binds integrin receptors expressed on conceptus trophoblast and endometrial LE, where it can stimulate changes in proliferation, migration, survival, adhesion and remodeling of the conceptus as it elongates, apposes and

adheres to the LE. OPN is hypothesized to serve as a bifunctional bridging ligand that mediates the adhesion between LE and trophoblast essential for implantation and placentation (Johnson *et al.* 1999a, 2003a,b).

### Conclusions and future directions

During the past decade, knowledge of the mechanisms and factors regulating conceptus implantation in mammals has benefited through insights from the ewe and other domestic animals. However, much remains to be discovered about the interactions that regulate blastocyst implantation. Our knowledge of the cellular and molecular mechanisms of BNC differentiation is also very limited. As illustrated in Figs 2 and 3, a number of potential adhesion factors and cascades are proposed to regulate implantation in sheep. These adhesion systems probably function only in the correct spatiotemporal sequence (Aplin 1997). Results from studies of rodents firmly support the hypothesis that implantation involves a multiplicity of receptor–ligand interactions that are organized into a cascade. Therefore, the individual and integrative roles of adhesion factors will need to be mechanistically determined by *in vivo*, *ex vivo* and *in vitro* experiments. Although gene knockouts and transgenics are technically possible in domestic animals, these techniques are not feasible as research tools to understand the specific roles of factors proposed to regulate implantation. Therefore, other strategies must be employed to conduct hypothesis-based research to determine the specific roles of candidate genes in regulating implantation in domestic animals. Promising technologies include the use of adenoviruses, antisense oligodeoxynucleotides, morpholinos and small inhibitory RNAs that could be used *in vivo* and *ex vivo* to perform gain-of-function and loss-of-function studies of specific gene(s) in the endometrial epithelia and trophoblast. Future research in sheep and other domestic animals must incorporate these types of approaches in order to determine the mechanistic roles of specific factors hypothesized to mediate implantation. The sequencing of the genomes of domestic animals is expected to generate knowledge and reagents useful to understand the basis of enhanced fertility in specific breeds of domestic animals (Finnish Landrace sheep and Meishan pigs) as well as infertility (Holstein dairy cattle and UGKO ewes). Understanding the signals that regulate uterine receptivity and implantation can be used to diagnose and identify the cause(s) of recurrent pregnancy loss and improve pregnancy rates in domestic animals and humans.

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