Molecular mechanisms facilitating bovine conceptus attachment to the uterine epithelium: a reexamination with insights from lymphocyte homing

ウシ胚の子宮内膜上皮細胞への接着の分子機構：リンパ球ホーミングからの解析

BAI　RU LAN

白　汝 嵐

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General Introduction

In the bovine, a significant fraction of embryonic losses occurs during peri-implantation period, resulting from insufficient biochemical communication between the elongating conceptus and the uterus (Diskin & Morris 2008). Implantation is generally considered a highly coordinated process including stages of hatching, apposition, attachment (pre-implantation period) and adhesion (implantation period), before placentation. During the peri-implantation period, blastocysts become oval (ovoid) shape on day 13 (day 0 = day of estrus) and start to elongate on day 14-15. Conceptus attachment is initiated when the blastocyst elongation slows on day 18-19, during which the maternal endometrium becomes "receptive" to conceptus attachment. Using two-dimensional gel electrophoresis, uterine fluids (histotroph) were investigated and found that uterine components differ between the stages of estrous cycle and/or pregnancy in the bovine species (MacLaughlin *et al.* 1986). Similar findings are observed when human uterine fluids from mid proliferative phase are compared to those of the mid secretory phase (Chen *et al.* 2009). These data indicate that uterine environments differ between pregnant and non-pregnant uteri, in particular during implantation initiation period. All the evidence strongly suggests that biochemical communication between the developing conceptus and uterus has to be characterized if one intends to find a way to reduce embryonic losses before placental formation.

Pre-implantation period

Progesterone is a steroid hormone, indispensable for the establishment and maintenance of pregnancy. Most mammalian species begin to undergo estrous or menstruation cycles once females reach puberty. Estrous cycles involve estrogen from the ovary and progesterone from corpus luteum; the latter is a transient endocrine organ. For a pregnancy to proceed, the corpus luteum must be protected from cyclical luteolysis; mammals have developed species-specific systems to provide the continuous production of progesterone throughout pregnancy. The protection of corpus luteum during the early

stage of pregnancy is generally termed as the maternal recognition of pregnancy. In the bovine, the maternal recognition of pregnancy must occur on days 14-15 of estrous cycle or pregnancy. In ruminants, one factor responsible for the maternal recognition of pregnancy was first identified as a conceptus protein, trophoblastin (Martal *et al*. 1979), which was later characterized and named as trophoblast protein-1, oTP-1, for the ovine (Godkin *et al.* 1982) and bTP-1 for the bovine species (Helmer *et al.* 1987). Characterization of its cDNA and amino acid sequences revealed that oTP-1 was a type I interferon (Imakawa *et al.* 1987), which was renamed as interferon-tau (IFNT) (Roberts *et al*. 1992). Progesterone and IFNT are considered two major factors required for pregnancy establishment in ruminant ungulates.

Progesterone

Progesterone is synthesized by a transient endocrine organ of corpus luteum, and acts on the uterus to support the developing embryo in mammals and maintain the pregnancy (Imakawa *et al.* 2013). Progesterone acts on its receptor (PGR), mainly localized in endometrial glands and sub-epithelial stroma at early stage of implantation, to regulate the expression and secretions of the endometrium (Robinson *et al*. 2001). Modulation of circulating progesterone alters the endometrial gene expression and the components of the histotroph required for uterine receptivity (Forde & Lonergan 2012). Supplementary progesterone also has been shown to increase the pregnancy rate in lactating dairy cattle. Cattle fitted with an intra-vaginal progesterone-releasing device (CIDR-B) post-artificial insemination (AI) improved pregnancy rates from 35% up to 48%, particularly in first and second lactation cows (Larson *et al*. 2007). However, supplemental progesterone does not always up-regulate the pregnancy rate (Villarroel *et al.* 2004); it is possible that the up-regulation is limited to low fertility cattle with low circulating concentrations of progesterone.

It was identified that for *in vitro*-produced blastocysts, post-hatching conceptus elongation does not

occur until the blastocyst is transferred into the uterus (Brandao *et al.* 2004, Alexopoulos *et al.* 2005). These observations suggest that the uterine environment plays a key role in embryo development, and one main regulator is undoubtedly progesterone. One that progesterone regulates at the uterine endometrium is the insulin-like growth factor (IGF) family. In this IGF family, there are two ligands, IGF1 and IGF2, two receptors, IGF1R and IGF2R, and also six binding proteins, insulin-like growth factor binding proteins IGFBP1 to IGFBP6 (Clemmons 1997). *IGF1*-ablation mice fail to ovulate (Lucy 2000), while in bovines the expression of IGF1 on the endometrium is not altered by progesterone either in estrous cycle or early pregnant period (McCarthy *et al.* 2012). In addition, it is identified that IGF2 and IGFBP2 are up-regulated in the endometrium of bovines when treated with progesterone after AI, and are suggested to be associated with conceptus elongation (McCarthy *et al.* 2012). IGF1R, the main receptor of IGFs, was significantly increased in endometrium at the same time (Robinson & Rosenzweig 2004). Not only in AI, but also for the bovine embryo transfer, different mutation in IGF1R gene leads to variations on the superovulation performance and pregnancy rate (Yang *et al.* 2013).

Other responsive molecules are Galectin family, which is recognized by conserved high affinity for β-galactosidases, such as lectin, galactoside-binding, soluble, 9 (LGALS9) and lectin, galactoside-binding, soluble, 3 binding protein (LGALS3BP) (Forde & Lonergan 2012). LGALS9 and LGALS3BP are expressed at low level in both cyclic and pregnant endometrium until Day 13 and involved in the uterine receptivity and implantation in the bovines species (Okumu *et al.* 2011). Although LGALS 15 is detected after day 10 of pregnancy in the ovine endometrium, and is essential for the conceptus growth (Satterfield *et al.* 2006), migration and attachment (Farmer *et al.*2008), it was not found in the bovine endometrium (Gray *et al.* 2004, Lewis *et al.* 2007). In bovines, progesterone supplementation increases the length of conceptus (Garrett *et al.* 1988) and interferon tau (IFNT) production (Mann *et al.* 2006). These observations suggest that kinds of gene expression and/or

response to those molecules are not the same between ovine and bovine species. Further experiments are much needed in the bovine species if we were to improve pregnancy rates in cattle.

IFNT

Using the Affymetrix microarray (Forde *et al*. 2010) and RNA-seq technology (Mamo *et al*. 2012), changes in endometrial gene expressions between the two stages of pre-implantation were compared; day 7 (day 0=estrous cycle) to those during the initiation of the conceptus elongation on day 13. Results of these studies have revealed similar changes in endometrial gene expressions during the cyclic and pregnant animals before the initiation stage of conceptus elongation. These observations suggest that although progesterone is absolutely required for pregnancy establishment, definitive uterine responses await sufficient embryo development, because the initiation of conceptus elongation coincides with its own production of the key cytokine IFNT and changes in uterine gene expression (Imakawa *et al*. 2013).

IFNT is one of the Type I interferons (IFNs), which is well known as a defense against pathogen infection and viruses, and the members share common receptors of Type I IFN (Li & Roberts 1994). IFNT is produced by trophectoderm cells of the ruminants' conceptuses at the pre-implantation stage. It is well known that IFNT is essential for preventing corpus luteum (CL) regression, in what is considered as the maternal recognition signal, and stimulates the endometrium to better accommodate for conceptus attachment through changing the expression of some related factors (Spencer & Bazer 1996). In comparisons of equivalent antiviral units of ovine IFNT and interferon alpha (IFNA), anti-luteolytic problems are found to be specific of IFNT but not IFNA, although they share similarities in nucleotides and functional homology (Green *et al.* 2005). In rats, IFNT is shown to exhibit other functions; for example, it is reported that IFNT is associated with obesity (Bazer *et al.* 2012).

The investigations of molecular mechanism of *IFNT* genes show that *IFNT* is up-regulated when the upstream is bound by cAMP response element binding protein-binding protein (CREBBP) (Xu *et al.* 2003), ETS2 (Ezashi *et al.* 2008), AP-1 (Ezashi & Roberts 2004), GATA2/3 (Bai *et al.* 2009) and CDX2 (Imakawa *et al.* 2006, Sakurai *et al.* 2009), while it is down-regulated when bound by EOMES (Sakurai *et al.* 2012). CDX2 also regulates the expression of *IFNT* through the recruitment of CREBBP, resulting in the histone modification of the *IFNT* gene (Sakurai *et al.* 2010). In the bovine genome (Btau4.0), there are 38 type I *IFN* genes registered, but the expression of only two *IFNT* transcripts, *IFNT1* and *IFNTc1*, by bovine conceptuses were found in utero during pre-implantation period by, and the amounts of *IFNT1* are found to be higher than those of *IFNTc1* in days 17 and 20 *in vivo* (Sakurai *et al.* 2013). The transfection experiment suggests that *IFNT1* and *IFNTc1* share almost the same transcription factors, except the binding site of far upstream AP-1.

Many factors induced by IFNT are identified as markers for uterine receptivity in bovines (Forde & Lonergan 2012). Some of these are the classical Type I IFN stimulated genes (ISGs); such as MX1, MX2 and ISG15 (Hicks *et al.* 2003, Austin *et al* 2004), while some others are associated with cell adhesion; such as connective tissue growth factor (CTGF), as well as cell remodeling related genes; such as matrix metalloproteinase 19 (MMP19) and tissue inhibitor of metalloproteinase 2 (TIMP2). Another factor induced by IFNT is a chemokine, IFN-γ-Inducible Protein 10 kDa (IP-10), secreted during early pregnancy period of ovine, which diffuses from the endometrium (Nagaoka *et al.* 2003). Secreted IP-10, binding to the receptor CXCR3 expressed on the trophoblast cells, is essential for trophoblast migration, apposition and initial adhesion.

Implantation period

After the sufficient communication between the conceptus and uterus during pre-implantation stage, a

stable attachment (implantation period) is followed.

Integrins

Integrins, a family of transmembrane glycoproteins, are composed of 24 αβ heterodimeric subtypes and play roles in the cell-cell and cell-extracellular matrix attachment involved in the migration and adhesion processes (Gille & Swerlick 1996). During bovine implantation period there are many subunits of integrins identified. In pregnant bovine trophoblast cells, Integrin α_1; is down-regulated as attachment proceeds (Maclntyre *et al.* 2002), and acting together with β_1 to form $\alpha_1\beta_1$, is reported associated with human embryo attachment process using an *in vitro* attachment model (Hall *et al*. 1990). It has been shown that integrin α_3, localized on the binucleate cells, may be associated with the binucleate fusion to the uterine epithelial cells (Maclntyre *et al.* 2002), and may possibly mediate heterotypic cell-cell adhesion in keratinocytes (Symington *et al*. 1993). Recently, Kimmins and co-workers (2004) confirmed that integrin $\alpha_v\beta_3$ is localized in the subepithelial stroma of intercaruncular regions during the peri-implantation period, but not in trophoblast cells at any stage of bovine pregnancy. These results suggest that integrin $\alpha_v\beta_3$ plays a role in the uterine epithelium-stromal signaling events that remodel the maternal environment for conceptus attachment.

ECM

Extracellular matrix (ECM) is generally recognized as the ligand of integrins, and is composed of collagen, fibronectin, laminin and osteopontin. Interacting with the ECM glycoproteins, integrins transduce signaling through the pathway from the extra domains to the intracellular domains, and remodel the cytoskeleton and other inner cell proteins (Copplino & Dedhar 2000). In the bovine, collagen IV and laminin are detected in the basal lamina and stroma, and are up-regulated as pregnancy proceeds, while in the trophoblast they are only observed on day 18 of pregnancy but not at later stages. These results suggest that collagen IV and laminin together with their receptor integrin α_1 are

associated with the conceptus attachment to the uterine epithelium and binucle cell migration and fusion (Maclntyre *et al.* 2002). Fibronectin 1 (FN1) is a glycoprotein component of the ECM causing cell migration and cell adhesion through cytoskeleton organization during cancer development/progression (Humphries 1993), wound healing (Nishida 2012), thrombosis (Maurer *et al.* 2010), and other physiological or pathological processes. The expression pattern of fibronectin investigated in the bovine embryos indicates that fibronectin is associated with blastocyst formation (Goossens *et al.* 2007). In many species, osteopontin (SPP1) is identified to be essential for embryo implantation through interactions with the receptor integrin $\alpha_v\beta_3$. For example, the SPP1-integrin $\alpha_v\beta_3$ complexes are demonstrated to promote trophectoderm cell adhesion and migration in the porcine (Erikson *et al.* 2009), and blocking integrin $\alpha_v\beta_3$ prevents embryo implantation in mice (Illera *et al.* 2000), as well as in humans (Apparao *et al.* 2001) and sheep (Johnson *et al.* 2001). However, SPPI and integrin $\alpha_v\beta_3$ do not co-localize at the fetal-maternal interface in the bovine species (Kimmins *et al.* 2004). Results from these observations reveal that molecular mechanisms of pregnancy establishments differ between species, even within the ruminant ungulates, and suggest that such mechanisms must be studied in the bovine species, if the pregnancy rate in this species is to be improved.

Problem

The pregnancy rate of bovines has been declining every year, and half of all failed pregnancies are due to the communication deficiency between early embryo/blastocyst and uterus during the peri-implantation period. Unfortunately, all of these investigations summarized above still do not fully explain implantation processes of the bovine species, because experiments to elucidate the mechanism, by which implantation processes proceed successfully, is very difficult to conduct. One major obstacle is the difficulty in getting appropriate tissues or cell samples at implantation stage in

cattle. As ruminants, the ovine shares similar pregnancy mechanism with bovine to a certain degree; for example, they have the same maternal recognition factor, IFNT, and form a similar type of placenta. There are however substantial differences between them; for example, in the subtype of IFNT (Sakurai *et al*. 2013), or in the expression patterns of other genes such as LGALS 15 (Gray *et al*. 2004, Lewis *et al.* 2007) and integrin $\alpha_v\beta_3$ (Erikson *et al.* 2009, Kimmins *et al.* 2004). On the other hand, it is hard to investigate the mechanism of implantation processes *in vivo*, and there are few real *in vitro* models to accurately simulate the implantation events *in vivo*.

Despite the issues mentioned above, the mechanisms of pre-implantation, not peri-implantation, have been well characterized. In particular, the endometrial gene expression pattern is unchanged between estrous and pre-implantation period before day 13 (Forde *et al*. 2010). These suggest that at least until this stage of pregnancy, the uterine gene expression between cyclic and pregnant animals is regulated by progesterone in a similar manner. However, once the conceptus starts to elongate, the difference in uterine gene expression surfaces. Furthermore, transcriptomic analysis of bovine conceptus and endometrium has characterized specific expression patterns of numerous genes stimulated by progesterone and IFNT directly or indirectly, which are associated with conceptus implantation and uterine receptivity (Mamo *et al*. 2012, Forde & Lonergan 2012). However, understanding the mechanism of peri-implantation processes is still far from complete. As bovine pregnancy rates continue to decline, new insights in experimental approaches are necessary.

Primary aim of this study

Therefore, based on the summary of the factors important for conceptus attachment to the uterus during peri-implantation period studied so far, the purpose of this study was to look at the implantation events from the perspective of lymphocyte homing, the phenomenon often seen in the blood stream. A

cascade of events similar to embryo implantation to the maternal endometrium can be found in lymphocytes; lymphocyte rolling in blood stream, attaching, arresting and invading to the endothelium, termed lymphocyte homing. It has been postulated that embryos undergo a similar process at the pre-implantation stage as leukocyte rolling around the endothelium (Dominguez *et al*. 2005). If this is the case, some key molecules between these two processes might be similar. I therefore hypothesized that embryo implantation, particularly the initial events associated with its attachment-adhesion to the uterine epithelium, could be similar to those of lymphocyte homing.

Contents of my dissertation

Based on my problem statement and primary aim of this study, my dissertation consists of three chapters. In chapter 1, I examined the molecular mechanisms associated with the initial event required for conceptus implantation to the maternal endometrium. The initial event seen during the implantation is conceptus attachment, which was studied from one of the cell adhesion molecules, L-selectin (SELL). In this chapter, I studied the conceptus and endometrial expression of SELL and their ligands *in vivo*. Expression and potential regulation was further investigated through the use of *in vitro* endometrial epithelial cells (EECs) and EECs/bovine trophoblast CT-1coculture systems, recently established in the laboratory. In chapter 2, I studied the event following the conceptus attachment to the uterine epithelium, called adhesion. Previously, vascular cell adhesion molecule (VCAM-1), a trans-membrane glycoprotein member of the immunoglobulin (Ig) gene super family required for lymphocyte homing, had not been studied within the context of conceptus adhesion to the uterine epithelium. In this chapter, I examined the expression of VCAM-1 and its receptor ITGA4 in the conceptus and endometrium *ex vivo*, and in order to identify the regulation of VCAM-1, the changes in VCAM-1 transcript and protein expression were further investigated through the use of the coculture system of EECs and trophoblast CT-1 cells. It has been thought that during the implantation

process, the trophectoderm adheres to the uterine epithelium, which is out of character for epithelial cells normally. Thus, the adhesion between trophectoderm and uterine epithelium has long been considered a cell biological paradox. Therefore, in chapter 3, I demonstrated that changes in gene expression associated with epithelial mesenchymal transition (EMT) occur in the bovine trophectoderm following conceptus attachment to the luminal epithelium. It should be noted that during the course of my dissertation studies, all bovine samples were collected at Zen-noh Embryo Transfer (ET) Center, Kamishihoro, Hokkaido, Japan. In particular, estrous synchronization, ovulation, *in vitro* fertilization (IVF), embryo transfer (ET), and slaughter were done at ET center. Endometrial samples, conceptus collection, uterine flushing collection, and uterine/conceptus fixation were initially done by me or Dr. Imakawa at ET Center, which were transferred to the Laboratory of Animal Breeding, Graduate School of Agricultural and Life Sciences, The University of Tokyo, for further processing and the execution of various experiments described in Chapter 1, 2, and 3.

Chapter 1

Endometrial selectins are associated with bovine conceptus attachment to the uterine epithelium

Abstract

In placental mammals, blastocyst implantation to the maternal endometrium is essential for pregnancy to succeed, however, initial interaction between blastocyst and uterine epithelium has not been well characterized. Objectives of this study were to determine if selectins and their ligands were expressed in the bovine conceptus and/or uterus during the peri-attachment period, and to study whether selectins were associated with conceptus attachment to the uterine epithelium. Through the RNA-seq analysis of bovine conceptuses on days 17, 20, and 22 (day 0=day of estrus), only L-selectin (SELL) ligand, podocalyxin (*PODXL*), was found in these conceptuses. Quantitative PCR analysis confirmed the presence of *PODXL* in these conceptuses, and revealed that SELL mRNA detected in the uterine epithelium, but not in conceptuses, increased during the peri-attachment period. In the cultured endometrial epithelial cells (EECs), *SELL* transcript was up-regulated when uterine flushings from day 20 pregnant animals were placed onto these cells. *SELL* was also up-regulated when cultured EECs were treated with EGF or bFGF, but not with IFNT, while the protein of SELL was only induced by EGF but not bFGF. In the coculture system with EECs and bovine trophoblast CT-1 cells, *SELL* expression in EECs was effectively reduced by its siRNA, however, IFNT, a marker for CT-1 cell attachment to EECs, was not reduced. These observations suggest that the conceptus could attach to the uterine epithelium through the interaction between endometrial SELL and conceptus PODXL, possibly initiating conceptus attachment process in the bovine species.

Introduction

During peri-implantation period, conceptuses must communicate with the uterine endometrium if they were to survive and proceed for attachment to the uterine epithelium. In cattle, embryonic and fetal losses are approximately 40%, major fraction of which occurs before day 16 following breeding (Dunne *et al.* 2000; Berg *et al.* 2010; Diskin *et al.* 2011). The steroid hormone progesterone (P4) plays a key role in the establishment and maintenance of pregnancy. Available evidence suggests that elevated concentrations of P4 during post-conception period generally result in higher conceptus survival (Garrett *et al.* 1988; Carter *et al.* 2008), although the mechanism of P4 action on conceptus development has not been definitively elucidated. The ruminant trophoblast floats within the uterine lumen following hatching from zona-pellucida, and the bovine blastocyst then elongates up to 20-30 cm before conceptus attachment is initiated. During the pre-implantation period, elongating conceptuses float in the uterine histotroph, resulting from P4-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 development, which in turn affects endometrial gene expression. Despite numerous studies executed in the bovine species, molecular mechanisms associated with their attachment processes, particularly the initial attachment to the endometrial epithelium have not been well characterized. This lack of information results in limited methods to reduce early embryonic losses.

A cascade of events similar to embryo implantation to the maternal endometrium can be found in lymphocytes (Dominguez *et al.* 2005); lymphocyte rolling in blood stream, attaching, arresting and invading to the endothelium, a process known as lymphocyte homing. It is well known that selectins with their ligands are essential for the initial processes of lymphocyte homing (Tedder *et al.* 1990, Lawrence *et al.* 1995). Selectins are a group of three carbohydrate-binding proteins, E-selectin (SELE), P-selectin (SELP) and L-selectin (SELL), encoded by three separate genes (Lasky 1992). SELE expression is restricted to activated endothelium, and SELP can be found on activated platelets and

endothelial cells. SELL was originally discovered as leukocyte adhesion molecule 1 (LAM1), which is expressed on leukocytes, and its ligands are present on endothelial cells. During lymphocyte homing, SELL expression on lymphocytes results in slowing down the rolling speed of lymphocytes in the blood stream. Once lymphocyte rolling slows down, cell adhesion molecules such as integrins (ITGs) become effective in further slowing down, finally leading to lymphocyte arresting (Butcher & Picker 1996). It is also reported that the absence of SELL on lymphocytes significantly affects their ability to migrate out of tall endothelial cells called high endothelial venules (HEV) (Subramanian *et al.* 2012).

SELL is also detected on the outer cellular surface of human blastocysts after hatching from the zona-pellucida, and has been identified as a scaffold molecule that plays a role in human embryo-endometrial apposition process (Genbacev *et al.* 2003; Wang *et al.* 2008; Liu *et al.* 2011). In addition, SELL ligand oligosaccharides are detected using MECA-79 and MECA-452 antibodies, whose epitope structures are closely related to the SELL ligand (Mitsuoka *et al.* 1998). MECA-79 and MECA-452 antigens are found widely on luminal and glandular endometrial epithelia in the human uterus (Lai *et al.* 2005). The subcellular localization of MECA-79 in the human pinopodes is associated with the window of implantation (Nejatbakhsh *et al.* 2012). MECA79 is more highly expressed on endometrial surface epithelium in donor egg recipients with embryonic implantation (Shamonki *et al.* 2006), while low expression has been noted in patients with repeated implantation failure (Foulk *et al.* 2007) and in infertile women (Margarit *et al.* 2009). SELL adhesion system is also identified to be essential for forming and maintaining cell columns during early stages of placenta development and mediating cytotrophoblast emigration from the placenta in humans (Prakobphol *et al.* 2006). The expression and role of SELL during the peri-implantation period in mammals other than those in humans and murine species (Taniguchi *et al.* 1998) have not been well characterized. Unlike these species, implantation in ruminants is non-invasive, and little information is available on whether selectins exist in the bovine uterus or if they play a role on conceptus attachment to the uterine epithelium.

Sequence reads obtained from the previous RNA-seq analysis in days 17, 20, and 22 bovine conceptuses (Nakagawa *et al.* 2013) identified transcripts of selectin ligands; glycosylation-dependent cell adhesion molecule 1 (*GlyCAM-1*) (Imai et al. 1993), *CD34* (Puri *et al.* 1995), podocalyxin (*PODXL*) (Sassetti *et al.* 1998), and *MADCAM1* (Berg *et al.* 1993). In this report, we studied the conceptus and endometrial expression of selectins and their ligands *in vivo*. Expression and regulation along with potential uterine factor was further investigated through the use of *in vitro* endometrial epithelial cells (EECs) monoculture and those with bovine trophoblast CT-1cocultures.

Materials and Methods

Animals, tissue collection, and uterine flushing preparation

All animal procedures in this study were approved by the Committee for Experimental Animals at Zennoh Embryo Transfer (ET) Center and the University of Tokyo. Estrous synchronization, superovulation, artificial insemination (AI) and ET processes were performed as previously described (Ideta *et al.* 2007). For ET processes, seven-day embryos (day 0 = day of estrus) were collected from super-ovulated and artificially inseminated Japanese black cattle. Eighteen blastocysts derived from the superovulation/AI procedure were transferred non-surgically into the uterine horn of nine Holstein heifers (two blastocysts/recipient), ipsilateral to the corpus luteum on day 7 of the estrous cycle. Elongated conceptuses on day 17, 20 or 22 (three animals each) were collected non-surgically by uterine flushing with 500 ml sterile phosphate-buffered saline (PBS, pH 7.2). Conceptuses in the uterine flushing media were obtained by centrifugation at 1,000 x g for 5 min and snap-frozen in liquid nitrogen. Following conceptus removal, the media and those from day 17 cyclic animals (n=3) were further centrifuged at 4,000 g for 5 min to remove cell debris, supernatants were filtered through 0.22 μm membrane, and then samples were stored at -80 ℃ until use. For endometrial tissue collection, uterine biopsy samples (10 x 5 x 5 mm) obtained from day 17 cyclic and days 17, 20 and 22 pregnant animals were washed in physiological saline and snap-frozen immediately. All tissue and uterine flushing samples were transferred to Laboratory of Animal Breeding at the University of Tokyo.

Cell preparation and culture conditions

Bovine uterine endometrial epithelial cells (EECs) (Skarzynski *et al.* 2000) were isolated from Holstein cows that were obtained from a local abattoir, according to protocols approved by the local institutional animal care and use committee as previously reported (Tanikawa *et al.* 2008). In brief, uteri of the early luteal phase (days 2 to 5) were used in this study. The hysterectomized uterine lumen was trypsinized (0.3% w/v) in order to detach the epithelial cells and then EECs were isolated. The isolated EECs were

cultured on collagen type I-coated culture dish in DMEM/F12 (1:1) medium supplemented with 10% (v/v) FBS, 40 units/ml of penicillin, and 40 μg/ml of streptomycin at 37 ℃ under 5% CO2 in humidified air. EECs were used within 4 passages to avoid changes in cell characteristics, specifically down-regulation of steroid receptor expression. The cultured EECs in this study maintained their characteristic intermediate filaments in that epithelial cells expressed an epithelial cell marker, cytokeratin, and a mesenchymal cell marker, vimentin. Cytokeratin-positive epithelial cells frequently acquire vimentin during culture (Zeiler et al. 2007).

Bovine trophoblast CT-1 cells (Talbot *et al.* 2000), a generous gift from Dr. Alan Ealy, Virginia Polytechnic Institute, were cultured on plastic plates coated with Matrigel (Japan BD Biosciences, Tokyo, Japan) at 37 °C in air with 5% CO2 in the medium of Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal bovine serum (FBS, JRH Biosciences, Lenexa, KS, USA) supplemented with 4.5 g/liter D-glucose (Invitrogen, Carlsbad, CA, USA), nonessential amino acids (Invitrogen), 2 mM glutamine (Invitrogen), 2 mM sodium pyruvate (Invitrogen), 55 μM-mercaptoethanol (Invitrogen) and antibiotic/antimycotic solution (Invitrogen).

To generate spheroids, CT-1 cells were cultured in low-attachment culture dishes (Corning, Tokyo, Japan) prior to mono- or coculture at 37 ℃ in air with 5% CO2 (Sakurai et al. 2012). For uterine flushing media preparation, the samples (10 ml from 500 ml uterine flushings) after thawing were concentrated and desalinated through the use of Micron filter device (Ultracel YM-3, Millipore, Billerica, MA). Protein concentrations were determined with the Bradford reagent (BioRad Laboratories, Hercules, CA) and the concentrations adjusted to 1 μg/μl with distilled water. In monocultures, the CT-1 cells placed onto collagen type I-coated 6-well plates were incubated with 10 μg proteins from ovine (day 17 pregnant; P17, Sakurai *et al.* 2012) or bovine uterine flushing (day 17 cyclic; C17, P17, P20, or P22) added in serum-free DMEM for the indicated period. Incocultures, EECs were initially

cultured on 6-well collagen type I-coated plates to reach more than 90% confluence in new born calf serum (NBCS)-containing DMEM, followed by maintenance in serum-free DMEM for 24 h prior tococulture. Thereafter, CT-1 cells (approximately 200 spheroids per well) and 10 μg protein from each uterine flushing were cultured onto a confluent layer of the EECs for the indicated incubation period. Protein concentrations of uterine flushings for the culture system were determined from preliminary examinations. Timing and the degree of force to remove CT-1 cells from the uterine cells were also predetermined, resulting in the collection of CT-1 cells with minimal contamination of uterine cells (Sakurai *et al.* 2012). In brief, after incubation, cells were washed twice with PBS and incubated in PBS for 5 min, and then CT-1 cells were separated from substratum of collagen type I-coated dish (monocultured) or adhering uterine cells (cocultured) with gentle pipetting of PBS. To collect cocultured CT-1 cells without contamination of uterine cells, the cells in PBS were passed through 70 μm cell strainer (BD Biosciences, Tokyo, Japan), and the cells which remained on the membrane were purified further with Percoll gradient method (Sakurai *et al*. 2012). The cell viability was more than 95% when tested for the ability of cells to exclude trypan blue. Mono- or cocultured CT-1 cells were then subjected to RNA isolation.

Transfection of SELL or SELP siRNA

The nucleotide structures of *SELL* and *SELP* siRNA were designed through the use of siDirect program (RNAi, Tokyo, Japan), and the siRNAs were prepared commercially (Sigma-Aldrich, St. Louis, MO, USA). The nucleotide sequences of bovine *SELL* (NM_174182.1) and *SELP* (NM_174183.2) were used to design the siRNA for *SELL* and *SELP* coding regions, respectively, while an unrelated sequence of *EGFP* (EU56363) was used as a negative control.

Bovine EECs were cultured in the medium described in the cell culture section. To evaluate the effects of *SELL* or *SELP* siRNA, the medium was replaced with reduced serum media (Opti-MEM, Invitrogen)

and siRNAs were transfected into EECs using Lipofectamine reagent (Invitrogen) according to the protocol provided by the manufacturer. The concentrations of each siRNA had been predetermined before experimentation.

RNA extraction and Quantitative RT-PCR

Using ISOGEN reagent (Nippon gene, Tokyo, Japan), total RNAs were extracted from days 17, 20 and 22 conceptuses and biopsied uterine endometrial tissues. Total RNAs were also isolated from monocultured EECs, mono- andcocultured CT-1 cells, and siRNA treated EECs. For PCR and real-time PCR analyses, isolated RNA (total 250 ng) was reverse-transcribed to cDNA using ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan) with random primers in a 10 μl reaction volume, and the resulting cDNA (RT template) was stored at 4 ℃ until use.

Reverse-transcribed cDNA (3 μl) was subjected to quantitative real-time PCR (qPCR) amplification using 0.1 units of ExTaq HS polymerase (Takara Biomedicals), ExTaq HS buffer, 0.5 μM of the oligonucleotide primers listed in Table 1, 2.5 mM of dNTP, SYBR green (SYBR Green I Nucleic Acid Gel stain, Takara Biomedicals) as fluorescence intercalater and Rox reference dye (Invitrogen) in a final volume of 20 μl, and PCR amplification was carried out on an Applied Biosystems 7900HT real-time PCR System (Applied Biosystems, Foster City, CA, USA). Amplification efficiencies of each target and the reference gene, bovine beta-actin (ACTB), were examined through their calibration curves and found to be comparable (Bustin *et al*. 2009, Sakurai *et al.* 2013). The thermal profile for qPCR consisted of 40 cycles at 95 ℃ for 10 sec, annealing at 60 ℃ for 20 sec, and extension at 72 ℃ for 40 sec. Average threshold (Ct) values for each target were determined by Sequence Detection System software v1.2 (Applied Biosystems). Each run was completed with a melting curve analysis to confirm the specificity of amplification and the absence of primer dimer.

Immunohistochemistry

The plate of cultured EECs was fixed with 4% paraformaldehyde (PFA) in PBS. After 30 min incubation with 10% normal goat serum, the sections were incubated with mouse monoclonal anti-bovine CD62L antibody, SELL (BAQ92A,1:50 dilution, BOV2046, Monoclonal Antibody Center, Washington State University, WA, USA) overnight at 4 ˚C, or normal mouse IgG (sc-2025, Santa Cruz Biotechnology, Inc.) as negative control. Subsequently, the plate was incubated with goat anti-mouse IgG biotin conjugate (1:400 dilution, B7401, Sigma-Aldrich). The immunoreactivity was visualized by means of fluorescent conjugates of Streptavidin 568 (1:200 dilution, S11223, Invitrogen) and DAPI (4',6-diamidino-2-phenylindole, 1:10000 dilution, D8417, Sigma-Aldrich) for 30 min. They were then examined under light microscope (BX-51, Olympus).

Statistical analysis

All experimental data from the bioassays represent the results obtained from three or more independent experiments each with triplicate assays, expressed as the mean ± SEM. Statistical analysis was performed utilizing ANOVA, followed by a Tukey-Kramer's test or Dunnett's test for multiple comparisons between experimental groups with the StatView statistical analysis software (version 5; SAS Institute Inc.). Differences of $P < 0.05$ were considered to be significant.

Results

Expression of selectin and their ligand transcripts in bovine uteri during the peri-attachment period

RNA-seq (SOLiD3) analysis revealed that among possible SELL ligands, *CD34, GlyCAM-1, MadCAM* and *PODXL*, examined, *PODXL* was expressed in days 17, 20, or 22 bovine conceptuses (Table 1). qPCR analysis revealed that *PODXL* mRNA increased in day 22 conceptuses (Fig. 1A), but significant changes in *PODXL* levels were not found in endometrial tissues during the time period examined (Fig. 1B). In the RNA-seq analysis, *SELL* transcripts were not found in days 17, 20, or 22 bovine conceptuses (Table 1), which was also confirmed by qPCR analysis (results not shown). Execution of qPCR was then directed toward RNA analyses extracted from uterine endometrial tissues. The qPCR analyses in RNAs extracted from bovine uterine endometrial tissues obtained from day 17 cyclic, and days 17, 20 and 22 pregnant animals revealed the presence of selectin transcripts in endometrial tissues. Uterine *SELL* expression was minimal in day 17 cyclic animals, but increased approximately 4-fold between days 17 and 20 of pregnancy and 2-fold in day 22 of pregnancy compared with those in cyclic animals (Fig. 2A), while increase in *SELP* was found in day 10 pregnant uterus, and in days 20 and 22 pregnant uteri (Fig. 2B). *SELE* expression was not found in uterine and conceptus samples during the same period (data not shown).

Up-regulation of SELL in EECs treated with uterine flushings or growth factors

qPCR was executed to determine *SELL* mRNA in RNA extracted from mono-cultured EECs that were treated with bovine uterine flushings from day 17 cyclic, or day 17, 20 or 22 pregnant animals. *SELL* expression was up-regulated 2-fold when cultured EECs were treated with the uterine flushing from day 20 pregnant animals, while the expression of *SELP* did not change in these treatments (Fig. 3A). These results together with those in Fig. 2 indicated that *SELL* was expressed in the uterine endometrial epithelium.

Instead of uterine flushings, EECs were then treated with epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) or IFNT for 48 h (Fig. 3B). *SELL* mRNA in EECs was up-regulated by progesterone, EGF or bFGF treatment, whereas estrogen or IFNT had minimal effects on *SELL* expression.

Up-regulation of SELL in EECs treated with EGF or FGF and the presence of EGFR or FGFR in conceptuses and uterine endometrium

To confirm if the increase in *SELL* transcripts (Fig. 3B) was associated with SELL protein, the EECs treated with EGF (10 ng/ml) was subjected to immunofluorescence staining (Fig. 4A). The expression of SELL in EECs treated with EGF was enhanced when compared with those in EECs without the EGF treatment. Since EGF treatment effectively increased SELL expression in EECs, we next examined the expression of EGF receptor (*EGFR*) transcripts in conceptuses and endometrial tissues. *EGFR* in days 17, 20 and 22 conceptuses increased as pregnancy progressed. Minute amounts of *EGFR* were found in all uterine tissues examined, however, the highest *EGFR* expression was found in day 22 pregnant endometrium (Fig. 4B). Experiments were executed with bFGF, but showing different results from EGF (Fig. 5A).

Expression of IFNT in mono- orcocultured CT-1 cells with uterine flushing-treated EECs, and incocultured CT-1 cells with SELL siRNA-treated EECs

To investigate the function of selectin on conceptus attachment to the endometrial epithelial cells, we used an *in vitro* attachment model of bovine trophoblast CT-1 cells to EECs with slight modifications. In this study, flushing media from bovine uteri were used and changes in IFNT in CT-1 cells were monitored because IFNT down-regulation was indicative of CT-1 cell attachment to EECs (Sakurai *et al.* 2012). Decrease in IFNT was found when uterine flushings from day 20 pregnant animals were tested for the cocultured CT-1 with EECs while decrease in IFNT was not found in monocultured CT-1 cells

(Fig. 6A), which was in agreement with our previous observations (Sakurai *et al.* 2012). *SELL* or *SELP* siRNA was then applied to EECs and its effect on mRNA levels was monitored. Both siRNAs were effective in suppressing respective mRNA (Fig. 6B). Although changes in *IFNT* mRNA were not observed in CT-1 cells cocultured with *SELL* siRNA treated EECs, decrease in *IFNT* mRNA was found in CT-1 cells cocultured with *SELP* siRNA or control siRNA treated EECs (Fig. 6C).

Discussion

In this study, SELL expression was detected on endometrial epithelia and a likely ligand, *PODXL*, was found in both conceptus and endometrium. Prior to this study, we expected that SELL expression would be at the conceptus side, similar to those found in human trophoblasts (Genbacev *et al*. 2003) and at the tips of microvilli of lymphocytes in the lymphocyte homing (Rosen 2004). Lymphocytes are in fact detected in uterine intra-epithelium and stroma of non-pregnant goats, and are decreased in the inter-placentomal areas and inter-caruncular epithelial regions of pregnant uteri (Martínez *et al.* 2005). In addition, SELL is localized on leukocytes located at the endothelial surface of placentomal vessels in day 50 pregnant goats (Muniz *et al.* 2006). These results indicate that SELL expression in lymphocytes is similar among humans, goats and cattle, however, SELL and its ligand expression in the bovine uterus differ from those of humans. In human or murine uteri, the blastocyst migrates until its apposition to the implantation site, whereas the ruminant conceptus elongates before its attachment to caruncular regions of the uterine epithelia, suggesting that slowing down of conceptus migration in the bovine species may not be as important as in human or murine pregnancy.

In the bovine, conceptus attachment to the uterine epithelium begins on day 19-20 of pregnancy (Wathes & Wodding 1980). In this study, the increase in endometrial SELL expression was found as early as on day 17 (Fig. 2), indicating that SELL expression appeared prior to the initiation of conceptus attachment to the uterine epithelium. It has been characterized that soon after elongated conceptus attaches to the uterine epithelium and trophoblast CT-1 cells attach to the EECs, IFNT expression decreases (Sakurai *et al.* 2010, 2012). However, down-regulation of *SELL* expression with its siRNA in the EECs of our coculture system did not cause any reduction in *IFNT* in CT-1 cells (Fig. 4A), indicating that CT-1 cell attachment to EECs did not occur. These results suggest that SELL expression proceeds prior to the conceptus attachment to the uterine epithelium.

Similar to our previous observations in which sheep uterine flushings were examined (Sakurai *et al.* 2012), the uterine flushing from day 20 pregnant cows was effective in the up-regulation of SELL in the uterine epithelial cells. Although components of the uterine flushing have not been carefully characterized, based on our previous observations (Tamada *et al.* 2002, Yamakoshi *et al,* 2012) and others (Sakuma *et al.* 2012), EGF, FGF, and IFNT are likely to be present in the uterine flushings. Each of these factors was then examined if they could up-regulate SELL expression in bovine uterine epithelial cell cultures. EGF and FGF were found to up-regulate SELL expression in our epithelial cell culture system (Fig. 3B). It was reported that EGF and its receptor (EGFR) are essential for the elongation of bovine conceptuses (Kliem *et al.* 1998). In this study, progressive increase in *EGFR* was found in days 17, 20 and 22 conceptuses while *EGFR* at the endometrial sides increased on day 22 (Fig. 5B). Although IFNT was undoubtedly present in day 20 uterine flushings, EGF and bFGF could also represent in the uterine flushing, which up-regulated endometrial epithelial SELL expression. It has been characterized that these steroid hormones and IFNT regulate endometrial gene expression necessary for the establishment of pregnancy in ovine and bovine species (Spencer *et al.* 2007, Forde & Lonergan 2012). It should be noted in this study that IFNT was not involved in endometrial epithelial SELL expression. In addition, bFGF has been shown to enhance the rate of blastocyst attachment in mice (Taniguchi *et al.* 1998). Moreover, among many factors that have been found to participate in EMT inducers (Said & Williams 2011), EGF and bFGF are also involved in EMT processes. Together with the observation of *PODXL* up-regulation, increases in EGFR found in both day 22 conceptuses and endometrial tissues suggest that EMT and related changes in endometrial environments are required for the conceptus implantation to the maternal endometrium.

Following initial attachment between trophectodermal epithelium and endometrial epithelium on day 19-20 of bovine pregnancy, stable adhesion occurs between days 20 and 22 (Wathes & Wooding 1980). These observations led us to identify a phenomenon, the epithelial mesenchymal transition (EMT),

occurring in bovine conceptuses on day 22 when the trophectoderm expresses both epithelial and mesenchymal markers (Yamakoshi *et al*. 2012). In this study, despite high *SELL* expression in day 17 pregnant endometrium, up-regulation of its ligand, *PODXL*, in the conceptus did not occur until day 22. This does not exclude the possibility that SELL is involved in the initial attachment process between the conceptus and endometrial epithelium. It has been shown numerous times that SELL is associated with the slowing down of lymphocyte rolling, the event necessary for lymphocyte homing (Dominguez *et al.* 2005). In addition, PODXL has been found as a markedly up-regulated protein in TGFB induced EMT of human lung adenocarcinoma A549 cells (Meng *et al.* 2011). These investigators concluded that PODXL is an important regulator of the EMT like process as it regulates the loss of epithelial features and the acquisition of a motile phenotype. Together with those found by Meng and coworkers (2011), our observations suggest that endometrial epithelial SELL and conceptus PODXL are involved in the initial aspects of trophoblast attachment to the uterine epithelium, and that PODXL is also involved in trophoblast EMT required for stable adhesion of two cell types.

In conclusion, results from this study suggest that one of the cell adhesion molecules, SELL, is involved in the initial step in conceptus attachment to the uterine endometrium. SELL and its ligand PODXL could also function as molecules necessary for EMT processes, resulting in stable attachment/adhesion between two cell types.

Table 1 Selectin and its ligand related transcripts found from RNA-seq analysis

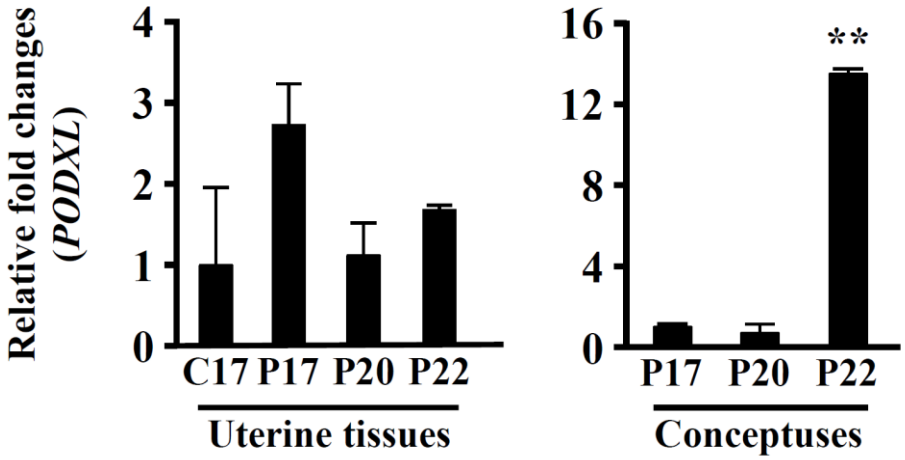
			RPKM		
	Transcript ID	**Gene name**	**D17**	**D20**	**D22**
SELL	ENSBTAT00000046865	LYAM1_BOVIN	0	0	0.119
SELE	ENSBTAT00000009612	LYAM2_BOVIN	0	0	0
SELP	ENSBTAT00000046869	LYAM3_BOVIN	0	0	0.069
SELPLG	ENSBTAT00000029578	NP_001032717.1	3.509	2.043	0.275
CD34	ENSBTAT00000013963	NP_776434.1	0.210	0.276	0.575
GlyCAM	ENSBTAT00000017854	GLCM1_BOVIN	0	0	0
MADCAM	ENSBTAT00000043399	NP_001032910.1	0	0	0.048
PODXL1	ENSBTAT00000013799	PODXL	29.130	1.342	186.821
PODXL2	ENSBTAT00000043559	IPI00841275.1	0.389	1.453	0.693

Table 2 Primers used to detect selectin, selectin ligand, EGF and bFGF

Name (GenBank accession No.)	Sequence
SELE (NM_214268.1)	F: 5'- CGTTGACGGGGCCAACCCAG -3'
	R: 5'-GGGACACAGTCGGAGCTTCACA -3'
SELL (NM_174182.1)	F: 5'-ACAGCCCTCTGCTACACAGCTTC -3'
	R: 5'-GGGGCCTCCAAAGGCACACA -3'
SELP (NM_174183.2)	F: 5'-GGACAGCCCCAACTCCGGTG -3'
	R: 5'-TCGCCAAAGGGATGCGAGCA -3'
PODXL (XM_002686960.2)	F: 5'-TCTGTCCTCCTCGACCAACCTC -3'
	R: 5'-TGCGACCTGTGTTTTCAGTGTGGT -3'
EGF (XM_002688103.2)	F: 5'- GATCCTGCGTCCTTGGAACA -3'
	R: 5'- ACAAAGGGTGAGCTGATGGG -3'
EGFR (XM_002696890.1)	F: 5'-TCCTTCACACGTACTGCACC -3'
	R: 5'-GAGAAAACTGGCCATGCTGC -3'
bFGF (NM_174056.3)	F: 5'- AAGCGGCTGTACTGCAAGAA -3'
	R: 5'- ACACTCGTCTGTAACACATTTAGAA -3'
FGFR1 (NM_001110207)	F: 5'- GATGATGACGACGATGACTC -3'
	R: 5'- CATAACGGACCTTGTAGCCT -3'
IFNT (AF238613)	F: 5'-CATCTTCCCCATGGCCTTCG -3'
	R: 5'-TCATCTCAAAGTGAGTTCAG -3'
ACTB (NM_173979.3)	F: 5'-TCCCTGGAGAAGAGCTACGA -3
	R: 5'-GGGCAGTGATCTCTTTCTGC -3'

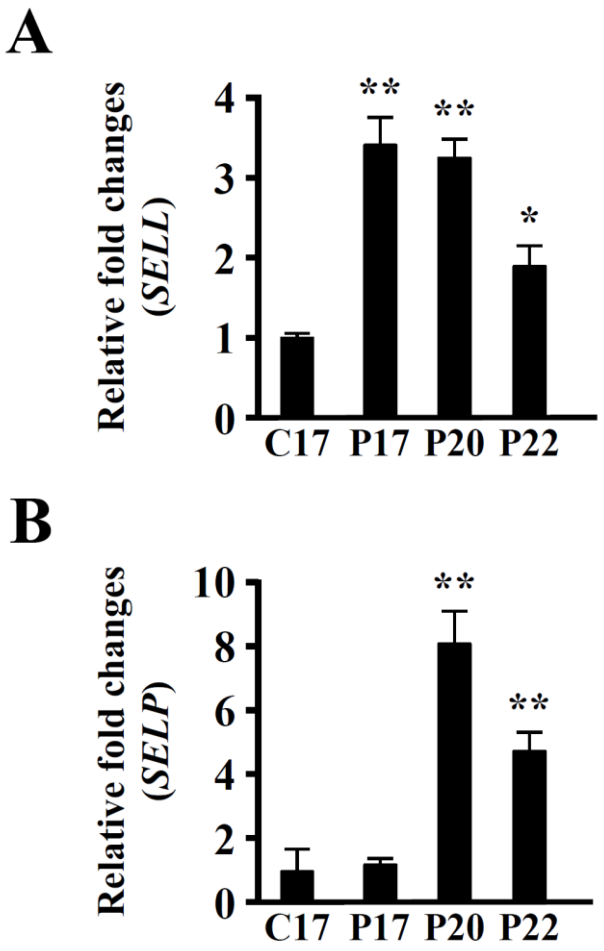
F: Forward primer; R: Reverse primer

Figure 1



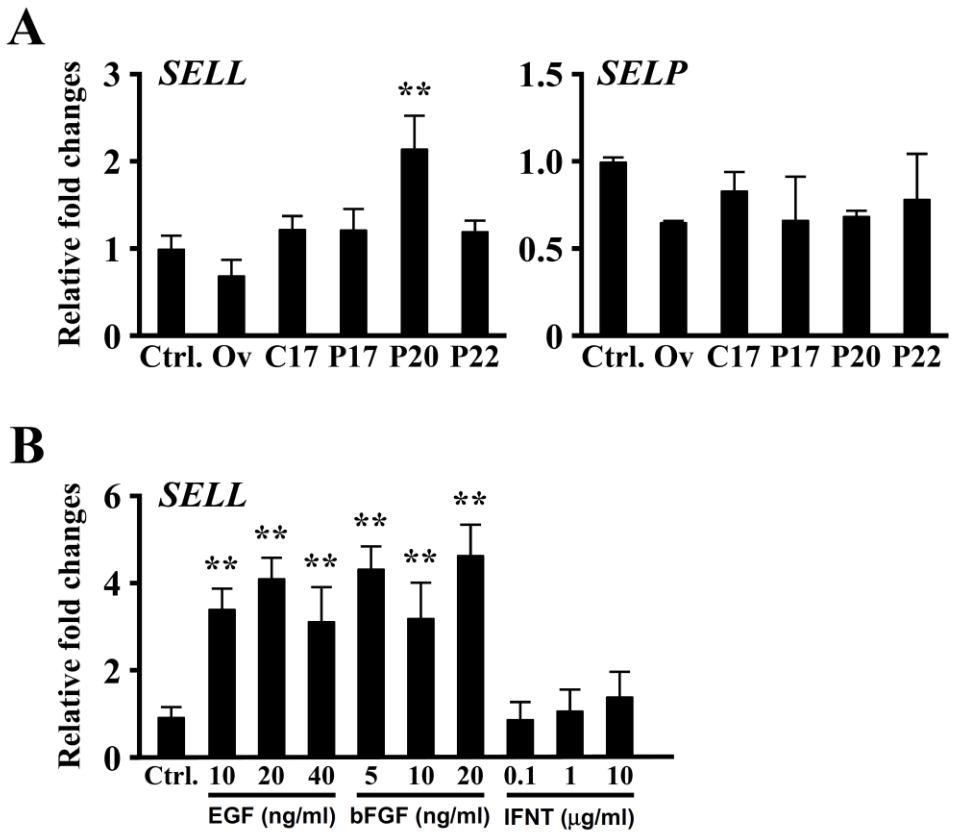
Detection of *PODXL* transcripts in conceptuses and endometria during the peri-attachment period. A: Total RNAs extracted from days 17, 20, and 22 conceptuses (n=3 each day) were subjected to qPCR for *PODXL* transcripts. B: Total RNAs extracted from day 17 cyclic, and days 17, 20 and 22 pregnant endometria (n=3 each day) were subjected to qPCR for *PODXL* transcripts. *ACTB* mRNA was used as an internal control for RNA integrity. Values represent mean ± SEM from three independent samples with duplicates within a day of conceptus or endometrial tissue sampling. **, statistically significant differences in mRNA levels ($P < 0.01$ vs. the value of day 17 conceptuses).

Figure 2.



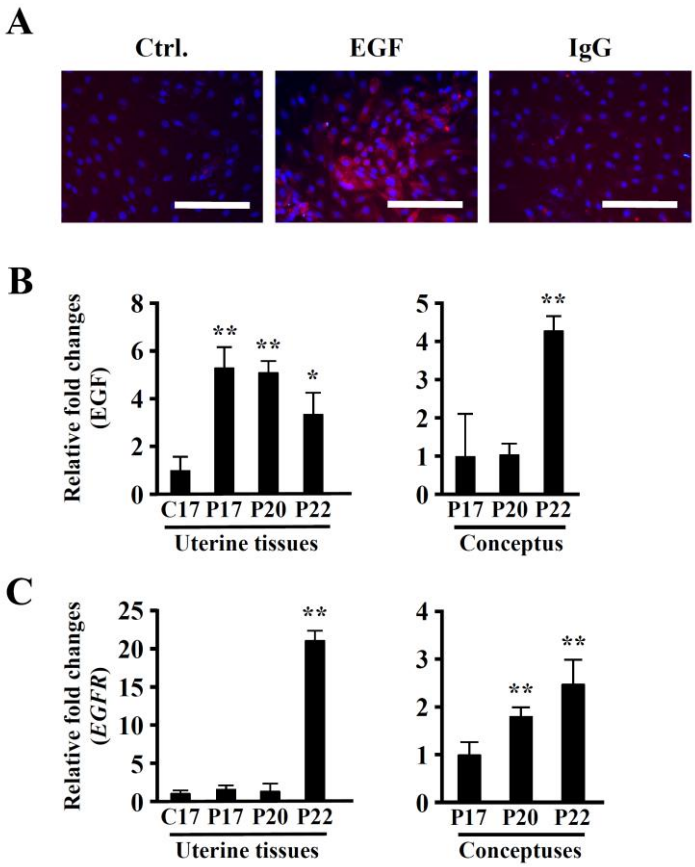
Selectin mRNAs in the bovine uterus during the peri-attachment period. Total RNAs extracted from non-pregnant (day 17; C17) and pregnant (days 17, 20 and 22; P17, P20 and P22, respectively) endometria (n=3 each day) were subjected to quantitative RT-PCR (qPCR) analyses for the detection of *SELL* (A), or *SELP* (B) transcripts. *ACTB* mRNA was used as an internal control for RNA integrity. Values represent mean ± SEM from three independent samples with duplicates within a day of endometrial tissue sampling. *and**, statistically significant differences in mRNA levels ($P < 0.05$ and $P < 0.01$, respectively vs. the value of day 17 cyclic animals).

Figure 3.



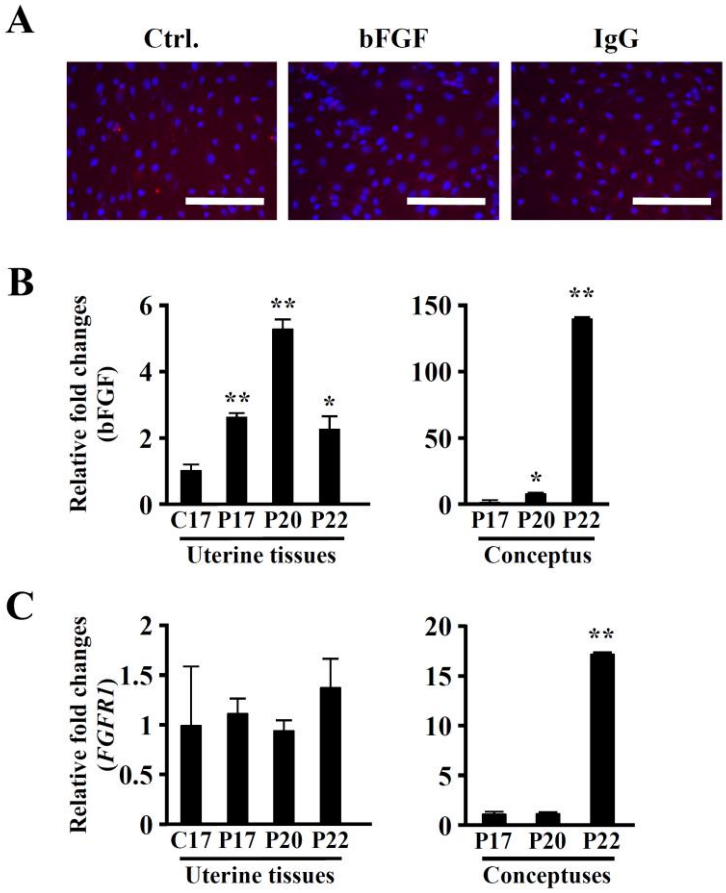
Changes in *SELL* or *SELP* transcripts in bovine endometrial epithelial cells cultured with uterine flushings. A: Levels of *SELL* and *SELP* mRNAs in monocultured endometrial epithelial cells (EECs) treated with ovine uterine flushing obtained from pregnant sheep (day 17; Ov), or bovine uterine flushing from cyclic (day 17; C17) or pregnant animals (day 17, 20 or 22; P17, P20 or P22, respectively). B: Up-regulation of *SELL* transcripts in monocultured EECs treated with EGF (10, 20, 40 ng/ml), bFGF (5, 10, 20 ng/ml), or IFNT (0.1, 1, 10 μg/ml) for 48 h in the serum-free medium. *ACTB* mRNA was used as an internal control for RNA integrity. Values represent mean ± SEM from three independent experiments with triplicates within each experiment. **Statistically significant differences in mRNA levels ($P < 0.01$) within a day of uterine flushing or dose of EGF or bFGF.

Figure4.



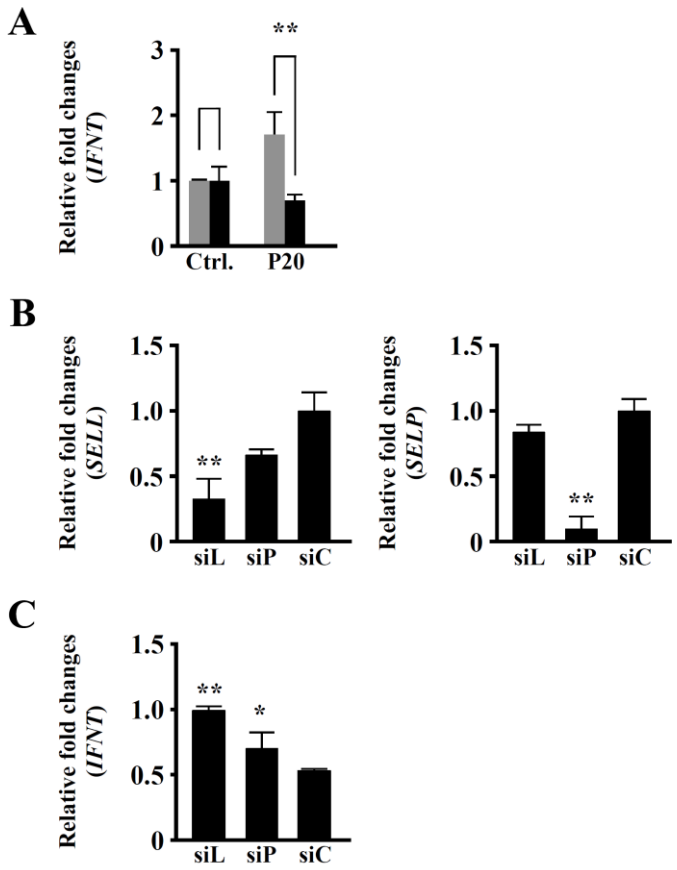
Immunofluorescence analysis of SELL in bovine endometrial epithelial cells (EECs) treated with EGF, and detection of EGFR transcripts in conceptuses and endometria during the peri-attachment period. A: Expression of *SELL* in monocultured EECs treated with EGF (20 ng/ml) for 48 h. Left: EECs cultured without EGF (Ctrl.), Middle: EECs cultured with EGF (EGF), and Right: a negative control (IgG). SELL expression is seen in red, nuclei was counterstained with DAPI, and the pre-immune serum was applied for the negative control. Three independent experiments were executed and the representative one is shown. Scale bar=200 μm. B: Changes in *EGF* expression in bovine uteri. Left: Changes in *EGFR* transcripts in endometria of day 17 cyclic (day 17; C17) or pregnant animals (day 17, 20, or 22; P17, P20, or P22, respectively). Right: Changes in *EGF* transcripts in days 17, 20 and 22 conceptuses (P17, P20, P22, respectively) (n=3 each day). C: Expression of *EGFR* in bovine uteri. Left: Changes in *EGFR* transcripts in endometria of day 17 cyclic (day 17; C17) or pregnant animals (day 17, 20, or 22; P17, P20, or P22, respectively). Right: Changes in *EGFR* transcripts in days 17, 20, and 22 conceptuses (P17, P20, P22, respectively) (n=3 each day). *ACTB* mRNA was used as an internal control for RNA integrity. Values represent mean ± SEM from three independent samples with duplicates within a day of conceptus or endometrial tissues. **Statistically significant differences in mRNA levels ($P < 0.01$ vs. the value of day 17 conceptuses or the endometrium from day 17 cyclic animals).

Figure 5.



Immunofluorescence analysis of SELL in bovine endometrial epithelial cells (EECs) treated with FGF, and detection of *EGFR* transcripts in conceptuses and endometria during the peri-attachment period. A: Expression of SELL in monocultured EECs treated with FGF (10 ng/ml) for 48 h. Left: EECs cultured without bFGF (Ctrl.), Middle: EECs cultured with FGF (FGF), and Right: a negative control (IgG). SELL expression is seen in red, nuclei was counterstained with DAPI, and the pre-immune serum was applied for the negative control. Three independent experiments were executed and the representative one is shown. Scale bar=200 μm. B: Changes in bFGF expression in bovine uteri. Left: Changes in *bFGFR* transcripts in endometria of day 17 cyclic (day 17; C17) or pregnant animals (day 17, 20, or 22; P17, P20, or P22, respectively). Right: Changes in *bFGF* transcripts in days 17, 20 and 22 conceptuses (P17, P20, P22, respectively) (n=3 each day). C: Expression of *bFGFR* in bovine uteri. Left: Changes in *bFGFR* transcripts in endometria of day 17 cyclic (day 17; C17) or pregnant animals (day 17, 20, or 22; P17, P20, or P22, respectively). Right: Changes in *bFGFR* transcripts in days 17, 20, and 22 conceptuses (P17, P20, P22, respectively) (n=3 each day). *ACTB* mRNA was used as an internal control for RNA integrity. Values represent mean ± SEM from three independent samples with duplicates within a day of conceptus or endometrial tissues. **Statistically significant differences in mRNA levels ($P < 0.01$ vs. the value of day 17 conceptuses or the endometrium from day 17 cyclic animals).

Figure 5.



Changes in *IFNT* transcripts in CT-1 cells mono- orcocultured with endometrial epithelial cells (EECs), and effects of *SELL* or *SELP* knock-downed EECs on *IFNT* expression in cocultured CT-1 cells. A: Changes in *IFNT* mRNAs in CT-1 cells mono- (grey bar) orcocultured (dark, solid bar) with EECs treated with uterine flushings obtained from day 17 cyclic (day 17; C17) or pregnant animals (day 20; P20). In the coculture, bovine EECs were grown to confluence, and CT-1 cells and uterine flushings (10 μg) were then placed on the EECs, which were further cultured for 48 h. Total RNAs extracted from the CT-1 cells were subjected to qPCR analysis for *IFNT* mRNA. *ACTB* mRNA was used as an internal control for RNA integrity. B: Effects of *SELL* or *SELP* siRNA on endogenous *SELL* or *SELP* transcripts in EECs. EECs were treated with *SELL* siRNA (siL), *SELP* siRNA (siP), or control siRNA (siC; *EGFP*), and were cultured for 48 h. Three independent experiments were executed and the representative one is shown. C: Bovine EECs treated with *SELL* siRNA (siL), *SELP* siRNA (siP) or control siRNA (siC) for 48 h were cocultured with CT-1 cells for another 48 h. Total RNAs were extracted from the CT-1 cells and subjected to qPCR analysis for *IFNT* mRNA. *ACTB* mRNA was used as an internal control for RNA integrity. Values represent mean ±SEM from three independent samples with duplicates within a day of uterine flushing (A), or siRNA treatment (C). *and**, statistically significant differences in mRNA levels ($P < 0.05$ and $P < 0.01$, respectively vs. the value of the control without uterine flushing or siRNA treatment; far left in the graph A or C).

Chapter 2

Involvement of vascular cell adhesion molecule (VCAM-1) in the bovine conceptus adhesion to the uterine endometrium

Abstract

Following biochemical communication, the conceptus and the uterus must establish the proper cell-cell interaction, resulting in the progression of implantation processes. Although conceptus implantation to the maternal endometrium is well- studied in murine species, those in the bovine have not been sufficiently characterized. To clarify the mechanism of conceptus attachment, we focused on vascular cell adhesion molecules (VCAM-1) to determine if it was expressed in bovine conceptuses or endometrium during the peri-attachment period. Uterine *VCAM-1* expression was minimal in day 17 (day 0 = day of estrus) cyclic and pregnant animals, but increased between days 20 and 22 of pregnancy. In cultured endometrial epithelial cells (EECs), *VCAM-1* expression was up-regulated when treated with growth factor or uterine flushings, while its protein was increased in the cell surface of EECs that werecocultured with bovine trophoblast CT-1 cells. *VCAM-1* expression in CT-1 cells was also up-regulated with the use of uterine flushings, and further increased when these cells werecocultured with EECs. Expression of VCAM-1 receptor, integrin alpha 4 (*ITGA4)*, increased significantly in day 22 conceptuses. Immunohistochemical detection revealed that in day 22 pregnant uteri, VCAM-1 protein was found in both EECs and conceptuses, but ITGA4 was localized only in trophoblasts. These observations indicate that in the bovine species, cell-cell interactions between conceptuses and uterine epithelial cells are required for sufficient VCAM-1 and ITGA4 expressions, and suggest that uterine VCAM-1 and conceptus ITGA4 play a role in the establishment of conceptus adhesion to the uterine endometrium.

Introduction

In the bovine, a significant fraction of embryonic losses occurs during peri-implantation period, resulting from insufficient biochemical communication between the elongating conceptus and the uterus (Diskin & Morris 2008). It is well documented that cell-cell interactions and integrin (ITG)-mediated signaling between the conceptus and endometrium are critical for successful implantation. Specifically, the extracellular domain of ITGs acts as a receptor for extracellular complements (ECMs) such as fibronectin, osteopontin (SPP), laminin, collagen-type IV, and complements (Akiyama 1996). In addition to ECMs, constituents of uterine histotroph, IP-10 (CXCL10), galactoside-binding, soluble, 15 (LGALS 15), and IGFBP1, have been characterized to activate ITGs through their RGD domain during the trophectoderm attachment period in goats, sheep, and cattle (Nagaoka *et al*. 2003, Farmer *et a*l. 2008, Simmons *et al*. 2009). In the bovine species, the expression of ITGs has been characterized at the uteroplacental interface during the periods of trophectoderm attachment (MacLaren & Wildemen 1995, MacIntyre *et a*l. 2002) and placentation (Pfarrer *et al*. 2003). In the stages of bovine trophoblast giant cell migration and fusion with the uterine epithelial cells–characterizes ITGs consist of five α subunits (*ITGA2B*, *ITGA3*, *ITGA5*, *ITGA8* and *ITGAV*) and two β subunits (*ITGB1* and *ITGB3*) (Pfarrer 2006). In our previous investigation (Yamakoshi *et al*. 2012), integrin subunits α (*ITGAV*, *ITGA5*) and β (*ITGB1*, *ITGB3* and *ITGB5*) are constitutively expressed in bovine peri-attachment trophoblast cells, whereas the expression of *ITGA4* and *ITGA8* subunits is induced after attachment of trophoblast cells to uterine EECs. In spite of these efforts, our knowledge is not sufficient to improve conceptus attachment and adhesion to the uterine epithelium in the bovine species.

Vascular cell adhesion molecule (VCAM-1), a trans-membrane glycoprotein member of the immunoglobulin gene superfamily (Osborn *et al*. 1989), is known to be present in the reproductive tracts of mammalian species. In humans, for example, VCAM-1 is present on the endometrial side,

specifically localized on decidual stromal cells in the areas where migrating blastocysts are present, but not present on vascular endothelial cells in decidua parietalis. Endometrial expression of VCAM-1 at the peri-implantation stage of patients with unexplained infertility was significantly lower than in control patients (Konac *et al.* 2009), suggesting that the expression of VCAM-1 might be essential for the preparation of the endometrium for invasive blastocyst implantation. In *VCAM-1* gene ablation study (Gurtner *et al*. 1995), most *VCAM-1*-deficient embryos suffer embryonic loss due to a failure in the formation of normal allantoic mesoderm, their distribution and/or the subsequent placental defects. In the study of early pregnancy in sheep, VCAM-1 is first found in endothelial cells on days 17-19 in both caruncular and intercaruncular areas of the endometrium, and becomes strongly induced in endothelial cells on 26-27 days (Rahman *et al.* 2004). However, VCAM-1 in the uterine endometrium and/or conceptuses in the context of cell-cell interactions has not been studied during early pregnancy period in limited-invasive implantation of ruminants.

VCAM-1, induced by various cytokines in different organs in mice (Henninger *et al.* 1997), always functions by means of Very Late Antigen-4 (*VLA4*), integrin α4β1 (*ITGA4*/ *ITGB1*), and integrin α4β7 (*ITGA4*/*ITGB7*). Homozygous loss of *ITGB1* expression in mice was lethal during early post-implantation development, resulting in inner cell mass failure (Stephens *et al.* 1995). It was also identified that homozygous *ITGA4* null knockout mice fail to complete fusion of the allantois with the chorionic membrane during placentation period (Yang *et al.* 1995). In our previous investigation on bovine conceptuses, *ITGA4* mRNA was found at elevated expression levels on day 22, 2-3 days after the initiation of trophoblast attachment to the endometrial epithelium (Yamakoshi *et al.* 2012). I also found that changes in trophoblastic gene expression, including *ITG*s, were seen when bovine trophoblast CT-1 cells werecocultured with endometrial epithelial cells (EECs), which was further enhanced with the addition of uterine flushings from pregnant animals (Sakurai et al. 2012). These results suggest that components of uterine flushings/histotroph, including ECMs and various cytokines, as well as cell-cell

interactions are important in the progression of conceptus attachment to the uterine epithelium in the bovine species.

In this study, I therefore examined the expression of VCAM-1 and its receptor ITGA4 in the bovine conceptus and endometrium during the peri-implantation period. Using two types of endometrial epithelial cultures; EECs alone (monoculture), or EECs cultured with bovine trophoblast CT-1 cells (coculture), the experiments were extended to examine if components of uterine flushings and/or cell-cell interaction were required for the regulation of *VCAM-1* transcript and protein expression.

Materials and Methods

Animals, tissue collection, and uterine flushing preparation

All animal procedures in the present study were approved by the Committee for Experimental Animals at Zennoh Embryo Transfer (ET) Center and the University of Tokyo. Estrous synchronization, superovulation and embryo transfer processes were performed as previously described (Ideta *et al*. 2007). Thirty embryos derived from the superovulation were transferred non-surgically into the uterine horn of fifteen Holstein heifers (two blastocysts/recipient), ipsilateral to the corpus luteum on day 7 (day 0 = day of estrus) of the estrous cycle. For RNA analyses, elongated conceptuses were collected non-surgically by uterine flushing on day 17, 20, or 22 (three animals each) with 500 ml sterile phosphate-buffered saline (PBS, pH 7.2). Conceptuses in the uterine flushing media were obtained by centrifugation at 1,000 x g for 5 min and snap-frozen in liquid nitrogen. Following conceptus removal, the media and those from day 17 cyclic animals (n=3) were further centrifuged at 4,000 x g for 5 min to remove cell debris, supernatants were filtered through 0.22-μm membrane, and then samples were stored at -80 ℃ until use. After thawing, the samples (10 ml from 500 ml uterine flushings) were concentrated and desalinated through the use of Micron filter device (Ultracel YM-3, Millipore, Billerica, MA). Protein concentrations were determined with the Bradford reagent (BioRad Laboratories, Hercules, CA) and the concentrations adjusted to 1 μg/ml with distilled water. For immunohistochemistry, hysterectomy was performed on days 20 and 22 (n=3), the uteri containing conceptus tissues were frozen in dry ice-cooled heptane. For endometrial tissue collection, uterine biopsy samples (10 x 5 x 5 mm) were obtained non-surgically from day 17 cyclic and days 17, 20 and 22 pregnant animals, all of which were frozen immediately. Samples were transferred to the Laboratory of Animal Breeding at the University of Tokyo and stored at -80 ℃ until use.

Cell preparation, culture condition

Bovine trophoblast CT-1 cells (Talbot *et al*. 2000), a generous gift from Dr. Alan Ealy, Virginia Polytechnic Institute, were cultured on plastic plates coated with Matrigel (Japan BD biosciences, Tokyo, Japan) at 37 ℃ in air with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (Wako Pure Chemical Industries, Ltd. Japan) containing 10% (v/v) fetal bovine serum (FBS, JRH Biosciences, Lenexa, KS, USA) supplemented with 4.5 g/liter D-glucose (Invitrogen, Carlsbad, CA, USA), nonessential amino acids (Invitrogen), 2 mM glutamine (Invitrogen), 2 mM sodium pyruvate (Invitrogen), 55 μM-mercaptoethanol (Invitrogen) and antibiotic/antimycotic solution (Invitrogen).

Primary bovine endometrial epithelial cells (EECs) were isolated and cultured as previously described (Skarzynski *et al*. 2000). In brief, uteri of healthy Holstein cows were obtained from a local abattoir in accordance with protocols approved by the local Institutional Animal Care and Use Committee (Tanikawa *et al.* 2008). Uteri of the early luteal phase (days 2 to 5) were excised and immediately transported to the laboratory. To detach EECs, the uterine lumen was trypsinized (0.3% w/v), from which EECs were isolated (Skarzynski *et al*. 2000). The isolated EECs were cultured on collagen type IA-coated culture dish in DMEM/F12 (1:1) medium supplemented with 10% (v/v) newborn calf serum (NBCS, Invitrogen), 40 units/ml of penicillin, and 40 μg/ml of streptomycin at 37 ℃ under 5% CO2 in humidified air. EECs were used within 4 passages to avoid changes in cell characteristics, specifically down-regulation of steroid receptor expression. The cultured EECs in this study maintained their characteristic intermediate filaments in that epithelial cells expressed an epithelial cell marker, cytokeratin, and a mesenchymal cell marker, vimentin. Cytokeratin-positive epithelial cells frequently acquire vimentin during culture (Zeiler *et al*. 2007; Sakurai *et al.* 2012). In monocultures, EECs placed onto collagen type I-coated 6-well dishes were incubated with 10 μg proteins from day 22 bovine uterine flushing, EGF (10, 20, 40 ng/ml), bFGF (0.1, 1, 10 ng/ml) or IFNT (0.1, 1, 10 μg/ml) in serum-free DMEM/F12 for 48 h.

Incoculture experimentations, EECs were initially cultured on 6-well collagen type IA-coated plates to reach more than 90% confluence in NBCS-contained DMEM, followed by maintenance culture in serum-free DMEM for 24 h. Thereafter, CT-1 cells (approximately 200 spheroids per well) were added onto a confluent layer of the EECs (Sakurai *et al*. 2012), which were then placed onto a shaker for 48 h. To collect CT-1 cells fromcocultured CT-1/EECs without uterine cell contaminations, the cells in the culture medium were passed through 70 μm cell strainer (BD biosciences, Tokyo, Japan), and the cells which remained on the membrane were purified further with Percoll gradient method (Sakurai *et al*. 2012). The cell viability was more than 95% when tested for the ability of cells to exclude trypan blue. CT-1 cells were then harvested from the culture medium by centrifugation at 400 x g for 4 min, and subjected to RNA isolation.

RNA extraction and Quantitative RT-PCR

Total RNA was isolated from conceptuses, uterine tissues, or cultured CT-1 or EECs with ISOGEN reagent (Nippon gene, Tokyo, Japan) according to the protocol provided by the manufacturer. For real-time PCR analyses, isolated RNA (total 0.5 μg) was reverse-transcribed to cDNA using ReverTra Ace qPCR RT Kit (Toyobo, Osaka, Japan) including 1 X RT buffer, Enzyme Mix, and primer Mx in a 10 μl reaction volume, and the resulting cDNA (RT template) was stored at 4 ℃ until use.

The cDNA reaction mixture was diluted 1:10 using DNase- and RNase-free molecular biology grade water and 3 μl was subjected to real-time PCR amplification using Thunderbird SYBR qPCR Mix Kit (Toyobo, Osaka, Japan) with primers listed in Table 1, and PCR amplification was carried out on an Applied Biosystems 7900HT real-time PCR System (Applied Biosystems, Foster City, CA, USA). The thermal profile for real-time PCR consisted of 40 cycles at 95 ℃ for 15 sec, annealing at 60 ℃ for 20 sec, and extension at 72 ℃ for 45 sec. Amplification efficiencies of each target and the reference gene, bovine beta-actin (*ACTB*), were examined through their calibration curves and found to be

comparable (Bustin *et al*. 2009, Sakurai *et al*. 2013). Average threshold (Ct) values for each target were determined by Sequence Detection System software v1.2 (Applied Biosystems). Each run was completed with a melting curve analysis to confirm the specificity of amplification and the absence of primer dimer.

Immunohistochemistry

Immunohistochemical analyses were performed on 10 μm fresh-frozen sections of days 20 and 22 uterine tissues. Frozen sections were fixed with 4% paraformaldehyde/PBS, and endogenous peroxidase was quenched by immersing in 0.3% (v/v) hydrogen peroxide/methanol, as previously described (Yamakoshi *et al*. 2012). Streptavidin/biotin blocking kit (Vector Laboratories, Burlingame, CA, USA) was used to block endogenous biotin according to the manufacturer's instructions. After 30 min incubation with 10% normal goat serum, the sections were incubated with rabbit anti-human VCAM-1 polyclonal antibody (1:100 dilution, ab106777, abcam), mouse anti-human CD49d antibody (1:100 dilution, MCA697GA, AbD serotec) or normal mouse IgG (1:100 dilution, sc-2025, Santa Cruz Biotechnology, Inc.,) or normal rabbit IgG (1:100 dilution, sc-2027, Santa Cruz Biotechnology, Inc.,) as negative control overnight at 4 ℃. Subsequently, the sections were incubated with either goat anti-mouse IgG biotin conjugate (1:400 dilution, B7401, Sigma-Aldrich) or goat anti-rabbit IgG biotin conjugate (1:800 dilution, B8895, Sigma-Aldrich). The immunoreactivity was visualized by means of avidin-peroxidase (1:400 dilution, E2886, Sigma-Aldrich) and AEC substrate kit (Invitrogen) according to the manufacturer's instructions. The sections were counter-stained with methylgreen and then examined under light microscope (BX-51, Olympus, Tokyo, Japan).

The plate of cultured EECs was fixed with 4% PFA (paraformaldehyde) in PBS. After 30 min incubation with 10% normal goat serum, the sections were incubated with rabbit anti-human VCAM-1 antibody (1:100 dilution, ab106777, abcam) overnight at 4 ℃, and normal rabbit IgG (1:100 dilution,

sc-2027, Santa Cruz Biotechnology, Inc.,) was used as negative control. Subsequently, the plate was incubated with goat anti-rabbit IgG biotin conjugate (1:800 dilution, B8895, Sigma-Aldrich). The immunoreactivity was visualized by means of fluorescent conjugates of Streptavidin 568 (1:200 dilution, S11223, Invitrogen) and DAPI (4',6-diamidino-2-phenylindole, 1:10000 dilution, D8417, Sigma-Aldrich) for 30 min. They were then examined under light microscope (BX-51, Olympus, Tokyo, Japan).

Statistical analyses

All experimental data from the bioassays represent the results obtained from three or more independent experiments each with triplicate assays, expressed as the mean ± SEM. Statistical analysis was performed utilizing Dunnett's test for multiple comparisons between experimental groups with the StatView statistical analysis software (version 5; SAS Institute Inc.). Differences of $P < 0.05$ were considered to be significant.

Results

Expression of VCAM-1 in the bovine uterus during the peri-attachment period

qPCR analyses in RNAs extracted from bovine uterine endometrial tissues obtained from day 17 cyclic (C17), and days 17, 20 and 22 pregnant animals (P17, P20 and P22) revealed that *VCAM-1* transcripts were found in endometrial tissues. Uterine *VCAM-1* expression was minimal in day 17 cyclic animals, was slightly up-regulated in day 17 pregnant animals, and increased approximately 4-5 fold (vs. day 17 cyclic) in days 20 and 22 pregnant animals (Fig. 1A). To confirm the up-regulation of *VCAM-1* associated with its protein, immunohistochemistry was carried out with frozen sections from day 20 pregnant uterine tissues. VCAM-1 protein was detected in the uterine luminal epithelium, middle glandular epithelium and endothelium of both caruncular and inter-caruncular regions of the endometrium (Fig. 1B).

Up-regulation of VCAM-1 in the endometrial epithelial cells (EECs) treated with uterine flushings or growth factor

qPCR was executed to determine *VCAM-1* mRNA in RNA extracted from EECs that were treated with or without bovine uterine flushing from day 22 pregnant animals. *VCAM-1* expression was up-regulated 2-fold when cultured EECs were treated with the uterine flushing (Fig. 2A). These results together with those in Fig. 1 indicated that VCAM-1 was expressed in the uterine endometrial epithelium. Instead of uterine flushings, EECs were then treated with epidermal growth factor (EGF), basic fibroblast growth factor (bFGF) or IFNT for 48 h (Fig. 2B). *VCAM-1* mRNA in EECs was found to be up-regulated in all cases. To confirm if the increase in *VCAM-1* transcripts was associated with VCAM-1 protein, the EECs treated with EGF (20 ng/ml) or bFGF (10 ng/ml) were subjected to immunofluorescence staining (Fig. 2C). The expression of VCAM-1 in EECs treated with EGF or bFGF was enhanced when compared with those in EECs without treatment, and the VCAM-1 protein was found only in the cytoplasmic area, but not at the cell surface of EECs.

Up-regulation of VCAM-1 in EECscocultured with bovine trophoblast CT-1 cells

The expression of *VCAM-1* transcripts was also examined in monocultured EECs as well as in the CT-1 coculture. The expression of *VCAM-1* was up-regulated 12-fold in EECscocultured with CT-1 (Fig. 3A), exceeding the effects of day 22 pregnant uterine flushings, EGF or bFGF treatment (Fig. 2A, Fig. 2B). To confirm whether the increase in *VCAM-1* transcripts was associated with VCAM-1 protein, the EECscocultured with CT-1 with shaking for 48 h were subjected to immunofluorescence staining (Fig. 3B). The expression of VCAM-1 in EECscocultured with CT-1 cells was enhanced when compared with monocultured EECs. Moreover, the protein was found not only in the cytoplasmic region of EECs, but also at their cell surface, revealing that cell-cell interaction with CT-1 cells could enhance VCAM-1 expression in the EECs.

Expression and regulation of VCAM-1 transcripts in the bovine conceptus during the peri-attachment period

qPCR analyses in RNAs extracted from bovine conceptuses obtained from day 17, 20 and 22 pregnant animals revealed that *VCAM-1* transcript expression was minimal in day 17 and 20 before increasing 200 fold in day 22 conceptuses (Fig. 4A). qPCR was also executed to determine *VCAM-1* mRNA in RNA extracted from monocultured CT-1 cells treated with bovine uterine flushing from day 22 pregnant orcocultured CT-1 cells with EECs. Although *VCAM-1* was not found in CT-1 cells without treatment, up-regulated transcripts were found when CT-1 cells were treated with the uterine flushing from day 22 pregnant animals, and further increases in VCAM-1 transcripts in CT-1 cells cocultured with EECs (Fig. 4B). These results indicated that similar to VCAM-1 expression in EECs, up-regulation of VCAM-1 in CT-1 cells could result from cell to cell interactions between trophoblasts and uterine endometrial epithelial cells.

The expression of VCAM-1 receptor, ITGA4 by bovine conceptus

qPCR analyses in RNAs extracted from bovine conceptus obtained from day 17, 20 and 22 pregnant animals revealed that *ITGA4* expression was minimal in day 17 and 20, while 6000-fold increase in *ITGA4* was found in day 22 conceptuses (Fig. 5A), but the expression is minimal at the uterine endometrium in days 17, 20 and 22 pregnant animals (data not shown). Immunohistochemistry was executed to determine ITGA4 and its ligand VCAM-1 in frozen sections from day 22 pregnant animals (Fig. 5B). ITGA4 was localized at the apical surface of trophoblast cells, but not at the maternal side, while VCAM-1 was found at both trophoblast cells and apical surface of endometrial epithelium.

Discussion

VCAM-1 is well-known as a cell adhesion mediator during the process of lymphocyte homing (May *et al.* 1993), angiogenesis (Ding *et al.* 2003) and even placentation (Gurtner *et al.* 1995). In this study, VCAM-1 expression was detected at the trophoblast and uterine epithelium, as well as uterine glandular epithelium and endothelium during the peri-implantation period. In endothelial cells, VCAM-1 is induced by cytokines such as Tumor Necrosis Factor alpha (TNF-α) produced by leukocytes. The increase in VCAM-1 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, increases in *VCAM-1* and its protein expression in the cytoplasma of cultured EECs were detected when treated with the uterine flushings from day 22 of pregnant animals, growth factor (EGF or bFGF), or IFNT. However, VCAM-1 was up-regulated at the surface of EECs only when EECs werecocultured with bovine trophoblast CT-1 cells. More importantly, the degree of up-regulation of *VCAM-1* mRNA was more significant incocultured EECs than in monocultured EECs treated with uterine molecules, and a similar degree of up-regulation was also found in *VCAM-1* transcript expression incocultured CT-1. Based on these observations, both cell-cell interactions between the conceptus and uterine epithelium and uterine molecules, found in the uterine flushings from day 22, could induce VCAM-1 production in the endometrium and/or conceptuses, particularly its expression at the surface of these cell types (Fig. 6). These results strongly suggest that the VCAM-1 is required for bovine conceptus attachment and/or adhesion to the uterine luminal epithelium.

VLA-4, composed of integrin alpha 4 (ITGA4, CD49d) and integrin beta 1 (ITGB1, CD29), has been found as a receptor for VCAM-1, and the complex is known to function in the process of arresting rolling monocytes within blood vessels (Chu *et al.* 2013), and cancer-endothelial cell adhesion (Song *et al.* 2012). Deletion of ITGA4 in mice results in embryo lethality with failure of allantois-chorion

fusion and thus placentation (Yang *et al.* 1995). In this study, *ITGA4* transcript expression was significantly up-regulated in day 22 conceptuses. At the same time, *VCAM-1* mRNA was also increased in uterine tissues (Fig. 1A, Fig. 5A), which agrees with our finding that the proteins of ITGA4 and VCAM-1 were localized in the apical surface of trophoblast cells and the apical surface of EECs, respectively, in the frozen sections from day 22 pregnant animals. The human patients with unexplained infertility exhibited a marked decrease of VCAM-1 expression in endometrium in the late secretory phase, coincident with the implantation window, which suggests that down-regulated uterine VCAM-1 may result in asynchrony of uterine development for conceptus implantation (Konac *et al.* 2009). Therefore, these results suggest that VCAM-1 and ITGA4 are associated with conceptus attachment to the uterine epithelium in the bovine.

In our previous observations of trophoblasts' epithelial mesenchymal transition (EMT) (Yamakoshi *et al.* 2012), up-regulation of N-cadherin, vimentin, some ITGs including ITGA4, and matrix metalloproteases (MMPs) occurs simultaneously on day 22. In this study, not only *ITGA4*, but also *VCAM-1* transcript expression were found to be up-regulated on day 22 (Fig. 4A), which was accompanied with the expression of ITGA4 and VCAM-1 proteins at the surface of trophoblasts. As E-cadherin (CDH1) declines in the trophoblast cell surface on day 22 (Yamakoshi *et al.* 2012), the up-regulation of the VCAM-1/ITGA4 complex may aid in attachment processes between trophoblasts and uterine epithelium. It is well-established that MMPs play a role in the process of trophoblast invasion during the implantation period in humans (Cohen *et al.* 2006). Although the bovine implantation is not as invasive as humans, the increase in MMPs in day 22 conceptuses was also noted previously (Yamakoshi *et al*. 2012). It is reported that VCAM-1 activates endothelial cell MMPs through reactive oxygen species (ROS) during lymphocyte migration (Deem & Cook-Mills 2004). It is possible that MMP expression in bovine conceptuses is up-regulated through ROS during the

attachment period. Thus, up-regulated VCAM-1 and possibly MMPs in day 22 conceptuses could also be associated with the attachment and/or adhesion processes in the non-invasive mode of implantation.

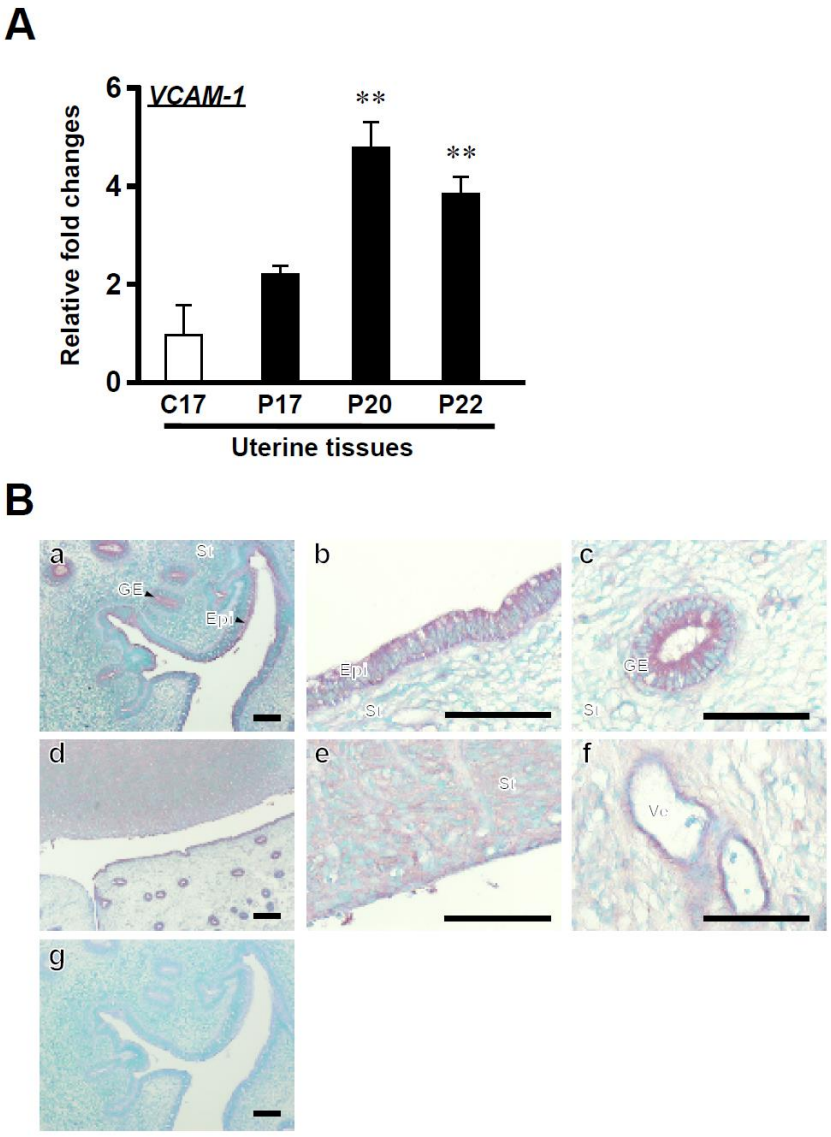
In conclusion, this study demonstrates that the expression of VCAM-1 can be up-regulated by the molecules present in uterine flushings, but the efficiency of VCAM-1 expression at the cell surface is significantly increased by the cell-cell interactions between conceptus and uterine epithelial cells. Therefore, following the initiation of cell-cell interaction (attachment) processes, VCAM-1 together with its receptor, ITGA4, is likely to be required for conceptus adhesion to the endometrial epithelium in the bovine species.

Table 1. Primers for quantitative RT-PCR analyses

Name (GenBank accession No.)	Sequence
VCAM-1 (XM_005204078.1)	F: 5'- AGAAGAGCCAACATGACAGGGTCA -3'
	R: 5'-TCAAGGAAGCCTGAACCCCCAGT -3'
ITGA4 (NM_174748.1)	F: TCTGGCATAGTGAGACTTGAC
	R: GGACTTACAAACCCATGAACTG
ACTB (NM_173979.3)	F: 5'-TCCCTGGAGAAGAGCTACGA -3'
	R: 5'-GGGCAGTGATCTCTTTCTGC -3'

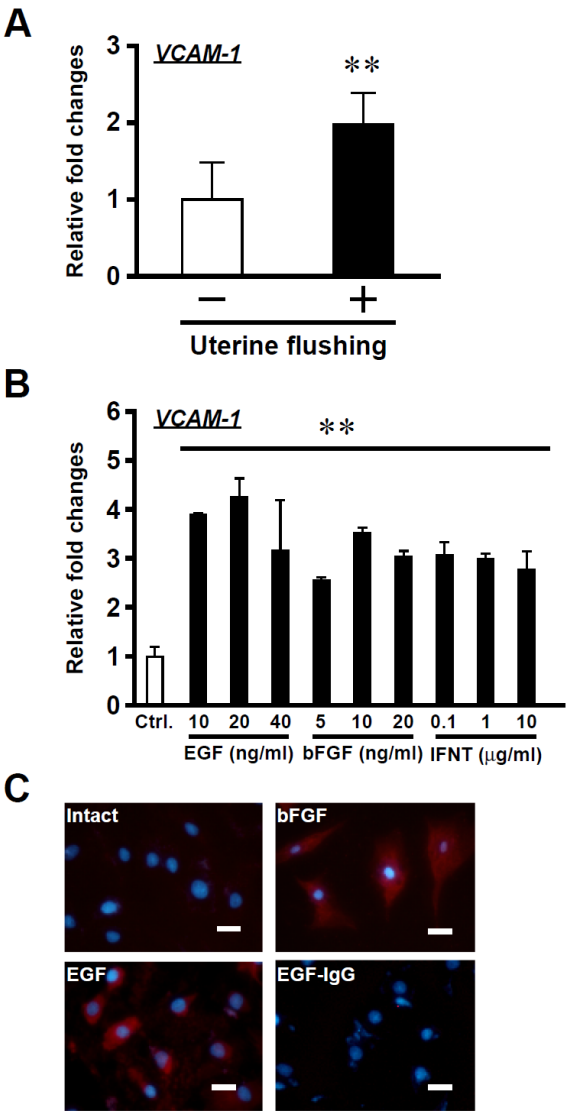
F: Forward primer; R: Reverse primer

Figure 1.



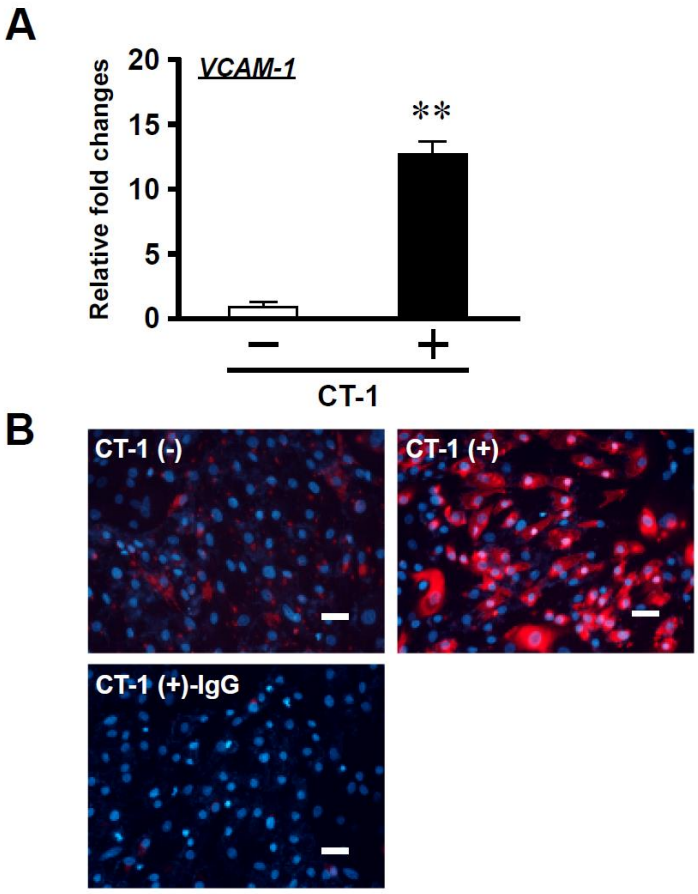
Expression of *VCAM-1* in the bovine uterus during the peri-attachment period. A: Changes in *VCAM-1* in endometria of day 17 cyclic (day 17; C17) or pregnant animals (day 17, 20, or 22; P17, P20, or P22, respectively) (n=3 each day). *ACTB* mRNA was used as an internal control for RNA integrity. B: Immunohistochemical analysis of VCAM-1 in the bovine uterus from day 20 pregnant animals. Tissue sections (10 μm) from day 20 uteri were immunostained for VCAM-1 using an anti-human VCAM-1 antibody (a-f), and a negative control with the normal rabbit IgG as primary antibody (g). a: low magnification showing a cross-section of the uterus, b: uterine epithelium, c: glandular epithelium, d: caruncle side of the endometrium, e: uterine stroma, f: uterine vessels. LE, endometrial luminal epithelium; GE, glandular epithelium; ST, endometrial stroma; Scale bar=200 μm.

Figure 2.



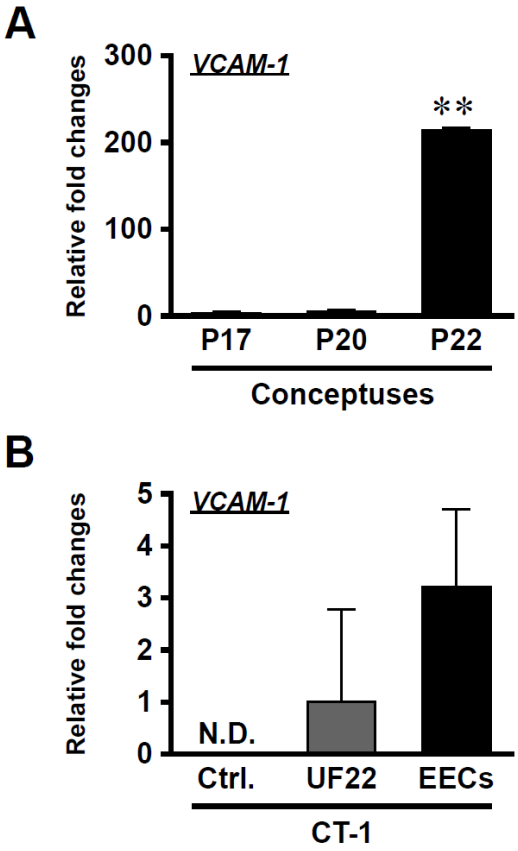
Up-regulation of VCAM-1 in bovine endometrial epithelial cells treated with uterine flushings, steroid or cytokine. A: Levels of *VCAM-1* mRNAs in monocultured endometrial epithelial cells (EECs) treated with bovine uterine flushing from day 22 pregnant animals. B: Up-regulation of *VCAM-1* transcripts in monocultured EECs in the serum-free medium treated with EGF (10, 20, 40 ng/ml), bFGF (5, 10, 20 ng/ml), or IFNT (0.1, 1, 10 μg/ml) for 48 h. *ACTB* mRNA was used as an internal control for RNA integrity. Values represent mean ± SEM from three independent experiments with triplicates within each experiment. **Statistically significant differences in mRNA levels (P<0.01). C: Immunofluorescence analysis of VCAM-1 in EECs treated with EGF (20 ng/ml) or bFGF (10 ng/ml) for 48 h. Intact: EECs cultured without EGF or bFGF, bFGF(+): EECs cultured with bFGF, EGF(+): EECs cultured with EGF, and EGF-IgG: a negative control (N.C.). VCAM-1 expression is seen in red, nuclei were counterstained with DAPI, and the normal rabbit IgG was applied for the negative control. Three independent experiments were executed and the representative one is shown. Scale bar=200 μm.

Figure 3.



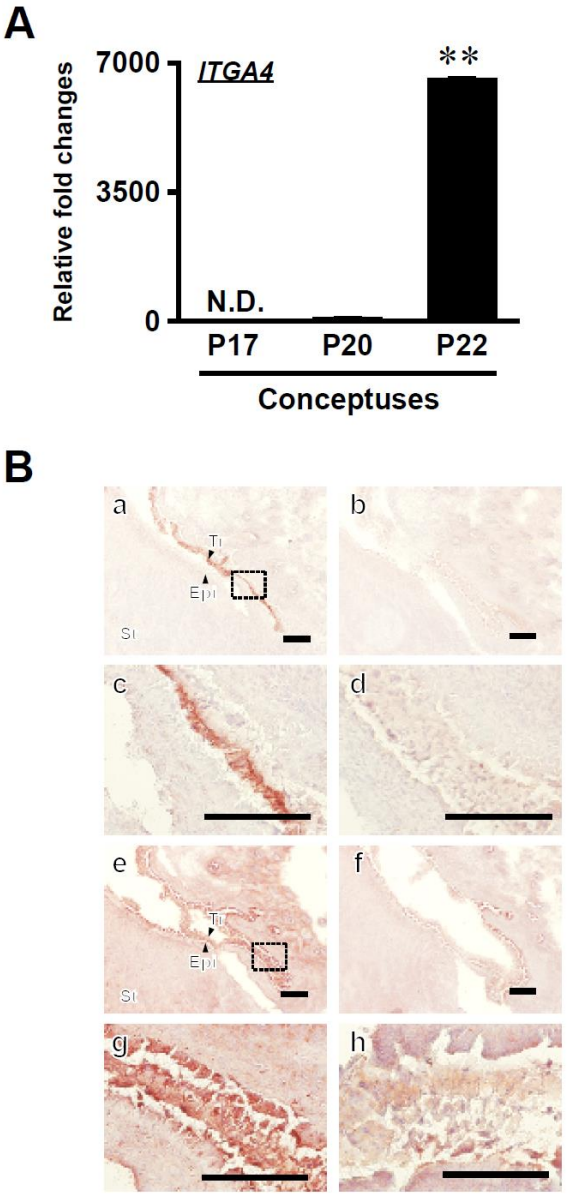
Up-regulation of VCAM-1 in bovine endometrial epithelial cellscocultured with bovine trophoblast CT-1 cells. A: Up-regulation of *VCAM-1* transcripts in bovine endometrial epithelial cells (EECs)cocultured with (+) or without (-) CT-1 cells. *ACTB* mRNA was used as an internal control for RNA integrity. Values represent mean ± SEM from three independent experiments with triplicates within each experiment. **Statistically significant differences in mRNA levels ($P < 0.01$). B: Immunofluorescence analysis of VCAM-1 in EECscocultured with CT-1 cells for 48 h. CT-1 (-): monocultured EECs, CT-1 (+): EECscocultured with CT-1 cells, and CT-1 (+)-IgG: a negative control (N.C.). VCAM-1 expression is seen in red, nuclei were counterstained with DAPI, and the normal rabbit IgG was applied for the negative control. Three independent experiments were executed and the representative one is shown. Scale bar = 200 μm.

Figure 4.



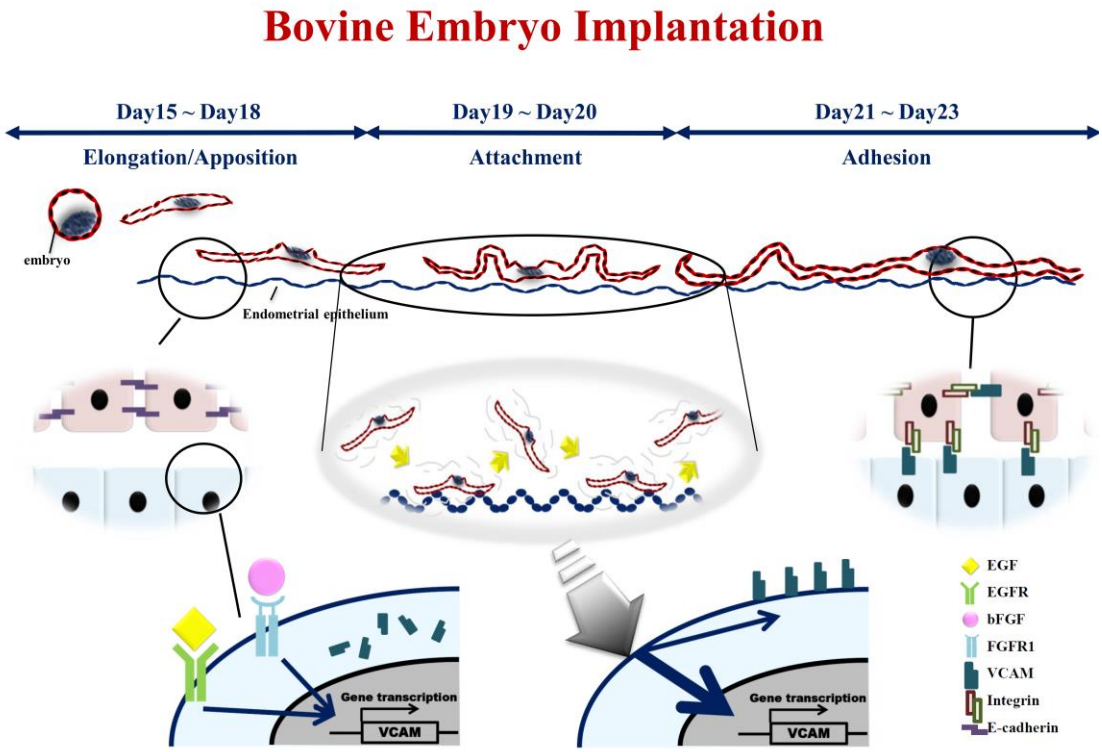
Expression and regulation of *VCAM-1* transcripts in bovine conceptuses during the peri-attachment period. A: Changes in *VCAM-1* mRNAs in days 17, 20, and 22 conceptuses (P17, P20, and P22, respectively). B: Up-regulation of *VCAM-1* mRNAs in CT-1 cells treated with uterine flushings from day 22 pregnant animals (UF22) orcocultured with EECs (EECs+). Values represent mean ± SEM from three independent samples with duplicates within a treatment. **Statistically significant differences in mRNA levels ($P < 0.01$).

Figure 5.



Expression of VCAM-1 receptor, integrin alpha 4 (ITGA4) by bovine conceptuses. A: Changes in *ITGA4* mRNAs in days 17, 20, and 22 conceptuses (P17, P20, and P22, respectively). Note that *ITGA4* expression was minimal at the uterine epithelium and stroma. Values represent mean ± SEM from three independent samples with duplicates within a day of conceptus collection. **Statistically significant differences in mRNA levels ($P < 0.01$). B: Immunohistochemical analysis of ITGA4 (a-d) or VCAM-1 (e-h) in the bovine uterus from day 22 pregnant animals. Tissue sections (10 μm) from day 22 uteri were immunostained for ITGA4 using an anti-ITGA4 antibody (a, c) or normal mouse IgG as a negative control (b, d), and for VCAM-1 using an anti-VCAM-1 antibody (e, g), or normal rabbit IgG as a negative control (f, h). Epi, endometrial luminal epithelium; Tr,trophoblast; St, endometrial stroma; Scale bar = 200 μm.

Figure 6.



A model of bovine embryo implantation. Upper: Morphological representation of bovine conceptuses during periods of conceptus elongation/apposition (from day 15 to day 18), attachment (from day 19 to day 20) and adhesion (from day 21 to day 23) to the uterine endometrium. Lower: Before or during the initial attachment processes between the conceptus and the uterine epithelium, VCAM-1 expression is restricted to the cytoplasm of endometrial epithelium. However, as the conceptus attachment progress, VCAM-1 expression is seen at the surface of epithelial cells, resulting in the adhesion of the conceptus to the uterine epithelium through VCAM-1 and ITGA4 complex formation.

Chapter 3

Expression of mesenchymal-related genes by the bovine trophectoderm following conceptus attachment to the endometrial epithelium

Abstract

In the course of experiments to identify and characterize factors that function in bovine conceptuses during peri-attachment periods, various transcripts related to the epithelial-mesenchymal transition (EMT) were found. In this study, RNA was extracted from different sets of days 17, 20 and 22 (day 0 = day of estrus) bovine conceptuses and subjected to real-time PCR analysis as well as western blotting, from which abundances of N-cadherin, vimentin, *MMP2* and *MMP9* mRNAs were determined on Day 22, concurrent with E-cadherin, mRNA and protein, down-regulation. Transcription factors in EMT processes were then analyzed and changes in *SNAI2*, *ZEB1*, *ZEB2*, *TWIST1*, *TWIST2* and *KLF8* transcripts were found in day 22 conceptuses, while confirming SNAI2 expression by western blotting. Immunohistochemical analysis revealed that the day 22 trophectoderm expressed mesenchymal markers N-cadherin and vimentin as well as an epithelial marker cytokeratin. In attempts to identify molecular mechanisms by which the trophectoderm expressed EMT-related genes, growth factor receptors associated with EMT were analyzed. Up-regulation of growth factor receptor transcripts, *FGFR1*, *PDGFRA*, *PDGFRB* and *TGFBR2* mRNAs, was found on day 22. The analysis was extended to determine integrin (ITG) transcripts and found high levels of *ITGA4*, *ITGA8*, *ITGB3* and *ITGB5* mRNAs on day 22. These observations indicate that after the conceptus-endometrium attachment, EMT-related transcripts as well as an epithelial marker cytokeratin were present in the bovine trophectoderm, and suggest that the implantation process for non-invasive trophoblasts requires not only extracellular matrix expression but also partial EMT.

Introduction

Embryo implantation is an essential process for viviparity (Amoroso 1968) that differs among species and includes both non-invasive and invasive trophoblasts. However, the implantation process invariably starts from blastocyst migration, apposition, attachment and adhesion to epithelial lining of the endometrium (Bowen & Burghardt 2000). At the preimplantation stage of embryo development, the first cell fate specification results in the segregation of inner cell mass and trophectoderm (Nishioka *et al*. 2009). The trophectoderm forms epithelial structure of the blastocyst and possesses epithelial characteristics, including apico-basal cell polarity, lateral junctions with neighboring cells and basal contact with the basement membrane proteins (Biggers *et al*. 1988, Kang *et al*. 1990, Thorsteinsdottir 1992, Fleming *et al*. 2001). Despite the fact that the apical plasma membranes of simple epithelia normally lack adhesive properties, the trophectoderm still manages to adhere to the uterine epithelium via their apical domains as part of the implantation process. Thus, the adhesion between trophectoderm and uterine epithelium has long been considered a cell biological paradox (Denker 1993).

Successive phases of implantation are classified as 1) shedding of the zona pellucida, 2) pre-contact stage and blastocyst orientation, 3) apposition and attachment, 4) adhesion, and 5) invasion (Chavatte-Palmer & Guillomot 2007, Bazer *et al*. 2009). With the exception of rodents and primates, in which the conceptus enters a receptive uterus and attaches immediately to the uterine epithelium, most domestic animals have a pre-receptive phase during which the conceptus does not physically interact with the uterine epithelium. Ruminant species such as sheep and cows have superficial/central implantation, in which a prolonged pre-attachment period is followed by incremental apposition and attachment of the conceptus to the luminal epithelium. In the bovine species, attachment is first observed in day 20 trophoblasts, which are characterized by the presence of multinucleate epithelial cells by day 24. Degenerative changes in many uterine epithelial cells are seen at the regions to which the trophectoderm contacts between 22 and 28 days (Wathes & Wooding 1980). A long pre-attachment period is

characterized, at least in part, by apical expression of mucin MUC1 with extensive glycosylation, which sterically inhibits cell-cell and cell-extracellular matrix (ECM) interactions (Wesseling *et al*. 1995, Komatsu *et al*. 1997). The involvement of ECM proteins, secreted phosphoprotein 1 (SPP1, osteopontin), and MUC1 and transmembrane receptor integrin subunits in the trophectoderm adhesion to luminal epithelium has been well characterized in sheep and pigs (Johnson *et al*. 2001). Integrins are dominant glycoproteins in adhesion cascades, owing to their ability to bind ECM ligands to mediate adhesion, cause cytoskeletal reorganization to stabilize adhesion, and transduce cellular signals through numerous signaling intermediates (Yoshinaga 1989, Burghardt *et al*. 1997, Giancotti & Ruoslahti 1999). However, gene expressions that determine the cell properties of the trophectoderm during this period have not been well characterized.

In humans and mice, trophoblast invasion into the endometrial matrix is subsequent to the blastocyst's adhesion to the uterine wall. To enable invasion competence, it has been postulated that the trophectoderm undergoes a partial epithelial-mesenchymal transition (EMT), which involves downregulation of apico-basal polarity, expression of mesenchymal-type adhesion proteins, reorganization of the cytoskeleton, and expression of matrix metalloproteinases (MMPs) (Denker 1993, Thie *et al*. 1996, Vicovac & Aplin 1996). In addition, the signaling involved in regulating EMT events aids in allowing adhesion at the apical membrane of trophectoderm (Hohn & Denker 2002). Yet, to date, it is unclear how exactly the trophectoderm acquires adhesive and/or invasive competence, and thus how EMT events relate to the non-invasive conceptus implantation still remains speculative.

Rodents have been the primary model for understanding the implantation process (Dey *et al*. 2004, Lee *et al*. 2007). In mice, however, the blastocyst commences the implantation process soon after hatching from the zona pellucida, and is implanted in the endometrial stroma following trophoblast penetration through the uterine epithelium. This rapid progress of murine blastocyst implantation makes it difficult

to elucidate the mechanisms associated with blastocyst attachment and adhesion to the uterine epithelium. In the domestic animals such as porcine, ovine and bovine species, on the other hand, the trophoblast cells do not penetrate the uterine epithelium, but rather maintain an apical-apical adhesion throughout the implantation process (Chavatte-Palmer & Guillomot 2007). Understanding the events associated with the apical-apical adhesion could provide a new insight into the relationship between trophoblast and uterine cells. Therefore, ruminants could serve as ideal models to study the physiological as well as molecular events through which attachment and adhesion between trophoblast and uterine epithelium proceed.

In the bovine, attachment between trophectodermal epithelium and endometrial epithelium is first seen on day 20 of gestation, and subsequent stable adhesion occurs between days 20 and 22 (Wathes & Wooding 1980). I hypothesized that molecular events similar to the EMT process would be required to make trophectoderm adhesion to the endometrial epithelium possible. In this report, I will demonstrate that changes in gene expression associated with EMT occur in the bovine trophectoderm following conceptus attachment to the luminal epithelium.

Materials and Methods

Animals and tissue preparation

All animal procedures in the present study were approved by the Committee for Experimental Animals at Zen-noh Embryo Transfer (ET) Center and the University of Tokyo. Estrous synchronization, superovulation and embryo transfer process were performed as previously described (Ideta *et al*. 2007). Seven-day embryos (day 0 = day of estrus) were collected from superovulated Japanese black cattle. Thirty embryos derived from the superovulation were transferred nonsurgically into the uterine horn of fifteen Holstein heifers (two blastocysts/recipient), ipsilateral to the corpus luteum on day 7 of the estrous cycle. For RNA analyses, elongated conceptuses were collected nonsurgically by uterine flushing on day 17, 20, or 22 (four animals each) with 500 ml sterile phosphate-buffered saline (PBS, pH 7.2). Conceptuses in the uterine flushing media were obtained by centrifugation at 1,000 rpm for 5 min and snap-frozen in liquid nitrogen. For immunohistochemistry, hysterectomy was performed on day 22 (n=3), and the uterine tissues containing conceptus were frozen in dry ice-cooled heptane. Samples were transferred to Laboratory of Animal Breeding at the University of Tokyo and stored at -80˚C until use.

RNA extraction and RT-PCR

Total RNA was prepared from days 17, 20 and 22 conceptuses with ISOGEN reagent (Nippon gene, Tokyo, Japan). cDNAs were each synthesized from total RNA (250 ng) with ReverTra Ace (TOYOBO, Osaka, Japan) and random primers, diluted ten times with water, and subjected to PCR amplification to estimate mRNA levels. PCR was carried out with 1 or 3 μl of diluted cDNA reaction mixture, 0.5 units of ExTaq polymerase (Takara Biomedicals, Tokyo, Japan), ExTaq buffer, 0.2 μM of the oligonucleotide primers described in Table 1 and 0.2 mM of dNTP in a final volume of 20 μl. The thermal profile for RCR was at 95˚C for 2 min, followed by 30 or 35 cycles of 95˚C for 30 sec, 57˚C for 30 sec and 72˚C for 30 sec. The PCR products were separated on a 2.0% agarose gel containing ethidium bromide and

visualized under UV light through an image analysis system (ATTO Corporation, Tokyo, Japan).

Quantitative RT-PCR

Reverse-transcribed cDNA (3 μl) was subjected to real-time PCR amplification using 0.1 units of ExTaq HS polymerase (Takara Biomedicals), ExTaq HS buffer, 0.5 μM of the oligonucleotide primers listed in Table 1, 2.5 mM of dNTP, SYBR green (SYBR Green I Nucleic Acid Gel stain, Takara Biomedicals) as fluorescence intercalater and Rox reference dye (Invitrogen, Carlsbad, CA) in a final volume of 20 μl, and PCR amplification was carried out on an Applied Biosystems 7900HT real-time PCR System (Applied Biosystems, Foster City, CA). The thermal profile for real-time PCR was at 95˚C for 10 min, followed by 40 cycles of 95˚C for 10 sec, 60˚C for 20 sec and 72˚C for 40 sec. Average threshold (Ct) values for mRNA were calculated and normalized to Ct values for *ACTB* mRNA (Sakurai *et al*. 2009). Each run was completed with a melting curve analysis to confirm the specificity of amplification and the absence of primer dimers.

Western blotting

Western blot analysis was performed using cell lysates from days 17, 20 and 22 conceptuses. Cell lysates (10 μg) were loaded into each lane and separated by 10% SDS-polyacrylamide gel electrophoresis, transferred onto PVDF membrane (Millipore, Bedford, MA), and then treated with rabbit anti-human CDH1 antibody (1:1000 Cell Signaling Technology), rabbit polyclonal SNAI2 antibody (1:1000, abcam), rabbit monoclonal anti-IFNT antibody (1:1000 Operon, Tokyo, Japan) or rabbit polyclonal ACTB antibody (1:1000, abcam) diluted with Can Get Signal Solution I (TOYOBO CO., LTD. Life Science Development OSAKA JAPAN). The proteins were detected using the secondary antibody conjugated with horseradish peroxidase (goat anti-rabbit, 1:10000, Irritant NA934V) diluted with Can Get Signal Solution II on an ECL western blotting detection system (Amersham Pharmacia Biotech, Buckinghamshire, UK) (Sakurai *et al*. 2010).

Immunohistochemistry

Immunohistochemical analyses were performed on 10 μm fresh-frozen sections of day 22 uterine tissues. Sections were fixed with 4% paraformaldehyde/PBS, and endogenous peroxidase was quenched by immersing in 0.3% (v/v) hydrogen peroxide/methanol. Streptavidin/biotin blocking kit (Vector Laboratories, Burlingame, CA) was used to block endogenous biotin according to the manufacturer's instructions. After 30 min incubation with 10% normal goat serum, the sections were incubated with rabbit anti-bovine cytokeratin polyclonal antibody (1:100 dilution, catalog no. Z0622, Dako, Hamburg, Germany), mouse anti-human vimentin monoclonal antibody (clone V9, 1:100 dilution, catalog no. M0725, Dako), rabbit anti-mouse E-cadherin polyclonal antibody (1:100 dilution, catalog no. 3195, Cell Signaling Technology, Danvers, MA), rabbit anti-human N-cadherin polyclonal antibody (1:100 dilution, catalog no. ab12221, Abcam, Cambridge, MA), or mouse anti-human CD49d (integrin alpha 4 chain, VLA-4) antibody (1:100 dilution, AbD Serotec - a division of MorphoSys, Kidlington, UK) overnight at 4˚C. Subsequently, the sections were incubated with either goat anti-rabbit IgG biotin conjugate (1:800 dilution, catalog no. B8895, Sigma-Aldrich, St. Louis, MO) or goat anti-mouse IgG biotin conjugate (1:1000 dilution, catalog no. B9904, Sigma-Aldrich). The immunoreactivity was visualized by means of avidin-peroxidase (catalog no. E2886, Sigma-Aldrich) and AEC substrate kit (catalog no. 00-2007, Invitrogen) according to the manufacturer's instructions. The sections were counter-stained with hematoxylin and then examined under light microscope (BX-51, Olympus, Tokyo, Japan).

Statistical analyses

Statistical analyses were performed using the R statistical package (http://www.r-project.org/). All quantitative RT-PCR results were presented as mean ± S.E.M., and data were analyzed by analyses of variance with general linear model followed by Tukey contrast tests.

Results

Up-regulation of EMT markers in day 22 bovine conceptuses

To evaluate the expression patterns of transcripts typical of the EMT process, E-cadherin (*CDH1*), N-cadherin (*CDH2*) and vimentin (*VIM*) mRNAs in days 17, 20 and 22 conceptuses were evaluated by RT-PCR. High levels of *CDH2* and *VIM* mRNA were found in day 22 conceptuses while that of *CDH1* decreased (Fig. 1A). Similar changes in these transcripts were also found when RNA extracted from different sets of conceptuses was analyzed by quantitative RT-PCR analysis (Fig. 1B). I also examined matrix metalloproteinases 2 (*MMP2*) and matrix metalloproteinase 9 (*MMP9*) mRNAs, both of which are implicated in the EMT event during cancer metastasis (Yilmaz & Christofori 2009). Although bovine implantation is non-invasive, levels of *MMP2* and *MMP9* transcripts in the bovine conceptus were nevertheless found to be higher on day 22 (Fig. 1B). To support the results obtained from the PCR analysis, western blot analysis was carried out for CDH1 as well as IFNT, a marker commonly used in this laboratory. CDH1 protein was low in day 22 conceptuses (Fig. 1C).

Up-regulation of EMT regulatory transcription factor mRNAs on day 22 of pregnancy

Recent studies on cancer metastasis in various cell types have revealed that the process of EMT could be regulated by a number of transcription factors, including *SNAI1*, *SNAI2*, *ZEB1*, *ZEB2*, *KLF8*, *TCF3*, *TWIST1* and *TWIST2* (Thiery *et al*. 2009). Hence I next examined changes in these transcription factor mRNAs in days 17, 20 and 22 conceptuses. RT-PCR analyses found that all of these transcription factor transcripts were present on day 22 (Fig. 2A). On days 17 and 20, *SNAI2*, *ZEB1*, *ZEB2*, *TWIST1*, *TWIST2* and *KLF8* mRNAs were low or undetectable, whereas consistent levels of *SNAI1* and *TCF3* mRNAs were found from days 17 through 22 (Fig. 2A). Up-regulation of *SNAI2*, *ZEB1*, *ZEB2*, *TWIST1*, *TWIST2* and *KLF8* mRNAs was also confirmed by quantitative RT-PCR (Fig. 2B). Levels of *SNAI2* and *ZEB1* transcripts on day 22 were approximately 40 times greater than those on day 20 (Fig. 2B). In addition, levels of *ZEB2* and *TWIST1* mRNAs increased approximately 20-fold on day 22 compared to

those on day 20 (Fig. 2B). Up-regulation of these transcription factor mRNAs in day 22 conceptuses was consistent with the occurrence of EMT event in the trophectoderm of these conceptuses. To support the results from the PCR analysis, western blotting was carried out to characterize changes in SNAI2 protein. On day 22 elevated SNAI2 protein levels were consistent with the high levels of *SNAI2* mRNA (Fig. 2C).

Expression of EMT marker proteins in day 22 bovine conceptuses

Up-regulation of transcripts associated with EMT process led us to believe that EMT marker proteins would also be present in day 22 conceptuses. I first performed immunohistochemistry for cytokeratin as an epithelial marker and vimentin as a mesenchymal marker on tissue sections from day 22 pregnant uteri. In the uterine endometrium, both luminal and glandular epithelia were positive for cytokeratin and negative for vimentin, and the stromal cells were cytokeratin-negative and vimentin-positive (Fig. 3). On the other hand, the trophoblast layer of conceptus was positive for both cytokeratin and vimentin (Fig. 3).

The expression of mesenchymal marker in the trophectoderm led us to evaluate changes in E-cadherin and/or N-cadherin proteins, which have been identified as key events in the EMT during embryogenesis and carcinogenesis (Thiery 2003, Katoh & Katoh 2008). Immunolocalization of E-cadherin and N-cadherin revealed that the trophoblast layer of day 22 conceptus expressed N-cadherin, whereas the expression of E-cadherin in the trophectoderm was not detected by immunohistochemistry (Fig. 4). The endometrial stromal cells expressed only N-cadherin, whereas both luminal and glandular epithelia of the endometrium expressed both E- and N-cadherin (Fig. 4). Relatively strong expression of N-cadherin was found on the region of the luminal epithelium where the trophectoderm attached (Fig. 4).

Expression of growth factor receptor mRNAs during the peri-attachment period

Unique protein expressions found in the trophectoderm layer, including the up-regulation of N-cadherin and vimentin on day 22, indicated that the epithelial trophectoderm exhibited protein expression commonly associated with mesenchyme (Fig. 3 and 4). However, EMT factor expression was not limited to the portion where the trophoblast layer attached to the luminal epithelium, but was found throughout the conceptus trophectoderm (Fig. 3 and 4). To elucidate molecular mechanisms associated with EMT in non-invasive trophoblasts, several growth factor receptors, through which EMT events are mediated by downstream signaling, were then examined. These included epidermal growth factor receptor (*EGFR*), fibroblast growth factor receptor 1 (*FGFR1*), insulin-like growth factor-1 receptor (*IGF1R*), platelet-derived growth factor receptors (*PDGFRA* and *PDGFRB*) and transforming growth factor β receptors (*TGFBR1* and *TGFBR2*) (Said & Williams 2011). The expression of *EGFR* and *TGFBR1* mRNAs was found from days 17 through 22 (Fig. 5A). The expression levels of *FGFR1*, *PDGFRA*, *PDGFRB* and *TGFBR2* mRNAs were low or undetectable on days 17 and 20 and became detectable on day 22 (Fig. 5A). The quantitative RT-PCR revealed the up-regulation of *FGFR1*, *PDGFRA*, *PDGFRB* and *TGFBR2* mRNAs on day 22, and the consistent expression of *TGFBR1* mRNA from days 17 through 22 (Fig. 5B).

Expression of α- and β-subunits of Integrin mRNAs in peri-implantation conceptuses

Integrins are also known to mediate EMT independently or in cooperation with growth factor receptors (Larue & Bellacosa 2005). I thus examined levels of α- and β-subunits of integrin (*ITGA4, ITGA5, ITGA8, ITGAV, ITGB1, ITGB3, ITGB5 and ITGB6*) mRNAs in the conceptuses. Higher *ITGA5*, *ITGAV* and *ITGB1* transcripts were found from days 17 through 22, whereas the expression of *ITGB6* mRNA was undetectable (Fig. 6A). Though the expression of *ITGA4*, *ITGA8*, *ITGB3* and *ITGB5* mRNAs was found on day 22, the expression levels on days 17 and 20 were low or undetectable (Fig. 6A). The up-regulation of *ITGA4* and *ITGA8* transcripts on day 22 and the consistent expression of *ITGAV* and

ITGB1 transcripts were also confirmed by quantitative RT-PCR (Fig. 6B). Using immunohistochemistry I further examined the expression of ITGA4 on tissue sections from day 22 pregnant uteri, and found positive immunostaining on that day (Fig. 7).

Discussion

The EMT has been recognized to occur as complete EMT or partial EMT, and the latter is characterized by transient loss of epithelial characteristics without full acquisition of mesenchymal characteristics (Leroy & Mostov 2007). In this study, I demonstrated that after bovine conceptus-endometrium attachment, the trophectoderm expressed vimentin while cytokeratin expression was still retained. Since the bovine trophoblast cells from mid-gestational placentomes do not express vimentin (Haeger *et al*. 2010), this co-existence of the epithelial and mesenchymal gene expression in the trophectoderm could be transient and possibly be prerequisite for steps from attachment to adhesion. Recently, co-expression of cytokeratin and vimentin was also found in the luminal epithelium of long-term progesterone-treated pigs (Bailey *et al*. 2010). It has been noted that many processes occurring during development and tissue remodeling, as well as in adult organisms involve a transient loss of epithelial polarity without full acquisition of mesenchymal characteristics. For example, the partial EMT has been found in mammary gland development and epithelial wound healing (Leroy & Mostov 2007). These findings and those in this study are consistent with the notion that there is a spectrum of partial EMT processes in which cells undergo only selected EMT steps for a transient period (Grunert *et al*. 2003, Huber *et al*. 2005).

Nakano *et al*. (2005) have reported that E-cadherin expression is distributed within the cytoplasm of trophoblast binucleate cells in the bovine placentome. They have also noted the translocation of β-catenin into the nuclei of trophoblast binucleate cells, indicating the role of E-cadherin-β-catenin expression in trophoblast differentiation. It is reported that tyrosine kinases induce the tyrosine phosphorylation and ubiquitination of the E-cadherin complex, which causes endocytosis of E-cadherin (Fujita *et al*. 2002, Janda *et al*. 2006). When N-cadherin is highly expressed in day 22 trophoblasts, the increase in E-cadherin degradation could also be involved in the further reduction of the trophectodermal E-cadherin expression. Hence, the loss of E-cadherin as conceptus attachment to luminal epithelium

progresses may play a role in the transition in gene expression required for the successful progression from implantation to placentation.

A number of transcription factors have been reported to regulate the transition in gene expression associated with EMT (Thiery *et al*. 2009). Among these EMT master regulators, I have identified *SNAI2*, *ZEB1*, *ZEB2*, *TWIST1*, *TWIST2* and *KLF8* mRNAs to be up-regulated concurrent with cytokeratin expression in the trophectoderm. It has been characterized that SNAIL, ZEB, and KLF8 factors bind to and repress E-cadherin promoter activity (Peinado *et al*. 2007, Wang *et al*. 2007), whereas TWIST1 and TWIST2 repress E-cadherin transcription indirectly (Yang & Weinberg 2008). One of the SNAILs, SNAI2, was discovered in the chick as a key regulator of mesoderm formation and neural crest migration, two developmental processes involving EMT (Nieto *et al*. 1994). In cell cultures, experimental overexpression of SNAI1 or SNAI2 is sufficient to induce epithelial cells to undergo EMT (Savagner *et al*. 1997, Batlle *et al*. 2000, Cano *et al*. 2000, Bolos *et al*. 2003). However, SNAI1 has been reported to be a more potent inducer of EMT and repressor of E-cadherin than SNAI2 (Batlle *et al*. 2000). The retention of cytokeratin expression in spite of vimentin expression in day 22 trophoblasts could be explained by the increase in SNAI2, not SNAI1, in this study. Moreover, EMT is induced and promoted by extracellular stimuli, including growth factors such as TGFβ and FGF and extracellular matrix protein, and the downstream signaling of their receptors (Said & Williams 2011). This observation, together with the up-regulation of various growth factor receptor *TGFBR2*, *FGFR1*, *PDGFRA*, and *PDGFRB* transcripts demonstrated in this study, suggests that intrauterine proteins secreted during the process of conceptus-endometrium attachment progression could be responsible for the regulation of EMT in the bovine trophectoderm.

SNAIL and ZEB factors are known to induce the expression of metalloproteases that can degrade basement membrane, thereby favoring invasion (Thiery *et al*. 2009). Although the bovine trophoblasts

do not penetrate into the endometrium, up-regulation of *MMP2* and *MMP9* transcripts suggest that these MMPs play a role in non-invasive trophoblasts. The ligand for FGFR1, FGF1, is known to up-regulate *MMP13*, resulting in EMT induction (Billottet *et al.* 2008). It is possible that up-regulation of *MMP2* and *MMP9* could be induced by various growth factors. In humans and rodents, MMPs play a pivotal role in the process of trophoblast invasion throughout the implantation and placentation periods, which require the degradation and remodeling of extracellular matrix at the feto-maternal interface (Cohen *et al.* 2006). MMPs play a similar role in bovine trophoblasts (Hirata *et al.* 2003, Kizaki *et al.* 2008, Dilly *et al.* 2010). Thus, further investigation on the upstream signaling of the up-regulation of *MMP2* and *MMP9* transcription and their roles during the bovine attachment processes may provide insights into trophoblast behavior in the non-invasive mode of implantation.

Our observation showed that the expressions of *ITGA4*, *ITGA8* and *ITGB5*, which encode integrin subunits α4, α8 and β5, respectively, were increased in day 22 conceptuses. The α4 and α8 subunits are known to form heterodimers with β1 subunit and interact with osteopontin (SPP1) (Bayless *et al.* 1998, Schnapp *et al.* 1995), and in combination with αV subunit, β5 subunit is also known to bind to SPP1 (Caltabiano *et al.* 1999). Among those up-regulated integrin subunits, the increase in the expression of integrin α4 was prominent. The integrin α4β1, also known as very late antigen-4 (VLA-4), is normally expressed in leukocytes (Yednock *et al.* 1992) and often seen in mesenchymal stem cells (Kumar & Ponnazhagan 2007). Although the majority of SPP1 binding integrins bind to the RGD (Arg-Gly-Asp) domain of SPP1, the α4β1 integrin interacts with SPP1 through its SVVYGLR (Ser-Val-Val-Tyr-Gly-Leu-Arg) motif (Ito *et al.* 2009). At the implantation sites in the porcine, αVβ6 trophectoderm and αVβ3 uterine epithelial cell integrins bind to SPP1 at the apical membranes, and those integrin-SPP1 bindings may have a critical role in conceptus implantation to the endometrium (Erikson *et al.* 2009). Our observation that the particular subunits of SPP1-binding integrin are up-regulated during the attachment process indicates the possible involvement of SPP1 and its binding partner

integrins in the trophoblast adhesion to endometrial epithelium in the bovine. The prominent expression of integrin α4 may also enable the trophoblast to interact with the SVVYGLR motif of SPP1 even when the RGD domain of SPP1 is occupied by the uterine epithelial cell integrins. These results suggest that both ECM bindings and partial EMT are required for trophoblast adhesion and possibly placental formation in the bovine species.

In conclusion, it has been thought that EMT is the event required for trophoblast migration and invasion to maternal endometrium in mammals with invasive placentation. Results from this study suggest that in addition to ECM bindings, partial EMT is required for proper adhesion of trophoblasts in non-invasive placentation.

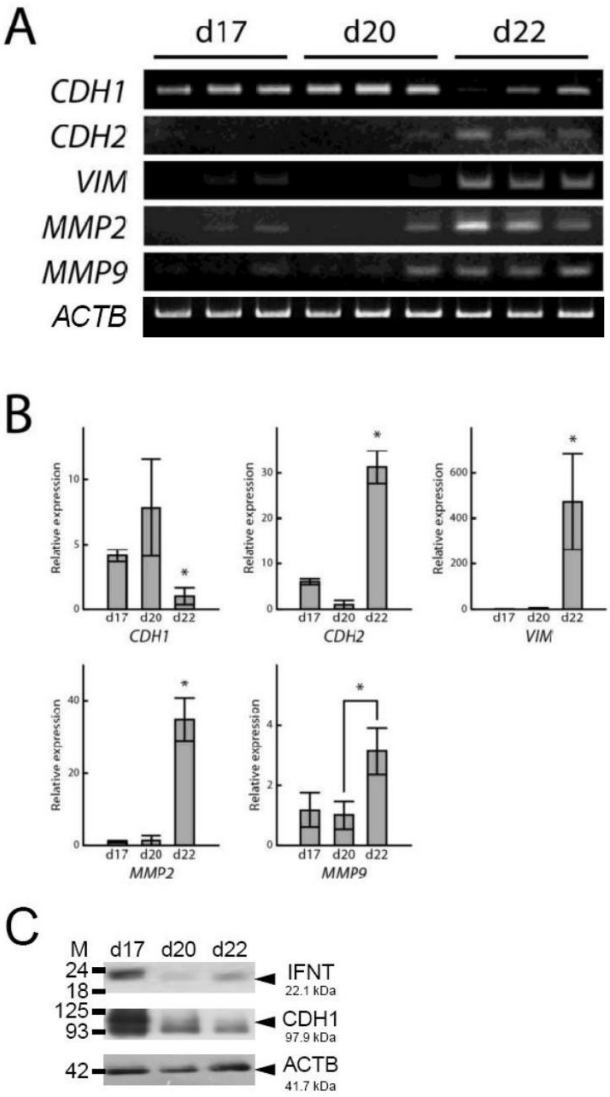
Table 1. Primers for RT-PCR and quantitative RT-PCR analyses

Name (GenBank accession No.)	Sequence	Product length (bp)
CDH1(NM_001002763)	F: GTGATAGATGTGAATGAAGCCC R: AATCCGATACGTGATCTTCTG	167
CDH2 (NM_001166492)	F: CCTCTGACAATGGAATCCCT R: GAGTTTCACAAATCTCTGCCT	137
MMP2 (NM_174745)	F: CTTCAAGGACCGATTCATCTG R: CAGTTAAAGGCAGCATCCAC	280
MMP9 (NM_174744)	F: TTCCTGGGCAAGGAATACTC R: AACAGGCTGTATCCTTGATCC	151
VIM (NM_173969)	F: CAAGTCCAAGTTTGCTGACC R: TCATGTTCTGAATCTCATCCTG	266
SNAI1 (NM_001112708)	F: TTCTCCAGAGCTCACTTTCC R: GAGAGTCCCAGATGAGTGTC	135
SNAI2 (NM_001034538)	F: CAAGGACACATCAGAACTCAC R: CTCTTGCACTGGTATTTCTTGAC	156
ZEB1 (XM_615192)	F: ATATTGCTATACCTACCGTCAC R: TTGCCTTTCATCCTGATTTCC	232
ZEB2 (NM_001076192)	F: AAACTGATGAAGAAGACAAGCC R: ACGAGGTAGGACAATTTGAG	299
KLF8 (XM_603911)	F: CAAGCATCCGTACTGTTCTG R: GGGTTCAACTTTAACTGATCCA	284
TCF3 (XM_609091)	F: CCGAAGATGAGAAGAAGGAC R: TTAGGGTTCAGGTTACGCTC	320
TWIST1 (XM_001250357)	F: GGTACATCGACTTCCTCTACC R: GGAAACAATGACATCTAGGTCTC	227
TWIST2 (NM_001083748)	F: GCCAGGTACATAGACTTCCTC R: GGGTTTCAGAAGTTGCAGAC	272
ITGA4 (NM_174748.1)	F: TCTGGCATAGTGAGACTTGAC R: GGACTTACAAACCCATGAACTG	241
ITGA5 (NM_001166500.1)	F: GTGACGTGGGCCCTGCTGTC R: CCGTCTTTGCAGCCGGTGGT	230

ITGAV (NM_174367.1)	F: ACTCATTTCCGATCAAGTAGCA R: GAAACGAAGTCATCTATGCCA	166
ITGA8 (XM_002701040)	F: ATGGACAATGTCACCCGAAT R: AATTGCTGTCTGGGTTGTCC	172
ITGB1 (NM_174368.2)	F: AAGCCTCTGGGCTTCACTGAGGA R: CCGACACGTCCCTCGTTGCA	155
ITGB3 (XM_616376.3)	F: CGTAAGCTCACCAGTAACCT R: GCCAATCTTCTCATCACAGAC	284
ITGB5 (NM_174679.2)	F: CGGGTCCTTCTGCGAGTGCG R: GCCCCTGGCTCTGTGCACTG	228
ITGB6 (NM_174698)	F: GAACAGCTCCAAGTGCAACA R: ACAAGTGGCAGACACACTGC	194
TGFBR1 (NM_174621)	F: GTTTACCATTGCTTGTTCAGAG R: GTGCCATTGTCTTTATTGTCTG	262
TGFBR2 (NM_001159566)	F: TTCTCCGAAGAGTACACCAG R: TGTTGTGGTTGATGTTGTTGG	291
FGFR1 (NM_001110207)	F: GATGATGACGACGATGACTC R: CATAACGGACCTTGTAGCCT	263
PDGFRA (NM_001192345)	F: GGACAAATGAAAGGCAAAGG R: CAGCACATTCGTAATCTCCA	143
PDGFRB (NM_001075896)	F: CTGTGAAGATGCTGAAATCC R: TAGATGGGTCCTCCTTTAGTG	159
IGF1R (XM_606794)	F: AGAGACATCTATGAGACGGAC R: CAGCTCAAACAGCATGTCAG	280
EGFR (XM_592211)	F: ATGCTCTATGACCCTACCAC R: TTCCGTTACAAACTTTGCCA	236
ACTB (NM_173979.3)	F: TCCCTGGAGAAGAGCTACGA R: GGGCAGTGATCTCTTTCTGC	255

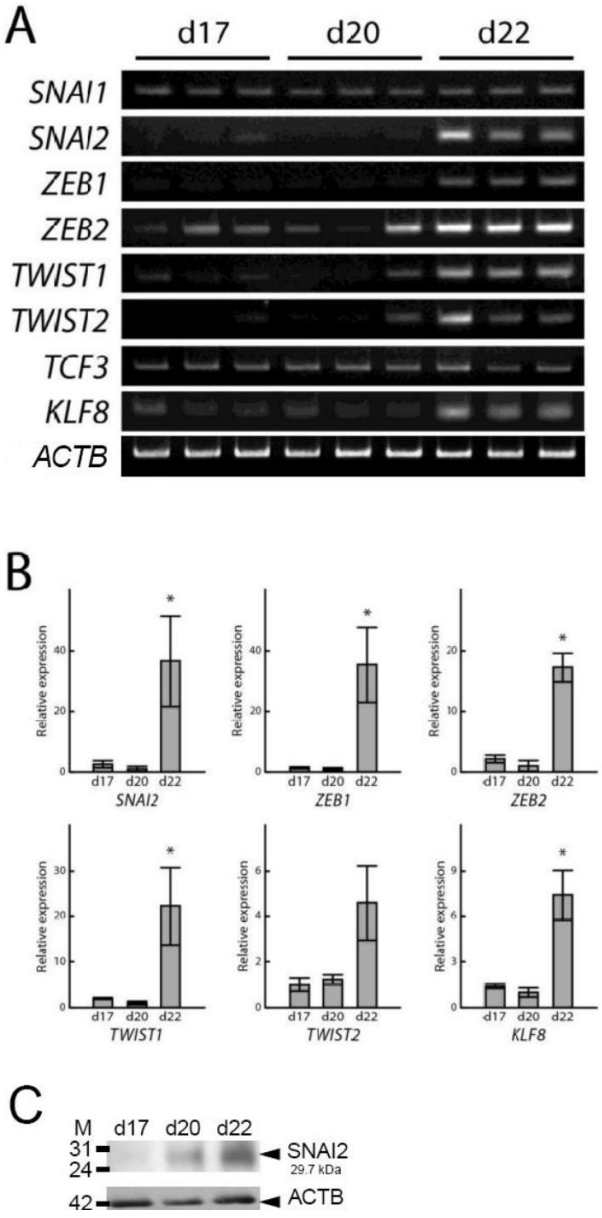
F: Forward primer; R: Reverse primer

Figure 1.



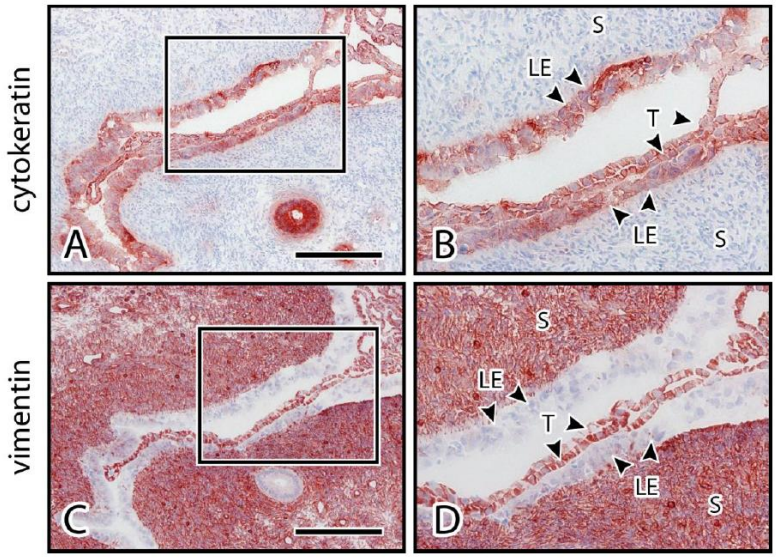
Epithelial and mesenchymal marker mRNAs in bovine conceptuses during peri-attachment period. A: Total RNAs extracted from days 17, 20 and 22 conceptuses (n=3 each day) were subjected to RT-PCR analyses for the detection of E-cadherin (*CDH1*), N-cadherin (*CDH2*), vimentin (*VIM*), *MMP2* and *MMP9* transcripts. *ACTB* mRNA was used as an internal control for RNA integrity. B: Levels of *CDH1*, *CDH2*, *VIM*, *MMP2* and *MMP9* mRNAs in days 17, 20 and 22 conceptuses (n=4 each day) were examined by quantitative RT-PCR. Reactions were performed in duplicate, and the data was analyzed by using the change in cycle threshold value method (Sakurai *et al*. 2009). *ACTB* mRNA served as the internal control. *P < 0.05. C: Expression of CDH1, ACTB and IFNT in days 17, 20 and 22 conceptuses was examined by western blotting. Three independent experiments were performed for each antibody.

Figure 2.



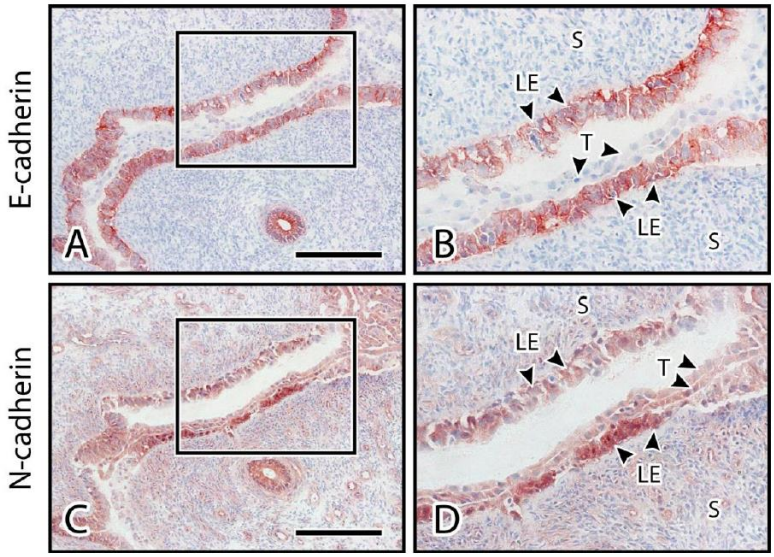
EMT related transcription factor mRNAs in bovine conceptuses during peri-attachment period. A: Total RNAs extracted from days 17, 20 and 22 conceptuses (n=3 each day) were subjected to RT-PCR analyses for the detection of *SNAI1*, *SNAI2*, *ZEB1*, *ZEB2*, *KLF8*, *TCF3*, *TWIST1* and *TWIST2* transcripts. *ACTB* mRNA was used as an internal control for RNA integrity. B: Levels of *SNAI2*, *ZEB1*, *ZEB2*, *TWIST1*, *TWIST2* and *KLF8* mRNAs in days 17, 20 and 22 conceptuses (n=4 each day) were examined by quantitative RT-PCR. Reactions were performed in duplicate, and the data were analyzed by using the change in cycle threshold value method (Sakurai *et al.* 2009). *ACTB* mRNA served as the internal control. *P < 0.05. C: Expression of SNAI2 and ACTB in days 17, 20 and 22 conceptuses were examined by western blotting. Three independent experiments were performed for each antibody.

Figure 3.



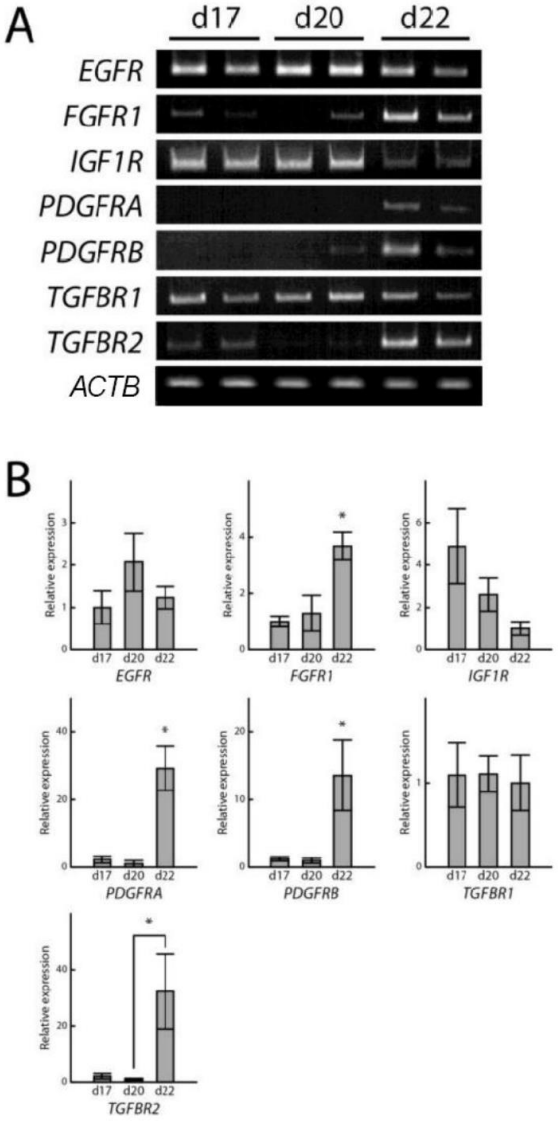
Expression of cytokeratin and vimentin in conceptus and endometrium on day 22. Tissue sections from day 22 uterus (n=3) containing conceptus were immunostained for cytokeratin (A and B) and vimentin (C and D). Boxed areas in A and C are presented at a higher magnification in B and D, respectively. T, trophectoderm; LE, endometrial luminal epithelium; S, endometrial stroma. Scale bar=200 μm.

Figure 4.



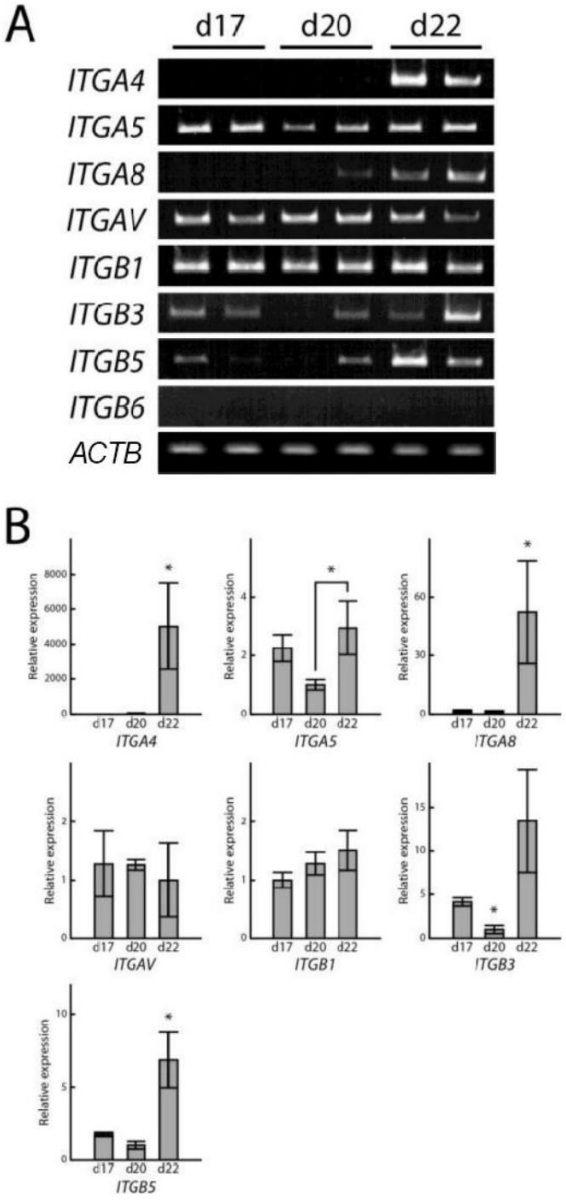
Expression of E-cadherin and N-cadherin in conceptus and endometrium on day 22. Tissue sections from day 22 uterus (n=3) containing conceptus were immunostained for E-cadherin (A and B) and N-cadherin (C and D). Boxed areas in A and C are presented at a higher magnification in B and D, respectively. T, trophectoderm; LE, endometrial luminal epithelium; S, endometrial stroma. Scale bar=200 μm.

Figure 5.



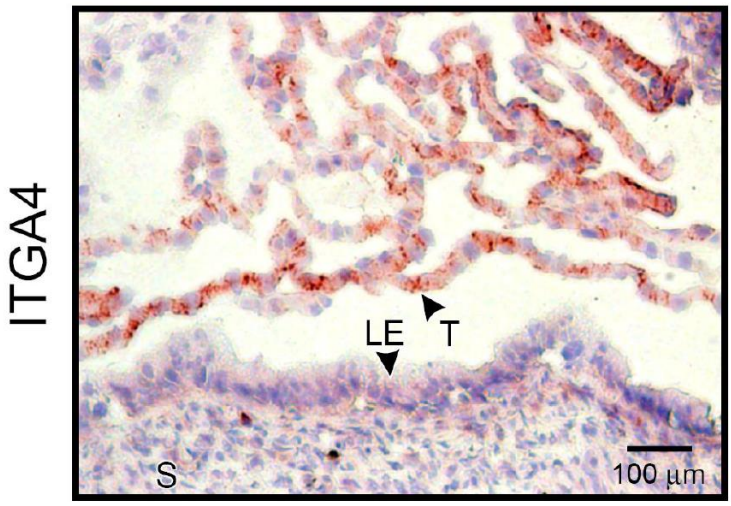
Detection of growth factor receptor mRNAs in bovine conceptuses during peri-attachment period. A: Total RNAs extracted from days 17, 20 and 22 conceptuses (n=3 each day) were subjected to RT-PCR analyses for growth factor receptor transcripts. These included epidermal growth factor receptor (*EGFR*), fibroblast growth factor receptor 1 (*FGFR1*), insulin-like growth factor-1 receptor (*IGF1R*), platelet-derived growth factor receptors (*PDGFRA* and *PDGFRB*) and transforming growth factor β receptors (*TGFBR1* and *TGFBR2*). *ACTB* mRNA was used as an internal control for RNA integrity. B: Levels of *EGFR*, *FGFR1*, *IGF1R*, *PDGFRA*, *PDGFRB*, *TGFBR1* and *TGFBR* mRNAs in days 17, 20 and 22 conceptuses (n=4 each day) were examined by quantitative RT-PCR. Reactions were performed in duplicate, and the data were analyzed by using the change in cycle threshold value method (Sakurai *et al*. 2009). *ACTB* mRNA served as the internal control. *P < 0.05.

Figure 6.



Detection of integrin α (ITGA) and β (ITGB) subunit mRNAs in bovine conceptuses during peri-attachment period. A: Total RNAs extracted from days 17, 20 and 22 conceptuses (n=3 each day) were subjected to RT-PCR analyses for *ITGA4*, *ITGA5*, *ITGA8*, *ITGAV*, *ITGB1*, *ITGB3*, *IITGB5* and *ITGB6* transcripts. *ACTB* mRNA was used as an internal control for RNA integrity. B: Levels of *ITGA4*, *ITGA5*, *ITGA8*, *ITGAV*, *ITGB1*, *ITGB3* and *ITGB5* mRNAs in days 17, 20 and 22 conceptuses (n=4 each day) were examined by quantitative RT-PCR. Reactions were performed in duplicate, and the data were analyzed using the change in cycle threshold value method (Sakurai *et al*. 2009). *ACTB* mRNA served as the internal control. *P < 0.05.

Figure 7.



Expression of integrin alpha 4 (ITGA4) in conceptus and endometrium on day 22. Tissue sections from day 22 uterus (n=3) containing conceptus were immunostained for integrin alpha 4. T, trophectoderm; LE, endometrial luminal epithelium; S, endometrial stroma. Scale bar=100 µm

General Discussion

Regardless of mammalian species, the maintenance of sufficient maternal-fetal communication throughout pregnancy is required for proper embryonic-fetal development as well as maternal health. It has been known that due to insufficient communication between the developing embryo and uterine endometrium, substantial number of embryonic losses occurs during the peri-attachment period. To successfully establish pregnancy in mammals, therefore, the appropriate communication between conceptus and maternal endometrium is necessary. Although numerous investigations have revealed many factors expressed in the uterus at the peri-implantation stage, the continual decline in bovine pregnancy rate has not yet been halted. Therefore, in this study, I attempted to examine the implantation events from different perspective, namely lymphocyte homing.

In chapter 1, I demonstrated that SELL could play a role for bovine implantation, but its role in slowing down conceptus migration in the bovine species may be less important than in humans (Genbacev *et al.* 2003) or in lymphocyte homing (Rosen 2004). To investigate the function of SELL on conceptus attachment to the endometrial epithelial cells, I used an *in vitro* attachment model of bovine trophoblast CT-1 cells to EECs. These observations indicated that down-regulation of *SELL* expression with its siRNA in the EECs of the coculture system resulted in failure of CT-1 cell attachment to EECs. The experiment of cultured EECs with various treatments also indicated that SELL was up-regulated by soluble factors, possibly cytokines and CAMs, present in uterine flushings from day 20 pregnant animals, and EGF could be one of the regulators for SELL expression in endometrium *in vivo* during peri-implantation period. These and the results from SELL ligand, Podocalyxin-like protein 1 (PODXL) (Dominguez *et al*. 2005), examinations suggest that secretion of EGF in the uterine lumen regulated the expression of SELL in the endometrium through the EGF receptor, and the up-regulated SELL, together with conceptus PODXL, is involved in initiating conceptus attachment process in the bovine species. It should be noted that the expression site of SELL would have been at the conceptus side, if the finding from the lymphocyte homing could directly be applied to those of bovine conceptus

implantation to the uterine endometrium. Differences in these expressions should further be examined in the context of bovine conceptus implantation, which may provide a key event for the initiation of conceptus implantation, previously unsuspected.

In chapter 2, I examined VCAM-1 expression, its effects and transcriptional regulation. The expression of VCAM-1 was detected at the trophoblast and uterine epithelium, as well as in uterine glandular epithelium and endothelium during the peri-implantation period. VCAM-1 is well-known as a cell adhesion mediator during the process of lymphocyte homing (May *et al.* 1993), angiogenesis (Ding *et al.* 2003) and even placentation (Gurtner *et al.* 1995). Among possible VCAM-1 receptors (Chu *et al.* 2013), ITGA4 was detected at the apical surface and inter-cell surface of day 22 trophoblasts. I demonstrated that *VCAM-1* transcript was up-regulated when treated with uterine flushings from day 22 pregnant animals, and that VCAM-1 protein in EECs treated with EGF or bFGF was enhanced in the cytoplasm. However, the expression of VCAM-1 could be found at the cell surface of EECs only whencocultured with bovine trophoblast CT-1 cells. These observations indicated that VCAM-1 in EECs was regulated by the soluble factors secreted in the uterine cavity, but the increases in cell surface expression of VCAM-1 protein required the interaction with CT-1 cells. These results strongly suggest that the up-regulation of the VCAM-1/ITGA4 complex may aid in attachment processes between trophoblasts and uterine epithelium, and therefore previously unsuspected VCAM-1 and ITGA4 are associated with conceptus attachment to the uterine epithelium in the bovine species. The expression of VCAM-1 was at the uterine epithelium, which would have been expected from the study of lymphocyte homing. It appeared that endometrial VCAM-1 would work through the conceptus ITGA4, however, the result in which VCAM-1 was also localized at the cell surface of trophoblast. The expression patterns of VCAM-1 in bovine implantation differed from those of lymphocyte homing, requiring further experimentation on the function of conceptus VCAM-1, if any, within the context of bovine trophoblast attachment/adhesion to the uterine epithelium.

In chapter 3, I demonstrated that after bovine conceptus-endometrium attachment, the trophectoderm expressed both vimentin and cytokeratin, which are the mesenchymal and epithelial cell markers, respectively, the phenomenon suggestive of epithelial-mesenchymal transition (EMT). Among the transcription factors associated with EMT (Thiery *et al*. 2009), I have identified *SNAI2*, *ZEB1*, *ZEB2*, *TWIST1*, *TWIST2* and *KLF8* mRNAs to be up-regulated concurrent with cytokeratin expression in the trophectoderm. Although SNAI1 has been reported to be a more potent inducer of EMT and repressor of E-cadherin than SNAI2 (Batlle *et al*. 2000), in this study changes in EMT-related gene expression could be caused by the increase in SNAI2 but not SNAI1. Expression of both epithelial and mesenchymal markers in the bovine trophoblast could be considered as a partial EMT. Together with highly expressed N-cadherin in day 22 trophoblasts, the increase in E-cadherin degradation suggested that the partial EMT occurring in day 22 trophoblast may play a role in the transition in gene expression required for the successful progression from implantation to placentation. The phenomenon of partial EMT was also found in the luminal epithelium of long-term progesterone-treated pigs (Bailey *et al*. 2010). Moreover, the up-regulation of various growth factor receptor *TGFBR2*, *FGFR1*, *PDGFRA*, and *PDGFRB* transcripts indicated that intrauterine proteins secreted during the process of conceptus-endometrium attachment progression could be responsible for the regulation of EMT in the bovine trophectoderm. Furthermore, the expressions of *ITGA4*, *ITGA8* and *ITGB5* were increased in day 22 conceptuses, which are potential receptors for the ECM (Bayless *et al*. 1998, Erikson *et al*. 2009). MMPs, particularly MMP2 and MMP9 (Hirata *et al*. 2003, Kizaki *et al*. 2008, Dilly *et al*. 2010), are well known to function in the process of trophoblast invasion throughout the implantation and placentation periods in humans and rodents (Cohen *et al*. 2006). Up-regulation of *MMP2* and *MMP9* transcripts in this study indicated that these MMPs could also play roles in non-invasive trophoblasts. These results suggest that both ECM bindings and partial EMT are required for trophoblast adhesion to the uterine endometrium in the bovine species.

Throughout the course of my studies, uterine flushings, particularly those from days 20-22 pregnant uteri, had significant contribution to mimic the cellular events associated with conceptus implantation to the maternal endometrium. Although molecules present in the uterine flushings are being analyzed in the laboratory, they are not available for further analysis at this time. However, I am more than certain that molecules present in uterine flushings during peri-implantation period such as EGF or bFGF induced SELL and VCAM-1 expression in the endometrium through their endometrial receptors. The up-regulated SELL together with the ligands expressed on conceptuses may be involved in the initial step in conceptus attachment to the uterine endometrium. More importantly, this cell-cell attachment stimulation between trophectoderm cells and uterine epithelial cells further up-regulated VCAM-1 expression at the cell surface. These cell surface expressions of VCAM-1 allow the interaction of the ligand with the receptor, ITGA4, which is likely to be required for stable conceptus adhesion to the endometrial epithelium in the bovine species. Moreover, the significantly up-regulated PODXL, VCAM-1, ITGA4, EGF, EGFR together with marker of EMT in trophectoderm cells during the implantation period suggest that in addition to ECM bindings, partial EMT is also required for proper adhesion of trophoblasts to the uterine endometrium. In these experiments, I modeled lymphocyte homing on the study of SELL and VCAM-1 in bovine implantation processes, however, the expression pattern of these factors were not the same between two events. Although expression patterns of SELL and VCAM-1 were determined, their roles in bovine implantation are far from understanding to the point where these knowledge could be used for the improvement of pregnancy rate in the bovine species.

The studies discussed here intend to examine and clarify the proteins associated with gene expression within the context of bovine conceptus attachment and adhesion to the uterine endometrium, so as to provide the framework needed to investigate the mechanisms of implantation in cattle. My studies

provide new insights for researches into the mechanisms of bovine implantation, during which significant fraction of embryonic losses occurs. Rather than the study of a single event or factor during the peri-implantation period, it is clear that the whole picture of how these factors interact with each other, and how they can work together spatio-temporally must be studied in the future. With those data, I am confident that application of more than one factor will increase chances of proper implantation, leading to the improvement of pregnancy in Japan and throughout cattle industry in the world.

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