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

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Abstract

In the course of experiments to identify and characterize the 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 estrous) bovine conceptuses and subjected to real-time PCR analysis as well as Western blotting, from which abundances of N-cadherin (CDH2), vimentin, matrix metalloproteinase 2 (gelatinase A, 72 kDa gelatinase, 72 kDa type IV collagenase) (MMP2), and matrix metalloproteinase 9 (gelatinase B, 92 kDa gelatinase, 92 kDa type IV collagenase) (MMP9) mRNAs were determined on day 22, concurrent with (CDH1) mRNA and protein downregulation. Transcription factors in EMT processes were then analyzed and changes in snail homolog 2 (*Drosophila*) (*SNAIL*), zinc finger E-box binding homeobox 1 (*ZEB1*), zinc finger E-box binding homeobox 2 (*ZEB2*), twist homolog 1 (*Drosophila*) (*TWIST1*), twist homolog 2 (*Drosophila*) (*TWIST2*), and Kruppel-like factor 8 (*KLF8*) transcripts were found in day 22 conceptuses, while confirming *SNAIL2* expression by Western blotting. Immunohistochemical analysis revealed that the day 22 trophoctoderm expressed the mesenchymal markers N-cadherin and vimentin as well as the epithelial marker cytokeratin. In attempts to identify the molecular mechanisms by which the trophoctoderm expressed EMT-related genes, growth factor receptors associated with EMT were analyzed. Upregulation of the growth factor receptor transcripts, fibroblast growth factor receptor 1 (*FGFR1*), platelet-derived growth factor receptor, alpha polypeptide (*PDGFR α*), platelet-derived growth factor receptor, beta polypeptide (*PDGFR β*), and transforming growth factor, beta receptor II (70/80 kDa) (*TGFR β 2*) mRNAs, was found on day 22. The analysis was extended to determine the integrin (ITG) transcripts and found high levels of integrin, alpha 4 (antigen CD49D, alpha 4 subunit of VLA-4 receptor) (*ITGA4*), integrin, alpha 8 (*ITGA8*), integrin, beta 3 (platelet glycoprotein IIIa, antigen CD61) (*ITGB3*), and integrin, beta 5 (*ITGB5*) mRNAs on day 22. These observations indicate that after the conceptus–endometrium attachment, EMT-related transcripts as well as the epithelial marker cytokeratin were present in the bovine trophoctoderm and suggest that the implantation process for noninvasive trophoblasts requires not only extracellular matrix expression but also partial EMT.

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Introduction

Embryo implantation is an essential process for viviparity (Amoroso 1968) that differs among species and includes both noninvasive and invasive trophoblasts. However, the implantation process invariably begins from blastocyst migration, apposition, attachment, and adhesion to the 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 the inner cell mass and trophoctoderm (Nishioka *et al.* 2009). The trophoctoderm forms the epithelial structure of the blastocyst and possesses

epithelial characteristics, including apicobasal cell polarity, lateral junctions with neighboring cells, and basal contact with the basement membrane proteins (Biggers *et al.* 1988, Kang *et al.* 1990, Thorsteinsdóttir 1992, Fleming *et al.* 2001). Despite the fact that the apical plasma membranes of simple epithelia normally lack adhesive properties, the trophoctoderm still manages to adhere to the uterine epithelium through its apical domains as part of the implantation process. Thus, the adhesion between trophoctoderm 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 (ZP), 2) precontact

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 prereceptive 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 preattachment period is followed by incremental apposition and attachment of the conceptus to the luminal epithelium. In bovine species, attachment is first observed in day 20 trophoblasts that 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 trophoblast contacts between 22 and 28 days (Wathes & Wooding 1980). A long preattachment period is characterized, at least in part, by apical expression of mucin 1, cell surface associated (*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 (ITG) subunits in the trophoblast adhesion to luminal epithelium has been well characterized in sheep and pigs (Johnson *et al.* 2001). ITGs are the dominant glycoproteins in adhesion cascades, owing to their ability to bind to 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 expression that determines the cell properties of the trophoblast during this period has 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 trophoblast undergoes a partial epithelial–mesenchymal transition (EMT), which involves downregulation of apicobasal polarity, expression of mesenchymal-type adhesion proteins, reorganization of the cytoskeleton, and expression of matrix metalloproteinases (MMPs; Denker 1993, Thie *et al.* 1996, Vičová & Aplín 1996). In addition, the signaling involved in regulating EMT events aids in allowing adhesion at the apical membrane of the trophoblast (Hohn & Denker 2002). Yet, to date, it is unclear exactly how the trophoblast acquires adhesive and/or invasive competence, and thus how EMT events relate to the noninvasive 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 ZP 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 domestic animals such as porcine, ovine, and bovine species, on the other hand, the trophoblast cells do not penetrate the uterine epithelium but

Figure 1 Epithelial and mesenchymal marker mRNAs in bovine conceptuses during the 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 cadherin 1, type 1, E-cadherin (epithelial) (*CDH1*), cadherin 2, type 1, N-cadherin (neuronal) (*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 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 *CDH1*, *ACTB*, and *IFNT* in days 17, 20, and 22 conceptuses was examined by Western blotting. Three independent experiments were performed for each antibody.

rather maintain an apical–apical adhesion throughout the implantation process (Chavatte-Palmer & Guillomot 2007). Understanding the events associated with 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 trophoctodermal 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). We hypothesized that molecular events similar to the EMT process would be required to make trophoctoderm adhesion to the endometrial epithelium possible. In this paper, we demonstrate that changes in gene expression associated with the EMT occur in the bovine trophoctoderm following conceptus attachment to the luminal epithelium.

Results

Upregulation of EMT markers in day 22 bovine conceptuses

To evaluate the expression patterns of transcripts typical of the EMT process, cadherin 1, type 1, E-cadherin (epithelial) (*CDH1*), cadherin 2, type 1, N-cadherin (neuronal) (*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 the level 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). We also examined *MMP2* and *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 RT-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).

Upregulation 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, we 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). Upregulation of *SNAI2*, *ZEB1*, *ZEB2*, *TWIST1*,

Figure 2 EMT-related transcription factor mRNAs in bovine conceptuses during the 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.

TWIST2, and *KLF8* mRNAs was also confirmed by quantitative RT-PCR (Fig. 2B). Levels of *SNAI2* and *ZEB1* transcripts on day 22 were ~40 times greater than those on day 20 (Fig. 2B). In addition, levels of *ZEB2* and *TWIST1* mRNAs increased ~20-fold on day 22 compared with those on day 20 (Fig. 2B). Upregulation of these transcription factor mRNAs in day 22 conceptuses was consistent with the occurrence of the EMT event in the trophoderm of these conceptuses. To support the results from the RT-PCR analysis, Western blotting was carried out to characterize changes in the 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

Upregulation of transcripts associated with the EMT process led us to believe that EMT marker proteins would also be present in day 22 conceptuses. We first performed immunohistochemistry for cytokeratin as an epithelial marker and VIM 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 VIM, and the stromal cells were cytokeratin-negative and VIM-positive (Fig. 3). On the other hand, the trophoblast layer of the conceptus was positive for both cytokeratin and VIM (Fig. 3).

The expression of the mesenchymal marker in the trophoderm led us to evaluate changes in the CDH1 and/or CDH2 proteins, which have been identified as key events in the EMT during embryogenesis and carcinogenesis (Thiery 2003, Katoh & Katoh 2008). Immunolocalization of CDH1 and CDH2 revealed that the trophoblast

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

Figure 4 Expression of CDH1 and CDH2 in conceptus and endometrium on day 22. Tissue sections from day 22 uteri ($n=3$) containing conceptuses were immunostained for (A and B) CDH1 and (C and D) CDH2. Boxed areas in A and C are presented at a higher magnification in B and D respectively. T, trophoderm; LE, (endometrial) luminal epithelium; S, (endometrial) stroma. Scale bar = 200 μ m.

layer of day 22 conceptus expressed CDH2, whereas the expression of CDH1 in the trophoderm was not detected by immunohistochemistry (Fig. 4). The endometrial stromal cells expressed only CDH2, whereas both luminal and glandular epithelia of the endometrium expressed both CDH1 and CDH2 (Fig. 4). Relatively strong expression of CDH2 was found on the region of the luminal epithelium where the trophoderm attached (Fig. 4).

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

Unique protein expressions found in the trophoderm layer, including the upregulation of CDH2 and VIM on day 22, indicated that the epithelial trophoderm exhibited protein expression commonly associated with the mesenchyme (Figs 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 trophoderm (Figs 3 and 4). To elucidate the 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 receptor, alpha and beta polypeptides (PDGFRA and PDGFRB), and transforming growth factor β receptors (TGFB1 and TGFB2) (Said & Williams 2011). The expression of *EGFR* and *TGFB1* mRNAs was found from days 17 through 22 (Fig. 5A). The expression levels of *FGFR1*, *PDGFRA*, *PDGFRB*, and *TGFB2* mRNAs were low or undetectable on days 17

Figure 5 Detection of growth factor receptor mRNAs in bovine conceptuses during the 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-I receptor (IGFIR), 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*, *IGFIR*, *PDGFRA*, *PDGFRB*, *TGFBR1*, and *TGFBR2* 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. * $P<0.05$.

and 20 and became detectable on day 22 (Fig. 5A). Quantitative RT-PCR revealed the upregulation 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 ITG mRNAs in peri-implantation conceptuses

ITGs are also known to mediate EMT independently or in cooperation with growth factor receptors (Larue & Bellacosa 2005). We thus examined the levels of α - and β -subunits of ITG (*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 upregulation 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, we further examined the expression of *ITGA4* on tissue sections from day 22 pregnant uteri and found positive immunostaining on that day (Supplementary Figure 1, see section on supplementary data given at the end of this paper).

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, we demonstrated that after bovine conceptus–endometrium attachment, the trophoblast expressed VIM while cytokeratin expression was still retained. Since the bovine trophoblast cells from mid-gestational placentomes do not express VIM (Haeger *et al.* 2010), this coexistence of the epithelial and mesenchymal gene expression in the trophoblast could be transient and possibly be a prerequisite for the steps from attachment to adhesion. Recently, coexpression of cytokeratin and VIM 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, 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 (Grünert *et al.* 2003, Huber *et al.* 2005).

Nakano *et al.* (2005) have reported that CDH1 expression is distributed within the cytoplasm of trophoblast binucleate cells in the bovine placental.

Figure 6 Detection of integrin α (ITGA) and β (ITGB) subunit mRNAs in bovine conceptuses during the 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*, *ITGB5*, 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 by using the change in cycle threshold value method (Sakurai *et al.* 2009). *ACTB* mRNA served as the internal control. * $P<0.05$.

They have also noted the translocation of β -catenin into the nuclei of trophoblast binucleate cells, indicating the role of CDH1- β -catenin expression in trophoblast differentiation. It is reported that tyrosine kinases induce the tyrosine phosphorylation and ubiquitination of the CDH1 complex, which causes endocytosis of CDH1 (Fujita *et al.* 2002, Janda *et al.* 2006). When CDH2 is highly expressed in day 22 trophoblasts, the increase in CDH1 degradation could also be involved in the further reduction of trophoblast CDH1 expression. Hence, the loss of CDH1 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, we have identified *SNAI2*, *ZEB1*, *ZEB2*, *TWIST1*, *TWIST2*, and *KLF8* mRNAs to be upregulated concurrently with cytokeratin expression in the trophoblast. It has been characterized that *SNAI1*, *ZEB*, and *KLF8* factors bind to and repress CDH1 promoter activity (Peinado *et al.* 2007, Wang *et al.* 2007), whereas *TWIST1* and *TWIST2* repress CDH1 transcription indirectly (Yang & Weinberg 2008). *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, Bolós *et al.* 2003). However, *SNAI1* has been reported to be a more potent inducer of EMT and repressor of CDH1 than *SNAI2* (Batlle *et al.* 2000). The retention of cytokeratin expression in spite of VIM 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 TGF β and FGF and ECM protein, and the downstream signaling of their receptors (Said & Williams 2011). This observation, together with the upregulation 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 trophoblast.

SNAI and *ZEB* factors are known to induce the expression of MMPs that can degrade the basement membrane, thereby favoring invasion (Thiery *et al.* 2009). Although bovine trophoblasts do not penetrate into the endometrium, upregulation of *MMP2* and *MMP9* transcripts suggests that these MMPs play a role in non-invasive trophoblasts. The ligand for *FGFR1*, FGF1, is known to upregulate *MMP13*, resulting in EMT induction (Billottet *et al.* 2008). It is possible that upregulation 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 ECM 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 upregulation 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.

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

Name (GenBank accession no.)	Sequence	Product length (bp)
<i>CDH1</i> (NM_001002763)	F: GTGATAGATGTGAATGAAGCCC R: AATCCGATACGTGATCTTCTG	167
<i>CDH2</i> (NM_001166492)	F: CCTCTGACAATGGAATCCCT R: GAGTTTCACAAATCTCTGCCT	137
<i>MMP2</i> (NM_174745)	F: CTTCAAGGACCGATTATCATCTG R: CAGTTAAAGGCAGCATCCAC	280
<i>MMP9</i> (NM_174744)	F: TTCCTGGGCAAGGAATACTC R: AACAGGCTGTATCCTTGATCC	151
<i>VIM</i> (NM_173969)	F: CAAGTCCAAGTTTGCTGACC R: TCATGTTCTGAATCTCATCCTG	266
<i>SNAI1</i> (NM_001112708)	F: TTCTCCAGAGCTCACTTTCC R: GAGAGTCCCAGATGAGTGTC	135
<i>SNAI2</i> (NM_001034538)	F: CAAGGACACATCAGAACTCAC R: CTCTTGCACTGGTATTTCTTGAC	156
<i>ZEB1</i> (XM_615192)	F: ATATTGCTATACCTACCGTCAC R: TTGCCTTTCATCCTGATTTC	232
<i>ZEB2</i> (NM_001076192)	F: AAAGTGTGAAGAAGACAAGCC R: ACGAGGTAGGACAATTTGAG	299
<i>KLF8</i> (XM_603911)	F: CAAGCATCCGTACTGTTCTG R: GGGTTCAACTTTAACTGATCCA	284
<i>TCF3</i> (XM_609091)	F: CCGAAGATGAGAAGAAGGAC R: TTAGGGTTCAAGTTACGCTC	320
<i>TWIST1</i> (XM_001250357)	F: GGTACATCGACTTCCTCTACC R: GGAAACAATGACATCTAGGTCTC	227
<i>TWIST2</i> (NM_001083748)	F: GCCAGGTACATAGACTTCCTC R: GGGTTTCAGAGTTGCAGAC	272
<i>ITGA4</i> (NM_174748.1)	F: TCTGGCATAGTGAGACTTGAC R: GGAATTACAAACCCATGAAGTG	241
<i>ITGA5</i> (NM_001166500.1)	F: GTGACGTGGGCGCTGCTGTC R: CCGTCTTTGCAGCCGGTGGT	230
<i>ITGAV</i> (NM_174367.1)	F: ACTCATTTCCGATCAAGTAGCA R: GAAACGAAGTCATCTATGCCA	166
<i>ITGA8</i> (XM_002701040)	F: ATGGACAATGTCACCCGAAT R: AATTGCTGTCTGGGTTGTCC	172
<i>ITGB1</i> (NM_174368.2)	F: AAGCCTCTGGGCTTCACTGAGGA R: CCGACACGTCCTCGTTGCA	155
<i>ITGB3</i> (XM_616376.3)	F: CGTAAGCTCACCAGTAACCT R: GCCAATCTTCTCATCACAGAC	284
<i>ITGB5</i> (NM_174679.2)	F: CCGGTCCTTCTGCGAGTGCG R: GCCCCTGGCTCTGTGCACTG	228
<i>ITGB6</i> (NM_174698)	F: GAACAGCTCCAAGTGCAACA R: ACAAGTGGCAGACACACTGC	194
<i>TGFB1</i> (NM_174621)	F: GTTTACCATTGCTTGTTGAGAG R: GTGCCATTGTCTTATTGTCTG	262
<i>TGFB2</i> (NM_001159566)	F: TTCTCCGAAGAGTACACCAG R: TGTGTGGTTGATGTTGTTGG	291
<i>FGFR1</i> (NM_001110207)	F: GATGATGACGACGATGACTC R: CATAACGGACCTTGATGCTC	263
<i>PDGFRA</i> (NM_001192345)	F: GGACAAATGAAAGGCAAAGG R: CAGCACATTCTGTAATCTCCA	143
<i>PDGFRB</i> (NM_001075896)	F: CTGTGAAGATGCTGAAATCC R: TAGATGGGCTCCTTTAGTG	159
<i>IGF1R</i> (XM_606794)	F: AGAGACATCTATGAGACGGAC R: CAGCTCAAACAGCATGTGAG	280
<i>EGFR</i> (XM_592211)	F: ATGCTCTATGACCTACCAC R: TTCCGTTACAACTTTGCCA	236
<i>ACTB</i> (NM_173979.3)	F: TCCCTGGAGAAGAGCTACGA R: GGGCAGTGATCTCTTTCTGC	255

F, forward primer; R, reverse primer.

Our observation showed that the expressions of *ITGA4*, *ITGA8*, and *ITGB5*, which encode the ITG subunits $\alpha 4$, $\alpha 8$, and $\beta 5$, respectively, were increased in day 22 conceptuses. The $\alpha 4$ and $\alpha 8$ subunits are known to form heterodimers with the $\beta 1$ subunit and interact with osteopontin (SPP1; Schnapp *et al.* 1995, Bayless *et al.* 1998), and in combination with the αV subunit, the $\beta 5$ subunit is also known to bind to SPP1 (Caltabiano *et al.* 1999). Among those upregulated ITG subunits, the increase in the expression of ITG $\alpha 4$ was prominent. ITG $\alpha 4\beta 1$, also known as very late antigen-4 (VLA-4), is normally expressed in leukocytes (Yednock *et al.* 1992) and is often seen in mesenchymal stem cells (Kumar & Ponnazhagan 2007). Although the majority of SPP1-binding ITGs bind to the RGD (Arg-Gly-Asp) domain of SPP1, $\alpha 4\beta 1$ ITG 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, the $\alpha V\beta 6$ trophoblast and $\alpha V\beta 3$ uterine epithelial cell ITGs bind to SPP1 at the apical membranes, and those ITG–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 ITG are upregulated during the attachment process indicates the possible involvement of SPP1 and its binding partner ITG in the trophoblast adhesion to the endometrial epithelium in the bovine. The prominent expression of ITG $\alpha 4$ 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 ITGs. These results suggest that both ECM binding 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 the maternal endometrium in mammals with invasive placentation. Results from this study suggest that, in addition to ECM binding, partial EMT is required for proper adhesion of trophoblasts in non-invasive placentation.

Materials and Methods

Animals and tissue preparation

All animal procedures in this 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 ET were performed as described previously (Ideta *et al.* 2007). Seven-day embryos (day 0 = day of estrous) were collected from superovulated Japanese black cattle. Thirty embryos derived from the superovulation were transferred non-surgically into the uterine horn of 15 Holstein heifers (two blastocysts/recipient), ipsilateral to the corpus luteum on day 7 of the estrous cycle. For RNA analyses, elongated conceptuses were collected non-surgically by uterine flushing on day 17, 20, or 22 (four animals each) with 500 ml sterile PBS (pH 7.2).

Conceptuses in the uterine flushing media were obtained by centrifugation at 180 *g* for 5 min and snap-frozen in liquid nitrogen. For immunohistochemistry, hysterectomy was performed on day 22 ($n=3$), and the uterine tissues containing the conceptus were frozen in dry ice-cooled heptane. Samples were transferred to the 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 Co., Ltd, 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 diluted cDNA reaction mixture, 0.5 units Ex Taq DNA polymerase (Takara Biomedicals, Tokyo, Japan), Ex Taq buffer, 0.2 μM oligonucleotide primers described in Table 1, and 0.2 mM deoxyribonucleotide triphosphate (dNTP) in a final volume of 20 μl . The thermal profile for PCR was at 95°C for 2 min, followed by 30 or 35 cycles of 95°C for 30 s, 57°C for 30 s, and 72°C for 30 s. The PCR products were separated on a 2.0% agarose gel containing ethidium bromide and visualized under u.v. 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 Ex Taq HS polymerase (Takara Biomedicals), Ex Taq HS buffer, 0.5 μM oligonucleotide primers listed in Table 1, 2.5 mM dNTP, SYBR green (SYBR Green I Nucleic Acid Gel Stain, Takara Biomedicals) as fluorescence intercalator, 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). The thermal profile for real-time PCR was at 95°C for 10 min, followed by 40 cycles of 95°C for 10 s, 60°C for 20 s, and 72°C for 40 s. Average threshold (C_t) values for mRNA were calculated and normalized to C_t 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-PAGE, transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Bedford, MA, USA), and then treated with rabbit anti-human CDH1 antibody (1:1000; Cell Signaling Technology, Danvers, MA, USA), rabbit polyclonal SNAI2 antibody (1:1000; Abcam, Cambridge, MA, USA), 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). The proteins were

detected using the secondary antibody conjugated with HRP (goat antirabbit, 1:10 000; Irritant NA934V) diluted with Can Get Signal Solution II on an ECL Western blotting detection system (Amersham Pharmacia Biotech; 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, Inc. 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-bovine cytokeratin polyclonal antibody (1:100 dilution, catalog no. Z0622; Dako Deutschland GmbH, Hamburg, Germany), mouse anti-human VIM MAB (clone V9, 1:100 dilution, catalog no. M0725; Dako), rabbit antimouse CDH1 polyclonal antibody (1:100 dilution, catalog no. 3195; Cell Signaling Technology), rabbit anti-human CDH2 polyclonal antibody (1:100 dilution, catalog no. ab12221; Abcam), or mouse anti-human ITGA4 (ITG 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 antirabbit IgG biotin conjugate (1:800 dilution, catalog no. B8895; Sigma–Aldrich) or goat antimouse 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 counterstained with hematoxylin and then examined under a light microscope (BX-51; Olympus, Tokyo, Japan).

Statistical analyses

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

Supplementary data

This is linked to the online version of the paper at <http://dx.doi.org/10.1530/REP-11-0364>.

Declaration of interest

The authors declare that there is no conflict of interest that could be perceived as prejudicing the impartiality of the research reported.

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