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 trophectoderm, which is coordinated by the embryonic expression and cell adhesive properties of E-cadherin. The trophectoderm 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 trophectoderm 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 trophectoderm 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 trophectoderm 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 Goodenough, 1988; Fleming and Johnson, 1988; Rodrigez-Boulán 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; Kemler and Ozawa, 1989; Gumbiner and McCreary, 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

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 trophoctoderm, required for the vectorial transport of fluids to form and sustain the blastocoel (Biggers *et al.*, 1988; Watson, 1992; Kidder, 1993). The events of trophoctoderm 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 inseminated 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 trophoctoderm 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 (Wiemer *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 μ g sodium pyruvate ml^{-1} (Sigma Chemical Co, St Louis, MO), 5 μ g FSH ml^{-1} (Follitropin; Vetrapharm, London, ON), 5 μ g LH ml^{-1} (Vetrapharm) and 1 μ g oestradiol ml^{-1} (Sigma) for 22 h at 39°C in a humidified 5%

CO_2 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 μ l drop) composed of bicarbonate-buffered modified Tyrodes solution under light paraffin oil (BDH Inc., Toronto, ON). COCs and spermatozoa (2.25×10^5 motile spermatozoa per drop) were incubated for 18 h at 39°C under 5% CO_2 in air before removal of the cumulus investment with a fine bored glass pipette. Inseminated oocytes were co-cultured in 50 μ l 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 μ l 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 Temeles *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 μ l of GITC buffer (4 mol guanidinium isothiocyanate l^{-1} ; Pharmacia, Quebec, PQ; 0.1 mol Tris-HCl l^{-1} , pH 7.4; 1 mol 2- β mercaptoethanol l^{-1} ; Sigma) in the presence of 20 μ g 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 μ l re-suspension buffer (40 mmol Tris-HCl l^{-1} , pH 7.9; 10 mmol NaCl l^{-1} ; 6 mmol MgCl_2 l^{-1}). Genomic DNA was degraded by incubating the samples with 1 unit of RQ1 DNase (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 μ l 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 μ g oviduct cell total RNA was used for reverse transcription.

Reverse transcription and polymerase chain reaction

Total RNA was reverse-transcribed (RT) using oligo (dT) priming and Superscript™ Reverse Transcriptase (Gibco, BRL; Harvey *et al.*, 1995; Watson *et al.*, 1992b, 1994). RNA samples were incubated with 0.5 μ g 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 l^{-1} , pH 8.3, 75 mmol KCl l^{-1} , 3 mmol MgCl_2 l^{-1} , 10 mmol dithiothreitol (DTT) l^{-1} , 0.5 mmol dNTPs l^{-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 $\times$ PCR buffer (200 mmol Tris-HCl l^{-1} , pH 8.8, 500 mmol KCl l^{-1} plus 1.0–2.0 mmol MgCl_2 l^{-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 $\times$ 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 $\times$ 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

PCR Product	Primer Sequences	Amplicon position and size (bp)	Genbank Accession Number	References
β -catenin	5'-primer = GGTGCCATTCCACGACTAGTT 3'-primer = CAGCAGTCTCATTCCAAGCCA	1791–2283 of human cDNA = 473	emb Z19054	Hulsken <i>et al.</i> , 1994 Nollet <i>et al.</i> , 1996
E-cadherin	5'-primer = TGAGGCCAAGCAGCAATACA 3'-primer = TGCTGTCTTCACATGCTCA	1405–1751 of mouse cDNA = 350	emb X06115	Nagafuchi <i>et al.</i> , 1987

plus 1% goat serum in PBS before a final transfer onto glass slides in 20 μ l of Fluoro-Guard (BioRad, Mississauga, ON) mounting medium under elevated 22 $\times$ 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 diaminobenzidine 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–diaminobenzidine (DAB) immunocytochemistry (Hay-Schmidt, 1995). In total, 15 embryos of each stage were examined, representing a total of 150 embryos for each antiserum. 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 l⁻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 antiserum were diluted 1:2000 in PBS with 0.1% (v/v) Triton X-100 (PBST). For these studies, a rabbit polyclonal ZO-1 primary antiserum (Zymed, San Francisco, CA) was diluted 1:3000 in PBST. For each antiserum, 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 μ m 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 antiserum first on untreated bovine tissue sections. Secondary antibody controls were conducted in which the primary antiserum 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. Detection of transcripts encoding E-cadherin and β -catenin in bovine pre-attachment embryos. cDNA samples were amplified by 40 cycles of polymerase chain reaction. (a) β -catenin lanes are: L, 100 bp DNA ladder (bands from top to bottom: 700 bp, 600 bp, 500 bp, 400 bp); 1, one-cell zygote; 2, 2–5-cell embryo; 3, 6–8-cell embryo; 4, morula; 5, blastocyst; 6, negative control (no cDNA). (b) E-cadherin lanes are: L, 1 kb DNA ladder (bands from top to bottom: 516/506 bp; 394 bp; 344 bp; 298 bp; 220/200 bp; 154/142 bp); 1, day 2 bovine oviduct monolayer; 2, one-cell zygote; 3, 2–5-cell embryo; 4, 6–8-cell embryo; 5, morula; 6, blastocyst; 7, negative control.

		β -Catenin	
		1-----5' primer-----	60
bovine β -cat		gggtgccattc cacgactagt tcagttgctg gttcgtgcac atcaggatac ccagcgtcgt	
human β -cat		gggtgccattc cacgactagt tcagttgctg gttcgtgcac atcaggatac ccagcgtcgt	
		61	120
bovine β -cat		acatctatgg gtggaacaca gcagcagttt gtggagggag tccgcatgga agaaatagtt	
human β -cat		acgtccatgg gtggaacaca gcagcaattt gtggaggggg tccgcatgga agaaatagtt	
		121	180
bovine β -cat		gaaggttgta cggagccct tcacatccta nctcgggatg ttcacaaccg aatcggtatc	
human β -cat		gaaggttgta cggagccct tcacatccta gctcgggatg ttcacaaccg aattggtatc	
		181	240
bovine β -cat		agaggactaa ataccattcc attgtttgtg cagctgcttt attctcctat tgaaaatatc	
human β -cat		agaggactaa ataccattcc attgtttgtg cagctgcttt attctcctat tgaaaatatc	
		241	300
bovine β -cat		caaagaatag ctgcaggggt cctctgtgaa cttgctcagg acaaggaagc tgcagaagct	
human β -cat		caaagaatag ctgcaggggt cctctgtgaa cttgctcagg acaaggaagc tgcagaagct	
		301	360
bovine β -cat		attgaagcgg agggagccac agctcctctg acagaattac ttcaattctag gaatgaaggt	
human β -cat		attgaagcgg agggagccac agctcctctg acagaattac ttcaattctag gaatgaaggt	
		361	420
bovine β -cat		gtggcaacat atgcaactgc tgttttggtc cgaatgtctg aggacaagcc acaggattat	
human β -cat		gtggcaacat atgcaactgc tgttttggtc cgaatgtctg aggacaagcc acaagattat	
		421	----3'
bovine β -cat		aagaaacggc tttcggttga gctgaccagt tctctcttca gaacggagcc aatggcttgg	
human β -cat		aagaaacggc tttcagttga gctgaccagg tctctcttca gaacagagcc aatggcttgg	
		primer-----473	
bovine β -cat		aatgagactg ctg	
human β -cat		aatgagactg ctg	
		E-Cadherin	
		1	60
Bov Ecad		cattccacag ccacgtcac tgtggaggtg atagatgtga atgaagcccc catctttgtg	
Mus Ecad		cattccacag ccacgtcac tgtggaggtg atagatgtga atgaagcccc catctttatg	
		61	120
Bov Ecad		cctgcggaaa agagagtoga agtgcccga gactttggcg tgggcttgga aatcacatcc	
Mus Ecad		cctgcggaaa agagagtoga agtgcccga gactttggcg tgggcttgga aatcacatct	
		121	180
Bov Ecad		tatacggccc gggagccgta cacatttatg gacagaaga tcacgtatcg gatttggagg	
Mus Ecad		tatacggccc gggagccgta cacatttatg gacagaaga tcacgtatcg gatttggagg	
		181	240
Bov Ecad		gacactgcca actggctgga gattaatcca gaaacgggtg ccatttccca ctgcggctga	
Mus Ecad		gacactgcca actggctgga gattaatcca gaaacgggtg ccatttttca cgcggctga	
		241	--3'primer----- 275
Bov Ecad		gttgacaga gaggatgtg agcatgtgaa gaaca	
Mus Ecad		gttgacaga gaagaggtg agcatgtgaa gaaca	

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.

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.

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 trophectoderm in the blastocyst (Fig. 5a,b), and confined to the basolateral membranes of trophectoderm 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 trophectoderm. 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 μ m thick confocal laser scanning projections. Scale bars represent 50 μ m.

Fig. 4. Detection of β -catenin in early bovine pre-attachment embryos. Treatment of embryos with β -catenin-specific antiserum revealed universal staining surrounding the cell margins of each blastomere from (a) one-cell, (b) two-cell, (c) four-cell, and (d) eight-cell embryos. (e) β -Catenin immunofluorescence in the 16–32-cell morula decreased in the free surface of outer blastomeres. (f) No fluorescence was observed in secondary antisera controls. Scale bars represent 50 μ m.

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. 6d–g). In hatched blastocysts, staining was localized over the basolateral cell regions of the trophectoderm and encircling all margins of the ICM cells (Fig. 6f,g).

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

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 μ m.

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. 6h-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. Vectorial 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. 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 μ m.

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 trophoblast cells, while maintaining an apolar distribution in ICM cell margins. The polarized E-cadherin distribution pattern observed in differentiating bovine trophoblast 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,

Fig. 7. Immunolocalization of zonula occludens protein 1 (ZO-1) polypeptides in bovine cleavage and morula stage embryos. Polypeptides encoding ZO-1 were undetectable by indirect immunofluorescence in (a) one-cell, (b) two-cell, (c) four-cell, and (d) eight-cell bovine embryos. (e) ZO-1 first became detectable as punctate points of immunofluorescence between the outer cells of the morula (arrows). (f) Controls displayed a consistent absence of fluorescence signal. Scale bars represent 50 μm .

generated through gene targeting and homologous recombination, that carry null mutations for E-cadherin (Larue *et al.*, 1994; Riethmacher *et al.*, 1995). Riethmacher *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 trophectoderm cell layer (Larue *et al.*, 1994; Riethmacher *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 trophectoderm 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

Fig. 8. Localization of zonula occludens protein 1 (ZO-1) during the morula-to-blastocyst transition of bovine development *in vitro*. (a) By the mid-late 16–32-cell morula stage (arrows), regions of continuous immunofluorescence become apparent between the outer differentiating trophoblast cells. (b) As cavitation progresses, ZO-1 fluorescence is restricted to the apical region of trophoblast (TE) cell contact (arrows). (c) In the blastocyst, ZO-1 immunofluorescence is restricted to apical TE contact points (arrows and inset) while remaining undetectable in the inner cell mass (ICM). (d) A cross-section plane through the apical TE surface of an expanded blastocyst displays the continuous ring of ZO-1 immunofluorescence that forms between these cells. Scale bars represent 50 μm (a–c) and 25 μm (d).

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 trophoblast (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 trophoblast 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 catenin–ZO-1 complexes with E-cadherin may play a role in the shuttling 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-1 α^+ isoform proteins first appear in compacting 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 shuttling 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 that 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-1 α^* isoform 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 trophoderm 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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