Gene expression regulating epithelial intercellular junction biogenesis during human blastocyst development in vitro

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We investigated gene expression associated with trophectoderm epithelial intercellular junction formation in single human embryos at different stages of cleavage using RT-PCR methods based upon magnetic bead separation of poly-A+ RNA. Trophectoderm tight junction (TJ) and desmosome biogenesis contribute to intercellular sealing and tissue integrity, critical for vectorial transport and blastocoel cavity formation. Expression of the various genes throughout human preimplantation development showed differing levels of sensitivity of detection; these genes included claudin-1, occludin (TM4+ and TM4 isoforms), ZO-1 (ZO-1α+ and ZO-1α– isoforms), ZO-2 and JAM (junction adhesion molecule), and the desmosome junction gene, DSC2 (desmocollin 2). Some transcripts appeared to be expressed throughout preimplantation development (claudin-1, JAM, occludin TM4+ and TM4, ZO-1α– isoform) while others tended to be expressed preferentially in later cleavage and associated with blastocyst formation (ZO-2, ZO-1α+ isoform, DSC-2), illustrating an expression pattern broadly similar to mouse cleavage stages. Human embryo transcript detection was significantly decreased when reverse transcription was performed in solid phase to generate a bead/cDNA transient library rather than after mRNA elution from beads. Transcript detection tended to be positively correlated with embryo morphological grade using the solid phase method. In blastocysts, occludin TM4–, ZO-1α+ and DSC2 transcripts were the most susceptible to failure of detection, indicative of low levels of expression which may impact on trophectoderm differentiation competence. Immunofluorescent microscopy analysis of selected adhesion and TJ proteins in human embryos indicated poor membrane assembly compared with mouse blastocysts, which may further affect embryo viability.

Key words: blastocyst/epithelium/human embryo/tight junction/trophectoderm

Introduction

The low rate of success of human assisted conception (~20% live birth rate per treatment cycle; Human Fertilisation and Embryo Authority, 2000) and the accompanying high risk of multiple pregnancy following uterine transfer of more than one embryo, are major concerns in the clinical treatment of infertility. The questionable viability of embryos transferred during early cleavage is one significant cause of failure. The rate of human blastocyst development in vitro, despite the application of various protocols in recent years involving either addition of supplements to culture medium or the use of sequential or co-culture strategies, has not improved much above 50% (Gardner and Lane, 1997; Jones et al., 1998; Lighten et al., 1998; Wiemer et al., 1998; Louttradis et al., 2000; Summers et al., 2000; Devreker et al., 2001). Moreover, variability in embryo developmental potential has recently been demonstrated by non-invasive analysis of amino acid turnover (Houghton et al., 2002). These data indicate the need to investigate molecular and cellular processes controlling human blastocyst development and to identify potential causes for failure of these processes. Such studies would also throw light on the variable potential of human embryos in the derivation of stem cell lines.

The mouse embryo has been used extensively as a model for the developmental mechanisms underlying blastocyst morphogenesis. A central process during preimplantation development is the differentiation of the trophectoderm epithelium which forms the wall of the blastocyst and engages in the transport activities responsible for blastocoel cavitation. The trophectoderm also protects and controls the environment of the internalized inner cell mass (ICM) from which the entire fetus will form after implantation. Studies on the mouse embryo have shown that trophectoderm differentiation during cleavage involves a temporally regulated programme of gene and protein expression required for generating an epithelial phenotype (reviewed in Watson and Barcroft, 2001; Fleming et al., 2001). The epithelial organization of trophectoderm cells permits polarized transport between maternal and blastocoelic environments driven by a
basolateral Na/K-ATPase. It is dependent upon intercellular adhesion and junctions to control the adhesiveness, signalling activity and integrity of the nascent epithelium.

The tight junction (TJ; zonula occludens) is a multi-protein complex located as a belt at the apex of the lateral contact regions between epithelial cells and forms a transepithelial seal as well as contributing to signalling activity and maintenance of cell polarity (reviewed in Stevenson and Keon, 1998; Balda and Matter, 2000). Key constituents of the TJ include three transmembrane proteins engaged in intercellular adhesion, occludin (Furuse et al., 1993), claudins (Furuse et al., 1998; Morita et al., 1999) and JAM (junction adhesion molecule; Martin-Padura et al., 1998). Molecular association between the cytoplasmic domain of occludin, claudins and JAM with several cytoplasmic plaque proteins, notably ZO-1, ZO-2, ZO-3 and cingulin, and the actin cytoskeleton have been identified and appear necessary for TJ assembly and function (Furuse et al., 1994; Fanning et al., 1998; Haskins et al., 1998; Cordenonsi et al., 1999; Itoh et al., 1999; Wittchen et al., 1999; Bazzoni et al., 2000). Human occludin has been shown to be expressed as two alternatively spliced isoforms either with (canonical, TM4+) or without (TM4-) the fourth transmembrane domain; TM4- occludin at the protein level appears to be a negative regulator of TJ integrity (Ghassemifar et al., 2002). ZO-1 is also expressed as two splice variants, either with (ZO-1α+) or without (ZO-1α-) a C-terminal α domain. ZO-1 isoforms exhibit differential tissue expression with ZO-1α+ predominating at stable TJ sites (Willott et al., 1992; Balda and Anderson, 1993; Sheth et al., 1997).

The desmosome is a small, disk-shaped, multi-protein assemblage between epithelial lateral surfaces where cytoskeletal intermediate filaments link to the membrane and which play a role in the integrity and signalling activity of the tissue layer (reviewed in Kowalczyk et al., 1999; Green and Gaudry, 2000). Desmosomes are therefore located below the apical lateral TJ network in epithelial cells and contribute to intercellular adhesion mediated by the transmembrane cadherin family members desmocollin and desmoglein.

Studies on mouse embryos have shown that trophoectoderm TJ and desmosomes are formed by the 32-cell stage when blastocoel cavitation begins. The TJ is constructed in a cell cycle-dependent manner during which different constituents assemble at the TJ membrane site in three phases, corresponding to 8-, 16- and 32-cell stages, initiated by the onset of E-cadherin-mediated cell-cell adhesion at embryo ‘compaction’ (reviewed in Fleming et al., 2001). The maturation steps partly represent different times at which TJ constituents are transcribed. For example, in the final phase of assembly, de-novo transcription and immediate translation of the TJ plaque protein, ZO-1α+ isoform, acts as a limiting factor for completion of TJ biogenesis. Thus, newly synthesized ZO-1α+ binds to the pre-existing pool of occludin in the cytoplasm, permitting assembly of the complex at the TJ membrane site (Sheth et al., 1997, 2000a). Similarly, de-novo transcription of the desmosome transmembrane glycoprotein, desmocollin 2 (DSC2), acts as a limiting factor for desmosome assembly at the 32-cell stage (Fleming et al., 1991; Collins et al., 1995).

The human embryo has been shown to express E-cadherin adhesion molecule (Campbell et al., 1995; Bloor et al., 2002) and to engage in embryo compaction around the 16-cell stage (Nikas et al., 1996). Tight junction and desmosome formation in the human embryo has been reported (Dale et al., 1991; Gualtieri et al., 1992; Hardy et al., 1996) and shown to include both ZO-1 and DSC2 expression (Bloor et al., 2002). In the present study, we have investigated the pattern of mRNA expression of TJ and desmosome constituents during human cleavage and blastocyst development using qualitative RT–PCR.

Materials and methods

Embryo and cell culture

Human preimplantation embryos were donated for research with informed patient consent from the Assisted Conception Unit at Leeds General Infirmary, Leeds, UK. Ethical approval for the research was granted by the Human Fertilisation and Embryology Authority (project licences R0067/-4 and R0067/-5) and local Ethics Committees. Oocytes were collected by follicular aspiration 36 h after hCG administration and cultured in Medi-Cult IVF medium (Medicell UK Ltd, UK) under oil at 37°C in 5% CO2. Oocyte–cumulus complexes were inseminated at ~40 h post hCG with 50 000 motile sperm per drop and incubated overnight at 37°C. Fertilization was confirmed by the presence of two pronuclei (day 1 post-insemination). Prior to being used for research, zygotes were cultured in 70 µl drops of Medi-Cult IVF medium under oil at 37°C in 5% CO2. Subsequently, embryos were transported in a mobile incubator at 37°C in Medi-Cult IVF medium in tubes gassed at 5% CO2, from the Assisted Conception Unit to University of York (HJL laboratory) and cultured in 4 µl drops of Earle’s balanced salt solution (EBSS) supported with 1 mmol/l glucose, 5 mmol/l lactate, 0.47 mmol/l pyruvate, 0.5% human serum albumin (Zenalb 20; Bioproducts Lab, UK) and a physiological concentration of amino acids (0.02–0.2 mmol/l depending on the amino acid) (Tay et al., 1997) at 37°C in 5% CO2. The embryos were transferred to fresh 4 µl drops of medium every 24 h and morphologically graded according to Houghton et al., 2002. After culture, embryos were processed for gene or protein expression which initiated at York (HJL laboratory) before completion and analysis at Southampton (TPF laboratory); for details, see below.

For comparison, mouse preimplantation embryos were derived from superovulated, mated MF1 mice (Biomedical Facility, University of Southampton) and, after collection from oviducts, were cultured in T6 medium plus 4 mg/ml bovine serum albumin as described previously (Sheth et al., 2000). Human Caco-2 colon epithelial cells (ECACC, UK) were cultured at 37°C in 5% CO2. The embryos were transferred to fresh 4 µl drops of medium every 24 h and morphologically graded according to Houghton et al., 2002. After culture, embryos were processed for gene or protein expression which initiated at York (HJL laboratory) before completion and analysis at Southampton (TPF laboratory); for details, see below.

RT–PCR

Individual human embryos were removed from culture, washed rapidly in phosphate-buffered saline containing 0.3% polyvinylpyrrolidone (PBS+PVP) and snap-frozen in <1 µl PBS+PVP before transport on dry ice from York to Southampton laboratories. PolyA+ RNA was extracted from single embryos after thawing using the Dynabeads mRNA Direct kit (Dynal A.S., Norway) as described previously (Eckert and Niemann, 1998; Holding et al., 2000) with modifications. Individual embryos were lysed in 150 µl lysis buffer for 10 min before 20 µl Dynabeads, previously washed in lysis buffer, were added and roller-incubated for 15 min at room temperature. Bead–mRNA complex was concentrated by a magnet and washed twice by removal of supernatant (kit buffers A and B) before cDNA synthesis either by (i) a solid phase method for multiple transcript analysis per embryo or (ii) an mRNA elution method usually for single gene expression (but including different splice variants) per embryo.

Solid phase method

PolyA+ mRNA from single embryos attached to beads was reverse-transcribed (using the bead oligo dT as primer) in 50 µl RT reactions into cDNA using 0.5 mmol/l of each dNTP, 50 IU RNAGuard (Pharmacia) and 250 IU Superscript II reverse transcriptase (Gibco-BRL). Conditions for RT were 10 min at 25°C, 1 h at 42°C and 5 min at 95°C. Supernatant containing mRNA was removed after the bead–cDNA complexes were concentrated down by magnet. The bead–cDNA complexes were washed in Tris–HCl (pH 7.5, 10 mmol/l). PCR analysis using up to six primer sets in sequence on each single embryo solid phase cDNA transient library was optimized using Caco-2 cell RNA (see below) and performed on the day of cDNA synthesis to avoid potential losses from longer storage. For each PCR reaction, beads were resuspended in 50 µl PCR master mix containing 0.2 mmol/l of sequence-specific primer (Table I), 2 mmol/l MgCl2, 1 mmol/l dNTP and 2.5 IU native Taq Polymerase (Gibco-BRL) and cycled twice (94°C 30 s, 58°C 1 min, 72°C 1 min) for second strand cDNA synthesis before incubation at 94°C for 2 min, placing tubes on ice to prevent re-annealing of strands, and removal of supernatant by magnet and its transfer to a new tube for a further 40 cycles of
PCR amplification as above. Beads were washed in Tris–HCl before re-use. A two stage PCR reaction was also employed, using nested primers and generally 2 μl (4%) of the first stage product. Primers for RNA polymerase A were used as a positive control in embryo samples (Eckert and Niemann, 1998). PCR products were separated by 1% agarose gel electrophoresis and directly sequenced using a BigDye Terminator kit (Applied Biosystems) and automated sequencing.

**mRNA elution method**

mRNA was eluted from beads in 20 μl RNAse-free water at 65°C for 2 min and cDNA synthesized as in the above method except using 20–50 μl RT reactions, 5 μmol/l random hexamers (Promega), 40 IU RNAguard and 200 IU Superscript II. To control for genomic DNA contamination of PolyA+ mRNA, 20% of the fraction eluted from the Dynabeads was treated similarly but in the absence of reverse transcriptase. 95% of the cDNA eluted from embryos was amplified in 50 μl single or two stage PCR reactions as described above for the solid phase method for the transcript of interest, and the remaining 5% of the cDNA was used for the RNA polymerase A positive control.

A positive control of RNA derived from Caco-2 cells was used in all RT–PCR analyses conducted on human embryos. Total RNA from Caco-2 cells was extracted using TRI-Reagent (Sigma) according to manufacturer’s instructions. A total of 1 ng Caco-2 RNA (approximately equivalent to a single embryo; Piko and Clegg, 1982) was subsequently treated with magnetic beads for polyA+ RNA isolation and run in all RT–PCR reactions alongside each embryo sample, either analysed by solid phase or eluted mRNA methods. In most cases (90% embryos analysed, n = 62), all transcripts were detected in the corresponding 1 ng Caco-2 RNA, otherwise the embryo was removed from the analysis of any transcript not detected in the Caco-2 RNA control.

**Antibodies and immunoconfocal microscopy**

Rabbit polyclonal antibodies to human occludin (Van Itallie and Anderson, 1997), mouse pan ZO-1 (Sheth et al., 1997), human ZO-1α+ and ZO-1α– isoforms (Balda and Anderson, 1993), human JAM (Martin-Padura et al., 1998), bovine DSC2 (Collins et al., 1995) and mouse E-cadherin (DECMA, Sigma) were used for immunostaining. Embryos were permeabilized in 0.25% Triton X-100 in PBS, washed three times in PBS and treated with a positive control in embryo samples (Eckert and Niemann, 1998). PCR Amplification as above. Beads were washed in Tris–HCl before re-use.

<table>
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<tr>
<th>Gene</th>
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<th>Sequence (5’→3’)</th>
<th>Position</th>
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<td>CACCACCATCTCCGTCCACA</td>
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<td>GACACGGGAAGACACTGGGACA</td>
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Sense primers are given above antisense primers.

**Statistical analysis**

Student t-test or one way analysis of variance were used for analysis. P < 0.05 was considered statistically significant.

**Results**

**Gene expression analysis**

A total of 62 human embryos (morphological grade 1 = 10, 16%; grade 2 = 28, 45%; grade 2.5 = 15, 24%; grade 3 = 9, 14%) were analysed qualitatively by RT–PCR for detection of eight mRNA encoding epithelial junction constituents (claudin-1; JAM; occludin TM4+ and TM4– isoforms; ZO-1α+ and ZO-1α– isoforms; ZO-2; DSC2). Embryos were analysed either by the solid phase (n = 33) or the mRNA elution method.
methods of cDNA synthesis. The solid phase method was deemed most suitable since repeated use of the single embryo cDNA transient 'library' should permit multiple transcript analysis. Guided by the reliable amplification of up to eight transcripts by repeated rounds of PCR from transient libraries generated by the solid phase method from 1 ng total RNA from Caco-2 cells, we analysed up to eight transcripts per embryo by one or two stage PCR using this method (eight transcripts in 24 embryos, three to seven transcripts in nine embryos, n = 33). The order of PCR amplification was consistently ZO-1 (α+, α−), ZO-2, occludin (TM4+, TM4−), JAM, claudin-1 and DSC2 in both embryo and Caco-2 samples. Where fewer than eight transcripts were analysed, this order was maintained but with individual transcripts omitted from analysis. This approach permitted 22–31 embryos to be used for analysis of each transcript. In contrast, to avoid depletion of limited first strand cDNA, the mRNA elution method was used predominantly with one primer set for detection of one or two (splice variants) transcripts. Two stage PCR was always used if the first stage reaction failed to detect the transcript analysed.

Embryos were classified into three stages of development: 3–8-cell, compact morula, and blastocyst stages. Results from the PCR analysis using the solid phase method are shown in Table II with representative gel photographs of individual embryo PCR products and their corresponding Caco-2 control products shown in Figure 1A–C. Although numbers of embryos are variable and in some cases too low to present an accurate percentage, certain trends are evident. The analysis, confirmed by sequencing of PCR products, revealed that all transcripts were expressed in human embryos but that the timing, duration of expression and sensitivity of detection of different transcripts was variable. Of the TJ transmembrane proteins, claudin-1 was readily detectable in nearly all embryos at each stage examined while both isoforms of occludin and JAM, although appearing to be present at each stage examined, were often not detectable in embryos. Of the tight junction cytoplasmic plaque proteins, a clear distinction was observed between the two isoforms of ZO-1. Whilst ZO-1α−isoform was readily detectable in most embryos throughout cleavage,

Table II. mRNA expression of cell junction genes in human embryos at different stages of development analysed by solid phase cDNA synthesis (%)

<table>
<thead>
<tr>
<th>Gene</th>
<th>Stage of development</th>
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<tbody>
<tr>
<td></td>
<td>3–8-cell</td>
</tr>
<tr>
<td>ZO-1α+</td>
<td>0/6(0)</td>
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<tr>
<td>ZO-1α−</td>
<td>5/6(83)</td>
</tr>
<tr>
<td>Occludin TM4+</td>
<td>1/6(17)</td>
</tr>
<tr>
<td>Occludin TM4−</td>
<td>2/6(33)</td>
</tr>
<tr>
<td>ZO-2</td>
<td>0/5(0)</td>
</tr>
<tr>
<td>JAM</td>
<td>2/5(40)</td>
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<tr>
<td>Claudin-1</td>
<td>6/6(100)</td>
</tr>
<tr>
<td>DSC2</td>
<td>1/5(20)</td>
</tr>
</tbody>
</table>

Figure 1. Representative composite gel images following RT–PCR analysis of individual human embryos and their positive control Caco-2 sample analysed in parallel using the solid phase (A–C) and mRNA elution (D, E) methods. Transcripts analysed are shown below each lane (A–C; Cld1 = claudin-1; Ocel = occludin) and arrowheads indicate respective size of PCR products where two isoforms are present per lane (α+/− for ZO-1, TM4+/− for occludin); MW = molecular weight marker lane. (A) 8-Cell embryo in which JAM, claudin-1, occludin TM4+ and ZO-1α− isoforms are detected but occludin TM4− and ZO-1α+ are not; all six transcripts are detected in Caco-2 control. (B) Blastocyst in which JAM, claudin TM4+, ZO-1α+ (faint) and ZO-1α− are detected but occludin TM4− is not; all transcripts present in control. (C) Blastocyst analysed for all eight transcripts but with ZO-1α+ and both isoforms of occludin undetected. Here embryo (e) and Caco-2 control (c) lanes for each transcript are adjacent to each other, with all transcripts detected in the control. (D) Compact morula (above) and blastocyst (below) analysed for ZO-1α+ and ZO-1α− alongside control Caco-2, showing absence of ZO-1α+ in the compact morula and no product detected in the controls lacking reverse transcriptase (−RT). (E) Compact morula and blastocyst stages analysed for occludin with both isoforms present; Caco-2 control on right.
Table III. mRNA expression of cell junction genes in human embryos at different stages of development analysed by mRNA elution methods (%)

<table>
<thead>
<tr>
<th>Gene</th>
<th>8-cell</th>
<th>Compact morula</th>
<th>Blastocyst</th>
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</thead>
<tbody>
<tr>
<td>ZO-1α+</td>
<td>1/3(33)</td>
<td>4/6(67)</td>
<td>11/12(92)</td>
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<tr>
<td>ZO-1α−</td>
<td>3/3(100)</td>
<td>6/6(100)</td>
<td>11/12(92)</td>
</tr>
<tr>
<td>Occludin TM4+</td>
<td>4/5(80)</td>
<td>2/4(50)</td>
<td></td>
</tr>
<tr>
<td>Occludin TM4−</td>
<td>1/5(20)</td>
<td>2/4(50)</td>
<td></td>
</tr>
<tr>
<td>DSC2</td>
<td></td>
<td>2/3(67)</td>
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</tbody>
</table>

Figure 2. Transcript detection index in embryos (mean % transcripts positive per embryo) in relation to morphological grade. Numbers of embryos analysed are shown in parentheses for each grade and total embryos on the right. *P < 0.05: difference between solid phase and mRNA elution methods for grade 2, 2.5 and total embryos.

The ZO-1α+ isoform was only evident in ~10% embryos, and from compaction stages onwards. The pattern of expression of ZO-2 was more clearly stage dependent, the transcript appearing to be present from compaction onwards. Similarly, the desmosome membrane protein constituent, DSC2, was detectable mainly only at the blastocyst stage (Table II).

Results of transcript expression in embryos at different stages of development following the mRNA elution method are shown in Table III with representative gel images shown in Figure 1D and E. This study was mainly limited to the isoforms of ZO-1 and occludin. In contrast to the solid phase data, expression of both isoforms of ZO-1 was evident in the majority of embryos analysed with ZO-1α− isoform appearing to be expressed ubiquitously and ZO-1α+ preferentially in compact morula and blastocyst stages. Similarly, the TM4+ isoform of occludin was detectable in a higher proportion of embryos than after solid phase analysis. Occludin isoforms were expressed in both compact morulae and blastocysts.

The gene expression data were also examined in relation to embryo grade (Figure 2). There was a tendency for a higher proportion of transcripts to be detected in embryos at higher morphological grades using the solid phase method (although not statistically significant) which was significantly less sensitive than the mRNA elution method ($P < 0.05$). The solid phase data for blastocysts were also used to determine which transcripts were most susceptible to failure of detection (Figure 3). This analysis indicated occludin TM4− isoform, followed by ZO-1α+ isoform and DSC2, were most susceptible to lack of detection.

**Protein localization analysis**

The localization of junctional proteins was investigated using immunofluorescence microscopy in a limited number of whole mount human blastocysts ($n = 17$) categorized either as morphological grade 1 ($n = 10$), 2 ($n = 4$) or 2.5 ($n = 3$) at the time of fixation (Figure 4). Primary antibodies were used that clearly recognized human homologues of junctional proteins localized at contact sites in Caco-2 epithelial cell monolayers, and mouse embryos were also routinely labelled alongside human embryos as positive controls. Immunolocalization of E-cadherin, pan ZO-1, ZO-1 α+ isoform, occludin (antibody recognizes both isoforms), JAM and DSC2 were investigated (Figure 4).

E-cadherin ($n = 4$ embryos) and JAM ($n = 2$) staining in human blastocysts was evident in isolated regions of cell contact in trophectoderm or ICM, or was present in diffuse cytoplasmic sites (Figure 4A, J). A similar pattern was evident for pan ZO-1 ($n = 2$) but with contact sites restricted to trophectoderm (Figure 4D). Occludin ($n = 4$) staining was mostly perinuclear in trophectoderm cells (Figure 4G). ZO-1α+ ($n = 4$) and DSC2 ($n = 1$) were not detected. In comparison, mouse blastocysts (Figure 4B, E, H, K) and human CaCO-2 cells (Figure 4C, F, I, L) showed clear membrane assembly of all these junctional proteins.

**Discussion**

Our study illustrates that human embryos engage in a programme of multiple gene expression associated with epithelial intercellular junction formation required for blastocyst expansion. The detection of analysed genes broadly fell into two patterns, indicating either an apparent ubiquitous expression throughout preimplantation development exemplified by claudin-1, the two occludin isoforms, JAM and the ZO-1α− isoform (although the relatively low detection of the two occludin isoforms limits interpretation of its expression pattern) or stage-specific expression as seen for ZO-2, DSC2 and ZO-1α+ isoforms. In the latter group, expression was predominant at late stages of cleavage, associated with blastocyst formation. The data confirm the ubiquitous expression of ZO-1 (isoforms not discriminated) and blastocyst-specific expression of DSC2 identified previously in morphologically and developmentally normal human embryos (Bloore et al., 2002). The gene expression pattern is also broadly similar to that identified for the mouse embryo during cleavage where ZO-1α− and occludin (TM4+ only, the TM4− isoform appears primate-specific; Ghassemifar et al., 2002) are ubiquitously expressed and ZO-1α+ and DSC2 are expressed close to the time of blastocyst formation (Collins et al., 1995; Sheth et al., 1997; 2000). However, in contrast to the human, in the mouse embryo, ZO-2 mRNA is detectable throughout cleavage while claudin-1 and JAM appear to...
express only from the embryonic genome (F. Thomas, B. Sheth, R. Nowak and T. P. Fleming, unpublished data).

A central theme to our study was the use of embryos of variable grade and cultured for different periods of time before analysis rather than to use exclusively embryos graded at highest quality. This is more likely to reflect the variable quality of embryos available in culture in assisted conception programmes. Also, we compared two methods of mRNA processing after extraction using magnetic beads, either by solid phase cDNA synthesis to construct transient cDNA libraries or by mRNA elution prior to cDNA synthesis. Using the solid phase method, which allows for multiple transcript analysis from a single embryo cDNA, we found that whilst some transcripts were readily detected in embryos throughout cleavage (e.g. claudin-1, JAM, ZO-1α– isoform), others were often undetectable (ZO-2, DSC2, occludin isoforms, ZO-1α+ isoform). In the case of ZO-2 and DSC2, the low incidence of detection can be partially explained by the late timing of expression at the blastocyst stage. However, for the occludin isoforms, TM4+ and TM4–, poor detection may either reflect low efficiency of PCR amplification or low expression/stability of the two mRNA species. A similar explanation could be given for the failure to detect the ZO-1α+ isoform in most blastocysts using the solid phase method since expression would be expected at this stage of development.

Figure 4. Immunoconfocal microscopy of human blastocysts (A, D, G, J), control mouse blastocysts (B, E, H, K) and Caco-2 cells (C, F, I, L) labelled for E-cadherin (A–C), pan ZO-1 (D–F), occludin (G–I) and JAM (J–L). Arrows indicate minimal membrane assembly of E-cadherin (A), ZO-1 (D) and JAM (J) and perinuclear cytoplasmic accumulation of occludin (G) in human blastocysts and normal membrane assembly of these proteins in mouse blastocysts (B, E, H, K). Bar = 20 μm.

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Certainly, in direct comparison between the solid phase and mRNA elution methods in the analyses of ZO-1 and occludin isoforms, the mRNA elution method was found to be significantly more efficient.

Previous studies have utilized different cDNA library approaches to examine the pattern of gene expression in individual human embryos (Adjaye et al., 1997; 1998; Holding et al., 2000; Salpekhar et al., 2001; Bloor et al., 2002). These reports not only a high variability in detection efficiency between individual embryos as expected biologically, but also found that a low amount of starting material can increase further the extent of variability (Holding et al., 2000). It is likely that our solid phase method shows a similar effect and a low amount of starting material may be one reason for reduced efficiency since the method was efficient when used with Caco-2 RNA extracted from a large number of cells and only subsequently diluted to amounts equivalent to single embryo levels. Why the solid phase method might be particularly sensitive in this respect is not known. This may derive from time-dependent degradation or loss of attached cDNA after synthesis to reduce cDNA template availability; however, all PCR amplifications from a single embryo were carried out rapidly in the current study (within 2–3 h after synthesis). The reduced efficiency of the solid phase method is unlikely to be due to the use of repeated rounds of PCR since a sensitive transcript (ZO-1-α+isoform) was analysed in the first run and an insensitive transcript (claudin-1) was analysed in a late run. Possibly, the difference in sensitivity of the two approaches may derive from the different RT priming methods (oligo dT in solid phase, random hexamers in mRNA elution). Given the opportunity to screen expression of several mRNA from single embryos using this method, it will be important to identify the precise cause of apparent loss of detection efficiency. However, in the case of ZO-1, using the same primers in the same solid phase RT–PCR reaction led to differential detection of isoforms. Here, the low level of detection of the ZO-1-α+ isoform is likely to reflect a genuine reduced expression or stability of this mRNA compared with the ZO-1-α- isoform.

Comparison of RT–PCR data against embryo morphological grade using the solid phase method revealed a trend (although not significant) for mRNA detection efficiency to be reduced as embryo quality declined. Comparison between transcripts indicated occludin TM4- isoform, ZO-1-α+ isoform and DSC2 to be the most susceptible for failure of detection. These data indicate that reduced mRNA availability, due either to reduced transcription or increased turnover, may be a contributory factor in loss of human embryo viability in vitro. Given that both ZO-1-α+ isoform and DSC2 have important regulatory roles in mouse blastocyst TJ and desmosome biogenesis respectively (Sheth et al., 1997; 2000; Collins et al., 1995; see Introduction), reduced availability of these transcripts may impact on the capacity of several junction proteins to assemble at the membrane.

In a limited study of junctional protein localization in human blastocysts using immunofluorescence microscopy, evidence of poor membrane assembly was found for E-cadherin, JAM, ZO-1 and occludin (protein isoforms not identified). Poor membrane assembly of E-cadherin and ZO-1 has also been reported in human blastocysts by Bloor et al. (2002). Whilst we found that membrane assembly of these proteins was extensive in Caco-2 cell and mouse embryo controls, only limited regions of individual embryos showed clear localization of proteins to cell contact sites. In the case of occludin, aggregates of protein surrounding the nucleus were apparent in most blastomeres examined. In the mouse blastocyst, de-novo expression of ZO-1-α+ isoform appears permissive for cytoplasmic binding and membrane assembly of occludin just prior to the time of cavitation at the 32-cell stage (Sheth et al., 1997; 2000). Moreover, TJ proteins are also dependent upon E-cadherin adhesion for membrane assembly (Fleming et al., 2001). Thus, low mRNA expression of the ZO-1-α+ isoform may exacerbate a broader deficiency in membrane assembly competence of junctional proteins in human embryos. Our data therefore support the conclusions of Hardy et al. (1996) that only embryos destined to survive display an organized pattern of intercellular junctions. Indeed, human blastocysts, in contrast to mouse blastocysts, are prone to collapse during in-vitro culture, indicative of weak or leaky TJ. Conversely, pulsatile blastocoeal collapse may assist in the zona hatching process (Gonzales et al., 1996).

In conclusion, our study indicates that human embryos undergo a temporal programme of gene expression required for intercellular junction biogenesis and blastocyst cavitation. We provide evidence of (i) impaired mRNA availability, based upon poor reliability of detection of specific transcripts particularly of key genes involved in regulation of junction formation, coupled with (ii) failure in membrane assembly of junctional components as potential contributors to loss of embryo viability in culture.

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