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Trophectoderm differentiation in the bovine embryo: characterization of a polarized epithelium

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Blastocyst formation is dependent on the differentiation of a transporting epithelium, the trophoderm, which is coordinated by the embryonic expression and cell adhesive properties of E-cadherin. The trophoderm shares differentiative characteristics with all epithelial tissues, including E-cadherin-mediated cell adhesion, tight junction formation, and polarized distribution of intramembrane proteins, including the Na–K ATPase. The present study was conducted to characterize the mRNA expression and distribution of polypeptides encoding E-cadherin, β-catenin, and the tight junction associated protein, zonula occludens protein 1, in pre-attachment bovine embryos, in vitro. Immunocytochemistry and gene specific reverse transcription–polymerase chain reaction methods were used. Transcripts for E-cadherin and β-catenin were detected in embryos of all stages throughout pre-attachment development. Immunocytochemistry revealed E-cadherin and β-catenin polypeptides evenly distributed around the cell margins of one-cell zygotes and cleavage stage embryos. In the morula, detection of these proteins diminished in the free apical surface of outer blastomeres. E-cadherin and β-catenin became restricted to the basolateral membranes of trophoderm cells of the blastocyst, while maintaining apolar distributions in the inner cell mass. Zonula occludens protein 1 immunoreactivity was undetectable until the morula stage and first appeared as punctate points between the outer cells. In the blastocyst, zonula occludens protein 1 was localized as a continuous ring at the apical points of trophoderm cell contact and was undetectable in the inner cell mass. These results illustrate that the gene products encoding E-cadherin, β-catenin and zonula occludens protein 1 are expressed and maintain cellular distribution patterns consistent with their predicted roles in mediating trophoderm differentiation in in vitro produced bovine embryos.

Introduction

Transporting epithelia differentiate from apolar cells during development 'in concert' with the formation of epithelial junctional complexes (Boller et al., 1985; Gumbiner and Simons, 1987; Gumbiner et al., 1988), resulting in the establishment of distinct apical and basolateral plasma membrane domains (Vestweber et al., 1987; D’Angelo Siliciano and Goodeough, 1988; Fleming and Johnson, 1988; Rodriguez-Boulan and Nelson, 1989; Wiley et al., 1990; Watson, 1992; Watson et al. 1992a; Collins and Fleming, 1995). The epithelial junctional complexes are macromolecular structures consisting of zonula occludens (that is, tight junctions), zonula adherens (that is, adherent junctions), macula adherens (that is, desmosomes), and gap junctions (Fleming et al. 1991, 1993; Citi, 1993; Kidder 1993). E-cadherin (uvomorulin) forms the main component of the adherent junction, which is located at the lateral region of epithelial cell contact. Stable cell contacts and adhesion plaques are maintained via anchorage of E-cadherin to the actin cytoskeleton through its cytoplasmic association with β-catenin, α-catenin and γ-catenin (Nagafuchi and Takeichi, 1988; Klemier and Ozawa, 1989; Gumbiner and McCrea, 1993; McNeill et al., 1993; Ranscht, 1994). The requirement for E-cadherin during epithelial differentiation has been demonstrated through the transfection of non-epithelial cell lines with cadherins (Nagafuchi et al., 1987; Marrs et al., 1993). While cells transfected with E-cadherin polarize and adopt an epithelial phenotype, those transfected with the brain-associated cadherin, B-cadherin, do not undergo these differentiative events (Marrs et al., 1993).

The tight junction consists of a complex of at least five proteins: zonula occludens protein 1 (ZO-1), ZO-2, 7H6, cingulin and occludin (for review see Citi, 1993). Occludin is the core integral membrane protein interacting with ZO-1 (a

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220 kDa peripheral membrane protein) and cingulin to form
a link between the tight junction and the cytoskeleton
(Stevenson et al., 1986, 1988; Anderson et al., 1988; Citi et al.,
1988, 1993; Furuse et al., 1993). At least two functions are
served by the tight junction: the regulation of paracellular
transport (the movement of water and solutes between
epithelial cells) and the maintenance of epithelial cell
polarity (Biggers et al., 1988; Stevenson et al., 1988; Watson,
1992; Citi, 1993).

Development of the early mammalian embryo to the
blastocyst stage is dependent upon the differentiation of a
transporting epithelium, the trophoderm, required for the
vectorial transport of fluids to form and sustain the
blastocoeel (Biggers et al., 1988; Watson, 1992; Kidder, 1993).
The events of trophoderm differentiation parallel those
involved in the differentiation of all epithelia and are
dependent upon the establishment of E-cadherin mediated
cell–cell adhesion (Vestweber et al., 1987; Fleming and
Johnson, 1988; Watson et al., 1990). While the expression
patterns of junctional complex genes are well characterized
in early mouse embryos (Vestweber et al., 1987; Larue et al.,
1994; Reithmacher et al., 1995), this type of analysis has only
just been initiated in embryos of other mammals (Reima et
al., 1993; Shehu et al., 1996). Shehu et al. (1996) characterized
the polypeptide distribution of a number of nuclear,
cytoplasmic and extracellular proteins, including E-cadherin
and ZO-1, in immunized bovine oocytes transferred to
ligated sheep oviducts. This system is reported to produce
embryos that display identical characteristics of in vivo
embryos with regard to morphology and pregnancy rates
after transfer to recipient cows (Shehu et al., 1996). The
present study examines the expression of these gene
products in embryos produced exclusively within a culture
environment. In addition, the expression of β-catenin gene
products during bovine pre-attachment development has
been examined for the first time. The present results
demonstrate that gene products encoding E-cadherin, β-
catenin and ZO-1 are expressed and maintain cellular
distribution patterns consistent with their predicted roles in
mediating trophoderm differentiation in bovine embryos
produced in vitro.

Materials and Methods

Bovine embryo culture

Bovine pre-attachment embryos were produced using
standard in vitro oocyte maturation, fertilization and embryo
culture methods (Wiener et al., 1991; Watson et al., 1994;
Winger et al., 1997). Cumulus–oocyte complexes (COCs)
excised by razor blade from ovaries within 4 h of removal
from the animal at an abattoir, were washed four times with
oocyte collection medium (Hepes-buffered TCM-199 plus
2% newborn calf serum (NCS); Gibco, BRL, Burlington, ON).
COCs were matured in TCM-199 medium plus 10% NCS
supplemented with 35 µg sodium pyruvate ml⁻¹ (Sigma
Chemical Co, St Louis, MO), 5 µg FSH ml⁻¹ (Follitropin;
Vetrapharm, London, ON), 5 µg LH ml⁻¹ (Vetrapharm) and 1
µg oestradiol ml⁻¹ (Sigma) for 22 h at 39°C in a humidified 5%
CO₂ in air atmosphere. Matured oocytes were fertilized in
vitro with frozen-thawed bovine semen (Semex Canada Inc,
Guelph, ON) prepared using a ‘swim-up’ method in sperm
TL medium (Hepes-buffered modified Tyrodes solution;
Parish et al., 1986). Matured COCs were washed in sperm TL
and placed in equilibrated fertilization drops (50 COCs per
300 µl drop) composed of bicarbonate-buffered modified
Tyrodes solution under light paraffin oil (BDH Inc., Toronto,
ON). COCs and spermatozoa (2.25 x 10⁶ motile spermatozoa
per drop) were incubated for 18 h at 39°C under 5% CO₂ in
air before removal of the cumulus investment with a fine
bored glass pipette. Inseminated oocytes were co-cultured in
50 µl culture micro-drops (TCM-199 plus 10% NCS) under
oil with 25–30 primary oviduct cell vesicles (Xu et al., 1992;
Harvey et al., 1995; Xia et al., 1996) and were supplemented
with an additional 50 µl TCM-199 plus 10% NCS medium
after 48 h of culture to support development to the blastocyst
stage.

RNA isolation

Total RNA was extracted from bovine embryos according
to the method of Temelles et al. (1994). Bovine embryos were
allocated into pools of one-cell zygotes, two–five cell
embryos, six–eight cell embryos, morulae (day 6 after
insemination), and blastocysts (day 8 after insemination).
Pools of 50–100 embryos were lysed at room temperature in
100 µl of GITC buffer (4 mol guanidinium isothiocyanate 1⁺;
Pharmacia, Quebec, PQ, 0.1 mol Tris–HCl 1⁻, pH 7.4; 1 mol 2-β
mercaptoethanol 1⁻; Sigma) in the presence of 20 µg of
Escherichia coli rRNA (Gibco, BRL). Samples were vortexed
vigorously and either frozen and stored at −70°C or
processed by ethanol precipitation before DNase digest. The
precipitated samples were centrifuged at 10 000 g for 20 min
at room temperature; the pellets were washed twice with
cold 70% ethanol and air dried before re-suspension in 20 µl
re-suspension buffer (40 mmol Tris–HCl 1⁻, pH 7.9; 10 mmol
NaCl 1⁻; 6 mmol MgCl₂ 1⁻). Genomic DNA was degraded by
incubating the samples with 1 unit of RNase (Promega,
Biotec, Madison, WI) for 30 min at 37°C. Samples were then
re-extracted with phenol and re-precipitated with ethanol
before the suspension of digested pellets in 10 µl autoclaved
MilliQ water. Oviductal total RNA was isolated by the same
method without addition of E. coli rRNA and was quantified
via spectrophotometry. Aliquots of 1 µg oviduct cell total
RNA was used for reverse transcription.

Reverse transcription and polymerase chain reaction

Total RNA was reverse-transcribed (RT) using oligo (dT)₁₂₋₁₈
priming and Superscript™ Reverse Transcriptase (Gibco,
BRL; Harvey et al., 1995; Watson et al., 1992b, 1994). RNA
samples were incubated with 0.5 µg Oligo (dT)₁₂₋₁₈ primer
(Gibco, BRL) for 10 min at 70°C. After cooling on ice, RNA
was incubated in First Strand Buffer (Gibco, BRL; containing
50 mmol Tris–HCl 1⁻, pH 8.3, 75 mmol KCl 1⁻, 3 mmol
MgCl₂ 1⁻, 10 mmol dithiothreitol (DTT) 1⁻, 0.5 mmol
dNTPs 1⁻) and 200 units of Superscript™ Reverse
Transcriptase (Gibco, BRL) for 1.5 h at 43°C. The reaction was terminated by heating at 94°C for 4 min and flash cooling on ice. Newly produced cDNA was further diluted with sterile distilled water to a concentration of two embryo equivalents per µl or the equivalent of 40 ng of oviduct RNA ml⁻¹. Polymerase chain reaction (PCR) was performed as described previously (Watson et al., 1992b, 1994; Betts et al., 1997). Aliquots of embryo and oviductal cDNA (2.5 µl of a 50 µl cDNA sample) were amplified with 1 unit of Taq DNA polymerase (Gibco, BRL) in a final volume of 50 µl containing 10 × PCR buffer (200 mmol Tris–HCl 1, pH 8.8, 500 mmol KCl 1, plus 1.0–2.0 mmol MgCl₂, 1, 0.2 mmol l⁻¹ of each dNTP and 2 mmol l⁻¹ of each gene-specific primer). The reaction mixture was covered with light paraffin oil and amplified for up to 40 cycles in a DNA thermal cycler (Perkin Elmer Cetus 480), with each cycle consisting of denaturation at 94°C for 1 min, re-annealing of primers to target sequences at 50–56°C for 30 s and primer extension at 72°C for 1 min. PCR products were resolved on 2% agarose gels containing 0.5 mg ethidium bromide ml⁻¹.

**Polymerase chain reaction primers**

Primer sequences for actin, E-cadherin, and β-catenin were designed from cDNA sequences retrieved from GENBANK and were synthesized by Gibco, BRL (Burlington ON; see Table 1 for sequences). cDNA samples were tested for the presence of genomic DNA contamination before use in gene-specific RT–PCR using a set of primers designed to bracket an intron of the β-actin cDNA. In the absence of genomic DNA, this primer set produces a 243 bp amplification product (Watson et al., 1992b, 1994; Harvey et al., 1995). All cDNA samples used in the present study displayed amplification of the appropriate sized β-actin cDNA PCR product. Identity of the products produced by PCR reaction was verified using dye-coupled sequencing performed by GenAlYTiC (University of Guelph, ON) for each primer set.

**Immunocytochemistry**

Characterization of E-cadherin, β-catenin and ZO-1 polypeptides was conducted simultaneously in Danish and Canadian laboratories. Two distinct methods of immunolocalization were used, consisting of wholemount confocal immunofluorescence and peroxidase diaminobenzidine immunocytochemistry. This strategy provided a unique opportunity to compare two methods of analysis and confirm the observed distribution patterns.

**Wholemount indirect immunofluorescence.** The following antisera were used: (1) a mouse monoclonal IgG2a (clone 36) raised against human E-cadherin (Transduction Laboratories, Mississauga, ON; 1:100 dilution); (2) a mouse monoclonal IgG1 (clone 14) directed against mouse β-catenin (Transduction Laboratories; 1:400 dilution); and (3) a rat monoclonal anti-ZO-1 antiserum (Chemicon, Mississauga, ON; 1:100 dilution). Wholemount immunofluorescence was applied to bovine embryos according to modifications of previous methods (De Sousa et al., 1993; Betts et al., 1997). Bovine embryos (from the one-cell stage to the blastocyst stage) were collected, washed twice in cold 1 × PBS. Embryos were fixed through a methanol–PBS series consisting of 1:1 MeOH–PBS for 2 min, 2:1 MeOH–PBS for 2 min and transfer into 600 µl PBS containing 0.002% (v/v) Triton X-100 for 5 min to allow the embryos to sink to the bottom of the chamber. Fixed embryos were washed in 1 × PBS and either stored at 4°C for up to 1 week or were further processed immediately. Embryos processed for ZO-1 and β-catenin immunofluorescence were permeabilized in blocking solution (0.01% (v/v) Triton X-100, 0.1 mol lysine l⁻¹ and 1% (v/v) goat serum in PBS) for 45 min at room temperature and washed three times in fresh PBS. Embryos processed for E-cadherin immunofluorescence were not subjected to Triton X-100 extraction. Embryos were incubated overnight at 4°C with primary antiserum (diluted in 0.002% (v/v) Triton X-100 plus 1% goat serum in PBS) and washed four times in 0.002% (v/v) Triton X-100 plus 1% goat serum, with the final wash lasting 4–6 h. Samples were then incubated in secondary antisera consisting of either fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse IgG secondary antibody (ICN Biochemicals, Montreal, PQ; 1:50 dilution) or FITC-conjugated goat anti-rat secondary antibody (ICN Biochemicals; 1:100 dilution) in 0.002% (v/v) Triton X-100 plus 1% goat serum in PBS for 2 h at room temperature. Embryos were washed three times in fresh PBS for 10 min and left overnight in a final wash of 0.002% (v/v) Triton X-100.

### Table 1. PCR primer sequences

<table>
<thead>
<tr>
<th>PCR Product</th>
<th>Primer Sequences</th>
<th>Amplicon position and size (bp)</th>
<th>Genbank Accession Number</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>β-catenin</td>
<td>5' primer = GGTGCCATTCCACGACTAGTT 3' primer = CAGCAGTCATCTCCAACCCA</td>
<td>1791-2283 of human cDNA = 473</td>
<td>embZ19054</td>
<td>Hulskens et al., 1994</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>Nolleteía/., 1996</td>
</tr>
<tr>
<td>E-cadherin</td>
<td>5' primer = TGAGGCACAAGCACAAATACA 3' primer = TGCTGTCTTCACATGCTCA</td>
<td>1405-1751 of mouse cDNA = 350</td>
<td>embIX06115</td>
<td>Nagafuchi et al., 1987</td>
</tr>
</tbody>
</table>
plus 1% goat serum in PBS before a final transfer onto glass slides in 20 μl of Fluoro-Guard (BioRad, Mississauga, ON) mounting medium under elevated 22 × 22 mm glass slides sealed with nail polish. Slides were viewed on a BioRad MRC 600 confocal laser scanning microscope (BioRad). The procedures were repeated as many as six times for each embryo stage and, in total, approximately 100 embryos of each stage were examined.

**Peroxidase diaminobenidine staining.** Embryos from the one-cell to the blastocyst stage were collected for analysis of E-cadherin, β-catenin, and ZO-1 distributions by application of peroxidase–diaminobenidine (DAB) immunocytochemistry (Hay-Schmidt, 1995). In total, 15 embryos of each stage were examined, representing a total of 150 embryos for each antisera. The zona pellucida was removed by incubating embryos in acid Tyrode’s buffer (pH 2.1) for 1–3 min. After removal of the zona pellucida, embryos were washed in TCM-199 media at 39°C for 10 min and fixed in 4% (w/v) paraformaldehyde in 0.1 mol phosphate buffer 1 for 24 h at 4°C. Fixed embryos were stored in PBS with 0.1% (v/v) Triton X-100 and 0.1% (w/v) sodium azide at 4°C. Identical primary antisera for E-cadherin and β-catenin were used in these studies as described above. For immunocytochemistry, these antisera were diluted 1:2000 in PBS with 0.1% (v/v) Triton X-100 (PBST). For these studies, a rabbit polyclonal ZO-1 primary antisera (Zymed, San Francisco, CA) was diluted 1:3000 in PBST. For each antisera, embryos were incubated overnight at 4°C with the primary antibody and washed three times in PBST. Embryos were then co-incubated with rabbit-anti-mouse-biotinylated and swine-anti-rabbit-biotinylated secondary antibodies (diluted 1:500 in PBST) for 2–4 h at 4°C and then incubated for 1 h at room temperature. Embryos were washed three times in PBST and incubated in avidin–biotin complex (ABC; Vector, Burlingame, CA) for 1–3 h at 4°C and 1 h at room temperature and then washed a further three times in PBST. ABC-treated embryos were incubated in 0.05% (w/v) DAB without perhydrol for 15 min, then incubated for 3–12 min in DAB plus perhydrol at room temperature. The DAB reaction was stopped by two washes in water. Subsequently, embryos were dehydrated, epon-embedded and serial sectioned in 2 μm sections, and every second section was stained with toluidine blue.

**Immunocytochemistry controls**

All of the antisera used in this study were obtained from commercial sources and their efficacy in specifically recognizing epitopes unique to their individual polypeptides has been determined by both western blot and in situ immunolocalization methods (Stevenson et al., 1986; Takeichi, 1988; Li and Poznansky, 1990; Ozawa et al., 1990; Behrens et al., 1993; Su et al., 1993). Routine controls were conducted to ensure that the experimental conditions were optimal. These controls included testing each antisera first on untreated bovine tissue sections. Secondary antibody controls were conducted in which the primary antisera was omitted to determine background fluorescence or DAB staining. Methanol fixation of whole-mount bovine embryos was used, as this method of fixation results in lower background autofluorescence and increased antigenicity compared with aldehyde fixatives (Davis, 1993). The immunofluorescence distributions were consistently observed among experimental replicate embryo pools for each embryo stage.

**Results**

**E-cadherin, and β-catenin transcripts during bovine pre-attachment development**

Transcripts encoding E-cadherin, and β-catenin (indicated by amplicons having the predicted sizes of 350 and 473 bp, respectively) were detected in one-cell bovine zygotes through to blastocyst stages (Fig. 1). In each case, the distribution of these transcripts suggests that these gene products are of both maternal and embryonic origin. Bovine RT–PCR products were sequenced to confirm the identity and contrast nucleotide sequence identity among species. Bovine RT–PCR products amplified using E-cadherin and β-catenin primers possessed 83% and 98% sequence identity with corresponding murine and human mRNA sequences, respectively (Fig. 2). An identical strategy for detecting transcripts encoding ZO-1 during this developmental interval was attempted. Experiments were conducted with three separate primer sets designed to recognize conserved regions of mouse and human ZO-1 cDNAs. For each primer set, a series of PCR amplicons from bovine pre-attachment embryo stages was produced (data not shown). However, these products were not of the expected size and, upon

![Fig. 1](attachment:fig1.png)
cloning and sequence analysis, did not share any homology with known ZO-1 cDNAs. For these reasons, it is the opinion of the authors that characterization of ZO-1 transcripts during early bovine development must await cloning and sequencing of the bovine ZO-1 gene.

Fig. 2. Bovine β-catenin and E-cadherin reverse transcription-polymerase chain reaction (RT-PCR) amplicon sequences. Nucleotide sequences of bovine embryo products were compared with the corresponding human (β-catenin) and mouse (E-cadherin) cDNA sequences. Specific primer sequences and areas of non-conserved bases are highlighted. The bovine β-catenin cDNA sequence shares 98% sequence homology with the human cDNA sequence. Analysis of 275 bp of the bovine E-cadherin cDNA by direct sequencing of PCR products demonstrated 83% homology to the known mouse cDNA sequence.

E-cadherin, β-catenin and ZO-1 polypeptides during bovine pre-attachment development

E-cadherin and β-catenin immunofluorescence was detected encircling the cell margins of each blastomere in
one-cell zygotes through to the morula stage (Figs 3 and 4a-f). The fluorescence was confined to the cell periphery and little cytoplasmic or nuclear signal was observed except in morulae where β-catenin was evident in the perinuclear cytoplasm (Fig. 4e). E-cadherin and β-catenin immunofluorescence diminished in the free apical surface of outer blastomeres in the morula; becoming undetectable in the apical surfaces of both mural and polar trophoectoderm in the blastocyst (Fig. 5a,b), and confined to the basolateral membranes of trophoectoderm cells and also encircling the cell periphery of each inner cell mass (ICM) cell (Fig. 5a,b). The fluorescent patterns displayed by E-cadherin and β-catenin antisera were consistently observed in all blastocysts and were never observed to include the apical membrane surfaces of the trophoectoderm. However, immunofluorescence for both E-cadherin and β-catenin did demonstrate variations in the intensity of immunostaining in the ICM of approximately 10% of blastocysts for both of these polypeptides (data not shown). In the peroxidase-AB stained embryos, perinuclear staining for E-cadherin was observed from the four-cell to the 16-cell stage (Fig. 6a–c). At the hatched blastocyst stage, E-cadherin was localized to the

Fig. 3. Immunofluorescence detection of E-cadherin polypeptides in cleavage-stage bovine embryos. Staining of (a) one-cell, (b) two-cell, (c) four-cell, and (d) eight-cell embryos with E-cadherin antiserum exhibit immunofluorescence surrounding the cell margins of each blastomere. (e) In the 16–32-cell morula, E-cadherin immunofluorescence was diminished in the free surfaces of outer blastomeres. (f) Embryos treated with secondary antibody alone did not display any fluorescence. All images are 1 μm thick confocal laser scanning projections. Scale bars represent 50 μm.
Lateral regions of trophectoderm cell margins extending from the ZO-1 staining to basal regions adjacent to the blastocoel cavity with sparse staining observed in the ICM cell periphery confined to cell contacts. Peroxidase-DAB staining for β-catenin was observed from the two-cell stage at cell-cell contacts (Fig. 6a–g). In hatched blastocysts, staining was localized over the basolateral cell regions of the trophectoderm and encircling all margins of the ICM cells (Fig. 6j–l).

In contrast to the distinct membrane associated localization of E-cadherin and β-catenin, no immunofluorescence signal was detected for ZO-1 in identical pools of early cleavage stage bovine embryos (Fig. 7a–f). ZO-1 immunofluorescence was first detected at the morula stage, appearing as punctate fluorescent points between the outer cells (Fig. 7e). The ZO-1 fluorescence underwent a marked transition from the morula to the blastocyst stage (Fig. 8a–d). By the late morula, regions of continuous fluorescence became apparent at regions of cell contact between the outer cells of the embryo (Fig. 8a). As cavitation progressed, ZO-1 immunofluorescence became distinctly localized to apical regions of cell contact (Fig. 8b), eventually forming a continuous fluorescent ring confined to the apical points of trophectoderm cell contact (Figs. 8d and 6k). No fluorescence
signal for ZO-1 was detected within the ICM (Figs 8c and 6k). ZO-1 staining was observed from the morula stage as cytoplasmic staining in peroxidase-DAB-stained embryos (Fig. 6b-k). The precise cellular distributions displayed by all antisera and the low amounts of background staining in controls (Figs 3f, 4f and 5f) ensured that specific immunolocalization patterns were observed.

**Discussion**

In mammalian preimplantation embryos, cell proliferation and differentiation after fertilization culminate in the formation of a fluid-filled blastocyst. Vectoral fluid transport and accumulation during blastocyst formation is attributed to the polarized epithelial characteristics of the trophectoderm, which arise as a consequence of differentiative events initiated during compaction. E-cadherin mediated cell-cell adhesion associated with compaction synchronizes and orients cell polarity in the embryo (Pratt et al., 1982; Johnson et al., 1986), representing a critical event in epithelial differentiation (Fleming et al., 1994; Larue et al., 1994; Reithmacher et al., 1995). The results of the present study show that transcripts and proteins encoding E-cadherin and its associated cytoplasmic protein, β-catenin, are present throughout bovine pre-attachment development in vitro. These results suggest that these gene products have both oogenetic and embryonic origins. The immuno-

**Fig. 5.** Localization of E-cadherin and β-catenin in bovine blastocysts produced in vitro. Both E-cadherin (a) and β-catenin (b) are restricted to the basolateral cell margins in trophectoderm cells (TE). This restricted distribution is more apparent in enlarged inserts of TE cell-cell contact regions (insets). The distribution patterns for these proteins remain apolar in the inner cell mass cells (ICM). Scale bars represent 50 µm.
Fig. 6. Peroxidase diaminobenzidine (DAB) staining of bovine pre-attachment embryos for E-cadherin, β-catenin and zonula occludens protein 1 (ZO-1). (a,d,g) E-cadherin; (b,e,h,j) β-catenin; (c,f,i,k) ZO-1. (a,b,c) Four-cell embryo stage; (d,e,f) 8-16-cell embryo stage; (g,h,i,j,k) hatched blastocysts. E-cadherin immunoreactivity was not strongly detected until the 8-16-cell embryo stage, where it assumed a distribution associated with cell–cell contacts (large arrowhead) and perinuclear regions (small arrowhead) of each blastomere. In hatched blastocysts, E-cadherin immunoreactivity was confined to cell–cell contact regions of the TE (arrowheads), remaining undetected in apical TE cell surfaces. β-catenin immunoreactivity mirrored (arrowheads) the distribution observed for E-cadherin throughout bovine early development. In contrast, ZO-1 immunoreactivity was first detected in compacting morulae confined to adjacent apical regions of TE (large arrowheads). Scale bar represents 20 μm.

Localization studies demonstrated that E-cadherin initially maintains an apolar distribution in blastomeres before compaction. Coincident with increased apposition of adjacent blastomeres and the onset of cavitation, these proteins adopt a polarized distribution in the basolateral membranes of trophoderm cells, while maintaining an apolar distribution in ICM cell margins. The polarized E-cadherin distribution pattern observed in differentiating bovine trophoderm is comparable with distributions reported for pig (Reima et al., 1993), and mouse early development (Vestweber et al., 1987; Reima, 1990; Larue et al., 1994; Riethmacher et al., 1995). In addition, Shehu et al. (1996) reported an identical E-cadherin distribution from the eight-cell stage through to the blastocyst in embryos transferred to ligated sheep oviducts. Therefore, the results obtained from in vitro derived embryos indicate that culture has little impact on embryonic E-cadherin distribution patterns. The present study reports the distribution of E-cadherin polypeptides in earlier stages than that reported by Shehu et al. (1996). It is now clear that E-cadherin is present in cell margins from the one-cell zygote stage onward during bovine early development.

The integral role played by E-cadherin in blastocyst formation has been demonstrated in transgenic mouse lines,
generated through gene targeting and homologous recombination, that carry null mutations for E-cadherin (Larue et al., 1994; Reithmacher et al., 1995). Reithmacher et al. (1995) reported that, initially, homozygous null embryos underwent compaction (an event contributed to residual oogenetic E-cadherin proteins) but failed to form normal blastocysts and never hatched from the zona pellucida. Removal of E-cadherin mediated cell–cell adhesion does not prevent cell polarization (Pratt et al., 1982; Johnson et al., 1986) but rather delays and randomizes the orientation of cell polarity (Johnson et al., 1986). Loss of ordered cell polarity in the embryo during compaction prevents the formation of a coherent trophoderm cell layer (Larue et al., 1994; Reithmacher et al., 1995). Further characterization of these null mutant embryos has revealed that expression of both α- and β-catenin is downregulated and that ZO-1 expression is not detectable (Ohsugi et al., 1997). These studies clearly demonstrate that E-cadherin plays a pivotal role in the differentiation of the trophoderm and, thus, plays a central role in supporting further embryonic development.

β-catenin binds to the cytoplasmic domain of the E-cadherin molecule and shares homology with the armadillo protein of Drosophila which is involved in the wingless
intracellular signalling pathway (Kemler and Ozawa, 1989; McCrea et al., 1991; Hynes, 1992; Kemler, 1993). This protein also shifts to a polarized distribution in differentiating mouse trophoderm (Haegel et al., 1995). The distribution pattern of β-catenin mRNAs and polypeptides has not been examined previously during bovine pre-attachment development. The results of the present study indicate that both mRNA and proteins encoding this gene are present in bovine embryos from the one-cell through to the blastocyst stage in patterns consistent with mouse development. Mouse embryos homozygous for β-catenin null mutations develop to the blastocyst stage and continue to progress until gastrulation (Haegel et al., 1995). It would appear that plakoglobin/γ-catenin interactions with E-cadherin are sufficient to mediate trophoderm differentiation (Haegel et al., 1995). It has been demonstrated that catenins associate with ZO-1 in Madin–Darby canine kidney cells during the early stages of tight junction assembly (Rajasekaran et al., 1996). Weak association of these catenins–ZO-1 complexes with E-cadherin may play a role in the shutdown of components of the tight junction to the lateral membranes mediating junction assembly (Rajasekaran et al., 1996). Bovine morulae immunostained with β-catenin antibodies in the present study demonstrated cytoplasmic localization of these polypeptides in addition to the membrane-bound distribution. Sheth et al. (1997) demonstrated that ZO-1α isoform proteins first appear in compaction mouse morulae as perinuclear foci and then accumulate in the membrane between the outer blastomeres. These apparent spatial similarities in the localization of β-catenin and ZO-1 further support a proposed shuffling role for β-catenin during tight junction assembly.

In contrast to E-cadherin and β-catenin proteins, ZO-1 polypeptides were not detected in early cleavage stage bovine embryos, but were first observed in morulae in differentiating outer blastomeres. These results are in contrast to previous findings in the bovine embryo, where ZO-1 polypeptides were not reported until the blastocyst stage (Shehu et al., 1996). The distribution pattern of ZO-1 consisted of punctate points of fluorescence that combined to form a thin fluorescent band confined to the apical contact regions of adjacent outer cells as the morula progressed towards the blastocyst stage. The two ZO-1 antibodies used in this study produced a similar detection pattern to those previously reported in mouse embryos (Fleming et al., 1989; Fleming and Hay, 1991), in which ZO-1 protein was localized at contact sites between outer blastomeres after compaction. Bovine embryos processed for peroxidase DAB staining with
the rabbit polyclonal antibody revealed cytoplasmic as well as membrane staining in morulae coincident with cytoplasmic staining for β-catenin at this stage. Sheth et al. (1997) demonstrated that ZO-1α isoforms proteins first appear in compacting mouse morulae as perinuclear foci and then accumulate in the membrane between the outer blastomeres. As compaction progresses, bovine embryos demonstrate a gradual establishment of continuous ZO-1 immunofluorescence along the apical regions of outer blastomeres. The establishment of zonular ZO-1 localization (Fleming et al., 1989, 1994) and tight junction formation coincide with the onset of cavitation (Ducibella and Anderson, 1975; McLaren and Smith, 1977; Pratt, 1985).

Our research is directed at providing an understanding of the mechanisms underlying blastocyst formation. These events are not well characterized during bovine early development and, owing to the limited availability of bovine early embryos derived in vivo for research, the majority of studies have investigated events in embryos derived in vitro. There are increasing concerns about the possible influence of varied culture environments on gene expression patterns. It is possible that the variation in the apparent intensity of ICM immunostaining observed for E-cadherin and β-catenin among blastocysts may also reflect in vitro effects on gene expression. Lower E-cadherin and β-catenin protein expression in the ICM may reflect embryo health and quality. However, direct comparison between embryos derived in vitro and in vivo is required to confirm this assumption.

In conclusion, the gene products encoding E-cadherin, β-catenin and ZO-1 have been shown to be expressed and to maintain distribution patterns consistent with their predicted role in coordinating the events of trophoectoderm differentiation in the early bovine embryo. Furthermore, the results of the present study indicate that bovine embryos derived in vitro express these important mediators of early development in patterns consistent with gene expression patterns associated with development in vivo.

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