Expression of cell adhesion molecules during human preimplantation embryo development

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Formation of a fully differentiated, implantation competent blastocyst requires the expression of a complex repertoire of molecules. However, the events that drive morphogenesis are poorly elucidated in the human embryo. In this work, we describe the amplification of representative cDNAs from morphologically and developmentally normal, individual human embryos at all stages from pronucleate to blastocyst. These cDNAs were probed to reveal the temporal expression pattern of cell adhesion molecules thought to play a key role in murine preimplantation embryo development. We demonstrated constitutive expression of β actin, β₁ and α₆ integrins, ZO-1 and E-cadherin, as shown previously in mouse embryos. No expression of β₃, α₂, α₃ or α₇ integrins nor of L or P selectin was detected at any stage of preimplantation development. β₅ integrin showed a regulated pattern of expression and was not expressed in blastocysts, while desmocollin-2 could only be detected at the blastocyst stage. Expression and localization of β₁, β₅ and α₆ integrins and ZO-1 and E-cadherin proteins was confirmed in blastocyst stage embryos by immunocytochemistry. We have identified differences in the expression of integrin molecules between mouse and human embryos, and propose a role for α₅β₅ and α₆β₁ integrin dimers in the human embryo at implantation.

Key words: cell adhesion/embryo/human/integrin/preimplantation

Introduction

Following fertilization, the embryo develops from a differentiated oocyte into a pluripotent zygote capable of cleavage and morphogenesis. In the first phase of development, maternally derived mRNA transcripts are gradually replaced with transcripts originating from the newly formed zygotic genome. In the human, zygotic genome activation (ZGA) occurs by the 4-cell stage (Braude et al., 1988; Taylor et al., 1997). Transcripts from the paternal genome have been detected in late pronucleate embryos (Ao et al., 1994), although the proteins are not detected at this stage. However, a number of experimental studies on the mouse embryo suggest that some maternal mRNAs are functional up to the morula or blastocyst stage (Larue et al., 1994; Riethmacher et al., 1995; Marek et al., 1999). Compaction occurs at the 4-cell stage in the mouse, slightly earlier than in human embryos. Following compaction, epithelial differentiation continues with the development of apicolateral tight junctions (TJ). This involves sequential synthesis and membrane assembly of the TJ component molecules, ZO-1α, rab13, cingulin and, finally, ZO-1α⁺ and occludin (Collins and Fleming 1995; Sheth et al., 1997, 2000a,b). Formation of functional TJs restricts paracellular movement of fluid and, in conjunction with incorporation of Na/K ATPase into the baso–lateral membranes of the TE, drives fluid transportation (Watson, 1992), resulting in the formation of a blastocoelic cavity. Desmosomes form during cavitation (32-cell stage) of the blastocyst, following sequential expression of desmoplaikins and the desmosomal cadherins, desmogleins and desmocollins (Fleming et al., 1991).

Also crucial to the formation of a fully differentiated, implantation competent blastocyst is the expression of a
complex repertoire of TE adhesion molecules that may mediate attachment of the embryo to the luminal epithelium of the uterus. A number of cell adhesion molecules expressed by murine TE have been implicated in attachment, including the trophinin/tasin/bystin complex (Suzuki et al., 1998; Aoki and Fukuda, 2000) and heparan sulphate proteoglycan (Carson et al., 1993; Smith et al., 1997). Potential candidates for the mediation of embryo attachment also include integrin family members. Integrins are heterodimers composed of an α and β membrane glycoprotein subunit. At least 25 functional integrin dimers can be formed from 19 α subunits and 8 β subunits identified to date and these have a diverse range of ligands (Humphries, 2000). Their primary function is in cell–cell and cell–matrix interactions, but they also have a role in cell signalling and survival. Integrin mRNA expression in mouse embryos has been determined; α5, α6B, αv, β1 and β3 subunits show constitutive expression throughout preimplantation development, α3 expression is detected at the 8-cell stage, and α2, α3, α7 and α6A are first detected in late blastocyst stage embryos (Sutherland et al., 1993). Of the molecules investigated, however, αvβ3 has been the only protein detected at the TE surface. Injection of antibodies to either αv or β3 integrins has been shown to reduce the number of implantation sites in mice, although only the Arg-Gly-Asp (RGD) integrin recognition fragment generated statistically significant results (Illera et al., 2000). In addition, embryos null for β1 integrin exhibit ICM retardation and peri-implantation lethality (Stephens et al., 1995). Considering this evidence, in mouse, integrin αvβ3 is a strong candidate for mediating primary trophoblast adhesion and migration and β1 integrin may play an important role in peri-implantation development. As differentiation of the TE continues and activation occurs, integrin α5β1 translocates to the apical surface of the abembryonic TE (the region which first contacts the uterine epithelium), suggesting a function in the mediation of embryo attachment (Schultz et al., 1997; Wang et al., 1998). In the human, there is little information on TE assembly or embryo attachment, although we have previously reported that human preimplantation embryos constitutively express α3, αv, β1, β3, β4 and β5 integrin subunit proteins (Campbell et al., 1995).

It has recently been confirmed that embryo morphology and even cleavage correlate with successful pregnancy outcome (Scott et al., 2000). Unlike some previous studies hampered by lack of research embryos, all of the early cleavage stages used in this study were cultured from a non-selected population of morphologically normal embryos frozen at the pronucleate stage, with the majority from cycles that resulted in clinical pregnancy. Morula and blastocyst stage embryos were all assessed to be of the highest quality grade. To elucidate the temporal expression pattern of key cell adhesion molecules in human embryos during preimplantation development, we have undertaken representative amplification of polyadenylated mRNAs from individual embryos at each stage of development. The resultant cDNA pools have been probed to determine the temporal expression pattern of a range of cell adhesion molecules thought to play a role in murine preimplantation TE development and differentiation.

Materials and methods

Embryos

Embryos were donated with fully informed consent by patients treated in IVF clinics at Leeds General Infirmary, Leeds, St Mary’s Hospital, Manchester, and Manchester Fertility Services, Whalley Range, Manchester, UK. All research was carried out with the permission of local ethical committees, and in accordance with the licence conditions of the Human Fertilisation and Embryology Authority (HFEA: project licence R0026). Early cleavage stage embryos (pronucleate to 8-cell stage) were cultured from unselected frozen pronucleate stage embryos donated to the programme. All the pronucleate, 2-cell and 4-cell embryos were from pregnant cycles. One of the 8-cell embryos was from a pregnant cycle, while the two other 8-cell embryos were siblings from a cycle that did not result in pregnancy. However, the donating parents were fertile since they later achieved a spontaneous pregnancy. Embryos lysed at morula and blastocyst stages had been transferred from Leeds General Infirmary to the University of York at early cleavage stage where they were cultured to blastocyst. These embryos had undergone some initial selection in that they had not been chosen for replacement or freezing, but at the time of lysis all were assessed morphologically to be of the highest grade.

Embryo thawing

Embryos were thawed using standard clinical protocols (Horne et al., 1997). Briefly, they were removed from liquid nitrogen and incubated at room temperature for 40 s then in a 30°C water bath for 1 min. Embryos were transferred sequentially through thawing solutions, T1 [1 mol/l 1,2 propanediol, 0.2 mol/l sucrose in phosphate-buffered saline (PBS)], T2 (0.5 mol/l 1,2 propanediol, 0.2 mol/l sucrose in PBS), T3 (0.2 mol/l sucrose in PBS) and T4 (IVF Universal; MediCult, Redhill, Surrey, UK) with 5 min incubation at each stage. Embryos were judged to be viable if they survived thawing followed by 1 h in culture. The rate of survival was 85%.

Embryo culture

For culture of early cleavage stage embryos (up to 8-cell), immediately post-thaw, embryos were transferred to 200 µl drops of pre-equilibrated medium (IVF Universal; MediCult) under oil and incubated at 37°C in 5% CO2 under the standard conditions used for IVF at St Mary’s hospital. For blastocyst stages, embryos were cultured to early cleavage stage in 70 µl drops of IVF medium under oil at 37°C in 5% CO2 in a humidified incubator, which are standard embryo culture conditions at Leeds General Infirmary. Embryos were then transferred to the University of York where they were cultured individually in 4 µl drops of medium (Earle’s Balanced Salt Solution (1X in Fresenius water for injection)), supplemented with 25 mmol/l NaHCO3, 0.47 mmol/l pyruvate, 1 mmol/l glucose, 1.13 mmol/l L-glutamine, 0.5% human serum albumin (Zenalb 20; Bio Products Laboratory, Herts, UK), 5 mmol/l L-lactate, 1 ml/100 ml MEM non-essential amino acids solution (Invitrogen, Paisley, UK), 2 ml/100 ml MEM amino acids solution without L-glutamine (Invitrogen), 50 IU/ml penicillin, 50 µg/ml streptomycin and incubated at 37°C in 5% CO2 with humidification. Embryos were transferred to a fresh, pre-equilibrated 4 µl drop of embryo culture medium every 24 h.

Lysis and cDNA amplification

The protocols were adapted from those previously used (Brady and Iscove, 1993; Nunez et al., 2000).

A single embryo was transferred in a minimum volume of culture medium (<0.5 µl) to 4.5 µl complete lysis buffer containing 50 mmol/l Tris–HCl, pH 8.3, 75 mmol/l KCl, 3 mmol/l MgCl2, 20 mmol/l dithiothreitol (DTT), 40 µg/ml bovine serum albumin.
Gene expression in preimplantation human embryos

(BSA), 0.5% NP40, 9.7 pmol/l dNTPs, 23 pmol/l oligo dT
, RNAse inhibitor 0.8 units/µl (Roche, Lewes, UK). The reaction was overlaid with mineral oil, heated to 65°C for 1 min, cooled to room temperature for 3 min then placed on ice. A total of 25 units (0.5 µl) of reverse transcriptase (Superscript RNaseH−: Invitrogen) was then added and the reaction was incubated at 37°C for 15 min, 65°C for 10 min then cooled on ice. Limitation of the reverse transcription step to 15 min resulted in the formation of cDNAs of 300–600 bp, reducing the possibility of size dependent preferential amplification during subsequent PCR amplifications. One volume of tailing buffer (0.33 mol/l potassium cacodylate, 6.7 mol/l COCl
, 0.67 mmol/l DTT, 0.16 mmol/l dATP, 0.45 units/µl rTdT; Invitrogen) was then added and the reaction was incubated at 37°C for 15 min, 65°C for 10 min and then cooled on ice. This step polyadenylates the first strand cDNA allowing subsequent global amplification of cDNA using a single NotI dT
 oligonucleotide primer.

PCR amplification of the polyA-tailed cdNA was carried out by the addition of two volumes of primary PCR reaction mix (22 mmol/l Tris–HCl pH 8.3, 6.3 mmol/l MgCl
, 110 mmol/l KCl, 2 mmol/l dNTPs, 0.04 mg/ml BSA, 0.2% Triton X-100, 8.5 µmol/l NotI dT
 oligonucleotide primer, 0.16 IU/µl Taq polymerase). The sequence of the NotI dT
 oligonucleotide primer is as follows: CATCTCGAGCGGCAGGGCTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTTT. Amplification was carried out using the following cycling profile: initial denaturation at 94°C for 2 min, 25 cycles of 1 min at 94°C, 2 min at 42°C and 6 min at 72°C; linked to a further 25 cycles of 1 min at 94°C, 1 min at 42°C and 2 min at 72°C. The optimal MgCl
 concentration was determined empirically for every batch of NotI dT
 oligonucleotide primer purchased. All primers used were obtained from Invitrogen.

Primary amplification products were then subject to a further amplification as follows: 1 µl of primary PCR reaction was used as template in a 50 µl final reaction volume containing 10 mmol/l Tris–HCl pH 8.3, 1.5 mmol/l MgCl
, 50 mmol/l KCl, 0.2 mmol/l dNTPs, 2 µmol/l NotI dT
 oligonucleotide and 0.025 IU/µl Taq polymerase. Amplification was carried out using the following cycling profile: initial denaturation at 94°C for 2 min followed by 50 cycles of 30 s at 94°C, 30 s at 54°C and 30 s at 72°C.

Embryos were lysed and subjected to the amplification protocol without reverse transcriptase (RT negatives) to confirm the absence of contaminating genomic DNA. Controls without embryo material (mRNA negatives); sperm (~50) and cumulus cells (~100) were also processed using the protocols described. Negative samples were probed for the presence of target genes in tandem with test samples.

Normalization of amplified cdNA

Serial dilutions of secondary amplification products were prepared and used as the template in a PCR reaction to amplify β actin: 1 µl of each dilution was used as template in a 25 µl final reaction volume containing 10 mmol/l Tris–HCl pH 8.3, 1.5 mmol/l MgCl
, 50 mmol/l KCl, 0.2 mmol/l dNTPs, 1 µmol/l β actin forward primer, 1 µmol/l β actin reverse primer, 0.025 IU/µl Taq polymerase. Amplification was carried out using the following cycling profile: initial denaturation at 94°C for 1 min followed by 17 cycles of 30 s at 94°C, 30 s at 62°C and 30 s at 72°C. A total of 10 µl of the resultant amplification products were visualized following electrophoresis on a 2% agarose gel stained with ethidium bromide. cDNA pools were discarded if no β actin signal was detected. In subsequent PCR experiments to probe cDNAs for the presence of test genes, 1 µl of a 10-fold concentration of the cdNA dilution at which β actin amplification products were first detected was used as template.

Gene specific PCR

Primers were designed to amplify target genes in the 500 bp immediately preceding the polyadenylation signal in the gene sequence. Primers were designed using PRIMER version 0.5 (copyright 1991, Whitehead Institute for Biomedical research). Primer pair sequences used in target gene amplification are detailed in Table I. Despite design and testing of multiple primer pairs, no primers were found to successfully amplify αv integrin. Amplification products were partially sequenced to verify identity using ABI Big dye technology (ABI, Warrington, UK). For target gene amplification, 1 µl of normalized cdNA was used as the template in a 25 µl final reaction volume containing 10 mmol/l Tris–HCl pH 8.3, 1.5 mmol/l MgCl
, 50 mmol/l KCl, 0.2 mmol/l dNTPs, 1 µmol/l forward primer, 1 µmol/l reverse primer and 0.025 IU/µl Taq polymerase. Amplification was carried out using the following cycling profile: initial denaturation at 94°C for 1 min followed by 50 cycles of 30 s at 94°C, 30 s at appropriate annealing temperature (Table I) and 30 s at 72°C. A total of 10 µl of the resultant amplification products was visualized following electrophoresis on a 2% agarose gel stained with ethidium bromide. All primer pairs successfully amplified DNA fragments of the appropriate size from human genomic DNA (data not shown).

Immunocytochemistry

Where target gene message was detected, immunocytochemistry was used to corroborate expression of protein in blastocyst stage embryos. Blastocysts for immunocytochemistry were obtained from the University of York where they were fixed in 1% paraformaldehyde in PBS prior to transportation to the University of Manchester. Embryos were recovered, washed through PBS supplemented with 4mg/ml immunoglobulin (IgG) free BSA (PBS/BSA; Stratex, Luton, UK) and permeabilized by incubation in 0.01% Triton X-100 in PBS/BSA for 3 min at room temperature. For E-cadherin staining, the zona pellucida was removed from embryos prior to permeabilization by sequential incubation in 5% pronase in PBS/BSA for 5 min then acid tyrosides for 1–2 min. Washing was repeated and the embryos were transferred to a 25 µl drop of primary antibody at the appropriate dilution (Table II), and incubated under oil for 1 h at room temperature with gentle agitation. Embryos were washed, then transferred to a 25 µl drop of secondary antibody at the appropriate dilution and incubated under oil for 1 h at room temperature with gentle agitation. After final washing, embryos were transferred to a 0.1 mm microslide (Camlab, Cambridge, UK), which was sealed then mounted onto a glass slide prior to visualization of staining by confocal microscopy. Microscopy was performed using a BioRad MRC 600 laser scanning attachment (BioRad Microscience, Hemel Hempstead, UK) linked to a 90 MHZ Pentium Compaq personal computer running COMOS Version 6 control software and N1 Zeiss microscope or a BioRad MRC1024 MP confocal head mounted on a Nikon eclipse TE300 fluorescence microscope (BioRad). Images were processed using Biorad LaserSharp software. Controls were either normal rabbit serum or rat or mouse IgG used at the same concentration as, and in place of, primary antibody. All images for test antibodies and controls were collected using identical confocal settings and manipulated identically after collection.

Results

Cell adhesion molecule gene expression

A panel of cdNAs was successfully amplified from three individually lysed embryos at each of pronucleate (PN, 1–3), 2-cell (2C, 1–3), 4-cell (4C, 1–3), 8-cell (8C, 1–3) and blastocyst (B1–3) stages of preimplantation development. All embryos expressed β actin. Analysis of cell adhesion molecule gene expression in these amplified cdNAs was undertaken and these results are summarized in Table III. Data are shown in Figure 1.
### Table I. Gene specific amplification primers

<table>
<thead>
<tr>
<th>Target gene</th>
<th>Primer pair sequences (5′–3′)</th>
<th>Accession number</th>
<th>Position in sequence</th>
<th>Fragment size (bp)</th>
<th>Annealing temp (°C)</th>
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<tbody>
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<td>GACACGACTGCGTTTGGAACC</td>
<td>M10277</td>
<td>3163–3179</td>
<td>387</td>
<td>62</td>
</tr>
<tr>
<td>β1 integrin</td>
<td>AGTATTTGCTGAATGGGGGACC</td>
<td>X07979</td>
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<td>300</td>
<td>62</td>
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<tr>
<td>β3 integrin</td>
<td>GTGCCAGGAGTTGGAATGTG</td>
<td>M20311</td>
<td>3500–3518</td>
<td>292</td>
<td>62</td>
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<tr>
<td>β5 integrin</td>
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<td>M35011</td>
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<td>62</td>
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<tr>
<td>α2 integrin</td>
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<td>X17033</td>
<td>4897–4916</td>
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<tr>
<td>α3 integrin</td>
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<td>207</td>
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<tr>
<td>α6 integrin</td>
<td>AGGAGCTAGTTATGAGAAGGG</td>
<td>X3586</td>
<td>5181–5202</td>
<td>152</td>
<td>62</td>
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<tr>
<td>α7 integrin</td>
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<td>AF032108</td>
<td>3549–3532</td>
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<tr>
<td>ZO-1</td>
<td>GCCATGTCACATTTACACACTGGAGG</td>
<td>L14837</td>
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<td>L14837</td>
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<tr>
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### Table II. Antibodies used in immunocytochemistry

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<th>Isotype</th>
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<th>Dilution</th>
<th>Secondary antibody</th>
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<td>Mouse IgG1</td>
<td>Ancell</td>
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<td>Anti mouse IgG TRITC</td>
<td>Stratech</td>
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<td>Anti rabbit IgG TRITC</td>
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<td>GoH3</td>
<td>Rabbit polyclonal</td>
<td>Serotec</td>
<td>1:50</td>
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<tr>
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### Table III. Cell adhesion molecule gene expression in cDNAs amplified from individual preimplantation human embryos: + denotes gene detected, – denotes not detected

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<td>β actin</td>
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<td>β1 integrin</td>
<td>+</td>
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<td>+</td>
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<td>α7 integrin</td>
<td>–</td>
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<td>ZO-1</td>
<td>+</td>
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<tr>
<td>E-cadherin</td>
<td>+</td>
</tr>
<tr>
<td>DSC-2</td>
<td>–</td>
</tr>
<tr>
<td>L selectin</td>
<td>–</td>
</tr>
<tr>
<td>P selectin</td>
<td>–</td>
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</table>
Expression of β actin, β1 integrin, ZO-1 and E-cadherin was constitutive, with embryos at all stages examined showing expression of these genes. β1 integrin product was reduced in blastocyst cDNAs, but a band could be detected. No expression of β3, α2, α3 or α7 integrins, nor L of P selectin was observed at any stage of preimplantation development. Of the remaining genes, β5 integrin showed a variable expression pattern in cleavage stage embryos, but was not detected at the blastocyst stage, while α6 integrin was expressed in all pronucleate, 2-cell, 8-cell and blastocyst samples, but in only 2/3 of the 4-cell samples. Desmocollin (DSC)-2 was only detected in a single blastocyst sample. We also amplified cDNAs from oocytes that failed to fertilize (data not shown). Expression of β actin, β1 and β5 integrin was studied in these samples; for β actin and β1 integrin, expression in the oocytes that had failed to fertilize was identical to that seen in pronucleate embryos. β5 integrin was detected in only a quarter of the oocytes that had failed to fertilize. No amplification of target genes was ever achieved from negative controls or from sperm and cumulus samples.

Immunolocalization of cell adhesion molecules

Immunocytochemistry was used to confirm mRNA translation and thus protein expression for β1, β5 and α6 integrins and E-cadherin and ZO-1 in fixed blastocysts. Staining for β1 integrin was detected in 4/4 blastocysts and showed a cytoplasmic distribution mainly confined to the TE. In later blastocysts, β1 integrin appeared to relocate to the surface of the TE and showed a punctate staining pattern. β5 integrin was detected in 5/6 blastocysts examined and appeared mainly in the cytoplasm of the TE, although there also appeared to be some cell surface staining in the polar trophectoderm. α6 integrin was detected in 3/3 blastocysts and showed a punctate staining pattern at the surface of the TE. ZO-1 expression was detected in 6/6 embryos. In early blastocysts ZO-1 expression was cytoplasmic, but localization of the protein to TE cell junctions was apparent in fully expanded blastocysts. Staining for E cadherin was punctate and extremely weak, but was detectable in 8/8 of the blastocysts examined. The protein appeared to be largely cytoplasmic, but in 2/8 blastocysts it was also localized to the TE cell junctions. No suitable antibody was available to allow the examination of DSC-2 protein. Representative images are shown in Figure 2.

Discussion

Although we know a considerable amount about gene expression patterns in mouse embryos, the same is not true for human preimplantation embryos. This reflects the scarcity of normal preimplantation embryos available for research: not all patients wish to consent to the donation of surplus embryos for use in research programmes. Thus, where material is donated for research, it is crucial to maximise the amount of data obtained. A number of techniques have been reported for investigating gene expression in human preimplantation embryos. These include conventional and quantitative RT–PCR methods (Sharkey et al., 1995; He et al., 1999; Taylor et al., 2001), construction of amplified cDNA libraries (Adjaye at al., 1999; Morozov et al., 1999) and the use of differential display and DNA sequence database interrogation (Holding et al., 2000; Choo et al., 2001; Stanton and Green, 2001). Conventional RT–PCR techniques only allow gene expression analysis of a small number of genes from the cDNA isolated from a single embryo (He et al., 1999). Furthermore, much of the published work has reported results based on the study of embryos that are morphologically abnormal or developmentally arrested (Adjaye at al., 1999; Krussel et al., 2001), and these results may not provide a true picture of gene expression during normal embryo development. In this study, we have used a method that allows amplification of all of the mRNAs within a single embryo whilst retaining the relative abundances of different mRNA species (Brady and Iscove, 1993). Embryo lysis, reverse transcription and subsequent primary amplification are all carried out in a single tube, avoiding purification steps that may lead to a loss of material. Also, unlike other protocols (Holding et al., 2000), reverse transcription is restricted to the most 3′ bases of the gene sequence, producing cDNA fragments of 300–600 bp. This limitation of fragment size prevents biased amplification of shorter mRNA transcripts, preserving the relative abundance of mRNAs (including rare species) in the resultant amplified cDNA pools. cDNA pools produced in this way are an almost limitless resource that can be repeatedly probed to produce a comprehensive gene expression profile for an individual embryo. This method does not allow the investigation of splice variant gene expression where the variation occurs in the 5′ region of the gene and as with any PCR-based technique,
cleavage stage embryos in this study were unselected since they were frozen at the pronucleate stage. The best indicator of embryo quality that we can obtain is the confirmation that sibling embryos produced normal children, and in this study all embryos up to the 4-cell stage had siblings in the same cohort that developed into normal babies. All of the 8-cell stage embryos had siblings that produced normal babies in the same or a subsequent cycle. Normality can never be guaranteed however, since the developmental potential of research embryos cannot be assessed. In common with another report on gene expression in the human embryo (Holding et al., 2000), we have analysed gene expression in single embryos. This avoids the possibility of a single aberrant embryo contributing mRNA species inappropriate to its morphological stage or the masking of individual embryo variations in gene expression. The heterogeneity in human development makes this essential.

In the current study, we have investigated whether key cell adhesion molecules expressed during mouse preimplantation development are also expressed in the preimplantation human embryo. In mouse embryos, a comprehensive examination of integrin gene and protein expression has demonstrated the constitutive expression of α6β1, α5β1 and αvβ3 dimers during preimplantation development. Other β1 associated subunits, α2, α3, α6A and α7, have been detected in late blastocysts and trophoblast outgrowths (Sutherland et al., 1993; Klaffky et al., 2001). The α5 and α6 subunits are distributed at the basal surface of the TE, with only the αvβ3 dimer localized to the TE surface and later at the site of focal contacts (Spanswick, 1998). It was proposed that the late expression of integrin subunits might expand the repertoire of potential extracellular matrix (ECM) interactions prior to outgrowth and invasion, but that only αvβ3 is in a position to mediate embryo attachment. However, exposure of murine embryos to immobilized or soluble fibronectin results in translocation of β1 and β3 integrin subunits to the apical surface of the TE (Schultz and Armanrt, 1995), indicating a potential functional role for these molecules in attachment. Integrin αvβ5 binds fibronectin, vitronectin and fibrinogen, while αvβ3 also binds laminin and a number of other ECM molecules. These molecules are all found in the trophectoderm invasion pathway (Kimber and Spanswick, 2000). Theoretically, various ECM components could act as bridging molecules binding the TE to the luminal surface of the uterus. However, both fibronectin and vitronectin null mouse embryos implant normally (George et al., 1993; Zheng et al., 1995) and laminin-1 null embryos implant although they die immediately after implantation due to failure of endoderm differentiation (Smyth et al., 1999; Murray and Edgar, 2000). Therefore, production of these ECM components by the preimplantation embryo is not essential for the process of implantation in mice, although a role for uterine laminin or osteopontin is still possible: laminin is present on the murine endometrial luminal epithelium and osteopontin is present on human endometrial epithelium and villus trophoblast (Carson et al., 1993; Daiter et al., 1996; von Wolff et al., 2001).

Human preimplantation embryos express a number of integrin subunits at the protein level. Cleavage stage embryos stain by immunofluorescence for α3, αv, β1, β3 β4 and β5 integrin subunit proteins, and at the blastocyst stage α3, αv,
β1, β3 and β5 have been demonstrated (Turpeenniemi-Hujanen et al., 1992; Campbell et al., 1995; Dubey et al., 2001). In the current work, despite the design of multiple sets of primers, it was not possible to amplify αv from control DNA, so no comment can be made about its mRNA expression. Interestingly however, contrary to mouse data, we were unable to detect a message for the β3 integrin subunit partner of αv at any stage during development. No mRNA for β3 or α3 integrin was detected in our study, although protein expression was detected in a previous study (Campbell et al., 1995). It is possible that the antibodies used cross-reacted with other integrins or that these proteins are long lived and are retained throughout cleavage following transcription in the oocyte, although if this were the case we would have expected to detect a signal in pronucleate stage cDNA pools. Importantly, in the previous work few embryos were used at each stage and they were generally morphologically abnormal, often unir or tri-pronucleate or fragmented, and the results may not reflect expression during normal development. However, as with any study using a scarce tissue, we cannot rule out the possibility that these genes may be expressed at levels below the limits of detection of our technique. Interestingly, neither α3 nor β3 protein was detected on first trimester cytotrophoblast, although β3 was present on syncytiotrophoblast (Campbell et al., 1995).

Despite the absence of β3, we did detect mRNA for another αv subunit partner, β5 integrin. β5 integrin was present in 1/4 failed-to-fertilize oocytes and all pronucleate stages, but was absent from 2/3 2-cell and 1/3 4-cell embryos. The lack of β5 integrin in oocytes may reflect their abnormality, with normal maternal expression seen in pronucleate embryos followed by degradation of maternal mRNA just at or before the time of genome activation. β5 integrin was then expressed in all 8-cell embryos (presumably from the embryonic genome), but was not detected at the blastocyst stage. Despite the absence of β5 mRNA in blastocysts, staining for the β5 subunit protein was detected in both the trophectoderm cytoplasm and at the outer surface of the TE. These data suggest that the heterodimer αvβ5 may be expressed in the human blastocyst and could be involved in initial interaction with luminal epithelium.

In our study, β1 was expressed constitutively and α6 was detected in all but 1/3 4-cell embryos. However, in contrast to that found in mouse, protein localization of these subunits clearly showed distribution of these molecules at the TE surface, suggesting a role in embryo attachment. However, interaction of α6β1 with a laminin-rich basement membrane has also been shown to provide cell survival signals in the presence of insulin-like growth factors (Farrelley et al., 1999), suggesting an alternative function. No expression of α2, α3 or α5 subunits was detected at any stage in preimplantation development. This suggests that either these molecules have no role in human preimplantation development or that expression occurs late in blastocyst maturation, as observed in the mouse.

We observed constitutive expression of E-cadherin mRNA in human preimplantation embryos, and the protein expression data showed a generally cytoplasmic distribution of the molecule in all but expanded blastocysts. This is not surprising since E-cadherin protein is detected throughout cleavage in mouse embryos (Sefton et al., 1992), where it provides the driving force for increased cell–cell adhesion at compaction (Vestweber et al., 1987). It is an absolute requirement for TE differentiation; embryos null for E-cadherin are unable to organise their TE or develop further (Larue et al., 1994; Riethmacher et al., 1995). Cell adhesion at compaction is regulated by post-transcriptional mechanisms, including relocation to the basal and lateral cell surfaces at the 8-cell stage (Vestweber et al., 1987) and protein kinase C (PKC)-mediated phosphorylation changes either in E-cadherin or associated catenins (Sefton et al., 1992; Pauken and Capco, 2000). These post-translational mechanisms are likely to apply in the human, but cannot be confirmed in human preimplantation embryos using current methodology.

DSC-2, one of the desmosomal cadherins, was detected in only one of the three blastocysts examined, suggesting late expression of the DSC-2 protein. Desmoplakin protein has been detected between the outer cells of the embryo, before cavitation (Hardy et al., 1996). Thus, desmosome assembly in human embryos may follow a similar pattern to that seen in the mouse, where some desmosomal proteins are synthesized earlier during cleavage, but assembly of desmosomes is driven by the synthesis of DSC-2 at the blastocyst stage (Collins and Fleming, 1995).

We also examined one component of the TJ system, ZO-1. ZO-1, a member of the membrane-associated guanylate kinase homologues (MAGuKs) (Anderson and Van Itallie, 1995) is present at the cytoplasmic face of the TJ where it binds to the membrane-embedded TJ component occludin and to another cytoplasmic MAGuK component, ZO-2 (Gumbiner et al., 1991; Furuse et al., 1994). ZO-1 occurs as two alternatively spliced isoforms that differ by the presence (α+) or absence (α−) of an 80 amino acid α domain (Willott et al., 1992). A systematic study of the expression of TJ components and the regulation of their assembly to form functional TJs in the mature blastocyst has been carried out in mouse embryos (Sheth et al., 1997, 2000a,b; Fleming et al., 2000). The ZO-1 α isoform is expressed during early cleavage while the α+ isoform appears at the late morula and blastocyst stage (Sheth et al., 1997). ZO-1α+ assembly into the TJ occurs as a late step, prior to blastocoele fluid accumulation, suggesting that it is required for genesis of the functional sealing TJ. Our methodology does not allow differentiation of the two splice variants of ZO-1, with primers identifying both ZO1 α+ and ZO1 α− isoforms. The apparently constitutive expression of ZO-1 observed in this study might therefore reflect a similar pattern of isoform expression to that seen in the mouse, although an alternative approach would be required to confirm this. The protein expression data would support relocation from the cytoplasm to cell junctions late in blastocyst differentiation.

Selectins are transmembrane carbohydrate binding proteins expressed by endothelial cells and leukocytes, with a primary function in the recruitment of leukocytes to sites of inflammation (Vestweber and Blanks, 1999). L and P selectin fragments were not amplifiable at any stage in embryo development in this study. L-selectin protein was detected previously up to the 4-cell stage (Campbell et al., 1995), but this protein may have been derived from oocyte mRNA. The data suggest that
these selectin molecules do not play a role, at least after the 4-cell stage, in early human embryo development.

Our study has confirmed expression in human embryos of mRNAs for a number of key cell adhesion molecules that may function in embryonic cell–cell adhesion or in interaction with ECM in the embryo or the uterine luminal epithelium. We have also demonstrated that integrin subunits implicated in mouse embryo development may not be expressed during human preimplantation development, suggesting potential species differences in the mechanism of development. By using a curtailed reverse transcription step prior to PCR amplification, we have been able to perform representative amplification of different mRNA species. We have applied this technique to single human embryos and produced a profile of cell adhesion molecule gene expression in individual embryos at various stages of development. The technique has the potential to allow the generation of an extensive profile of gene expression for individual embryos and we are currently probing the amplified cDNAs of these same embryos for genes associated with other processes.

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References


Gene expression in preimplantation human embryos


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