

Early placentation and local immune regulation

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Abstract

It was once thought that processes of conceptus implantation and placentation vary among mammalian species. However, physiological and biochemical processes including gene expression show more similarities than differences. In fact, recent progress has identified that in addition to the hormones, cytokines, proteases and cell adhesion molecules classically characterized, epithelial-mesenchymal transition (EMT), epigenetic regulation and the expression of endogenous retroviruses (ERV) are all required for the progression of conceptus implantation to placentation. Thus, continued research into EMT, epigenetic regulation, the expression of ERVs and immunoregulatory systems will aid in enhancing understanding of their impact on reproductive physiology in humans and domestic animals.

Introduction

The uterine structures in mammalian species as we know them today are the product of a long and complex evolutionary process. In a novel innovation, not only fertilization but embryonic growth occurs inside the body (Amoroso 1968). The uterus could then provide an adequate environment for conceptus growth; however, this arrangement presented new challenges, most immediately immunogenic ones because the conceptus carries paternal genes allogenic to the mother. Although the exact sequence of events remains unclear, the means of protecting the conceptus took the form of the trophoctodermal layer, while the ordeal of supporting and nourishing the conceptus was enabled by a tertiary structure called the placenta. However, extensive variation in trophoctoderm (TE) and placental structures exists across different mammalian species (Ramsey 1982). Trophoctodermal cells also play a major role during the process of conceptus implantation to the maternal uterine endometrium. In this review, new information on TE, its gene regulation, and immunological regulation has been integrated.

Processes of implantation

Bazer et al. (2009) have suggested that there are five phases of blastocyst implantation: (1) Migration and shedding of zona pellucida (ZP, hatching), (2) Pre-contact, blastocyst orientation

and apposition, (3) Attachment, (4) Adhesion, and (5) Endometrial invasion. These processes are followed by placental formation. During Phase 1, the blastocyst enters and migrates within the uterus. Shedding of the zona pellucida allows the expansion of the spherical blastocyst, including migration and changes in its shape from spherical to a tubular and filamentous form as in domestic animals. Phase 2 is a pre-contact period during which the blastocyst migrates or elongates without definitive contact between the TE and endometrial epithelium. In domestic animals, this is the period when the process of maternal recognition of pregnancy is initiated for the prevention of corpus luteum (CL) demise, resulting from biochemical communication between the developing conceptus and mother. Phase 3 is the attachment period, during which the TE establishes definitive contact with the uterine epithelium. Phase 4 is the time of firm adhesion between the TE and uterine epithelium and in some cases, superficial glandular epithelium, during which mononucleate TE cells differentiate into trophoblast multinucleate cells. Phase 5 is when many mammalian species begin to diverge greatly in their development as invasive TE causes the formation of decidualized endometrium, whereas noninvasive TE does not. For the first four phases, however, implantation processes appear fairly similar among mammalian species (Bazer et al. 2009).

Maternal recognition of pregnancy

In mammalian species, the maintenance of CL function and the continued secretion of a steroid hormone, progesterone (P4), are required for the establishment and maintenance of pregnancy. P4 is involved directly and/or indirectly in numerous uterine functions including endometrial secretions, alteration of blood flow at implantation sites and promotion of suitable physiological and/or immune environments for normal embryonic development. Despite its critical importance, the biochemical mechanisms by which the CL is maintained for continued P4 production differ from species to species. In humans, luteolysis is prevented by a luteotrophic factor, chorionic gonadotropin (CG), produced by the TE as it begins implantation (Hearn et al. 1991). In rodents, CL function is prolonged through the release of copulation-induced pituitary prolactin surges (Soares et al. 1991). In ruminant species, including cows, sheep and goats (Short 1969), interferon tau (IFNT), a major cytokine produced by the peri-implantation trophoblast, is the anti-luteolytic factor essential for the prolongation of CL life span (Godkin et al. 1982; Imakawa et al. 1987; Stewart et al. 1987; Charpigny et al. 1988; Roberts et al. 1992).

IFNT exhibits structural and functional similarities to those of type I IFNs such as IFNA and IFNB (Imakawa et al. 1989). These include antiviral and anti-proliferative activities, but IFNT shows much less cytotoxic activity than do IFNA or IFNB (Pestka et al. 1987; Roberts et al. 1989; Pontzer et al. 1991; Pontzer et al. 1997). It was found that type I IFNs bind to a common receptor complex with two polypeptide subunits (IFNAR1 and IFNAR2) (Pestka et al. 2004), both of which are present in ovine uterine epithelial cells (Rosenfeld et al. 2002). The surface epithelium of the uterine endometrium is the primary target for IFNT (Imakawa et al. 2002), but accumulated evidence suggests that IFNT can reach the stroma, and even the uterine myometrium (Ott et al. 1998; Johnson et al. 1999; Hicks et al. 2003). It has also been shown that IFNT probably reaches circulating immune cells and the ovaries (Hansen et al. 2010; Nitta et al. 2011). It was characterized that upon binding to the receptor, type I IFNs activate the janus kinase-signal transducer and activator of transcription-interferon regulatory factor (JAK-STAT-IRF) signaling pathway (Stark et al. 1998; Kim et al. 2003), causing the activation of a group of interferon-stimulated genes (ISGs) (Chen et al. 2007; Spencer et al. 2008). In addition to ISGs, IFNT induces wingless-type MMTV integration site family (WNTs) and *LGALS* gene expression

(Gray et al. 2004; Mohamed et al. 2005), as well as several chemokines in endometrial tissues, including chemokine ligand 10 (CXCL10) and CXCL9 (Nagaoka et al. 2003a; Imakawa et al. 2006a). Through the CXCL10 receptor, CXCR3, endometrial CXCL10 in turn attracts immune cells, particularly NK cells, to the implantation site of the endometrium (Nagaoka et al. 2003b), and regulates TE cell migration and integrin expression (Imakawa et al. 2006a). These changes may be in part involved in conceptus migration, apposition and initial attachment to the uterine epithelial cells in ruminants (Nagaoka et al. 2003b; Imakawa et al. 2005).

Transcriptional regulation of *IFNT*

Expression of IFNT is not induced by viruses or double stranded RNA, but produced by the early trophoblast (Godkin et al. 1982; Imakawa et al. 1995). Low expression levels of IFNT can be detected from the first day following hatching (Farin et al. 1989). The production of IFNT increases remarkably on day 13, when the blastocyst starts to elongate (Ashworth and Bazer 1989) and reaches the maximum level on day 16 of pregnancy, 100 μ g per cultured conceptus during 24 hours, while the blastocyst initiates its attachment to the uterine epithelial cells (Godkin et al. 1982; Imakawa et al. 1995). Following this event, however, IFNT expression decreases rapidly as the process of implantation proceeds, and at day 22 IFNT is no longer detected (Godkin et al. 1982).

Intensive experimentation has been conducted to elucidate the molecular mechanisms by which *IFNT* transcription is regulated. Although IFNT production could be initiated after *in vitro* fertilization and maturation (Hernandez-Ledezma et al. 1992; Stojkovic et al. 1999),

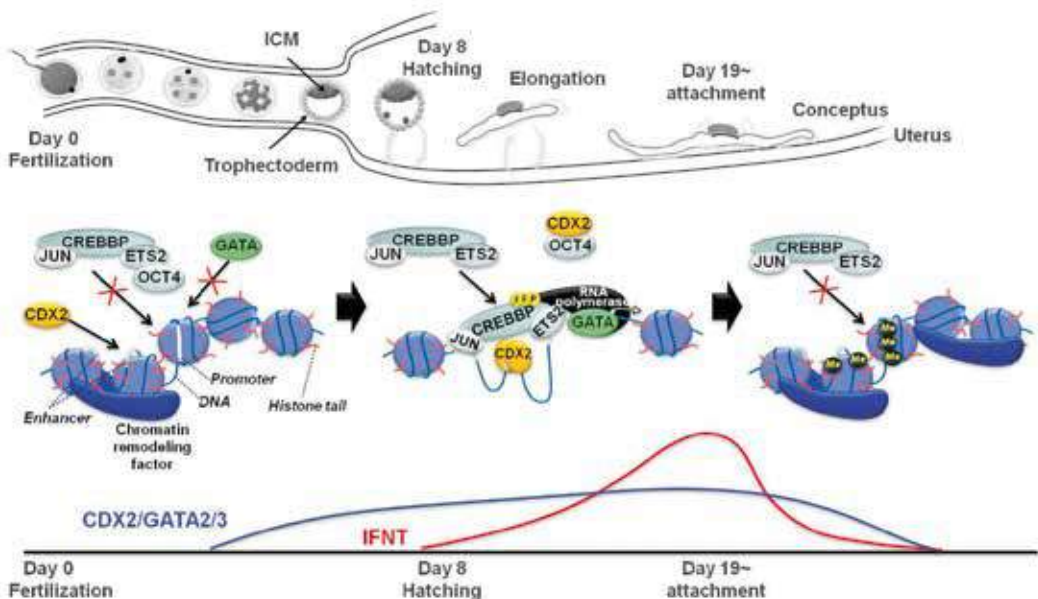


Fig. 1. Events leading to the establishment of pregnancy in cattle.

Upper: Events associated with early pregnancy. Biochemical communication between elongating conceptus and uterine endometrium leads to its attachment to the uterine epithelium.

Middle: Transcription factors and epigenetic changes associated with the regulation of the *IFNT* gene.

Lower: Expression profiles of transcription factors CDX2, GATA2/3 and IFNT.

substantial production of IFNT seen *in utero* could not be achieved without interaction with the uterine environment (Hernandez-Ledezma et al. 1992). It has been demonstrated that the endometrial cytokines and growth factors, GM-CSF, IL3 and FGF2, of which expression increases in the pregnant endometrium (Imakawa et al. 1993; Ocon-Grove et al. 2008), enhance IFNT expression in conceptus tissues and bovine trophoblast (CT-1) cells (Rooke et al. 2005; Michael et al. 2006).

Numerous transcription factors thus far found are potential regulators of the *IFNT* gene including ETS2 (Ezashi et al. 1998; Ezashi et al. 2001), activating protein 1 (AP-1, official symbol JUN) (Yamaguchi et al. 1999), CDX2 (Imakawa et al. 2006b; Sakurai et al. 2009), homeobox protein distal-less 3 (DLX3) (Ezashi et al. 2008), and the co-activators, cAMP-response element binding protein (CREB)-binding protein (CREBBP) and p300 (Xu et al. 2003; Das et al. 2008). While identifying Gata3 as another factor for trophoblast lineage specification, we additionally found that GATA2/3 could enhance (Bai et al. 2009) and EOMES down-regulate (Sakurai et al. 2013) *IFNT* gene transcription.

Epigenetic regulation of IFNT

Epigenetic alterations such as variation in covalent histone modification and DNA methylation regulate gene expression by altering chromatin conformation. While it is known that IFNT production is normally limited to either TE or trophoblast CT-1 and BT-1 cells (Roberts et al. 1992; Talbot et al. 2000; Shimada et al. 2001), Sakurai and coworkers (2009) investigated whether *IFNT* gene transcription could be induced in a cell type not related to trophoblast cells. These investigators demonstrated that significant increases in endogenous *IFNT* transcription in non-IFNT producing, bovine kidney epithelial MDBK cells could be induced through CDX2 over-expression and associated high H3K18 acetylation. They also noted that lowering H3K9 methylation appears to be another condition required for the degree of *IFNT* transcription seen in trophoblast cells. In addition, co-activators CREBBP and p300 with their intrinsic histone acetyl-transferase (HAT) activity are recruited to enhance *IFNT* transcription (Sakurai et al. 2010). However, the observation that the use of HAT inhibitor reduced histone acetylation at the *IFNT* gene even after *CDX* transfection indicates that CDX2-facilitated histone acetylation could be a triggering event necessary for gene expression unique to TE. Moreover, CREBBP/p300 recruitment is known to be associated with greater acetylation of the gene (Sakurai et al. 2010). These results suggest that induction of endogenous *IFNT* transcription in bovine trophoblast cells results from partial decondensation of chromosomal domains by histone acetylation and sufficient CDX2 expression, allowing additional transcription factor binding to the upstream region of *IFNT* genes for higher transcription of the gene.

Ovine genomic DNAs extracted from uterine endometrium (absence of IFNT production), white blood cells (WBC, no IFNT production), day 14 trophoblast (high IFNT production) and day 20 trophoblast (low IFNT production) were examined for methylation status of the *IFNT* upstream region containing 14 CpG sites (Nojima et al. 2004). Genomic DNA from uterine endometrium and WBC displayed higher methylation than day 14 and 20 trophoblasts. Day 14 trophoblasts, which had the highest *IFNT* transcription, were less methylated than day 20 trophoblasts, which possessed minute amounts of *IFNT* mRNAs, and day 17 trophoblasts contain half as much *IFNT* mRNA as day 14 trophoblasts. When cultured *in vitro* with the demethylation reagent 5-aza-dC, amounts of *IFNT* mRNA in day 17 trophoblasts were similar to those of day 14 *IFNT* mRNAs. These findings suggest that changes in the degree of DNA methylation in the upstream sequences of the *IFNT* gene could be one of the major mechanisms

leading to down-regulation of its expression and possibly its silencing in non-trophoblast tissues (Nojima et al. 2004).

Epithelial and mesenchymal transition

The TE forms the epithelial structure of the blastocyst and possesses epithelial characteristics, including apicobasal cell polarity, lateral junctions with neighboring cells, basal contact with the basement membrane proteins, and a distinct basal lamina (Biggers et al. 1988; Kang et al. 1990; Fleming et al. 2000). Despite the fact that the apical plasma membranes of simple epithelia normally lack adhesive properties, the TE still manages to adhere to the uterine epithelium through its apical domains as part of the pre-implantation process. Thus, the adhesion between TE and uterine epithelium has long been considered a cell biological paradox (Denker 1993). With the exception of rodents, in which the conceptus enters a receptive uterus and attaches immediately to the uterine epithelium, primates and most domestic animals have a pre-receptive phase during which the conceptus does not physically interact with the uterine epithelium. In cattle, attachment between trophoblastic epithelium and endometrial epithelium is first seen on day 20 of gestation, and subsequent stable adhesion occurs between days 20 and 22 (Wathes and Wooding 1980).

Another surprising finding was that changes in gene expression associated with epithelial-mesenchymal transition (EMT) occurred not before attachment, but rather on day 22, two to three days after the initiation of conceptus attachment to the uterine epithelium (Yamakoshi et al. 2012). Positive signals for both the epithelial marker cytokeratin and the mesenchymal marker vimentin were seen in the elongated TE on day 22. Increased transcripts of N-cadherin, vimentin, matrix metalloproteinase 2 (*MMP2*), and *MMP9* were also found on day 22, concurrent with E-cadherin mRNA and protein down-regulation. These observations indicate that after the conceptus-endometrium attachment, EMT-related transcripts, as well as cytokeratin, are present in the bovine TE, and suggest that in addition to extracellular matrix expression, partial EMT is required for proper adhesion of trophoblasts in noninvasive implantation.

In this study, we also identified that transcription factors (TF) *SNAI2*, *ZEB1*, *ZEB2*, *TWIST1*, *TWIST2*, and *KLF8* transcripts were up-regulated concurrent with cytokeratin expression in the TE (Yamakoshi et al. 2012). It has been shown that SNAIL, ZEB, and KLF8 TF bind to and repress E-cadherin promoter activity (Peinado et al. 2007; Wang et al. 2007), whereas TWIST1 and TWIST2 TF repress E-cadherin transcription indirectly (Yang and Weinberg 2008). In addition, SNAIL and ZEB TF are known to induce the expression of MMPs that can degrade the basement membrane, thereby favouring invasion (Thiery et al. 2009). Although the bovine trophoblasts do not penetrate into the endometrium, the confirmation that *MMP2* and *MMP9* transcripts are up-regulated not only suggests that they play a role in noninvasive trophoblasts, but also confirms further the similarity between invasive and non-invasive modes of implantation.

Endogenous retroviruses and pregnancy

Endogenous retroviruses (ERVs) are now known to be factors implicated in the development and differentiation of TE in humans, rodents, dogs/cats, rabbits, sheep and cattle (Mi et al. 2000; Blaise et al. 2003; Be et al. 2005; Arnaud et al. 2007; Heidmann et al. 2009; Cornelis et al. 2012; Koshi et al. 2012). During the course of evolution, all vertebrates have been exposed to multiple waves of cross-species infection by exogenous retroviruses, some of which infected germ cells and are inherited in an integrated, proviral form (Boeke and Stoye 1997). They were

once considered junk DNAs; however, it is now realized that ERVs have biological roles in protection against retroviral infection (Best et al. 1997) and in placental development (Harris 1998; Rawn and Cross 2008). Recently, it was found that high levels of transcripts found in embryonic stem (ES) cells, most of which are expressed in two-cell stage embryos, are induced by long terminal repeats of ERVs, suggesting the possibility that the foreign sequences have helped to drive cell-fate regulation in placental mammals (Macfarlan et al. 2012).

Trophoblast cells are very invasive in nature, and as uncontrolled invasiveness could cause abnormal destruction of uterine structures, invasion must be regulated for the protection of uterine endometrial integrity. When the cell cycles of TE cells are restricted, they go through endoreduplication, resulting in the formation of giant trophoblast cells. Although human syncytiotrophoblast cells result from cell fusion, these cells do not go through cell cycles, and thereby their invasiveness is held under control (Huppertz et al. 2002). There is no doubt that tissue inhibitors for MMPs (TIMPs) play a role in controlling the activity of MMPs *in utero* (Das et al. 1997; Itoh et al. 1998). However, inhibition of cell cycles through cell fusion and/or endoreduplication may also contribute to the regulation of TE invasiveness.

Syncytin-1 and -2 are products of the two human ERV envelop (*env*) genes, and are involved in the fusion of trophoblast cells, resulting in formation of multinucleated syncytiotrophoblast (Mi et al. 2000; Blaise et al. 2003). It was suggested that Syncytin-2 entered the primate lineage more than 40 million years ago (MYA) while Syncytin-1 entered the lineage 25-40 MYA (Blaise et al. 2003). In rodents, there are Syncytin A and Syncytin B, both of which are homologous to those of human Syncytin-1 and -2 (Be et al. 2005). Recent studies have shown that syncytin-like, putative fusogenic proteins are also expressed in the placenta of rabbits (Heidmann et al. 2009). In humans, cytotrophoblast cell fusion starts on days 7-11 pregnancy, the time corresponding to the implantation period (Boeke and Stoye 1997).

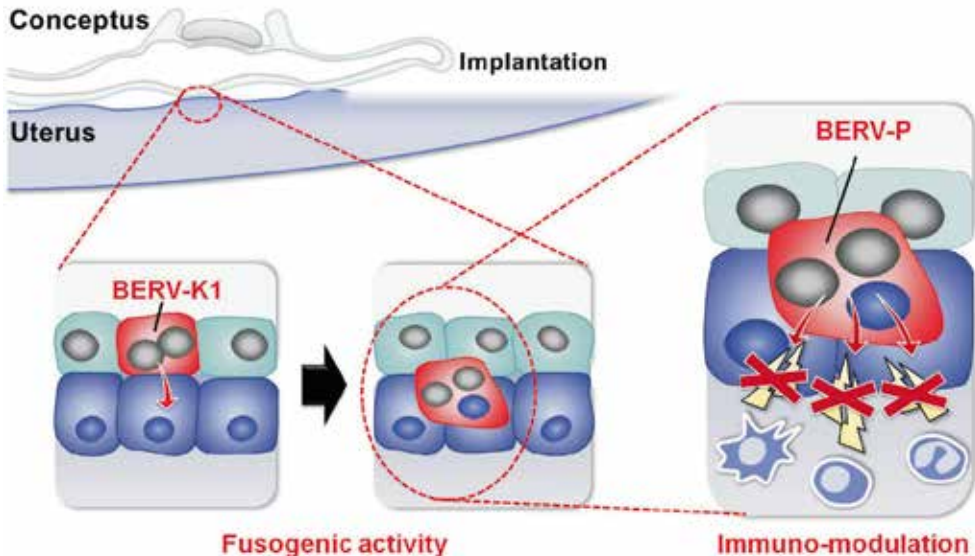


Fig. 2. A model of endogenous retrovirus actions during the period of bovine pregnancy establishment.

Left to Middle sections: Following conceptus attachment to the uterine epithelium, BERV-K1, expressed in binucleate trophoblast, facilitates its fusion with uterine epithelial cell resulting in the formation of trinucleate cells.

Right: BERV-P expressed by the binucleate and possibly trinucleate cells becomes the front line immunoregulatory molecule protection against various maternal immune cells.

In sheep, Jaagsiekte sheep retrovirus (JSRV) is a pathogenic exogenous retrovirus and is known as the causative agent of ovine pulmonary adenocarcinoma (Pötgens et al. 2002; Arnaud et al. 2007). The sheep genome contains a minimum of 27 copies of endogenous JSRV (*enJSRV*), some transcripts of which are found to be abundant in reproductive tracts, particularly in the uterine luminal and glandular epithelium, and epithelium of the oviducts and cervix (Dunlap et al. 2006). In the conceptus, expression of *enJSRV env* begins on day 12 of pregnancy, coincident with the onset of conceptus elongation, the increase in IFNT production and the period of maternal recognition of pregnancy (Pötgens et al. 2002). Transcripts for *enJSRV* are detected in mononucleate TE, but more abundant in trophoblast binucleate cells located at the fetal side of placentomes, and multinucleated syncytia located in the uterine endometrium (Palmarini et al. 2004; Dunlap et al. 2006). In addition, a cell surface receptor for the exogenous *enJSRV* and *enJSRV* envelope protein is hyaluronidase 2 (HYAL2) (Rai et al. 2001), which is expressed by binucleate trophoblast cells and syncytial plaques in the ovine placenta, but not in uterine epithelia, stroma or myometrium (Dunlap et al. 2005).

While it has not been determined whether binucleate cells result from cell fusion or endoreduplication, it is clear that trinucleate cells or syncytia are products of fusion between binucleate cells and uterine epithelial cells (Wooding and Beckers 1987; Dunlap et al. 2005; Baba et al. 2011; Nakaya et al. 2013). Unlike primates and rodents, TE cells of ruminants are not invasive, and thus do not penetrate deep into uterine stroma or spiral arteries; however, the facts that binucleate cells from bovine placenta possess *BERV-K1* (Baba et al. 2011), with fusogenic activity (Nakaya et al. 2013), and that trinucleate cells and syncytia are located in the endometrium (Wooding 1992) suggest that they may strengthen the adhesion between conceptus and uterine endometrium in the placentomes. Perhaps more importantly, these cells represent the foremost trophoblast population, which contacts the maternal immune cells, for the protection of the allogenic embryo during the course of pregnancy.

Pregnancy immunology

Evolution of the placenta has allowed mammals to maintain fetuses within the reproductive tract for prolonged periods, resulting in a greater rate of embryonic survival. However, this mode of pregnancy has also presented them with a considerable immunological challenge, since the genomes of fetuses are composed of maternal and paternal antigens. It was found that most of the maternal antibodies generated against the fetus during pregnancy are directed toward the paternally inherited major histocompatibility complex (MHC). Early studies (Redman et al. 1984) indicated that human trophoblast cells do not express two classical MHC (human leukocyte antigen, HLA) products HLA-A and -B, but they express non-classical MHC product HLA-G (Ellis et al. 1990). HLA-G protein expression is restricted almost entirely to trophoblast cells that are exposed to the maternal immune system. Other than the primates, including humans, the only groups of eutherian mammals in which placental immunology has been studied in detail are the rodents. These species exhibit hemochorial placentation, in which the maternal placenta is eroded away by invading trophoblast and thus these trophoblast cells are in direct contact with maternal blood. In many species, invasive trophoblast cells increase their expression of MHC antigens as they become more exposed to the maternal immune system. Although this has not been definitively resolved, it can be speculated that: (a) MHC on trophoblast cells may help them adhere to and invade into maternal tissues, and (b) trophoblasts isolated in a maternal environment may express MHC antigens to protect themselves from the maternal immune system (Bainbridge, 2000).

It is likely that ungulate trophoblast cells, separated from maternal immune cells by several layers of tissues, are less at risk than those of primates and rodents. In cattle, the MHC is known as the bovine leukocyte antigen (*BoLA*) and four non-classical bovine MHC class I genes, *NC1*, *NC2*, *NC3* and *NC4*, have been characterized (Birch et al. 2008). In common with HLA-G, *NC1* exhibits very limited polymorphism and has an early termination codon, leading to a molecule with a truncated cytoplasmic domain. Unlike HLA-G, *NC1* is transcribed in many cell types and its transcript is detected in bovine trophoblast cells at a higher level than in peripheral mononuclear cells. However, *NC1* protein expression in these cell types has not been demonstrated because of a lack of a specific monoclonal antibody (Davies et al. 2006).

It has been thought that most bovine trophoblast cells do not express classical MHC class I proteins (HLA-A and -B in humans) before day 120 of pregnancy (Davies 2007). During the last third of gestation, however, trophoblast cells in the interplacentomal and arcade regions of the placenta express classical MHC class I proteins, which have no adverse effects on pregnancy maintenance. It is thought that the classical MHC class I expression most likely contributes to placental separation at parturition, avoiding placental retention. In contrast, trophoblast cells in somatic cell nuclear transfer (SCNT) conceptuses express classical MHC class I antigens before day 34 of pregnancy (Pfister-Genskow et al. 2005). This unusual expression of classical MHC class I expression, in conjunction with a marked increase in the number of stromal lymphocytes in the uteri of surrogate dams, is thought to result in the abortion of SCNT conceptuses between days 30 and 90 pregnancy. This suggests that in addition to non-classical MHCs, appropriate regulation of classical MHC class I gene expression is critical for the establishment and maintenance of pregnancy with an allogeneic conceptus even in the non-invasive mode of bovine placentation.

Classical MHC class I is also expressed on bovine binucleate trophoblast cells, which are derived from uninucleate trophoblast cells, and are extruded from the fetal to maternal side of the placenta. As they develop, binucleate cells exhibit increased transcription of maternal and paternal classical MHC class I mRNA and protein expression (Ellis et al. 1998; Bainbridge et al. 1999). This process may have important immunological implications, as bovine binucleate cells are destined to fuse with maternal cells, although it is not known what mixture of transplantation antigens and MHC-bound peptides are expressed by the resulting fetomaternal hybrid cells, or what effect they may have on maternal immune cells.

It should be noted that human Syncytin-1 and Syncytin-2 do not possess similar fusogenic activities, rather Syncytin-1 possesses more fusogenic activity than that of Syncytin-2. Similar findings were made in BERVs in which BERV-K1 possesses more fusogenic activity compared to the others. In fact, BERV-P, is a newly discovered ERV, which structurally is similar to syncytin Car-1 in Carnivora (Car-1, Cornelis et al. 2012), has no fusogenic activity (Nakagawa et al. 2013). However, portions of the amino acid structure of BERV-P are similar, if not the same, as that of Car-1, suggesting that this bovine ERV may play a role in immunomodulation. Because of these recent findings, we propose that BERV-K1 with its fusogenic activity plays a role in the fusion between binucleate trophoblast cells and uterine epithelial cells, whereas BERV-P acts as an immunological modulator during the course of bovine pregnancy (Fig. 2).

Conclusion

The placenta is considered to be a fairly recent invention in mammals, of which the conceptus side contains TE epithelial cells. These cells play an important role in preventing immunological rejection from the beginning of the implantation process, when paternal gene products are

directly exposed to the maternal system. Until recently, the processes underlying conceptus implantation to the maternal endometrium have been studied from the standpoint of attachment and invasion of extracellular matrices, cell adhesion molecules, cytokines, and/or proteinases and their inhibitor expression. Recent progress suggests that although implantation is a complex phenomenon, it can be analyzed as a whole as well as in specific events. In particular, the study of implantation must include ERV genes and their specific expression in genital tracts. However, ERV research in reproduction is fairly new and with various ERV genes yet to be found, our current understanding of implantation and placental formation may be far from finalized. We must therefore treat our understanding of these processes as a work still in progress, and prepare for much work ahead in the elucidation of implantation, placentation processes as well as immunoregulation in mammalian reproduction.

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