

Implantation and Placentation in Ruminants



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Abstract In comparison to many other mammalian species, ruminant ungulates have a unique form of placentation. Ruminants initially display an epitheliochorial type of placentation; however, during the period of placental attachment, trophoblast giant binucleate cells (BNC) develop within the chorion to migrate and fuse with the uterine surface epithelium to form syncytial plaques. Binucleate cell migration and fusion continues throughout pregnancy but never appears to breach the basal lamina,

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beneath the uterine surface or luminal epithelium. Therefore, the semi-invasive type of placentation in ruminants is classified as synepitheliochorial. The endometrium of ruminant species also contains unique specialized aglandular structures termed “caruncles” in which the chorioallantois (cotyledons) interdigitates and forms highly vascularized fetal–maternal “placentomes.” This chapter will discuss the current knowledge of early conceptus development during the peri-attachment period, establishment of pregnancy, conceptus attachment, and placentation in ruminant ungulates. The features of placentomes, BNCs, fetomaternal hybrid cells, and multinucleated syncytial plaques of the cotyledonary placenta of ruminant species will be reviewed to highlight the unique form of placentation compared to the placentae of other artiodactyls.

Keywords Ruminant · Bovine · Ovine · Pregnancy · Conceptus · Uterus · Implantation · Placentation

1 Introduction

Attachment of the embryo or conceptus (embryo and extraembryonic membranes) to the maternal uterine endometrium followed subsequently by development of the placenta is the means by which eutherians provide the environment needed to support the prolonged period of in utero fetal development until parturition. It is notable that the early stages of embryo development between fertilization of the oocyte and blastocyst formation are conserved among mammalian viviparous species. It is only following hatching of the blastocyst from its zona pellucida coat that distinct deviations in the steps of mammalian implantation and placental development become apparent. Indeed, there is great variation in the area and depth of placental interactions with the maternal endometrium. Examples of such differences range from the noninvasive, epitheliochorial attachment in the pig and horse to the invasive, endotheliochorial, and hemochorial types of implantation in canines, rodents, and primates (Grosser 1909; Mossman 1937; Amoroso 1952; Wimsatt 1974). The acquisition of the epitheliochorial placenta in some larger species provides an interesting contrast relative to any advantages that an invasive placental form might offer (Mossman 1974; Carter and Enders 2013). Ruminants have a unique semi-invasive type of attachment in which formation of trophoblast giant binucleate cells (BNCs) migrate and fuse with the luminal epithelium (LE). The “invasiveness” of the BNC appears to be limited to fusion with the uterine epithelium to form syncytial plaques that do not penetrate the basal lamina present beneath the LE. The “synepitheliochorial” type of attachment is functionally intermediate between the noninvasive placentation in the pig and the invasive endotheliochorial placenta in canines (Chavatte-Palmer and Guillomot 2007). In addition to the unique form of conceptus attachment, the endometrium of the bicornuate uterus of ruminant

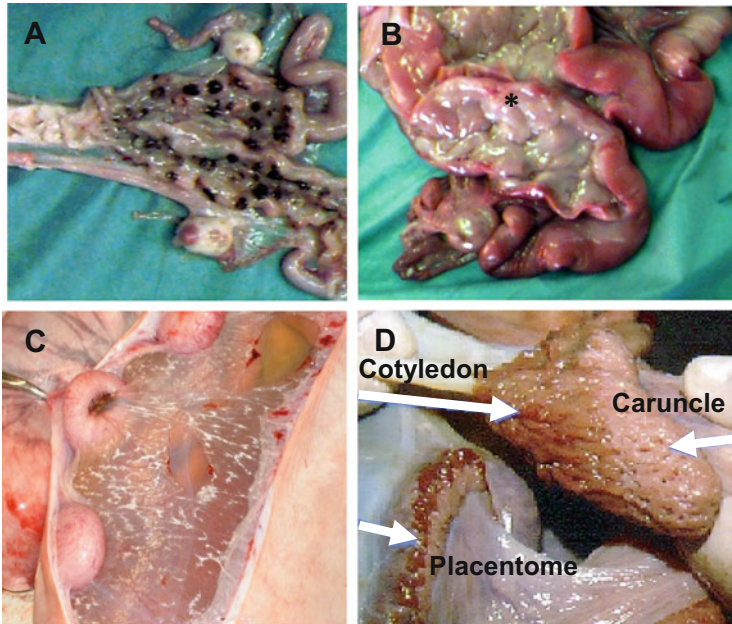


Fig. 1 Bicornuate uterus of the ewe (a) and heifer (b) opened to show the endometrial caruncles (asterisk) within the uterine lining. The presence of melanocytes in caruncles of blackface ewes results in the dark-pigmented color of the caruncles in panel a. (c) Sheep “concave” placentomes on day 120 of pregnancy. (d) Fetal cotyledons and maternal caruncles which form the “convex” placentome of the bovine placenta on day 240 of pregnancy

species is lined with specialized aglandular structures termed “caruncles” (Fig. 1a, b). Interdigitation of chorioallantois (cotyledons) and formation of crypts within the caruncles ultimately leads to the formation of highly vascularized fetal-maternal “placentomes” (Fig. 1c, d). The caruncles of ruminants develop in the endometrium after birth, and the total number varies between individual animals and species (Wiley et al. 1987; Hradecky et al. 1988). During pregnancy, the bovine, ovine, caprine, and *Antilopinae* (gazelles, blackbucks) placenta can have 75 to 125 placentomes, while *Cervidae* (deer) and other ruminant species may contain only 4–6 placentomes (Hradecky 1983). The number of cotyledons present in the giraffe can range from 123 to 191. The overall shape of the placentomes ranges from primarily flat (deer, antelope), convex (cattle) to concave (sheep, goat) (Mossman 1987). The camel is considered a ruminant. However, it is quite distinct evolutionarily from the *Ruminantia* and its placenta resembles the diffuse form of pigs and horses (Skidmore et al. 1996).

In this chapter, the current knowledge related to early embryonic development, implantation, and placentation in the cattle and sheep is discussed. The synepitheliochorial type of uterine attachment and the cotyledonary placenta are unique adaptations in nearly all ruminant ungulate species. The ruminant placenta represents a form of placental interaction with the maternal uterus that is distinct in comparison to other mammals.

2 Early Conceptus Development

After fertilization, the embryo undergoes cell divisions to reach the morula stage, which occurs around day 5 of gestation in cattle (Brackett et al. 1980). Initially both outer and inner cells of the morula do not have well-developed intercellular junctions. Instead, they are in contact only through cellular focal and gap junctions. Subsequently, the outer cells establish tight junctions, with a concomitant increase in the extent of cell-cell contacts. Meanwhile the inner cells retain the focal and gap junction. This process represents compaction of the morula. A blastocoelic cavity is formed through fluid movement into the intercellular spaces of the compact morula. The osmotic gradient driving blastocoel formation is established by Na^+/K^+ -ATPase (a “sodium pump”) present within the basement membrane of the outer (trophectoderm) cells. Formation of outer polarized and inner nonpolarized cells of the blastocyst triggers differential cellular activity of the Ras and Hippo signaling pathways to induce lineage segregation (see Telugu, Chapter “Development of Pre-Implantation Mammalian Blastocyst”).

In mice, cellular expression of NANOG and SOX2 prevents induction of a trophectoderm lineage and leads to the formation of the inner cell mass (ICM), while activation of the caudal-related homeobox gene *Cdx2* by Tead4/Yap stimulates trophectoderm lineage (Cockburn and Rossant 2010; see Telugu, Chapter “Development of Pre-Implantation Mammalian Blastocyst”). However, care must be taken in extending the mouse model to other mammals as there is variability among species (Spencer et al. 2006; Berg et al. 2011; Negrón-Pérez et al. 2018; Llobat 2020). In mice, expression of the octamer-binding transcription factor Pou5f1, also known as Oct4, is restricted to the ICM, which is not the case during early bovine blastocyst development (Berg et al. 2011). Pou5f1 is co-expressed with *Cdx2* in the trophectoderm, even after blastocyst development. The possible co-expression of Pou5f1 in the trophectoderm may be related to the extended period of peri-attachment and growth that is observed in the conceptuses of ungulates.

The bovine blastocyst hatches from its zona pellucida (Fig. 2a) on day 9–10 of gestation by weakening the zona through enzymes produced by both the blastocyst and endometrium as well as expanding and contracting to create a fracture in the zona glycoprotein coating. After hatching, the conceptus takes on a spherical and



Fig. 2 Photomicrographs of (a) hatching day-10 bovine blastocyst, (b) ovoid 1 mm day-14 bovine conceptus, and (c) histological section of ICM and trophoblast of a day-15 bovine conceptus

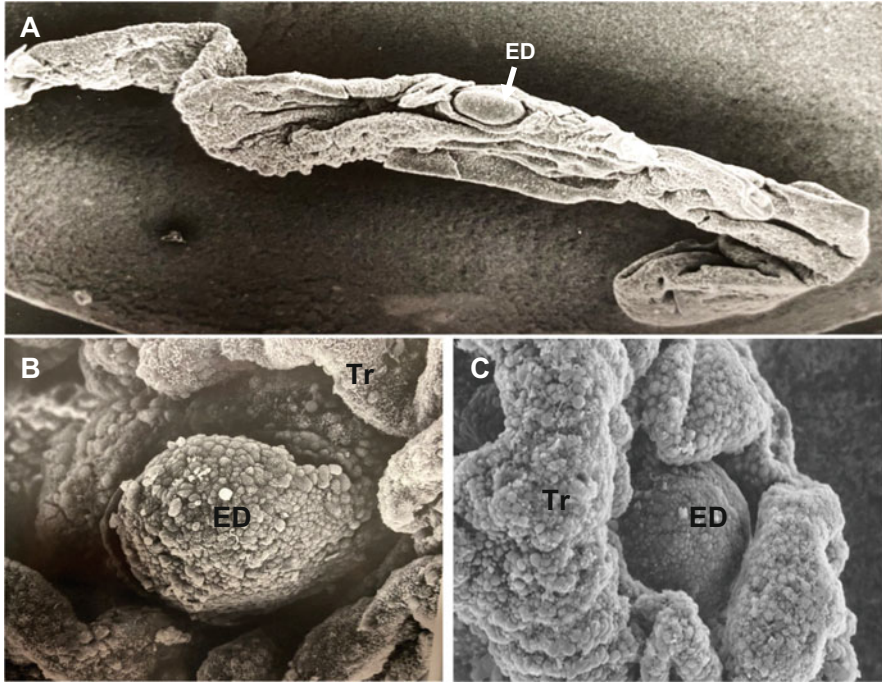


Fig. 3 Scanning electron microscopy photograph of (a) elongated 15 mm day-15 bovine conceptus, (b) embryonic disc of a day-15 bovine conceptus, and (c) embryonic disc descending into the trophoblast folds to form the amniotic sac of a bovine conceptus. *ED* embryonic disc, *Tr* trophoblast. (SEM photomicrographs courtesy Michael F. Smith University Missouri)

then an ovoid morphology (Fig. 2b). Differentiation of germ layers (Fig. 2c) begins in the ICM soon afterwards (Perry 1981). The establishment of the germ layers during gastrulation is the prelude to formation of the chorion, amnion, yolk sac, and allantoic membranes typical of the chorioallantoic type of placenta in ungulates (Perry 1981). During this period, the spherical conceptus slowly grows from 1 mm to 8 mm between day 12 and 14 of gestation (Betteridge et al. 1980). By day 15, endoderm completely lines the basal membrane of the trophectoderm of the ovoid conceptus (Fig. 2b). Mesoderm from the ICM migrates between endoderm and trophectoderm layers to form a trilaminar trophoblast (Fig. 2c). The mesoderm separates to associate with the endoderm (forming the vascular splanchnopleure yolk sac) and the outer trophectoderm (to form the avascular somatopleure) of the developing chorion. Thus, the origin of the extraembryonic membranes begins to form in ruminants prior to firm attachment to the uterine luminal surface epithelium.

The trophectoderm covering the ICM (Raubert's layer) is lost during the period of early conceptus expansion (Betteridge and Flechon 1988). The growing embryonic disc appears as a rounded sphere on the surface of the day 16 elongated conceptus (Fig. 3a). On days 16 to 18, the embryonic disc appears to descend (Fig. 3b) into the conceptus as the developing trophoblast layer (somatopleure) begins to fold around

and engulf it (Fig. 3c). Closure of the trophoblast fold over the embryonic disc establishes the amnion and forms the cavity for fluid accumulation within the amniotic sac. Development of the vascular yolk sac, which is in close apposition to the mesenchyme of the chorion (Fig. 2c), serves an essential function to transfer nutrients from the chorion surface (choriovitelline) to the embryos during early development.

3 Conceptus Elongation, Apposition, and Establishment of Pregnancy

The changes in early embryo development, attachment, and placental expansion in cattle and sheep have been documented and reviewed (Leiser 1975; King et al. 1982; Betteridge and Flechon 1988; Assis Neto et al. 2010). Growth and elongation of the bovine conceptus within the uterine horn is accelerated from day 15 to 18 of gestation. During this period, the 2 mm diameter filamentous conceptus reaches lengths over 200 mm (Fig. 4) to extend into the contralateral horn by day 18 to 19 of gestation (Greenstein et al. 1958; Betteridge et al. 1980). Conceptus elongation in sheep is initiated on day 12, and the conceptus membranes extend into the contralateral horn by day 17 (Rowson and Moore 1966). Unlike the rapid morphological



Fig. 4 Photomicrograph of an elongated 220 mm day-19 bovine conceptus. Insert: early embryo with yolk sac and outgrowth of allantois from the hindgut of a bovine conceptus. (Photo courtesy of Sofia Ortega, University of Missouri)

elongation of pig conceptuses (Geisert et al. 2015), the increase in the length of cattle and sheep conceptuses occurs through cell proliferation, migration, and morphological change in shape over 2–3 days (Wang et al. 2009; Bazer et al. 2010).

The bovine conceptus is rather weakly attached to the uterine surface epithelium during the period of elongation until approximately day 23 of gestation. During this timeframe, connections with the endometrium begin near the embryonic disk and spread from there (King et al. 1980). For the most part, apposition and attachment is similar among ruminants. A few differences exist, however. In sheep, the apposition steps begin with a decrease in trophoblast microvilli between days 13 and 15 (Guillomot et al. 1981, 1982, 1993). The transient loss of microvilli may promote apposition of trophoblasts with the LE. Besides cellular contacts between trophoblast and endometrial surface epithelium, the portion of the trophoblast overlying the uterine gland openings extend papillae into the superficial mouths of the glands between days 15 and 17 of pregnancy (Fig. 5a). Development of the trophoblastic papillae is proposed to anchor the peri-attachment conceptus to the uterine surface (Fig. 5b) during elongation throughout the uterine horns in the cow and sheep (Guillomot et al. 1981; Guillomot and Guay 1982). The trophoblastic papillae may also serve to absorb gland secretions until the formation of the areolae in the developing chorion.

The increase in the rate of conceptus elongation during this peri-attachment period is regulated by changes in the transcriptome and secretions of the endometrium which are programmed by progesterone following ovulation (Forde et al. 2011; Spencer and Hansen 2015; Spencer et al. 2016). In cattle, changes in endometrial secretions are sensitive to the rise of progesterone following ovulation and timing of the downregulation of progesterone receptor (PGR) in the endometrial luminal (LE) and glandular (GE) epithelium (see Lonergan et al. 2016). An earlier increase in circulating progesterone following ovulation is associated with advancing conceptus development and elongation. Exogenous treatment of bred cows with progesterone immediately after ovulation advances conceptus elongation on day 15 of gestation (Garrett et al. 1988) which is similar to the timing of elongation in sheep (Satterfield et al. 2006). Interactions between the conceptus and the endometrium during the peri-implantation period of conceptus elongation are sensitive to endometrial changes because dysregulation of these changes leads to downstream effects on conceptus survival and later embryonic loss in subfertile cows (Moraes et al. 2018).

During the peri-attachment period, sheep and bovine endometrium and conceptuses secrete prostaglandins as expression of prostaglandin-endoperoxide synthase 2 (PTGS2) is enhanced (Charpigny et al. 1997a, b). Prostaglandins modify the endometrial transcriptome and influence endometrial secretions to stimulate conceptus growth and survival (Simmons et al. 2009). Brooks et al. (2015) demonstrated that peroxisome proliferator activator receptor gamma (PPARG), a nuclear factor that binds to prostaglandins, is an essential regulator of conceptus proliferation and survival. In addition, conceptus development and elongation in sheep is severely comprised when endometrial and conceptus prostaglandin production is inhibited

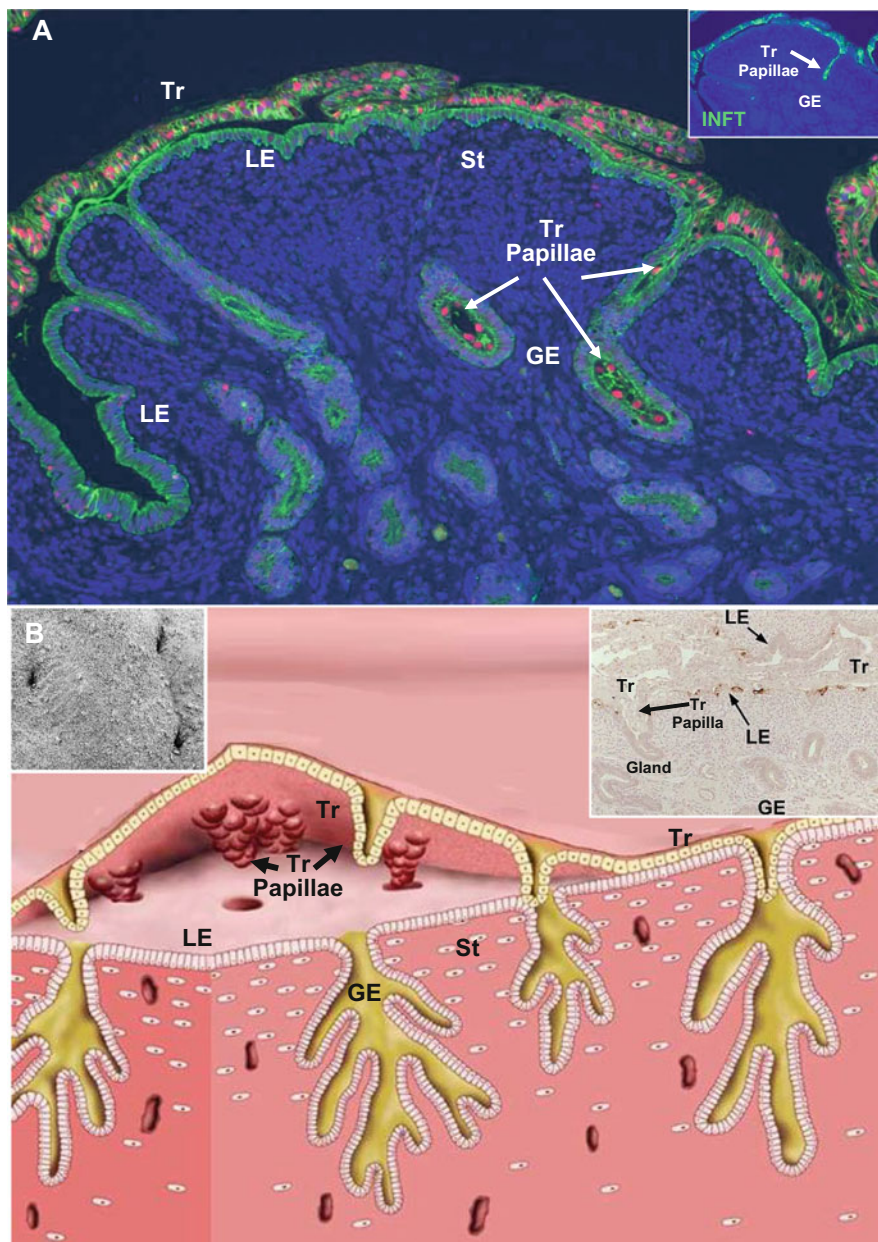


Fig. 5 During the peri-attachment period of conceptus elongation and expansion within the uterine lumen, apposition of the conceptus trophoblast (Tr) to the uterine luminal epithelium (LE) is aided by the development of trophoblastic papillae into the endometrial glands of sheep and cattle. (a) Immunofluorescence staining E-cadherin (green) and PCNA (red) of a serial section from a day-18 sheep pregnant uterus containing an expanded conceptus (10 \times). The intensive PCNA staining of the growing conceptus shows the close apposition of the trophoblast to the LE and the extension of a trophoblastic papilla into the mouth of an endometrial gland. The insert image displays the trophoblast expression of IFNT (green fluorescence) to illustrate the depth of the papilla penetration

with a PTGS2-specific inhibitor during the peri-attachment period (Dorniak et al. 2011). The progesterone-driven uterine transcriptome, proteome, lipidome, and metabolome play a critical role in conceptus growth and elongation essential to downstream placental development and survival.

Elongation of the bovine and ovine conceptus through the ipsilateral uterine horn on days 15 to 18 of pregnancy is critical to not only provide the essential uterine surface area for adequate nutrient and oxygen exchange but to inhibit luteolysis and extend progesterone production beyond the length of the estrous cycle. Maternal recognition of pregnancy in ruminants requires not only rapid growth, elongation, and apposition across the uterine lumen surface (Fig. 6a), but conceptus expression and secretion of the type 1 interferon tau (IFNT) (Fig. 6d) to prevent regression of the CL (see Spencer and Hansen 2015).

During the estrous cycle and early pregnancy, progesterone receptor (PGR) is expressed in the endometrial stroma cells, but no PGR expression is observed in the LE or GE (Fig. 6c). Loss of epithelial PGR in cyclic females allows upregulation of estrogen receptor, and follicular estrogen production stimulates an increase in endometrial oxytocin receptor expression. The release of prostaglandin F₂α (PGF₂α) initiates release of oxytocin from the CL which forms a positive feedback loop for increased pulsatile release of endometrial PGF₂α leading to luteolysis (Spencer and Hansen 2015). Briefly, during establishment of pregnancy (days 12–15 in sheep and 15–21 in cattle), conceptus growth (Fig. 5b) drives expansion and apposition to the endometrial LE (Fig. 6a) and positions the trophoblast so that secreted IFNT (Fig. 6a, d) can bind to type 1 IFN receptors present on the endometrial LE inhibiting the transcription of estrogen receptor alpha (ESR1) (Fig. 6e). Inhibition of ESR1 prevents establishment of the positive feedback loop necessary for the pulsatile release of PGF₂α from the endometrium preventing luteolysis (Spencer and Hansen 2015). Conceptus secretion of IFNT can also help regulate the endometrial transcriptome and possibly regulate immune function to prevent rejection of the semi-allograft fetus (Bazer et al. 2015, 2020).



Fig. 5 (continued) down into the endometrial gland (10×). **(b)** Cartoon illustration of apposition of the ruminant conceptus before attachment of the trophoblast to the uterine LE on day 20 of gestation. The development of the cone-shaped trophoblastic papilla into the mouth of the uterine glands is proposed to anchor the conceptus during elongation and expansion and provides absorption of glandular secretions. Left insert: scanning ECM of the openings of uterine glands on the endometrial surface of a heifer. Right insert: immunohistochemical section of a papilla extending into uterine gland during early pregnancy. *LE* luminal epithelium, *GE* glandular epithelium, *St* stroma

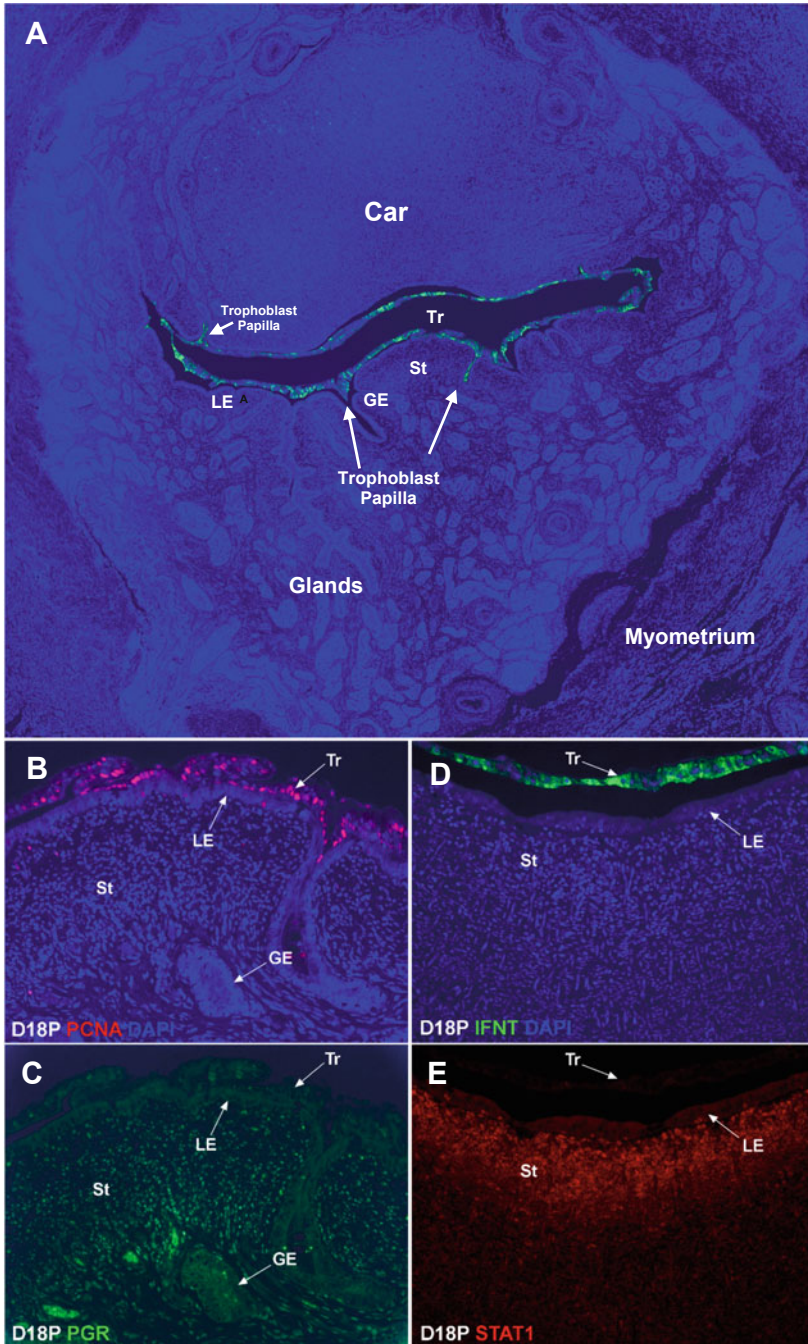


Fig. 6 Top panel: During early pregnancy in sheep, the mononuclear cells of the placental trophoblast synthesize and secrete interferon tau (IFNT), the signal for maternal recognition of pregnancy. (a) Paraffin-embedded thin section of an elongated, attaching conceptus within the uterine lumen of a day-18 pregnant sheep that has been immunofluorescence stained for IFNT

4 Conceptus Attachment and Placental Formation

The initial attachment between bovine trophoblast and uterine surface epithelium is evident on days 18 to 21 (King et al. 1980). With continued development and differentiation of the chorion, firm attachment occurs through interdigitation of the microvilli with the epithelium of the caruncular and intercaruncular areas of the uterine lining. At this point in development, the tips of the chorion extend throughout both uterine horns (King et al. 1982).

During implantation in the mouse, attachment to the uterine luminal epithelial surface involves progesterone induced loss of the antiadhesive MUC1 which serves as a physical interference at the LE for attachment (Surveyor et al. 1995). Expression of MUC1 is not downregulated by progesterone in the LE of sheep during the midluteal stage of the estrous cycle but is reduced at the conceptus–endometrial surface interface in association with conceptus IFNT production (Raheem et al. 2016). Loss of MUC1 allows cell to cell interactions with adhesion factors such as integrins, which are needed to facilitate attachment (Kim et al. 2010; Johnson et al. 2001, 2014). Multiple molecules such as GlyCAM (Spencer et al. 1999a, b), cadherins, galectin-15 (LGALS15), and secreted phosphoprotein 1 (SPP1) are proposed to form complexes with integrins and glycoconjugates expressed on the aligned apical surfaces of the trophoblast and LE (Johnson et al. 2018; Spencer et al. 2004a, b).

The apical surfaces of the interface between the endometrial LE and placenta can express integrin receptors $\alpha\beta3$, $\alpha\beta1$, $\alpha v\beta5$, $\alpha4\beta1$, and $\alpha5\beta1$ during conceptus attachment and throughout gestation in sheep (Johnson et al. 2001; Burghardt et al. 2009). Endometrial expression of SPP1, also known as osteopontin, serves as a crosslinker to bind the cellular integrin receptors together (Fig. 7a). Seo et al. (2020) found that $\alpha\beta3$, $\alpha\beta5$, and $\alpha4\beta1$ (Fig. 7b, c) serve as the integrin receptors for conceptus attachment during the peri-implantation period of pregnancy in sheep. Only $\beta3$ is exclusively limited to the apical surface of endometrial LE during the period of peri-attachment suggesting a specific role of $\beta3$ during attachment of conceptus. Interestingly, $\beta5$ expression is present in the LE and extends down into the openings of the GE. It is possible that $\beta5$ integrin associates with trophectoderm



Fig. 6 (continued) (green), and stained with DAPI (blue) for histological reference. The conceptus is attached to the LE of a glandular intercaruncular area of the endometrium with the papillae extending in the mouth of uterine glands and luminal epithelium (LE) of the nonglandular caruncle (Car). Higher magnification of the same implantation site illustrating (b) multiple proliferating conceptus trophoctoderm cells [proliferating cell nuclear antigen (PCNA)], but few proliferating endometrial LE cells, (c) progesterone receptors (PGRs) are expressed by the endometrial stromal cells, but no PGR expression is observed in the LE or glandular epithelium (GE), (d) IFNT expression by trophoctoderm cells, and (e) The classical interferon stimulated gene [signal transducer and stimulator of transcription 1 (STAT1)] expression by cells in the stroma (St), but no expression of STAT1 by LE cells. (Immunofluorescence staining was performed by Dr. Heewon Seo, Department of Veterinary Integrative Biosciences, Texas A&M University)

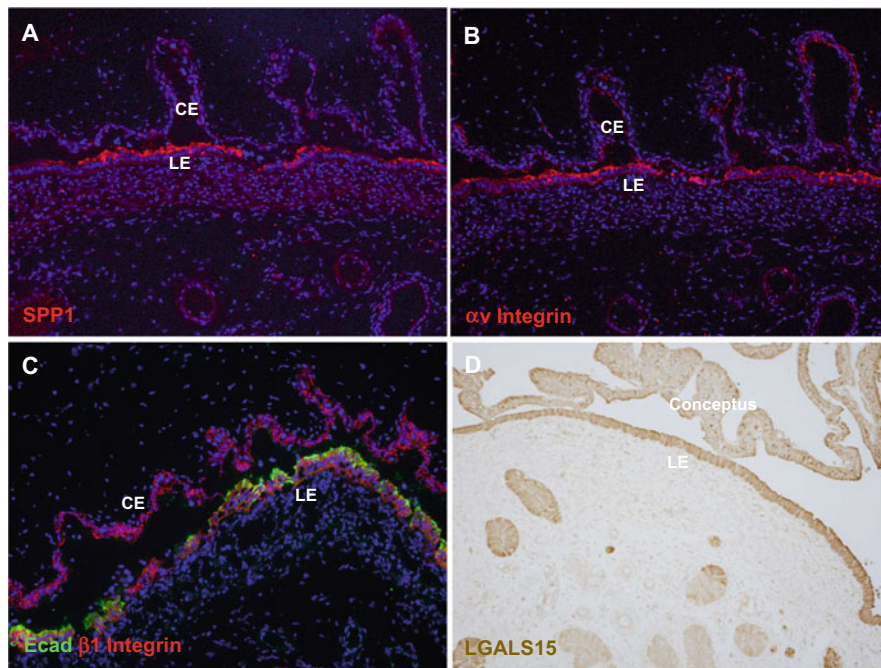


Fig. 7 Immunofluorescence microscopy illustrating the distribution of (a) SPPI, (b) αv integrin, and (c) $\beta 1$ integrin (red) and E-cad (green, immunostained endometrial luminal epithelial (LE) cells) at the endometrial–placental interface on day 60 of pregnancy in the sheep (10 \times). (d) Immunohistochemical localization of galactin-15 (LGALS15) in the sheep endometrium and conceptus on day 16 of pregnancy. CE chorionic epithelium. (The images in this figure adapted from Seo et al. 2020 and Gray et al. 2004)

papillae that anchor the conceptus during elongation and help provide absorption of glandular secretions during early development.

Galectin-15 has a carbohydrate recognition domain that binds to beta-galactosides (Cooper 2002) and contains sequences that can interact with integrins (Spencer et al. 2004a, b). In sheep, the conceptus and placenta do not produce LGALS15 which is specifically expressed by endometrial LE and superficial GE (Gray et al. 2004). The endometrial LGALS15 is secreted into the uterine lumen throughout pregnancy where it is absorbed by trophoblasts and utilized in cell adhesion or internalized to regulate cell growth, differentiation, and apoptosis (Fig. 7d). LGALS15 is absent in placentomes when the caruncular surface is devoid of LE during syncytial formation (Gray et al. 2004). However, LGALS15 is present in the intercaruncular area of the endometrium when the LE are reestablished.

During the peri-attachment period, the yolk sac develops from days 18 to 23 of gestation (Wrobel and Suess 1998) to provide the initial vascular system to transfer nutrients to the developing embryo (Assis Neto et al. 2010). The yolk sac provides the vascular transport of blood and nutrients to the developing embryo and it is also the source of primordial germ cells (Kritzenberger and Wrobel 2004). It also

contributes specific proteins and metabolites to the developing embryo (Mançanares et al. 2013; Galdos-Riveros et al. 2015). Attachment of the chorion to the uterine surface is closely followed by a projection of extraembryonic splanchnopleure from the embryo hindgut (Fig. 4a) which initiates allantois formation on day 21 of gestation (Maddox-Hyttel et al. 2003). The fluid-filled vascular allantois rapidly expands throughout the extraembryonic coelom (Fig. 8), while the yolk sac concurrently regresses becoming vestigial by day 55 although it is still present at term (Assis Neto et al. 2010). The chorion and allantois mesenchyme fuse to form the vascular chorioallantoic placenta from day 26 to 60 of gestation which will be present throughout the remainder of gestation (Fig. 8). The accumulation of fluid in the allantois serves to expand the chorioallantoic membranes forcing apposition into the intercaruncular and caruncular surface of the maternal endometrium. Fluid within the amnion is a different composition compared to the allantois (Fig. 8) where it functions to provide a protective environment for embryo/fetal development. Ligation of the urachus from the fetal kidney within the umbilical cord results in the loss of allantoic fluid during late pregnancy in sheep. The passage of fluid from the embryonic kidney through the urachus in the umbilical cord has led to the misconception that the allantois functions as a reservoir for fetal waste. However, the kidney only redistributes fluid as the maternal vasculature provides the water for the fetus and placenta (Bazer 1989). The allantois contains ions, carbohydrates (glucose, fructose), and amino acids as well as selective proteins and hormones (Bazer et al. 1981; Bazer and Johnson 2018). Therefore, the allantois provides a reserve of nutrients essential for the rapid demands of fetal growth throughout gestation in ungulates. Fluid in the amnion and allantois may also be transported directly through aquaporin water channels expressed in the chorioallantois (Zhu et al. 2015). Expression of transporters for ions, glucose, and amino acids in the chorioallantoic membranes contributes to maternal transfer of electrolytes, glucose, amino acids, and nutrients (Bazer et al. 2012).

As the placental membranes continue to grow, fluid volumes of the amnion and allantois increase and change throughout gestation in cattle and sheep. In cattle, allantoic fluid volume increases from 50 mL on day 30 to 600 mL by day 100 while amnion fluid volume reaches 450 mL (Eley et al. 1978). In cattle, fluid volume is over 5 L within the allantois and 2 L in the amnion during late gestation (day 260) with total volumes reaching 9 L near term (Rasby et al. 1990). In sheep, there is a similar increase in allantoic fluid volume from day 25 to 40 (21 to 91 mL) as the allantois expands within the chorion, but the fluid volume declines by day 70 (32 mL) (Gresham et al. 1972). A second increase in allantoic fluid volume continues to near term on day 140 (438 mL). These dynamic changes in allantoic fluid volume likely impact development and growth of the placenta and fetus.

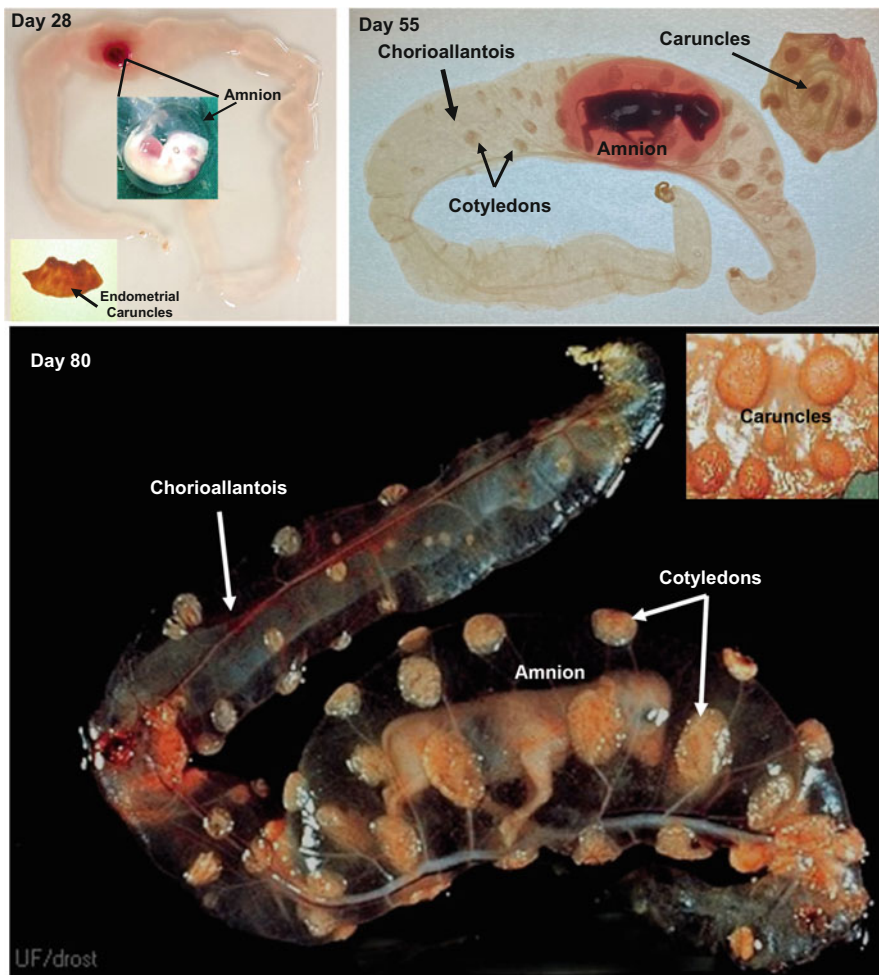


Fig. 8 Placental development during pregnancy in cattle. The fluid-filled allantois expands throughout the chorion which is attached to the entire uterine lumen by day 28 (Insert demonstrates the embryo enclosed in amnion). Although there is some growth of the maternal caruncles, chorionic cotyledons are not visually obvious at this time. The developing embryo is contained within the fluid-filled amnion (the red color which helps highlight the amnion is caused by hemolysis during handling). Cotyledons, which are largest near the amnion, are present on the chorion by day 55. Growth of the chorionic cotyledons occurs from the amnion toward the tips of the chorioallantois. The cotyledons are well developed in the contralateral uterine horn at this time. By day 80, the growing cotyledons and uterine caruncles forming the placentomes are developed throughout both uterine horns. (Photo of day 28 bovine embryo (insert) and day 80 bovine placenta were provided by The Drost Project The Visual Guides of Animal Reproduction (visgar, vetmed.ufl.edu))

5 Placentome Development

The expansion of the chorioallantois brings the chorion into close contact with glandular intercaruncular regions and aglandular caruncular (consisting of connective tissue formations covered by columnar epithelium) areas of the ruminant endometrium (Fig. 9a). The firm attachment of the chorion involves interdigitation at the microvillar junctions between the LE and chorionic epithelium (Fig. 9b). The chorion overlaying the openings to the uterine glands differentiate into areolae (Fig. 9c), which are involved with uptake of secretions into the capillaries of the chorioallantois (Johnson et al. 2018). In cattle, the chorion does not significantly infiltrate into the endometrial caruncles until after day 30 of gestation (King et al. 1979), and the cotyledonary attachments are not visible on the chorioallantois at this time (Fig. 8). From day 33 to 50 of gestation, the initial attachments between trophoblasts and caruncles are reinforced, and the surface area increases as the chorionic villi (cotyledons) penetrate into septa within the caruncular endometrium. The villi increase in length and undergo extensive branching (Figs. 8 and 9d–f). Simultaneously, development of an extensive blood supply in the villi produces mature “placentomes,” which serve as the main site of exchange for easily diffusible nutrients, waste disposal, and for meeting the oxygen demands of the developing fetus (King 1993; Leiser et al. 1998). The size of the placental cotyledons is largest near the amnion where cotyledon formation is first initiated and then spreads towards the tips of the chorion (Fig. 8). Since cotyledons represent the most visually apparent aspect of the mature ruminant placenta, it is also described as a “cotyledonary placenta.” The only animals in *Ruminantia* that do not have a cotyledonary placenta are in the *Tragulidae* family (e.g., the mouse deer); however, these animals do have giant binucleate trophoblast cells or BNC (described below) (Kimura et al. 2004). Potentially, the tragulid placenta represents an intermediate form between the epitheliochorial and the cotyledonary placentas of swine and most ruminants, respectively.

6 Trophoblast BNC Differentiation and Syncytial Formation

Grosser initially described the ruminant placenta as “syndesmochorial” (Grosser 1909, 1927). He had concluded that the uterine epithelium was lost during ruminant placental establishment and that there was direct apposition of trophoblast with the maternal connective tissue. Subsequently, Wooding and others concluded that the uterine epithelium was altered, but appeared to have remained, albeit sometimes in altered form (Wooding 1982a, b, 1984). Instead of an erosion of uterine epithelium, these histological scientists suggested that there was a fetomaternal syncytial cell layer. Consequently, the term “syndesmochorial placenta” was revised to “synepitheliochorial placentation”; where “syn” stands for the syncytium and

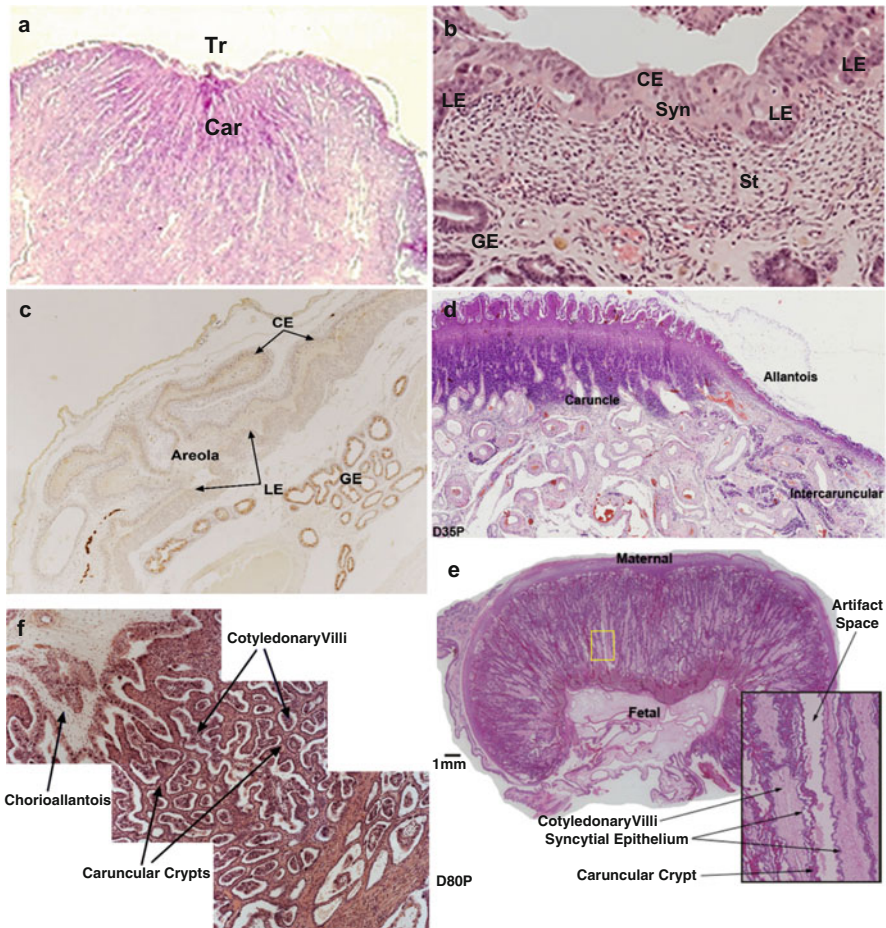


Fig. 9 Attachment and development of the chorioallantois to the intercaruncular and caruncular regions of the uterus. (a) Attachment of the trophoblast to the aglandular caruncular region of the uterus on day 21 of pregnancy in cattle (Reprinted/adapted with permission from Journal of Reproduction and Fertility, King et al. 1980), (b) bovine chorionic epithelium attachment to the uterine intercaruncular LE and syncytial plaque formation on day 20 of gestation, (c) day 60 chorionic areola of a sheep showing absorption of SPP1 secreted by the uterine glands (see Frank et al. 2020 for a detailed description of this pattern of expression), (d) day 35, (e) 80 placentome development in sheep, and (f) Penetration of the cotyledonary villi into the caruncular crypts of a bovine placentome. *Tr* trophoblast, *LE* luminal epithelium, *GE* glandular epithelium, *St* stroma, *CE* chorionic epithelium, *Syn* syncytial plaque

“epitheliochorial” signifies the persistence of recognizable fetal and maternal epithelia at the fetal–maternal interface (Wooding 1992).

During the peri-attachment period of conceptus development, a number of mono-nuclear cells within the trophoctoderm begin to differentiate into BNC (Fig. 10a) (Spencer and Hansen 2015). The BNCs appear around day 16 pc in sheep (Boshier

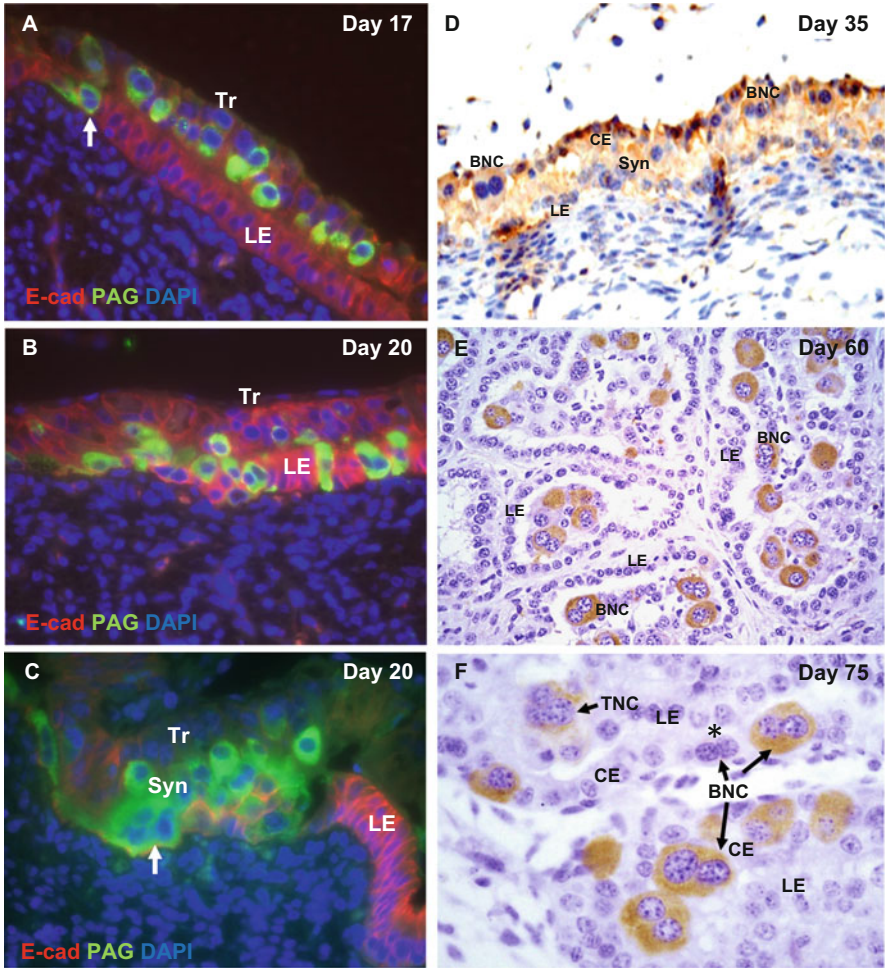


Fig. 10 Interactions between trophoblast giant “binucleated” cells (BNC) and uterine LE cells at the interface between fetal trophoblasts and maternal endometrium. Panels **a**, **b**, and **c** are interface images from sheep that were harvested during early pregnancy (after attachment) which were double immunofluorescence stained for PAG (green) and E-cadherin (red) at attachment sites between day 17 and day 20 of pregnancy. Panels **d**, **e**, and **f** are interface images from cattle that were harvested at various times during the first trimester of pregnancy which were immunostained for PAG with eosin counterstaining to reveal the cells present at the interface. (**a**) Within the trophoblast layer, multiple PAG-stained BNCs are present on day 17 of pregnancy. A BNC has migrated through the microvillar junction between the trophoblast and LE into the uterine LE cell layer (white arrow). (**b**) On day 20, there is extensive migration of BNCs into the uterine LE. (**c**) Uterine attachment site on day 20 where the uterine LE cells are being absorbed and replaced by migrating BNC forming a syncytial plaque. (**d**) Immunostaining of PAG at the bovine caruncular interface of the chorion and LE on day 35 of pregnancy. At this stage, cotyledons are just beginning to project into caruncles to begin placentome formation. A syncytium is present at the interface but syncytia are rarely observed after approximately day 40 of gestation in cattle. However, BNC fusion resulting in the formation of short-lived trinucleated cells continues throughout the remainder of the pregnancy. (**e**) Immunostaining of BNC PAG within a day-60 placentome. The section illustrates the cotyledonary villi containing BNC that have projected into the crypts of the uterine caruncles.

1969), day 18 pc in goat (Wango et al. 1990), and day 19 to 20 pc in cattle (Greenstein et al. 1958). The BNC arise from the mononuclear trophoblast cells through karyokinesis without cytokinesis (endoreduplication) or from mitotic polyploidy (Wooding and Flint 1994; Wooding et al. 1997; Spencer et al. 2010).

After formation of a vascularized chorioallantois, the BNCs represent approximately 15–20% of the chorionic epithelium, and these numbers remain relatively constant throughout gestation (Wooding et al. 1986; Wooding 1992; Wooding and Flint 1994). The BNC is a terminally differentiated cell, but it does undergo additional maturation. Along with two nuclei, it has an extensive rough endoplasmic reticulum and large golgi bodies that give rise to large numbers of cytoplasmic granules that make up almost 50% of the cell volume. When fully matured, the BNC migrates through the microvillar junctions between the trophoblast and luminal epithelia (Fig. 10a, b and f). They fuse with an uterine epithelial cell (Fig. 10b, f) to create a fetomaternal trinucleate cell (TNC) (Wooding 1987) (Fig. 10c, f). After the fusion, the secretory granules of the BNC transit towards the maternal side of the nascent TNC (Wooding 1987) to be expelled toward the maternal stroma. Following fusion, the TNC reforms part of the apposition with the adjacent trophoblast cells, which maintains an intact barrier between the two interfacing epithelia (Wooding 1987).

After the formation of trinucleate cells, binucleate cell migration and fusion can continue, which leads to the establishment of syncytial plaques (Fig. 10c, d) (Wooding 1984). The extent of syncytium formation differs between species. In sheep, the syncytial plaques are quite extensive; these contain up to 20–24 nuclei and they persist throughout pregnancy (Morgan and Wooding 1983). In cattle, syncytial formation is actually limited (Fig. 10d). No extensive syncytium is present beyond approximately day 40 of gestation (King et al. 1979; Wathes and Wooding 1980; King et al. 1982). After that time, only short-lived TNCs are formed (Fig. 10e, f) and these are soon replaced by epithelial cells (Wooding and Wathes 1980). The situation differs somewhat in small ruminants, such as sheep and goats. In the intercotyledonary regions, syncytial plaques are found initially, but they are soon replaced by uterine epithelium (King et al. 1981; King and Atkinson 1987; Wooding and Flint 1994). Afterwards, migrations and fusion of BNCs with uterine epithelia result in the formation of TNCs, but not a syncytium. In the placental regions of sheep, the syncytia persist at the interface throughout the pregnancy (Fig. 10c). The origins of the syncytia continue to be studied, and recent work suggests that further

Fig. 10 (continued) Note the LE of the crypts is intact and there are no syncytial plaques as occurs throughout pregnancy in sheep. (f) Immunostaining of BNC PAG within a day-75 placentome. Note a newly formed BNC (*) that has initiated PAG synthesis. A trinucleated cell arising from recent migration of a BNC that has integrated within the LE of the caruncular crypt is indicated by black arrow. *Tr* mononuclear trophoblast cells, *BNC* binucleated trophoblast giant cell, *TNC* trinucleated cell, *LE* luminal epithelium, *CE* cotyledonary epithelium (trophoblast), *Syn* syncytium, *E-cad* E-cadherin (red fluorescence, stains mononuclear trophoblasts, and LE), *PAG* pregnancy-associated glycoproteins

refinements of our understanding of ruminant placentation may be warranted. There is some evidence that uterine epithelia in the caruncles may not be part of the syncytial plaques at the interface of sheep (Seo et al. 2019). Of note, colocalization studies provided some evidence that the LE are eliminated and the syncytium itself is comprised of multinucleated syncytiotrophoblast entirely of placental origin (Seo et al. 2019).

As was mentioned previously, the giant trophoblast BNCs represent a cell type that contributes to the unique aspects of the placental form of ruminants. Numerous studies have started to reveal some of the mechanisms underlying the origins and functions of the ruminant-specific BNCs. For example, endometrial and conceptus expression of endogenous retroviruses (ERVs) are proposed to serve a role in placenta morphogenesis (Black et al. 2010) and the migration and fusion of BNC to the surface epithelium in the caruncular and intercaruncular areas of the uterus (Dunlap et al. 2006a; Spencer et al. 2010). Syncytins, which are expressed by envelope genes of retroviruses and have fusogenic activity, may have a role in placentation. *Syncytin-Rum1*, for which expression is specific to BNC, is associated with the synepitheliochorial placenta of ruminants and is not detected in other *Cetartiodactyla* or primates and rodents (Cornelis et al. 2013; Mi et al. 2000). Endogenous Jaagsiekte sheep retrovirus (enJSRVs) is expressed by both uterine luminal and glandular epithelia (Palmarini et al. 2001; Spencer et al. 2010) as well as in the conceptus trophoblast (Dunlap et al. 2005). enJSRVs are abundantly expressed in the trophoblast giant BNC and the syncytial plaques that arise from them. The receptor for both JSRV and enJSRVs Env, hyaluronoglucosaminidase 2 (HYAL2), is detectable in the BNC and the multinucleated syncytial plaques (Dunlap et al. 2005). Spencer et al. (2010) proposed that the formation of trinucleated fetomaternal hybrid cells arose when co-expressed enJSRVs env and HYAL2 initially fuse with endometrial luminal epithelial (LE) cells that are expressing enJSRVs env. It is worth noting that loss of enJSRVs env expression compromises conceptus growth and implantation in sheep (Dunlap et al. 2006b).

In addition to their contribution to the syncytia and formation of placentomes, it is important to note that BNCs produce steroid (progesterone), prostaglandin (PGI₂, PGE₂), and protein hormones [placental lactogens or chorionic somatomammotropin hormone one (CSH1)], as well as proteins without a clearly understood function (e.g., pregnancy-associated glycoproteins; PAGs) (Duello et al. 1986; Green et al. 2000; Reimers et al. 1985; Wallace et al. 2015; Wooding et al. 2005; Xie et al. 1991) (Fig. 10d–f). The latter are packaged in the BNC secretory granules. After fusion, the granules are released toward the uterine connective tissue. The PAGs can accumulate in the uterine stroma; they can also enter the maternal circulation (Wooding et al. 2005; Green et al. 2005). Their accumulation in the maternal blood within a few days after BNCs begin to appear in the chorion has made these proteins useful markers of pregnancy in ruminants (Green et al. 2005; Sasser et al. 1986; Zoli et al. 1992). Indeed, the circulating abundance of the PAGs appears to be correlated with placental function or viability of the pregnancy. The amount of certain PAGs at key stages in early pregnancy of cattle can serve to predict which cows are likely to spontaneously abort the pregnancy (Pohler et al. 2013,

2016a, b). Recent evidence supports a dual role for placental lactogen (CSH1) for paracrine effects to stimulate uterine gland secretions for embryonic and placental development (Spencer et al. 1999a, b, 2004a, b; Kelleher et al. 2019).

Thus, trophoblast BNCs have two principal functions: (1) primary contributors to the fetomaternal syncytium required for successful implantation and placentome formation and (2) production and delivery of protein and steroid hormones and numerous other proteins of unknown function to the maternal and fetal systems.

7 Summary

It is hoped that this chapter has provided the reader with an appreciation of the similarities and differences in embryo/conceptus development, attachment to the uterine endometrium, and subsequent placental development, between ruminant ungulates and the other mammals described in this book. Placentomes, BNCs, fetomaternal hybrid cells, and multinucleated syncytial plaques are distinguishing features of the cotyledonary placenta of *Ruminantia*. These modifications relative to other epitheliochorial forms within the *Artiodactyla* order likely reflect the rapid evolution of placental forms, even within the same phylogenetic order. The placenta is considered one of the most rapidly evolving mammalian organs (Roberts et al. 2016; Haig 1996). Among other things, these differences are probably driven by the selection of large, rapidly evolving gene families, the acquisition of endogenous retrovirus-derived genes, and the rapid evolution of placenta-specific enhancers (Roberts et al. 2016; Telugu and Green 2007). There are no structures comparable to placentomes in the placentas of other artiodactyls. Likewise, giant trophoblasts and the syncytial plaques they form are unique aspects of this placental form.

The fusion of BNCs with uterine epithelial cells or the establishment of syncytial plaques in place of an intact uterine epithelium is the extent of invasive implantation in ruminant ungulates. Even so, these alterations would change how the ruminant conceptus interacts with the maternal system. The nature of these interactions (immunological, endocrinological, etc.) is very different in many ways to that observed in pigs, whales, and camels, where erosion of the uterine epithelium does not occur (Roberts et al. 2016; Telugu and Green 2007).

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