Contribution of JAM-1 to epithelial differentiation and tight-junction biogenesis in the mouse preimplantation embryo

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Summary

We have investigated the contribution of the tight junction (TJ) transmembrane protein junction-adhesion-molecule 1 (JAM-1) to trophectoderm epithelial differentiation in the mouse embryo. JAM-1-encoding mRNA is expressed early from the embryonic genome and is detectable as protein from the eight-cell stage. Immunofluorescence confocal analysis of staged embryos and synchronized cell clusters revealed JAM-1 recruitment to cell contact sites occurred predominantly during the first hour after division to the eight-cell stage, earlier than any other TJ protein analysed to date in this model and before E-cadherin adhesion and cell polarization. During embryo compaction later in the fourth cell cycle, JAM-1 localized transiently yet precisely to the apical microvillous pole, where protein kinase Cζ (PKCζ) and PKCδ are also found, indicating a role in cell surface reorganization and polarization. Subsequently, in morulae and blastocysts, JAM-1 is distributed ubiquitously at cell contact sites within the embryo but is concentrated within the trophectoderm apicolateral junctional complex, a pattern resembling that of E-cadherin and nectin-2. However, treatment of embryos with anti-JAM-1-neutralizing antibodies indicated that JAM-1 did not contribute to global embryo compaction and adhesion but rather regulated the timing of blastocoel cavity formation dependent upon establishment of the trophectoderm TJ paracellular seal.

Key words: Junction adhesion molecule, Tight junction, Trophectoderm epithelium, Cell polarity, Blastocyst, Adherens junction.

Introduction

During mammalian cleavage, the egg divides to form a spherical blastocyst comprising an outer unilaminar epithelium, the trophectoderm, which generates the blastocoel cavity by polarized ion and water transport, and surrounds the undifferentiated inner cell mass (ICM; fetal progenitor). Subsequently, the trophectoderm initiates attachment and invasion of uterine endometrium at implantation and gives rise to the chorio-allantoic placenta. Trophectoderm differentiation in the mouse begins at compaction at the eight-cell stage when both intercellular adhesion between blastomeres mediated by E-cadherin and apico-basal cell polarity occur and is complete by the 32-cell stage, some 24 hours later, during which time a mature, fully polarized epithelial phenotype is generated, culminating in blastocoel fluid accumulation in the embryo interior (for reviews, see Fleming et al., 2001; Watson and Barcroft, 2001). Mouse trophectoderm is a useful model for unravelling steps in the epithelial differentiation process that cannot realistically be investigated in established cell lines (e.g. MDCK cells), in which cells are already mature and manipulated experimentally. Here, we consider cell polarization and particularly the construction of the zonular tight junction (TJ) between trophectoderm cells, an intercellular barrier essential for maintenance of membrane polarity and regulation of paracellular permeability (for a review, see Gonzalez-Mariscal et al., 2003). In the context of early morphogenesis, TJ biogenesis provides the means to control the microenvironment of the ICM via trophectoderm transport pathways for ions, amino acids, energy substrates, growth factors and other metabolites.

TJs are composed of several interacting proteins localized as a belt around the apex of each epithelial cell, above the E-cadherin-rich zonular adherens junction (AJ). In freeze-fracture P-face replicas, TJs appear as rows of branching strands composed of intramembraneous particles that, in thin section, constitute sites of close cell-cell membrane apposition (Gonzalez-Mariscal et al., 2003). So far, three groups of transmembrane proteins have been identified within TJs: occludin (Furuse et al., 1993); claudin family members (Furuse et al., 1998); and junctional adhesion molecule (JAM) (Martin-Padura et al., 1998; Bazzoni, 2003).

Occludin and claudins are unrelated proteins that traverse the membrane four times with two extracellular loops and intracellular N- and C-termini, the latter interacting with a

series of cytoplasmic plaque proteins that link the TJ to the actin cytoskeleton. Claudins form a multigene family with different members expressed and localized to TJs in distinct tissues, where they constitute the major component of the freeze-fracture strands and the inherent tissue-specific variability in paracellular barrier function (Tsukita et al., 2001; Gonzalez-Mariscal et al., 2003). However, occludin is encoded by a single gene and has also been shown to contribute to TJ intercellular adhesion and membrane polarity (Van Itallie and Anderson, 1997; Wong and Gumbiner, 1997; Gonzalez-Mariscal et al., 2003).

JAM-1 (36-41 kDa) is present at epithelial and endothelial TJs (Martin-Padura et al., 1998) as well as on platelets and cells of the peripheral lymphoid organs (Malergue et al., 1998; Williams et al., 1999). Unlike occludin and claudins, it is a single-membrane-span immunoglobulin (Ig)-like protein with two variable extracellular Ig domains and a short C-terminal cytoplasmic tail through which it interacts with TJ plaque and associated proteins, notably AF6 (Ebnet et al., 2000), ASIP/Par-3 (Itoh et al., 2001; Ebnet et al., 2001), ZO-1 (Bazzoni et al., 2000a), MUPP1 (Hamazaki et al., 2002), CASK (Martinez-Estrada et al., 2001) and cingulin (Bazzoni et al., 2000a; Ebnet et al., 2000). Structural and biochemical studies indicate that JAM-1 forms inverted U-shaped dimers within the plane of the membrane via interactions between adjacent extracellular N-termini; the dimers in turn engage in homophilic interactions that might form the basis for homotypic intercellular adhesion (Bazzoni et al., 2000b; Kostrewa et al., 2001; Bazzoni, 2003). The intercellular adhesive function of JAM-1 at the TJs contributes to the restriction of paracellular permeability in epithelia (Liu et al., 2000) and appears to facilitate leukocyte transmigration between vascular endothelial cells, possibly mediated by JAM-1 binding to integrins, because a monoclonal antibody against JAM-1 extracellular domain (BV11) inhibited such migration (Martìn-Padura et al., 1998; Bazzoni, 2003). JAM-1 is also implicated in the development of epithelial cell and membrane polarity through its association with ASIP/Par-3, which can recruit atypical protein kinase C (PKC) to the junction site (Itoh et al., 2001; Ebnet et al., 2001). Furthermore, JAM-1 might contribute to AJ formation, because it associates with the nectin calcium-independent adhesion complex, which in turn is involved in the organization and velocity of assembly of the E-cadherin-rich AJ (Takai and Nakanishi, 2003; Tachibana et al., 2000; Fukuhara et al., 2002; Honda et al., 2003). JAM-1 homologs (JAM-2, JAM-3 and JAM-4) have recently been identified in endothelial/epithelial cells and leukocyte membranes, and might contribute to paracellular transmigration (Cunningham et al., 2000; Arrate et al., 2001; Aurrand-Lions et al., 2001; Hirabayashi et al., 2003). A new nomenclature for JAMs has been proposed in which JAM-1 is referred to as JAM-A (Bazzoni, 2003).

The relevance of the mouse trophectoderm model for epithelial differentiation is evident in that cell polarization and TJ biogenesis are multistep processes that allow the contribution of specific gene products to specific events to be evaluated. Thus, TJ biogenesis in the mouse embryo occurs in three phases corresponding to the eight-cell, 16-cell and early-32-cell stages when constituents assemble at the apicolateral membrane contact site, largely regulated by temporal control of constituent gene expression and post-translational events (Fleming et al., 2001). Notably, E-cadherin adhesion at compaction is crucial for polarized membrane recruitment of TJ proteins and, until the 32-cell stage, TJ constituents localize with the AJ, the two junctions only becoming molecularly distinct once occludin and specific claudins assemble as a final event in TJ construction, apparently mediated by late transcription of the cytoplasmic binding partner ZO-1α+ isoform (Sheth et al., 1997; Sheth et al., 2000a; Sheth et al., 2000b; Fleming et al., 2001) (F.C.T., B.S. and T.P.F., unpublished).

Here, we report the contribution of JAM-1 in the mouse embryo to epithelial differentiation and offer new insight into the mechanisms of cell polarization and biogenesis of AJ and TJ in this model. Significantly, we find that JAM-1 membrane assembly occurs before that of any other recognized TJ constituent so far investigated in this model and, indeed, before compaction, indicating independence of its assembly from E-cadherin adhesion. JAM-1 also becomes intimately associated with the process of cell polarization at the eight-cell stage, when it localizes transiently and precisely with the apical microvillous pole, coincident with apical membrane localization of specific PKC isoforms. Subsequently, JAM-1 is expressed and localized to contact sites between cells in both trophectoderm and ICM lineages of the morula and blastocyst, coincident with trophectoderm expression of the nectin adhesion system, suggesting a broad role in early morphogenesis. However, through the use of neutralizing antibodies, we show that JAM-1 does not contribute to global intercellular adhesion within the embryo but rather to trophectoderm TJ paracellular sealing and the timing of blastocoel cavitation.

Materials and Methods

Embryo collection, culture and manipulation

4-5-week-old MF1 mice (Olac-derived, Southampton University Biomedical Facility) were superovulated by intraperitoneal injections of 5-10 IU per 0.1-0.2 ml pregnant mares' serum (PMS; Folligon, Intervet) and then 5-10 IU per 0.1-0.2 ml human chorionic gonadotrophin (hCG; Chorulon, Intervet) 48 hours later. Immediately following the hCG injection, females were either mated with sexually mature males of the same strain or left unmated for unfertilized egg collection. Cumulus masses were collected at 18-20 hours after hCG injection for isolation of unfertilized or fertilized eggs by removal of cumulus cells using a flame-polished micropipette following incubation with hyaluronidase [300 μg ml^{-1} in H6 medium with 4 mg ml^{-1} bovine serum albumin (BSA)] for 10 minutes at 37°C as described previously (Fleming et al., 2002).

Embryos were flushed at the late two-cell stage (approximately 48 hours after hCG) from dissected oviducts using H6 medium containing 4 mg ml^{-1} BSA and cultured in T6 plus 4 mg ml^{-1} BSA until required at 37°C in 5% CO_2 in air (Fleming et al., 2002). Two-cell, four-cell (~55 hours after hCG injection) and early and compact eight-cell embryos (70-75 hours after hCG injection) were identified using morphological criteria. To obtain synchronized pools of recently divided eight-cell embryos, late four-cell embryos (~65 hours after hCG injection) were observed hourly and newly formed eight-cell embryos (designated 0 hours after division) were collected and separately cultured. Synchronized pools of newly compact eight-cell embryos were similarly obtained by hourly inspection of stock cultures. The 16-cell embryos were staged by selecting newly compacting eight-cell embryos and culturing them separately for 10-12 hours (~80-85 hours after hCG injection); late morula (~32-cell stage; ~90 hours after hCG injection) were staged by culturing compact eight-cell embryos for 20-24 hours. Early and late blastocysts were timed from hCG injection (~96 and ~116 hours, respectively).

To obtain synchronized embryo-cell clusters, late four-cell embryos were disaggregated to single cells as described previously (Fleming et al., 2002). Briefly, embryos were treated with acid Tyrodes medium to remove the zona pellucida and incubated in Ca^{2+}-free medium for 15 minutes before disaggregation into single blastomeres (now called 1/4 blastomeres) using a small, heat-polished glass micropipette. Groups of five 1/4 blastomeres were cultured in individual 10 μl drops of T6 plus BSA and checked hourly for division into 2/8 couplets, which were separately cultured until required.

Reverse-transcription polymerase chain reaction

For the reverse-transcription polymerase chain reaction (RT-PCR), tissue samples were dissected from freshly culled mice, cut into ~1 mm cubes and snap frozen in microfuge tubes. Total RNA was isolated from them using the RNeasy miniprep kit (Qiagen, UK) after samples were homogenized in the kit buffer. Eggs and embryos were processed to extract either poly(A)$^+$ RNA or total RNA. For poly(A)$^+$ RNA, groups of five or ten eggs or embryos of appropriate stage were washed three times in PBS containing 0.3% polyvinylpyrrolidone (PBS+PVP), placed in a 1 μl drop of sterile PBS, snap frozen on dry ice in a siliconized 0.5 ml microfuge tube (Costar) and stored at –70°C. Poly(A)$^+$ RNA was extracted from freshly thawed samples using the Dynabeads mRNA DIRECT Kit (Dynal, UK) according to the manufacturer's instructions with minor modifications (Eckert and Niemann, 1998). Poly(A)$^+$ RNA was eluted in 9 μl nuclease-free water before addition to a 20 μl RT reaction comprising 1 mM of each dNTP (Amersham), 2.5 μM random hexamers (Perkin-Elmer) or 1 μM specific antisense primer, 10 U RNase inhibitor (Perkin-Elmer) and 25 U Moloney murine leukemia virus reverse transcriptase (Perkin-Elmer) or Sensiscript (Qiagen) enzyme. RT reactions were incubated for 10 minutes at 25°C, 60 minutes at 42°C and 5 minutes at 99°C, and then cooled to 4°C. For total RNA extraction, batches of five eggs and blastocysts were lysed and the RNA extracted using the Absolutely RNA™ Nanoprep Kit (Stratagene) according to manufacturer's instructions. The 20 μl RT comprised 0.5 mM dNTP mix (Qiagen), 0.16 μM random hexamers (Perkin-Elmer), 0.1 μM oligo-dT (Invitrogen), 10 U RNase inhibitor (Perkin-Elmer) and 1 μl Sensiscript enzyme (Qiagen). The reaction was incubated at 37°C for 1 hour and 5 minutes at 93°C, and then cooled to 4°C. cDNA equivalent to one egg per embryo was then used in a PCR reaction (described below).

After the RT reaction, 2-20 μl was transferred as template to a 50 μl PCR reaction comprising 1× PCR buffer (Gibco BRL), 1.5 mM $MgCl_2$, 200 μM each dNTP and 1 μM each sequence-specific primer. These comprised the sense (GAGGGGAAAGCCGGGAGGAAACT-GT, positions 80-104) and antisense (CAAAGGCTGTCACGGGGT-CAAAGAT, positions 662-686) primers based on the mouse cDNA sequence (GenBank accession number U89915), which spanned intron sequences to ensure that there was no amplification of genomic DNA. During the hot start (99°C for 5 minutes and 72°C for 2 minutes), 2 U Taq DNA polymerase (Gibco) were added at 72°C. Amplification of cDNA was carried out for 35-40 cycles comprising 95°C for 30 seconds, 60°C for 30 seconds and 72°C for 45 seconds, followed by a 5 minute final extension at 72°C. Reaction products were run on 0.5× TBE 2% agarose gels stained with ethidium bromide. Reaction products were prepared for sequencing using a QIAquick gel extraction protocol (Qiagen) after removal from the agarose gel and used in BigDye cycle sequencing protocols according to the manufacturer's instructions on an ABI377 sequencer (Applied Biosystems). Sequences were analysed using the Chromas software and identified by Blast search on the NCBI website (http://www.ncbi.nlm.nih.gov/BLAST/).

Immunofluorescence and confocal microscopy

Zona-free embryos were fixed in either 1% or 4% formaldehyde in PBS or in ice-cold methanol, attached to coverslips within processing chambers pretreated with 1 mg ml^{-1} poly-L-lysine hydrobromide (Sigma) or 0.1 mg ml^{-1} Concanavalin A (Sigma) in PBS, respectively, and processed for immunocytochemistry as described previously (Fleming et al., 2002). Staged embryos were stained with DECMA-1 (rat monoclonal anti-mouse E-cadherin, 1:1000; Sigma), BV12 (rat monoclonal anti-mouse-JAM-1 antibody, 0.1 μg ml^{-1}) (Martin-Padura et al., 1998), BV20 (rat monoclonal anti-mouse JAM-1 antibody, 10 μg ml^{-1}) (Bazzoni et al., 2000b), PKC isoform-specific polyclonal antibodies against rat sequences (atypical PKCs ι/λ and ζ and novel PKCs δ, μ and θ; 1:200 or 1:500) as detailed elsewhere (Eckert et al., 2004a) or anti-nectin-2 antibody (rat monoclonal anti-Nectin-2 antibody, 1 μg ml^{-1}; Cambridge Bioscience) in PBS containing 0.1% Tween 20 (PBS:Tween-20) overnight at 4°C. Chambers were washed three times over 30 minutes and labeled with a 1:500 dilution of Alexa-488- or -546-labelled anti-rat secondary antibody (Molecular Probes) for 1 hour. Chambers were rewashed three times over 30 minutes in PBS:Tween-20 and samples mounted in anti-fadant PBS-Citifluor (Citifluor, London). Rhodamine phalloidin (Molecular Probes; 6.6 μM stock in methanol) was used for co-labeling of actin filaments when required during the second PBS:Tween-20 wash (0.5 μl ml^{-1}) after the secondary antibody incubation. Embryos were visualized using a 63× oil-immersion objective on a Nikon inverted microscope linked to a Bio-Rad MRC-600 series confocal imaging system, equipped with a Krypton-Argon laser and K1/K2 filter sets. Images were analysed and processed by the Bio-Rad software system (Confocal assistant, Version 4.01).

Electrophoresis and immunoblotting

Mouse lung tissue was snap frozen in liquid nitrogen, ground to powder on dry ice, immediately boiled in 1% sodium dodecyl sulfate (SDS) in PBS for 5 minutes followed by centrifugation at 10,000 ***g*** for 10 minutes and storage of supernatant at –70°C. Pools of precisely staged embryos were washed three times in H6 medium plus 6 mg ml^{-1} polyvinylpyrrolidone and solubilized either in boiling 2× SDS sample buffer or 4× Novex sample buffer (Invitrogen) for 3-5 minutes, centrifuged at 10,000 ***g*** and stored at –70°C. Protein concentrations of tissue samples were determined using the Lowry method (DC Protein Assay, BioRad). Embryo and tissue samples were heated to 95°C for 5 minutes and briefly centrifuged at 10,000 ***g*** before electrophoresis on pre-cast 4-12% gradient TB-buffered gels with MOPS buffers (Invitrogen) and blotted onto Hybond-C nitrocellulose (Amersham). Blots were blocked with 5-10% non-fat dried-milk powder in PBS containing 0.05-0.10% Tween 20 before incubation in BV20 antibody (10 μg ml^{-1}) in blocking buffer for 1 hour. Blots were washed three times in PBS containing 0.05-0.10% Tween 20, incubated in horseradish-peroxidase (HRP)-conjugated rabbit anti-rat secondary antibody (Sigma) for 1 hour, and washed three further times before being developed with SuperSignal® West PICO (Pierce) kit and visualized on ECL hyperfilm (Amersham Biosciences).

Functional analyses

Embryos were staged from the onset of the eight-cell stage or from the start of compaction and incubated in T6 plus BSA containing 5 μg ml^{-1} BV11 or BV12 anti-JAM-1 antibodies (mouse monoclonal IgG2b class) for various culture periods. Control medium contained an equivalent concentration of mouse IgG2b anti-CD21 (lymphocyte-specific) kindly provided by A. Al-Shamkhani (University of Southampton, School of Medicine). Alternatively, embryos were treated with anti-E-cadherin antibody ECCD-1 (1:50 dilution of serum) (Shirayoshi et al., 1983), a known inhibitor of embryo compaction (Johnson et al., 1986) kindly provided by M. Takeichi (University of Kyoto, Kyoto, Japan). Antibody-containing media were changed every 12 hours. During treatment, embryos were scored depending on stage for either state of compaction or cavitation, at 1

hour or 2 hour intervals. In addition, nascent blastocysts after treatment were placed in fresh medium containing experimental or control antibody plus 1 mg ml^{-1} FITC/dextran 4 kDa (Sigma) and examined by confocal microscopy as described previously to determine the state of paracellular permeability (Sheth et al., 2000a).

Statistics

Rates of compaction and cavitation in embryo groups were compared by 'time to event' analysis using a Wilcoxon test stratified for experiment. *P* values less than 0.05 were regarded as significant.

Results

JAM-1 is expressed at the onset of epithelial differentiation

JAM-1-encoding mRNA expression was not detected by RT-PCR using poly(A)$^+$ RNA extracted from unfertilized or fertilized eggs but was readily amplified from embryos at all stages from two-cell to late blastocyst (five eggs or embryos per sample) with stronger signals evident from the eight-cell stage onwards (Fig. 1A). JAM-1 transcripts before the two-cell stage were also undetectable using poly(A)$^+$ RNA in ten embryo samples with an increased PCR cycle number (data not shown). However, using total RNA as starting material, JAM-1-encoding mRNA was readily identified in unfertilized eggs and blastocysts even at one egg or embryo per sample (Fig. 1A), suggesting that polyadenylation of JAM-1 transcripts might occur as development proceeds. JAM-1 protein production in fertilized eggs was examined by immunoblotting up to late blastocyst stages. JAM-1 protein was not detected in samples up to and including the pre-compact eight-cell embryo. Weak expression was evident in compact eight-cell embryos and increased in later stages examined (Fig. 1B).

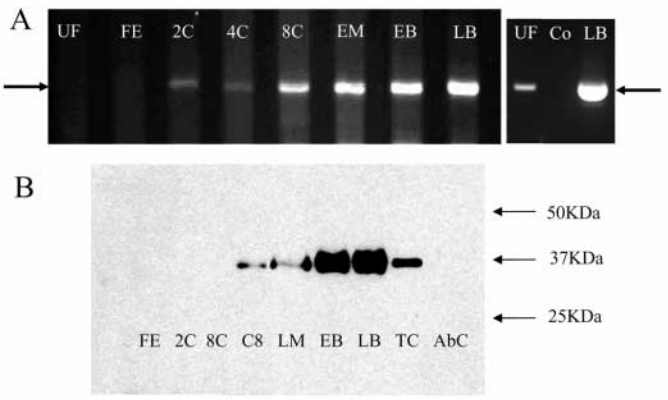

Fig. 1. (A) RT-PCR analysis of the expression of JAM-1-encoding mRNA in egg and embryo stages. The 600 bp cDNA product is indicated by arrows. (Left) Produced using poly(A)$^+$ RNA with five eggs or embryos per sample. (Right) Total RNA using one egg or embryo equivalent per sample. 'Co' indicates the control without the reverse transcription step. (B) Immunoblot analysis of JAM-1 protein expression in eggs and embryos using BV20 antibody, showing approximately 500 eggs or embryos per lane. Mouse lung tissue (18 μg protein) was used as positive tissue control (TC). The stages are unfertilized eggs (UF); fertilized eggs (FE) and embryos at two-cell (2C), four-cell (4C), precompact eight-cell (8C), compact eight-cell (C8), early morula (EM), late morula (LM), early blastocyst (EB) and late blastocyst (LB) stages. AbC, secondary-antibody-only control.

JAM-1 membrane assembly is independent of E-cadherin adhesion, associated with cell polarization, and initiates TJ biogenesis

JAM-1 localization was examined by immunfluorescence confocal microscopy using the BV12 anti-JAM-1 antibody in unfertilized eggs to late-blastocyst stages with E-cadherin staining used as a marker of AJ protein in a parallel set of samples (Fig. 2). Unfertilized eggs, two-cell and four-cell embryos showed no detectable JAM-1 staining (*n*=8-22 per stage), despite the presence of E-cadherin protein in eggs or embryos of the same developmental stage (Fig. 2A-C,I-K). JAM-1 staining was first detected in all pre-compact eight-cell embryos (*n*=6) at sites of cell-cell contact (Fig. 2L). At the onset of compaction, all eight-cell embryos (*n*=14) also revealed intense JAM-1 staining at the apical poles (outward facing) of blastomeres in addition to contact sites (Fig. 2M), a pattern not seen for E-cadherin (Fig. 2E). At the 16-cell morula stage, JAM-1 was again localized along lateral cell-cell contacts with minimal or no staining evident at microvillous poles (Fig. 2N; *n*=9). Cell contact membrane localization of JAM-1 was also seen in early and late blastocysts (*n*=11 for each) in both trophectoderm and ICM lineages, and with an apicolateral concentration of JAM-1 between trophectoderm cells (Fig. 2O,P). Contact-free trophectoderm basal membranes adjacent to the blastocoel were also stained for JAM-1. A similar staining pattern was evident for E-cadherin in morulae and blastocysts (Fig. 2F-H).

Verification of polar membrane localization of JAM-1 in compact eight-cell embryos was achieved by double labeling for actin filaments using phalloidin to identify microvillous poles (Johnson et al., 1986). Merged images revealed that apical surface JAM-1 was localized with the actin-rich microvillous poles (Fig. 3A). To determine the relative timing of cell contact and apical membrane assembly of JAM-1, embryos were examined at 0 hours, 3 hours, 6 hours and 9 hours after cleavage into eight-cell stage. This analysis revealed that JAM-1 contact staining was present in most or all embryos at all time points and preceded apical surface staining, which was evident mainly from 6 hours after division when compaction was occurring (Fig. 3B,C). The presence or absence of JAM-1 staining at cell-cell contact and blastomere poles was also investigated in timed 2/8 couplets (Fig. 4). Again, it was evident that contact staining preceded apical-pole staining and was present in most couplets from immediately after division onwards. By contrast, apical staining appeared as couplets compacted during mid-cycle (Fig. 4A,B).

JAM-1 membrane assembly coincides with maturation of epithelial cell polarity

To explore the potential role of JAM-1 in de novo establishment of epithelial polarity in the embryo, we examined whether JAM-1 apical membrane assembly at compaction was associated with a similar polarization of PKC isoforms that might occur through JAM-1 interaction with ASIP/Par-3 (Itoh et al., 2001; Ebnet et al., 2001). Of the two atypical PKC isoforms, PKCζ was found to localize to the apical

surface membrane at compaction, although it was usually not confined precisely to the central microvillous domain (Fig. 5A). PKCζ was also present at cell contact and intracellular sites, including the nuclear region. During later cleavage, PKCζ is localized with the TJ marker ZO-1α+ at cell contact sites in the trophectoderm (Eckert et al., 2004b). By contrast, the atypical PKCι/λ displayed only intracellular cytoplasmic staining in compact eight-cell embryos (Fig. 5A), although it later localizes with the TJ in blastocyst trophectoderm (Eckert et al., 2004b). Compact eight-cell embryos were also screened for the localization of the novel PKC isoforms δ, μ and θ. Of these, PKCδ revealed a striking staining pattern, localizing predominantly to the polar domain of the apical surface as well as to cell contact and intracellular sites (Fig. 5A). These data indicate that polarized JAM-1 staining at compaction coincides with a polarized distribution of select atypical and novel PKC isoforms.

We also investigated whether the relatively ubiquitous distribution of JAM-1 during morula and blastocyst formation (present at cell contact sites in both trophectoderm and ICM, like E-cadherin, and not restricted to trophectoderm TJs) might signify a role in maturation of the AJ during this period. The calcium-independent nectin adhesion complex has been shown to enhance E-cadherin-mediated adhesion and AJ maturation in non-developmental models and interacts directly with JAM-1 through the nectin cytoplasmic domain protein, afadin (Takai and Nakanishi, 2003; Tachibana et al., 2000; Fukuhara et al., 2002; Honda et al., 2003). We therefore examined the expression of nectin-2, a ubiquitous member of the nectin family, during early development. It was undetectable by immunofluorescence before compaction, when faint, discontinuous staining at cell contact sites was apparent (Fig. 5B). In morula and blastocyst stages, stronger nectin-2 staining was present, mainly within apicolateral cell contact sites within trophectoderm but also weakly at contact sites in the ICM (Fig. 5B). These data show a close correlation between JAM-1, E-cadherin and nectin-2 localization during later cleavage, indicative of a maturation in AJ structural identity. However, early assembly of JAM-1 during the eight-cell stage and E-cadherin throughout cleavage are unlikely to be mediated by nectin-2.

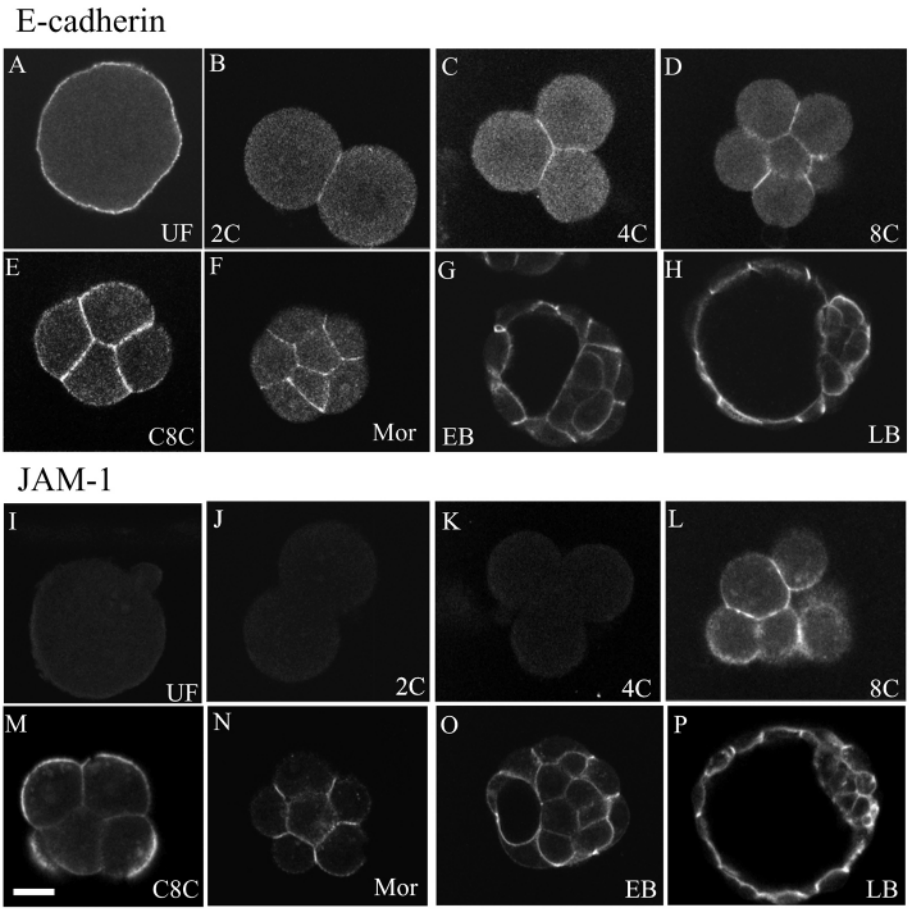

Fig. 2. Immunofluorescence confocal microscopy of E-cadherin (A-H) and JAM-1 (I-P) staining patterns during preimplantation development. The stages are unfertilized eggs (UF) and embryos at two-cell (2C), four-cell (4C), precompact eight-cell (8C), compact eight-cell (C8C), morula (Mor), early blastocyst (EB) and late blastocyst (LB) stages. Bar, 20 μm.

JAM-1 stimulates tight junction sealing and blastocyst formation but not global cell-cell adhesion in the embryo

Neutralizing-antibody incubation experiments were conducted to evaluate the relative contribution of JAM-1 to AJ and TJ adhesion in the embryo. Embryos were staged from the beginning of the eight-cell stage and cultured for up to 9 hours in the presence of anti-JAM-1 neutralizing antibodies BV11 or BV12, or in control medium containing an equal concentration of another IgG2b-class antibody (against CD21, a lymphocyte antigen not expressed in preimplantation embryos). Embryos underwent compaction at equivalent rates during the first 6 hours of culture in all three treatments (Fig. 6A). Newly compacted eight-cell embryos and later-stage morulae were also cultured in the presence of JAM-1 or control antibodies and, in all cases, the compacted state was maintained, whereas compact embryos cultured in an E-cadherin-neutralizing antibody (ECCD-1) fully decompacted within 20 minutes of treatment (data not shown). These results indicate that JAM-1 cell-surface expression does not contribute to initiation or maintenance of cell adhesion within the embryo.

To evaluate the role of JAM-1 in blastocoel formation dependent upon TJ integrity, newly compacted eight-cell embryos were cultured in the presence of anti-JAM-1 or control antibodies for up to 48 hours (Fig. 6B). Here, both BV11 and BV12 treatment had no effect on global embryo adhesion but significantly reduced the rate of cavitation compared with control embryos ($P<0.0001$ and $P<0.005$, respectively). Nascent blastocysts (up to 2 hours after the onset of cavity formation) incubated with anti-JAM-1 or control antibody from compaction were examined by confocal

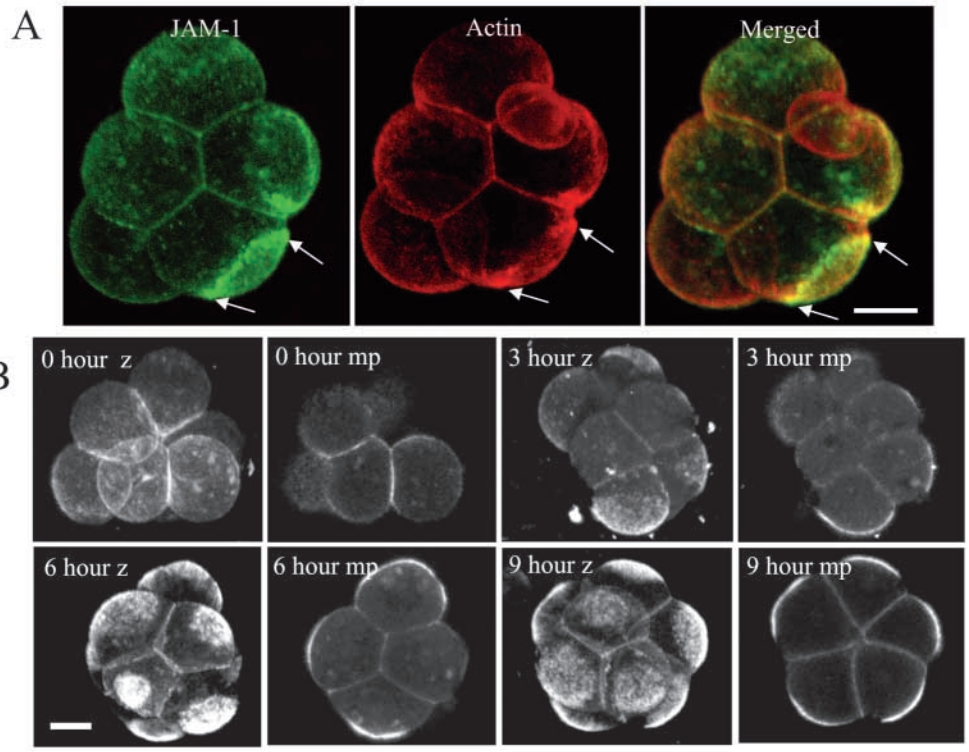

Fig. 3. (A) Co-localization of JAM-1 and actin (phalloidin staining) at the apical pole (arrows) in compact eight-cell embryos. (B) JAM-1 localization in representative eight-cell embryos at 0 hours, 3 hours, 6 hours and 9 hours after division in *z*-axis series (z) and single mid-plane (mp) sections. Bar, 20 μm. (C) Timing of cell-cell contact and apical polar staining of JAM-1 in eight-cell embryos at different times during the fourth cell cycle (*n*=30-40 embryos per time point; experiment repeated four times).

microscopy for evidence of paracellular permeability by exclusion of FITC-dextran. FITC-dextran penetration into the blastocoel cavity was evident in a similar proportion of JAM-1 (BV11, 33%, *n*=52; BV12, 23%, *n*=47) and control (IgG2b, 29%, *n*=68) antibody-treated embryos. These data indicate that JAM-1 intercellular adhesion contributes to the trophectoderm TJ permeability barrier required for blastocoel cavity formation but that, once blastocysts are formed in the presence of anti-JAM-1 antibodies, they have normal effective TJs.

Discussion

We have investigated the contribution of JAM-1 to mouse trophectoderm differentiation and blastocyst formation, and identified several novel features in its expression and activity that give new insight into mechanisms of differentiation and TJ biogenesis. First, detection of JAM-1-encoding mRNA within unfertilized eggs was only possible using total rather than poly(A)$^+$ RNA; using the latter material, JAM-1 transcripts were first detected at the two-cell stage, when the embryonic genome is mainly activated, with increasing cDNA amplification apparent at progressively later stages of cleavage (Fig. 1A). These data might indicate maternal expression of a poorly adenylated JAM-1-encoding mRNA, with polyadenylation coinciding with embryonic transcription. Deadenylation and subsequent polyadenylation of mRNAs are recognized mechanisms of translational repression and initiation, respectively, in the mouse oocyte during maturation, following fertilization and at the onset of embryogenesis (for a review, see Eichenlaub-Ritter and Peschke, 2002). The apparent depletion of polyadenylated JAM-1-encoding mRNA in mouse eggs is unexpected because transcripts for most other epithelial TJ genes analysed to date (ZO-1α– isoform, ZO-2, rab13, cingulin, occludin) are readily detectable in mouse egg poly(A)$^+$ RNA (Sheth et al., 1997; Sheth et al., 2000a; Sheth et al., 2000b) (B.S. and T. P. F., unpublished).

We recently examined JAM-1 expression in bovine eggs and embryos using poly(A)$^+$ RNA and found a similar temporal pattern, with JAM-1-encoding transcripts detectable in only a small minority of germinal vesicle and in-vitro-matured metaphase-II oocytes and, when present, barely detectable using semiquantitative methods (Miller et al., 2003). Likewise, we have found that only a minority of cultured human embryos before the eight-cell stage (presumed maternal genome) (Braude et al., 1988) exhibit JAM-1-encoding mRNA within poly(A)$^+$ RNA compared with later cleavage when the transcript is readily detectable (Ghassemifar et al., 2003). Collectively, these data suggest that JAM-1-encoding mRNA across species might exhibit stage-dependent differential processing to regulate the early timing of its translation and protein membrane assembly relative to other TJ constituents. This might reflect a critical role for JAM-1 protein in cell polarization at compaction and subsequent TJ biogenesis (discussed below).

Data obtained from embryo immunoblotting (Fig. 1B) demonstrated that JAM-1 protein expression is first detectable in compact eight-cell embryos, increasing in late morulae and in early and late blastocysts. Immunolocalization data demonstrated unambiguously that JAM-1 protein is first assembled at the membrane in pre-compact eight-cell embryos,

where it is found weakly localized to cell-cell contact sites. Both in synchronized, intact eight-cell embryos and in timed 2/8 couplets, JAM-1 membrane staining at cell contacts occurred rapidly (within an hour) after division from the four-cell stage and clearly in advance of activation of E-cadherin adhesion at compaction and cell polarization (Figs 3, 4). However, once compaction occurred, JAM-1 transiently localized to the apical microvillous pole (Fig. 3A). During later cleavage, JAM-1 was present on all cell contact sites in both trophectoderm and ICM lineages, although it was concentrated within the trophectoderm apicolateral domain junctional complex (Fig. 2).

The early expression and membrane localization of JAM-1 at contact sites before compaction is unusual in that all other TJ proteins studied to date in the embryo differentiation model undergo membrane assembly only after E-cadherin adhesion at compaction has initiated (Fleming et al., 1989; Fleming et al., 1993; Fleming et al., 2001; Sheth et al., 1997; Sheth et al., 2000a; Sheth et al., 2000b). Indeed, experimental manipulation of E-cadherin adhesion in embryos has indicated its requirement for normal spatial and temporal control of TJ protein membrane assembly and stability (Fleming et al., 1989; Javed et al., 1993; Sheth et al., 2000b). Moreover, JAM-1 membrane recruitment occurs substantially ahead of other TJ transmembrane proteins, with occludin and claudin assembly occurring from the late morula stage, some 24 hours later (Sheth et al., 2000a; Fleming et al., 2001) (F.C.T., B.S. and T.P.F., unpublished). The early assembly of JAM-1 indicates that it might contribute to an early phase in epithelial differentiation, supporting the findings from cell culture studies. Thus, JAM-1 recruitment to membranes in Caco-2 cells after calcium addition to initiate 'differentiation' occurred rapidly (within minutes) and preceded that of other junctional markers (Martinez-Estrada et al., 2001). Early JAM-1 membrane assembly also enhanced recruitment of other TJ proteins (ZO-1, occludin) in JAM-1-transfected CHO cells (Bazzoni et al., 2000a).

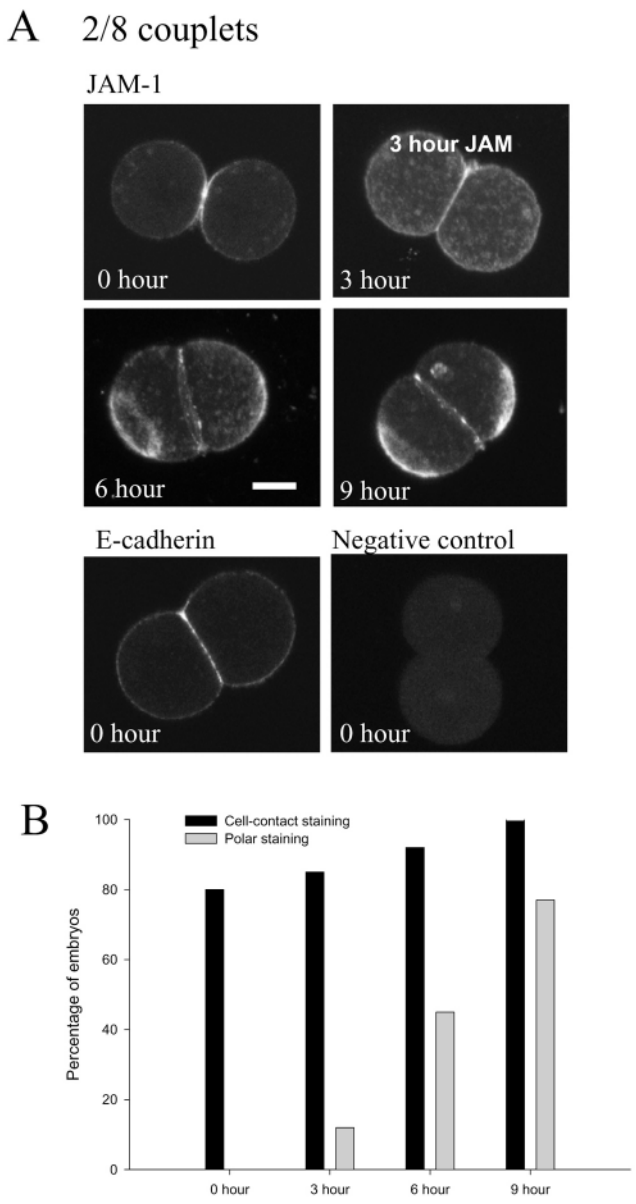

Fig. 4. (A) JAM-1 localization in representative 2/8 couplets at 0 hours, 3 hours, 6 hours and 9 hours after division from 1/4 blastomeres. E-cadherin and negative control (secondary antibody only) are shown at the bottom. Bar, 20 μm. (B) Timing of cell-cell contact and apical polar staining of JAM-1 in 2/8 couplets at different times after division from 1/4 cells (data from single experiment comprising 39 couplets).

An early role for JAM-1 in epithelial differentiation in the embryo is also indicated by its colocalization with the newly formed apical pole at compaction. We believe that apical membrane localization of JAM-1 during epithelial cell polarization has not been reported previously, although apical (luminal) surface localization of JAM-1is required in vascular endothelial cells to mediate leukocyte transmigration (Martin-Padura et al., 1998). JAM-1 recruits ASIP/PAR-3 and atypical PKC to junction sites for establishment of cell polarity in cultured epithelial cells and JAM-1-transfected fibroblasts and CHO cells (Itoh et al., 2001; Ebnet et al., 2001). To examine whether JAM-1 performs a similar role in the early embryo, we investigated the distribution of atypical (ι/λ, ζ) and novel (δ, μ, θ) PKC isoforms at the time of compaction. Although PKC ι/λ displayed only intracellular cytoplasmic staining at compaction, PKCζ was found at the apical membrane, suggesting that JAM-1 might facilitate this localization. Apical surface PKCζ at compaction has also been reported by Pauken and Capco (Pauken and Capco, 2000). Subsequently, both atypical PKCs associate with the trophectoderm junctional complex in blastocysts (Eckert et al., 2004b), to which JAM-1 is mainly localized.

What might be the functional significance of the transient apical membrane localization of JAM-1 and PKCζ at compaction? In addition to establishment of the epithelial phenotype in the embryo, cell polarity at compaction is also required for asymmetric cell divisions to occur in a proportion of eight-cell blastomeres. These divisions give rise to phenotypically distinct daughter cells that occupy outer and inner locations, and are the progenitors of trophectoderm and ICM lineages, respectively (for a review, see Fleming et al., 2004). Thus, experimental evidence suggests that the cytocortex associated with the apical microvillus pole at compaction acts as a stable memory of epithelial polarity in outer cells through subsequent mitoses as trophectoderm differentiation continues (Johnson et al., 1988). At the molecular level, the Par gene complex (including atypical PKCs) is implicated in the generation of cell lineage diversity in *Caenorhabditis elegans*

and *Drosophila* models, and is localized asymmetrically during differentiative divisions (for a review, see Knoblich, 2001). In these models, cell fate diversification can occur by atypical PKC phosphorylation of cytoskeletal components, resulting in different inheritance of determinant proteins (Betschinger et al., 2003). Similar to the current study, atypical PKC is localized to the apical membrane of blastomeres during cleavage of *Xenopus* embryos, in which a role in asymmetric cell division has been proposed (Chalmers et al., 2003).

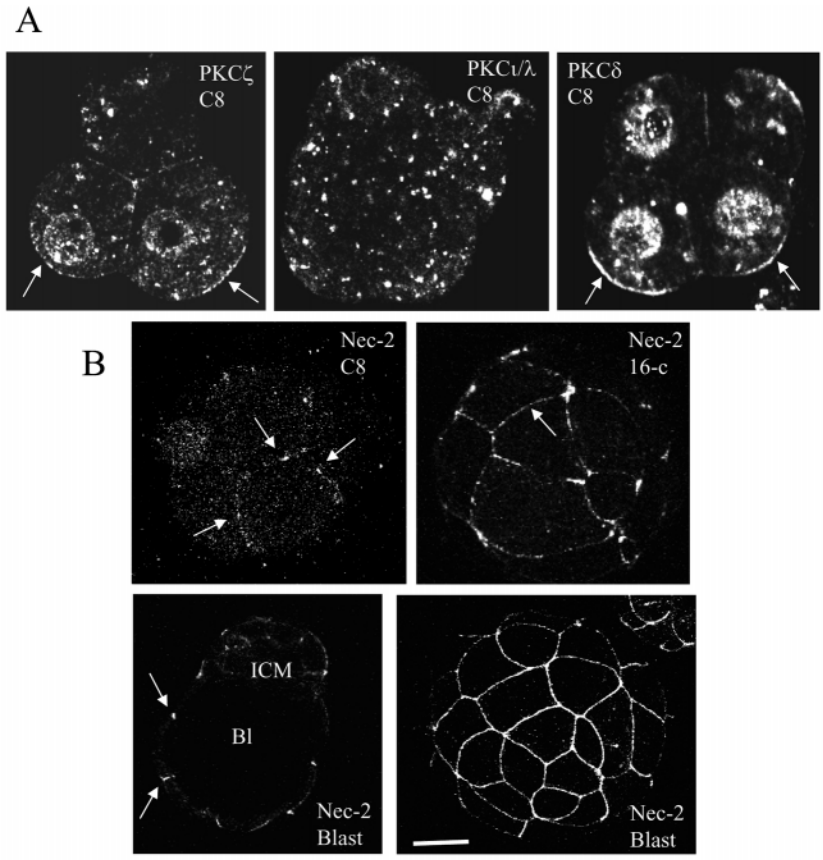

Fig. 5. (A) Localization of PKC isoforms ζ, ι/λ and δ within representative compact eight-cell embryos (C8). Arrows indicate immunostaining at the apical membrane domain of polarized blastomere for PKCζ and δ. (B) Immunolocalization of nectin-2 (arrows) within representative compact eight-cell embryo (C8), 16-cell morula (16-c) and blastocyst (Blast; left, mid-plane showing staining at trophectoderm junctions and at ICM cell contacts; right, *z*-axis series showing trophectoderm in tangential plane; Bl, blastocoel). *n*=10-20 embryos per stage or treatment in three replicates. Bar, 20 μm.

These data on the Par gene complex indicate that apical membrane PKCζ at compaction might contribute similarly to the process of early lineage diversification in the mouse embryo through cytoskeletal reorganization and cell polarity. Extensive cytoskeletal reorganization occurs during compaction and cell polarization in the embryo (Johnson and Maro, 1984; Maro and Pickering, 1984) with the submembraneous microvillous pole comprising actin and actin-binding proteins, including myosin (Sobel, 1983), tropomyosin (Clayton and Johnson, 1998) and ezrin (Louvet et al., 1996), as well as the TJ protein cingulin inherited from the oocyte (Fleming et al., 1993). In addition to PKCζ polarisation, we found the novel PKCδ to concentrate at the apical surface pole of blastomeres at compaction. PKCδ has been shown to bind F-actin at its C2 domain in neutrophils and epithelial cells, with experimental evidence of a function in dynamic restructuring of the membrane-associated actin cytoskeleton (Lopez-Lluch et al., 2001; Liedtke et al., 2003). Conventional PKCs, in particular PKCα at basolateral contact sites, have also been implicated in regulating compaction through β-catenin phosphorylation and activation of E-cadherin adhesion by linkage to the actin cytoskeleton (Winkel et al., 1990; Pauken and Capco, 1999; Pauken and Capco, 2000). Cell polarity at compaction therefore signifies a major reorganization of the cytoskeleton with key roles for select PKC isoforms that might contribute to both epithelial differentiation and cell lineage

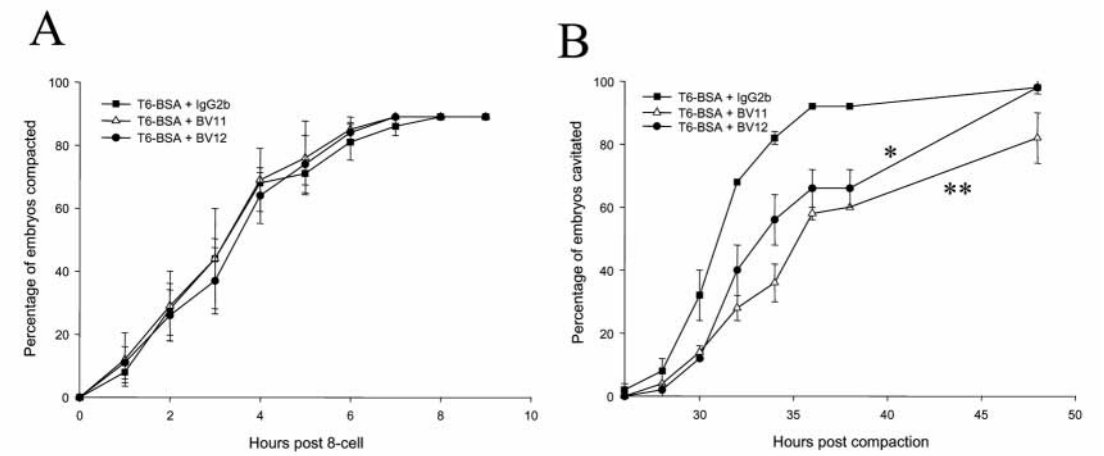

Fig. 6. (A) Effect of embryo culture during the eight-cell stage in T6 plus BSA plus BV11 or BV12 anti-JAM-1 antibodies compared with control IgG2b antibody (all at 5 μg ml^{-1}) on timing of compaction (mean of 89 embryos per treatment). (B) Effect of embryo culture from compaction in media as above on timing of cavitation (mean of 50 embryos per treatment). Rates of cavitation significantly lower than in control medium are indicated with asterisks: *P<0.005; **P<0.0001.

diversification. It will be of interest to determine whether transient JAM-1 polarization at compaction is required for PKC-mediated cellular reorganization at this time.

The co-distribution of JAM-1 and E-cadherin during morula and blastocyst stages within trophectoderm and ICM lineages (Vestweber et al., 1987; Clayton et al., 1993) suggests that JAM-1 might participate in AJ formation and global adhesion within the embryo in addition to TJ biogenesis. The E-cadherin adhesion system is known to associate with that of the calcium-independent immunoglobulin-like membrane protein nectin (Takai and Nakanishi, 2003; Tachibana et al., 2000). These two adhesion systems co-localize via interactions between their cytoplasmic-domain binding partners (α-catenin, afadin) and JAM-1 has recently been shown to be recruited to the nectin complex via afadin (Fukuhara et al., 2002). Nectin adhesion is also thought to enhance the maturation and velocity of assembly of the E-cadherin complex (Takai and Nakanishi, 2003; Honda et al., 2003). Thus, to explore the possibility that the broad distribution of JAM-1 during later cleavage might reflect a maturation of the AJ, we examined nectin expression (hitherto not investigated in the embryo model) using an antibody against nectin-2, a ubiquitous member of the nectin family (Takai and Nakanishi, 2003). A close correlation was found between JAM-1, E-cadherin and nectin-2 localization during morula and blastocyst stages, with contact sites within trophectoderm and ICM labeling for all three proteins. This suggests that JAM-1 membrane recruitment to these sites might be facilitated by the nectin complex and is indicative of a structural maturation of the AJ. However, de novo nectin-2 membrane recruitment (late eight-cell stage) follows that of both E-cadherin (two-cell stage) and JAM-1 (early eight-cell stage), indicating that it is not involved in early AJ adhesion from compaction. Examination of expression of other nectin family members in the embryo will be required to substantiate the functional relationship between these adhesion systems. Moreover, the early assembly of JAM-1 relative to other TJ transmembrane proteins in the embryo might explain the capacity for TJ plaque proteins (e.g. ZO-1α–isoform, cingulin) to assemble before occludin and claudin (Sheth et al., 1997; Sheth et al., 2000a; Sheth et al., 2000b; Fleming et al., 2001).

Our functional analysis demonstrated that, despite the co-distribution of JAM-1, E-cadherin and nectin-2, the onset and maintenance of E-cadherin-mediated adhesion at compaction was unaffected by anti-JAM-1 antibody embryo culture, whereas anti-E-cadherin antibody (ECCD-1) rapidly reversed intercellular adhesion. Thus, JAM-1 does not appear to contribute to any significant extent to global adhesive processes in the embryo, although molecular manipulation of JAM-1 will be required to confirm this. By contrast, anti-JAM-1 antibody treatment did indicate a role for JAM-1 in TJ formation in the embryo, with evidence of significant delay in timing of blastocoel cavity formation compared with controls indicating a requirement for JAM-1 in the establishment of the TJ paracellular seal. The capacity of experimental embryos to cavitate, albeit slowly, might indicate an exclusion or titration of antibody from basolateral surfaces owing to close cell contact.

In conclusion, our study has given new insight into the contribution of JAM-1 to epithelial differentiation. JAM-1 expression during early development provides evidence of involvement in cell polarity and differentiative divisions, the structural maturation of the AJ, and the biogenesis and function of the TJ. Although molecular analyses are now required to determine precisely the role of JAM-1 in each of these processes, this study has demonstrated the earliest known role for JAM-1 in blastocyst morphogenesis.

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