Integration of molecules to construct the processes of conceptus implantation to the maternal endometrium

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ABSTRACT: During the peri-implantation period, ruminant conceptuses go through rapid elongation, followed by their attachment to the uterine endometrial epithelial cells, during which interferon-tau (IFNT), a trophoctodermal cytokine required for the process of maternal recognition of pregnancy, is expressed in a temporal and spatial manner. On day 22 (day 0 = day of estrus), 2 to 3 d after the initiation of bovine conceptus attachment to the uterine epithelium, when IFNT production begins to subside, the expression of molecules related to epithelial-mesenchymal transition, zinc finger E-box binding homeobox 1, snail family transcriptional repressor 2, N-cadherin, and vimentin was found in the trophoctoderm. Through the use of in vitro coculture system with bovine trophoblast CT-1 and endometrial epithelial cells, a series of experiments have been conducted to elucidate mechanisms associated with the regulation of *IFNT* gene transcription and conceptus implantation, including epithelial-mesenchymal transition processes.

Expression of IFNT, both up- and downregulation, during the peri-implantation period is tightly controlled. Cytokines and cell adhesion molecules such as epidermal growth factor, basic fibroblast growth factor, transforming growth factor beta, activin A, L-selectin-podocalyxin, and vascular cell adhesion molecule 1-integrin $\alpha 4$ expressed in utero all contribute to the initiation of epithelial-mesenchymal transition in the trophoctoderm. These results indicate that conceptus implantation to the uterine endometrium proceeds while elongated conceptuses and endometria express cell adhesion molecules and their receptors, and the trophoctoderm experiences epithelial-mesenchymal transition. Data accumulated suggest that while the conceptus and the endometrial epithelium adhere, trophoctodermal cells must gain more flexibility for binucleate and possibly trinucleate cell formation during the peri-implantation period, and that understanding and constructing the conditions throughout implantation processes is key to improving ruminants' fertility.

Key words: conceptus implantation, epithelial-mesenchymal transition, interferon-tau, pregnancy, ruminants

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INTRODUCTION

Unlike those vertebrates that provide their embryos with nutrients solely from a yolk sac, eutherians nurture fetuses in the uterus, requiring trophoblast development and placental formation,

a means for gas and nutrient exchanges. Before reaching the stage of placental formation, the conceptus must implant to the maternal uterine endometrium, triggering the interaction between allogenic fetal and maternal cells. Failures in implantation or a lack of proper trophoblast development lead to insufficient placental development or functioning, resulting in pregnancy complications and/or conceptus or fetal losses. Implantation is a complex cascade of processes

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through which the developing conceptus undergoes apposition, attachment, adhesion, and in some cases, invasion to the uterine endometrium. Extensive studies have identified numerous molecules that function during the pre-implantation period. It has become apparent that implantation requires proper development of conceptus as well as the uterine environment.

MECHANISMS ASSOCIATED WITH CONTINUATION OF PROGESTERONE SECRETION AND THE REGULATION OF INTERFERON-TAU EXPRESSION

After successful fertilization in the oviduct, the fertilized ovum moves toward the uterus while undergoing several cell divisions. At the morula stage, it reaches the uterus where the blastocyst formation occurs. The blastocyst consists of 2 distinct cell types: the outer layer trophoblast and undifferentiated cuboidal cells, inner cell mass (ICM). The trophoblast becomes the trophoblast layer of the placenta, while the ICM contributes to the embryo and extra-embryonic membranes such as the allantois and amnion. The blastocyst then hatches from zona pellucida and acquires the ability to attach to the uterus, from which trophoblast and uterine development toward the placental formation begin to differ considerably: many blastocysts start attachment and invasion shortly after the hatching, as seen in rodents and primates, whereas, in ruminant ungulates, the blastocyst floats in the uterus for several days before rapid elongation on day 12 and 13 (day 0 = day of estrus) in sheep and on day 13 and 14 in cattle. After several days to a week of elongation, the ruminant conceptus occupies almost the entire surface of the uterine luminal epithelia and begins its attachment to specialized regions, known as caruncles, of the uterine endometrium.

In rodents and primates, the blastocyst begins its implantation processes immediately following its hatching, but biochemical communication between blastocysts and the uterine endometrium is now thought to exist and function, if pregnancy is to continue. However, the ruminant blastocyst undergoes a longer pre-implantation period, up to 2 wk, during which the trophoblast goes through extensive cell proliferation as well as differentiation. During this period, ruminants require a longer duration for the acquisition of physical and biochemical communications between conceptus and the endometrium, resulting in the establishment of an appropriate uterine environment for the attachment

of 2 cell types, semi-allogenic trophoblast and the maternal luminal epithelium.

Mechanisms Associated With Continuation of Progesterone Secretion

In mammals, most female animals of reproductive age have estrous cycles, during which a sequence of events in the ovary such as follicular growth, ovulation, corpus luteum (CL) formation, and functioning are periodically repeated. Progesterone, produced by CL, is involved in the regulation of uterine environment necessary for conceptus growth, implantation, and placental formation and maintenance. If pregnancy does not begin, CL regresses at the end of each luteal phase (luteolysis) and progesterone synthesis and secretion decline, allowing a new round of follicular growth, ovulation, and the continuation of estrous cycles. At the end of the luteal phase of the estrous cycle, prostaglandin F₂ α (PGF₂ α), produced by the uterine endometrium, reaches the CL and regresses CL structurally and functionally. Therefore, for a pregnancy to proceed, CL must be maintained, and rescue of CL from its regression is termed as the process of maternal recognition of pregnancy (Moor et al., 1969; Short, 1978).

In ruminant ungulates, interferon-tau (IFNT), produced by the mononuclear trophoblast of conceptuses, is a cytokine required for the process of maternal recognition of pregnancy. Discovery and characterization of IFNT have been reviewed elsewhere (Roberts et al., 1992; Bazer and Thatcher, 2017; Hansen et al., 2017; Imakawa et al., 2017). Expression of IFNT occurs at the time of blastocyst hatching (Ashworth and Bazer, 1989) and as the conceptus elongates, IFNT secretion begins to increase, and peaks on day 16 in sheep or day 20 in cattle (Godkin et al., 1982; Farin et al., 1989; Guillomot et al., 1990), both of which correspond to the time of CL regression during the estrous cycle or conceptus attachment to the maternal endometrial epithelium. Following peak IFNT production, its expression declines rapidly and by day 22 (sheep) and 25 (cattle), IFNT production is no longer detected, suggesting that up- and down-regulations of *IFNT* gene transcription are tightly controlled. Furthermore, it has been suggested that there may be 2 types of trophoblast cell populations in the elongating blastocyst: terminally differentiated and less differentiated. We suspect that the terminally differentiated trophoblast cells produce IFNT, whereas less differentiated cell populations proliferate during the pre-attachment period.

Since the late 1960s and 1970s, results from embryo transfer and related studies provided the idea that the CL is maintained through a local effect of the conceptus during early pregnancy (reviewed in [Bazer and Thatcher, 2017](#); [Hansen et al., 2017](#)). It should be noted that as compared to cyclic ewes, more PGF2 α is released from the uterus in day 13 pregnant ewes, and that the CL is more resistant to the lytic effects of higher PGF2 α ([Arosh et al., 2016](#)). Endometrial PGF2 α production is regulated through the expression of uterine epithelial estrogen receptor 1 and oxytocin receptor, both of which are increased on day 11 to 17 (day 0 = day of estrus) in cyclic or nonpregnant sheep, but not in pregnant animals. This cytokine, IFNT, is known as a primary factor in rescuing CL function from endometrial production of luteolytic PGF2 α ; however, this is not by direct action; rather, the antiluteolytic action of IFNT is to prevent increases in uterine epithelial estrogen receptor 1 and oxytocin receptor, resulting in the prevention of pulsatile PGF2 α release ([Spencer and Bazer, 1995, 1996](#); [Hansen et al., 2017](#)). It was demonstrated that an intracellular signaling molecule, interferon responsive factor 2 (IRF2) is responsible for mediating IFNT's effects on the bovine uterine endometrial oxytocin receptor gene ([Telgmann et al., 2003](#)); however, an intracellular signaling system other than IRF2 has not been well characterized.

Trophoblast IFNT is known to act on the endometrium in a paracrine manner; however, it has often been suspected that IFNT may exit from the uterus ([Schalue-Francis et al., 1991](#)). For the last 10 yr, data have been accumulated, providing evidence that IFNT could exit from the uterus via the uterine vein ([Oliveira et al., 2008](#); [Romero et al., 2015](#)). Although IFNT outside of the uterus has not been definitively quantitated ([Hansen et al., 2017](#)), the evidence suggests that IFNT could leak out of the uterus because the expression of interferon-stimulated genes has been detected in maternal tissues and cells including peripheral blood mononuclear cells, liver, and CL, in which IFNT is the only interferon expressed ([Bott et al., 2010](#); [Ribeiro et al., 2014](#); [Meyerholz et al., 2016](#); [Sinedino et al., 2017](#)). In addition, recent studies proposed another mechanism of IFNT transport into deeper endometrium as well as extra-endometrium. Three studies have identified IFNT in extracellular vesicles recovered from uterine luminal fluid of pregnant ewes and cows ([Ruiz-González et al., 2015](#); [Nakamura et al., 2016](#); [Kusama et al., 2018](#)). While interferon-stimulated gene expression found in peripheral blood mononuclear cells definitively results from IFNT

in the blood stream, further research is required to establish existence of IFNT outside of the uterine lumen.

If IFNT is present in the blood stream, the next question is whether IFNT directly protects the CL in an endocrine manner. To determine the direct effect of IFNT on the CL, numerous IFNT infusion studies have been conducted ([Bott et al., 2010](#); [Antoniazzi et al., 2013](#)). Rather than a single high dose of IFNT, multiple low doses of IFNT, which closely resemble the in vivo conditions, are more effective on the induction of interferon-stimulated genes in the CL and the resistance to the luteolytic PGF2 α treatment during the period of maternal recognition of pregnancy ([Antoniazzi et al., 2013](#); [Romero et al., 2013](#)). In addition to interferon-stimulated genes, IFNT induces several other genes including chemokine, cell adhesion, cytoskeletal and angiogenic pathways, as well as cell survival genes in the CL, some of which may play important roles in the inhibition of luteal cell apoptosis, thereby sustaining luteal steroidogenesis ([Antoniazzi et al., 2013](#)). Further evidence to support CL steroidogenesis is provided by [Miyamoto and coworkers \(2014\)](#), who showed that pregnancy induces lymphoangiogenesis in the bovine CL via vascular endothelial growth factor, followed by neutrophil influx and increase in interleukin 8 expression, both of which complement continued steroidogenesis in the CL ([Shirasuna et al., 2015](#)). However, further study is required to obtain definitive evidence on the endocrine effects of IFNT on CL maintenance.

Transcription Factors Functioning in the Trophectoderm During Early Pregnancy

As blastocyst development proceeds, the POU transcription factor, POU class 5 homeobox 1 Pou5f1 (**Oct4**), becomes progressively restricted to ICM and then to the epiblast ([Ralston and Rossant, 2005](#)). Several transcription factors in the trophectoderm have been observed to promote cell growth as well as enable further differentiation. Expression of a transcription factor caudal-related homeobox 2 (**CDX2**), restricted to the trophectoderm, plays a role in the repression of *Oct4* expression ([Beck et al., 1995](#); [Niwa et al., 2005](#)). However, unlike in the murine species, the presence of *OCT4* mRNA has been detected in bovine and ovine trophoblasts ([Degrelle et al., 2005](#); [Berg et al., 2011](#); [Kim et al., 2013](#)). [Berg et al. \(2011\)](#) showed that the upstream conserved region 4 (**CR4**) of *OCT4* genes differs between bovine and murine species, and that bovine

CR4 is able to enhance *Oct4* expression in mouse trophectoderm. Nevertheless, *Cdx2* acts early in the trophectoderm lineage differentiation as over-expression of *Cdx2* causes embryonic stem cells to differentiate into trophoblast stem cells (Niwa et al., 2005). In mice, expression of a transcription factor, T-box protein eomesodermin (EOMES), is subsequently detected in the trophectoderm cells of the blastocyst and continues in the extra-embryonic ectoderm of the early post-implantation embryos (Russ et al., 2000). The development of Eomes null mutant zygotes is arrested soon after implantation and organized embryonic or extra-embryonic structures are not formed. In blastocyst cultures, mutant homozygotes did not exhibit trophectoderm outgrowth, although they hatched normally and maintained the typical blastocyst morphogenesis until 7 d after the initiation of in vitro culture (Russ et al., 2000). These results indicate that CDX2 is required for initial lineage determination, and that EOMES functions in cell growth and possibly further trophectodermal cell differentiation (Fig. 1).

Upregulation of *IFNT* Expression

Drastic increases in *IFNT* gene transcription and its protein expression begin as the conceptus

starts to elongate, and high expression is maintained until the conceptus attachment to the uterine epithelium is initiated (Godkin et al., 1982; Guillomot et al., 1990). It has been shown that CDX2 is expressed in the ovine trophoblasts during the conceptus elongation period (Imakawa et al., 2006), and the degree of *IFNT* gene transcription is reflective of the degree of *CDX2* gene transcription in the bovine trophectoderm (Sakurai et al., 2009). We also reported that CDX2 and chromatin structure could be key elements that determine *IFNT* gene expression in bovine cells other than trophoblast cells or in human choriocarcinoma JAR or JEG3 cells (Sakurai et al., 2009). We then demonstrated that the degree of *IFNT* gene transcription is reflective of that found in CDX2 in the uterine flushed-bovine trophectoderm, and *IFNT* transcription is reduced when bovine trophoblast CT-1 cells are treated with *CDX2* siRNA (Sakurai et al., 2010), further supporting the role CDX2 plays in trophectoderm-specific expression of *IFNT*. In addition, several transcription factors for *IFNT* gene expression have been identified: v-ets avian erythroblastosis virus E26 oncogene homolog 2 (ETS2), distal-less 3 and GATA binding protein 2 (GATA2) and GATA3 bind to the proximal promoter region of *IFNT* gene, and activator

Figure 1. Cellular and molecular events associated with conceptus implantation to the maternal endometrial epithelium during the peri-attachment period. Upper: Conceptus development in utero. Middle: Mechanisms associated with the regulation of interferon-tau (*IFNT*) gene transcription. As chromatin structures are loosened up during the pre-attachment period, the transcription factor complex is formed and bound to the upstream region of *IFNT* gene, which drives the high levels of gene transcription. As the expression of a transcription factor CDX2 subsides, *IFNT* gene transcription declines, while phosphorylated YAP/TEAD translocate to the cytoplasm and another transcription factor EOMES is expressed. Lower: Progesterone secretion is maintained when a pregnancy is established (solid green line) or progesterone declines at the end of the luteal phase (dotted green line). Interferon-tau production is controlled in a spatial and temporal manner (red line).

protein 1 binds to the distal enhancer region of this gene (Ezashi et al., 1998; Yamaguchi et al., 1999; Ezashi et al., 2001; Imakawa et al., 2006; Das et al., 2008; Bai et al., 2009; Ezashi and Imakawa, 2017). Moreover, *IFNT* expression is upregulated by cyclic adenosine 3',5-monophosphate-response element binding protein-binding protein with intrinsic histone acetyltransferase activity (Xu et al., 2003). Furthermore, histone H3 lysine 18 is highly acetylated and histone H3 lysine 9 is less methylated before conceptus attachment, but this epigenetic status is reversed soon after the initiation of conceptus attachment to the uterine epithelium (Sakurai et al., 2010).

Downregulation of IFNT

Despite numerous studies conducted on upregulation of *IFNT* gene transcription, the mechanisms by which downregulation of *IFNT* gene transcription occurs, or its significance for progression of pregnancy have been well characterized. We previously showed that EOMES downregulates *IFNT* expression after conceptus attachment; however, EOMES alone does not completely downregulate the transcriptional activity of the *IFNT* gene (Sakurai et al., 2013). Recently, involvement of TEA domain transcription factor (TEAD) and cofactor yes-associated protein (YAP) in *IFNT* gene transcription has been demonstrated (Kusama et al., 2016b). Yes-associated protein is localized in the nucleus of trophoctoderms; however, as YAP phosphorylation increases on day 20 and 22 of bovine pregnancy, phosphorylated YAP is found in the cytoplasm and TEAD becomes less effective in the transcription of *IFNT* gene (Kusama et al., 2016b). These results indicate that similar to the upregulation of *IFNT* transcription, *IFNT* downregulation is also controlled by several mechanisms, suggesting that both up- and downregulation of *IFNT* gene transcription are tightly controlled during the peri-implantation period.

ESTABLISHMENT OF CELL TO CELL INTERACTION REQUIRED FOR IMPLANTATION TO PROCEED

The steroid hormone progesterone plays a key role in the establishment and maintenance of pregnancy. Although the mechanism of progesterone action on conceptus development has not been definitively elucidated, elevated concentrations of progesterone during the post-conception period generally result in higher conceptus survival

(Garrett et al., 1988; Carter et al., 2008; Clemente et al., 2009). In ruminant species, the trophoblast floats within the uterine lumen, and several days following hatching from zona pellucida, the bovine blastocyst then elongates up to 20 to 30 cm during a week-long period before conceptus attachment is initiated. During the pre-implantation period, elongating conceptuses still float in the uterine histotroph, of which quality and quantity of constituents are regulated by progesterone-induced developmental changes in endometrial gene expression (Spencer et al., 2008; Forde et al., 2009). These data indicate that the composition of uterine histotroph plays a major role in conceptus gene expression and development, which in turn affects endometrial gene expression. Despite extensive studies performed on the bovine species and numerous molecules identified (Forde and Lonergan, 2017), molecular mechanisms associated with the establishment of proper uterine environment that leads to the conceptus attachment to the uterine epithelium have not been well characterized (Dorniak et al., 2013).

Molecules Functioning in Conceptus Attachment

Following the initial attachment between trophoctodermal epithelium and endometrial epithelium on day 19 and 20 of bovine pregnancy, stable adhesion occurs between day 20 and 22 (Wathes and Wooding, 1980). In our bovine experimental systems, the whole conceptus can be flushed non-surgically on day 17 and 20, while tissue debris can be flushed from the uterus in the morning of day 22, but not much cell debris can be obtained using the same uterine flushing procedure in the afternoon of day 22, indicating that the conceptus has progressed to the adhesion stage between the trophoctoderm and uterine epithelium.

In humans and murine species, cell-to-cell interactions and integrin (ITG)-mediated signaling between the conceptus and endometrium are critical for successful implantation (Aplin et al., 1998; Armant, 2005). In goats, sheep, and cattle, interferon gamma-inducible protein 10, galactoside-binding, soluble 15, and insulin-like growth factor-binding protein 1 present in uterine histotroph have been characterized to activate ITGs through their Arg-Gly-Asp (RGD) domain during the trophoctoderm attachment period (Nagaoka et al., 2003; Farmer et al., 2008; Simmons et al., 2009). In the bovine species, the expression of ITGs is found at the uteroplacental interface during the peri-attachment period (MacLaren and Wildeman, 1995; MacIntyre et al., 2002) and placentation (Pfarrer et al., 2003).

Bovine trophoblast ITGs characterized for their migration and fusion with the uterine epithelial cells consist of 5 α -subunits (ITGA2B, ITGA3, ITGA5, ITGA8, and ITGA4) and 2 β -subunits (ITGB1 and ITGB3) (Pfarrer, 2006). We previously identified that integrin subunits α (ITGA4 and ITGA5) and β (ITGB1, ITGB3, and ITGB5) are constitutively expressed in bovine peri-attachment trophoblast cells, whereas the expression of ITGA8 is induced following the initiation of trophoblast cell attachment to the uterine endometrial epithelial cells (Yamakoshi et al., 2012). In spite of these efforts, our knowledge is not sufficient to regulate bovine conceptus attachment and adhesion to the uterine epithelium.

Recently, 2 more proteins have been characterized as essential for conceptus attachment to the maternal uterine epithelium in ruminants; L-selectin (SELL) and vascular adhesion molecule 1 (VCAM1). L-selectin is known as a molecule essential for lymphocyte rolling (Tedder et al., 1990). L-selectin is detected on the outer cellular surface of human blastocysts after hatching from the zona pellucida, where it plays a role as a scaffold molecule in the human embryo-endometrial apposition process (Genbacev et al., 2003; Wang et al., 2008; Liu et al., 2011). In the bovine, SELL expression was detected in endometrial epithelia, and its potential ligands, podocalyxin (PODXL) and selectin P ligand, were found in both conceptus and endometrium (Bai et al., 2015). In addition, SELL is localized on leukocytes located at the endothelial surface of placentomal vessels in day 50 pregnant sheep (Muniz et al., 2006). These results indicate that SELL expression in lymphocytes is similar among humans, goats, and cattle; however, the expression profiles of SELL and its ligand during the peri-implantation period in the bovine uterus differ from those in humans. In human uteri, the blastocyst migrates until it is apposed to the implantation site, whereas the ruminant conceptus elongates before its attachment to caruncular regions of the uterine epithelia. Although endometrial epithelial SELL expression is not orthologous to that in humans, cessation of conceptus movement and/or apposition through SELL expression is required for the implantation processes in humans (Genbacev et al., 2003) as well as the bovine species.

Vascular adhesion molecule 1 (Osborn et al., 1989) is known as a cell adhesion mediator during the process of lymphocyte homing (May et al., 1993), angiogenesis (Ding et al., 2003), and allantoic membrane fusion to the chorion (Gurtner et al., 1995). In humans, for example, VCAM1 is present

on the endometrial side, specifically localized to decidual stromal cells in the areas where migrating blastocysts are present, but not on vascular endothelial cells in decidua parietalis. In *Vcam1* gene ablation study (Gurtner et al., 1995), the allantois fails to fuse with the chorion, resulting in abnormal placental development and embryonic losses at 9.5–11.5 days of gestation. However, a minority of *Vcam1*-deficient mice survive, although these mice suffer from abnormal distribution of allantoic mesoderm over the chorionic surface. In the study on early pregnancy in sheep, VCAM1 is first found in endothelial cells on day 17 to 19 in both caruncular and intercaruncular areas of the endometrium and becomes strongly induced in endothelial cells on day 26 to 27 (Rahman et al., 2004). In our study, VCAM1 expression was detected at the trophoblast and uterine epithelium as well as uterine glandular epithelium and endothelium during the peri-implantation period (Bai et al., 2014). In endothelial cells, VCAM1 is induced by cytokines such as tumor necrosis factor- α produced by leukocytes (Henninger et al., 1997). The increase in VCAM1 leads to the production of more chemokines by the endothelial cells, which attract and proceed with the migration of leukocytes into these cells (Wittchen 2009). During the process of conceptus attachment to the endometrium in cattle, increases in *VCAM1* and its protein expression in the cytoplasm of cultured endometrial epithelial cells were detected when treated with either the uterine flushing media obtained from day 22 pregnant animals, epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), or IFNT (Bai et al., 2014). However, VCAM1 expression was further upregulated when endometrial epithelial cells were cocultured with bovine trophoblast CT-1 cells. More importantly, the degree of upregulation of *VCAM1* mRNA expression was more significant in cocultured endometrial epithelial cells than in mono-cultured endometrial epithelial cells treated with uterine flushing media, and a similar degree of upregulation was also found in *VCAM1* transcript expression in cocultured CT-1. Based on these observations, both cell–cell interactions between the conceptus and uterine epithelium and uterine molecules, found in the uterine flushings obtained from day 22 conceptuses, could induce VCAM1 production in the endometrium and/or conceptuses. Furthermore, during lymphocyte homing, once the rolling speed of lymphocytes slows down, VCAM1 and its receptor integrin α 4 (ITGA4) become effective in further slowing down and arresting lymphocyte movement (Butcher and Picker, 1996). These results strongly

suggest that VCAM1 is at least one of the cell adhesion mediators for bovine conceptus attachment and/or adhesion to the uterine luminal epithelium.

The results from these studies suggest that one of the cell adhesion molecules, SELL, is involved in the initial step in conceptus attachment to the uterine endometrium, likely through binding with conceptus PODXL under the regulation of EGF and bFGF. Moreover, VCAM1 is expressed in both uterine luminal and glandular epithelial cells and conceptuses, and its receptor, ITGA4, is found only in conceptuses at the peri-implantation stage in the bovine (Bai et al., 2014). All of these molecules functioning for conceptus-endometrial attachment are also known to be associated with the event called epithelial-mesenchymal transition (Fig. 2).

Epithelial-Mesenchymal Transition and Its Regulation in Ruminant Conceptuses

We previously reported that in day 22 bovine conceptuses, 2 to 3 d after the initiation of conceptus attachment to the uterine epithelium, positive signals for N-cadherin, vimentin (VIM), matrix metalloproteinase 2 (MMP2) and MMP9, often seen in epithelial-mesenchymal transition processes, were detected, whereas no or weak signal for E-cadherin (CDH1), found in epithelial cells, was detected (Yamakoshi et al., 2012).

Epithelial-mesenchymal transition is a process characterized by downregulation of apical-basal polarity, loss of cell to cell adhesion, reorganization of the cytoskeleton, and expression of MMPs. Epithelial-mesenchymal transition is essential in metastasis of cancer cells, is associated with repair processes including wound healing, tissue regeneration, and organ fibrosis, and is often seen in stem cell development, gastrulation, and trophoblast invasion (Jordan et al., 2011; Lim and Thiery, 2012; Lamouille et al., 2014). In each of these cases, cells or tissues that underwent epithelial-mesenchymal transition subsequently go through mesenchymal-epithelial transition (MET) to recover their epithelial nature, and the transition of epithelial-mesenchymal transition-MET processes are often repeated. In humans and mice, the trophoblast undergoes epithelial-mesenchymal transition process after the blastocyst adhesion to the uterine wall, followed by its invasion to the endometrial compartment (Jordan et al., 2011; Lim and Thiery, 2012; Lamouille et al., 2014). These observations indicate that the epithelial phenotype of these trophoblasts switches to mesenchymal phenotype, which acquires invasive cell characteristics, allowing cell migration into the maternal system. The next question is whether epithelial-mesenchymal transition is required for noninvasive trophoblasts of the ruminant ungulates for their conceptus attachment

Figure 2. Cellular and molecular events associated with epithelial-mesenchymal transition (EMT) during the post-attachment period in the bovine species. On day 20, high expression of follistatin (FST) inhibits the effect of activin A/TGFβ. L-selectin (SELL) and vascular cell adhesion molecule 1 (VCAM1), downstream molecules of EGF/FGF, are expressed on the surface of the uterine epithelium. As FST expression decreases, activin A/TGFβ become effective in the activation of SMADs, followed by the expression of EMT-related transcription factors, TWIST1, ZEB1, and SNAI2. As SELL and VACM receptors for podocalyxin (PODXL) and integrin α4 (ITGA4), respectively, are expressed on day 22, the association between trophoderm and uterine epithelium becomes tighter. However, trophoblasts go through EMT process, allowing trophodermal cells to become more flexible and motile.

to the predetermined luminal epithelium of uterine caruncles?

Two unexpected events need to be addressed: 1) why does the noninvasive bovine trophoblast experience epithelial-mesenchymal transition? 2) why does epithelial-mesenchymal transition occur after the expression of cell adhesion molecules such as SELL and VCAM as well as the initiation of cell-cell contact? To study mechanisms by which trophoblast epithelial-mesenchymal transition progresses, our coculture system with trophoblast CT-1 cells and bovine endometrial epithelial cells (Sakurai et al., 2012) was used to mimic those happening in utero (Kusama et al., 2016a). It should be noted that more than 2 decades ago, the expression of transforming growth factor B1 (TGFB1), TGFB2, or TGFB3, known to induce epithelial-mesenchymal transition, in ovine conceptuses and endometrium was found to increase on day 18 and onward, during which IFNT expression begins to decline (Doré et al., 1996; Imakawa et al., 1998), but the significance or function of TGFs has not yet been characterized. Using the iTRAQ analysis system, uterine flushing media were studied for the presence of epithelial-mesenchymal transition inducing molecules, and 7 TGFB-related factors, including follistatin (FST), heparanase, vasorin, osteoglycin, myostatin, HtrA serine peptidase 1, and connective tissue growth factor, were identified. In particular, high levels of FST, an inhibitor of activin A, present in day 20 uterine flushing media were decreased on day 22, whereas elevated activin A found in day 20 uterine flushing media and endometria was further increased on day 22 (Kusama et al., 2016a).

It was reported that epithelial-mesenchymal transition in human trophoblast cells can be induced by activin A (Li et al., 2014), which binds to type 2 activin A receptor (ACVR), resulting in the recruitment of type 1 ACVR, followed by the activation of downstream factors SMAD family members (SMADs) (Jones et al., 2002). In the study by Kusama et al. (2016a), *ACVR1*, *ACVR1B*, *ACVR2A*, and *ACVR2B* were upregulated in day 22 bovine conceptuses. The study was extended to examine whether SMAD2, SMAD3, and SMAD4 were upregulated and/or phosphorylated, finding that SMAD3 and SMAD4 were increased while phosphorylation level of SMAD2 was elevated in day 22 conceptuses (Kusama et al., 2016a). Moreover, the treatment with day 22 uterine flushing media or activin A upregulated the expression of epithelial-mesenchymal transition markers, snail family transcriptional repressor 2 (*SNAIL2*), zinc

finger E-box binding homeobox 1 (*ZEB1*), and twist family bHLH transcription factor 1 (*TWIST1*), in CT-1 cells, suggesting that epithelial-mesenchymal transition in day 22 bovine conceptuses could be induced by activin A secreted from the endometrium to the uterine lumen.

It is reported that epithelial-mesenchymal transition is induced by NOTCH signaling, an intracellular signaling pathway induced by cell attachment (Gonzalez and Medici, 2014). On blastocyst implantation, NOTCH signaling is activated by cell-cell interaction, in which NOTCH receptors expressed on the surface of endometrium interact with ligands present on the surface of blastocysts in humans and mice (Cuman et al., 2014). Activin A or TGFB upregulates several transcription factors such as *SNAIL2*, *ZEB1*, or *TWIST1*, which are mediated by phosphorylated SMAD2 or SMAD3 (Lamouille et al., 2014; Li et al., 2014), while NOTCH signaling upregulates the SMAD3 expression, enhancing TGFB-induced epithelial-mesenchymal transition marker expression (Niessen et al., 2008; Fu et al., 2009; Chen et al., 2014). These and our results (Kusama et al., 2016a) suggest that attachment of CT-1 cells to endometrial epithelial cells could activate the NOTCH signaling, which enhances activin A-induced epithelial-mesenchymal transition marker expression via SMAD2, SMAD3, and/or SMAD4. Despite epithelial-mesenchymal transition inducing and associated molecules exist in the uterine luminal fluid, activation of NOTCH signaling through conceptus attachment to the uterine epithelium is a key event in inducing epithelial-mesenchymal transition in trophoblast cells.

Why Both Cell Adhesion and Epithelial-Mesenchymal Transition Occur in Day 22 Bovine Conceptuses

The next question is, why is trophoblast epithelial-mesenchymal transition required when stable adhesion of the 2 cell types takes place? Constituents of uterine luminal fluid such as EGF and bFGF are among many factors that have been found to be involved in epithelial-mesenchymal transition processes (Said and Williams, 2011). Podocalyxin has been found as a markedly upregulated protein in TGFB-induced epithelial-mesenchymal transition of human lung adenocarcinoma A549 cells (Meng et al., 2011). These investigators concluded that PODXL is an important regulator of the epithelial-mesenchymal transition-like process as it regulates the loss of epithelial features and the acquisition of a motile phenotype. Results

from a series of our studies agree with those previously published, revealing that 1) EGF and bFGF are expressed in day 20 endometrium and conceptuses (Bai et al., 2015), 2) PODXL expression increases in day 22 conceptuses (Bai et al., 2015), and 3) VCAM1 and integrin $\alpha 4$ expression increase in day 22 endometrium and conceptuses, respectively (Bai et al., 2014). All these findings add up to the induction of epithelial-mesenchymal transition processes in day 22 bovine conceptuses, establishing stable adhesion between the 2 cell types (Imakawa et al., 2017). Moreover, acquisition of trophoblast cells with the mesenchymal morphology and(or) functionality allow the adhesion between the trophoblast and uterine epithelial cells, not those between epithelial and epithelial cells. During this period, trophoblast cells must be more flexible, enabling the formation of binucleate cells as well as trinucleate cell formation. It was found that binucleate trophoblast cells exhibit intermediate characteristics between epithelial and mesenchymal phenotypes (Pfarrer, 2006). Ruminant trophoblasts are known not to be invasive as those in humans and murine species, however, the trophoblastic cells need to be flexible and motile, which could be required for the formation of tertiary structure and subsequent placental formation, consisting of both cell types.

CONCLUSIONS

Progesterone plays a major role in the establishment and maintenance of pregnancy, and species' specific mechanisms have been identified for the continuous production of progesterone by the CL. During the peri-implantation period, progesterone regulates uterine environment through uterine gene expression and therefore conceptus development. The expression of IFNT, essential for the maintenance of CL, is tightly controlled genetically and epigenetically, which affects endometrial gene expression, resulting in the establishment of a proper endometrial environment. While IFNT is beginning to be downregulated, expression of many genes in both endometrium and conceptus change, during which the conceptus goes through attachment and adhesion to the uterine endometrium. Trophoblasts have been characterized as epithelial cells and upon undergoing epithelial-mesenchymal transition, they adhere to the uterine endometrium. This transition is required if the trophoblastic cells gain their flexible and perhaps motile nature, allowing the tighter communication with the maternal tissues and the construction of tertiary

structures and placenta. Progression of these events is regulated through the integration of various gene expression in a coordinated and concurrent manner, resulting in the establishment of an intimate and unique structure, the placenta.

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