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CELL ADHESION IN THE PREIMPLANTATION MAMMALIAN EMBRYO AND ITS ROLE IN TROPHECTODERM DIFFERENTIATION AND BLASTOCYST MORPHOGENESIS

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1. ABSTRACT

Cell adhesion plays a critical role in the differentiation of the trophectoderm epithelium and the morphogenesis of the blastocyst. In the mouse embryo, E-cadherin mediated adhesion initiates at compaction at the 8-cell stage, regulated post-translationally via protein kinase C and other signalling molecules. E-cadherin adhesion organises epithelial polarisation of blastomeres at compaction. Subsequently, the proteins of the epithelial tight junction are expressed and assemble at the apicolateral contact region between outer blastomeres in three phases, culminating at the 32-cell stage when blastocoel cavitation begins. Cell adhesion events also coordinate the cellular allocation and spatial segregation of the inner cell mass (ICM) of the blastocyst, and the maintenance of epithelial (trophectoderm) and non-epithelial (ICM) phenotypes during early morphogenesis.

2. INTRODUCTION

Cell adhesion in the early embryo is of fundamental importance for the subsequent development and viability of the conceptus. Indeed, it has been demonstrated using gene targeting methods that in the absence of cell adhesion, the blastocyst fails to develop (1-4). Cell adhesion acts in two broad ways to guide the early steps in morphogenesis. First, adhesion is a primary regulator of epithelial differentiation. The trophectoderm is the first epithelium to form during mammalian development and constitutes the wall of the blastocyst. This epithelium generates the blastocoel from the 32-cell stage in the mouse by vectorial transport driven by Na^+,K^+-ATPase enzyme, localised along basolateral membranes. By so doing, the trophectoderm also provides protection and controls the environment of the inner cell mass (ICM), the second lineage of the blastocyst and progenitor of the entire foetus. The trophectoderm is also critical for implantation, being the tissue which interacts directly with the uterine surface to achieve embryo attachment and then invasion. Later, the trophectoderm contributes exclusively to extra-embryonic lineages, principally the chorio-allantoic placenta. Cell adhesion events are critical throughout the period of trophectoderm differentiation during cleavage and regulate the initiation of epithelial cell polarity, cell signalling activity, the maturation of a transepithelial permeability seal, and the integrity of the epithelium during blastocyst expansion.

A second, perhaps more subtle, role for cell adhesion in the early embryo is in the allocation of blastomeres to the two cell lineages of the blastocyst. This contribution ensures that appropriate numbers of cells enter trophectoderm and ICM cell populations. Subsequently, adhesion events maintain these divergent phenotypes such that, if adhesion patterns are altered, ICM cells can be redirected into the trophectoderm differentiation pathway. Evidence has shown that the normal pattern of lineage allocation may be disturbed in different species by environmental influences such as growth factor and serum levels in culture and the level of maternal dietary protein, which may lead to adverse long-term consequences (5-9). This indicates the importance of adhesion processes in early morphogenesis.

Here, we will first review the role of adhesion in trophectoderm differentiation and second consider its

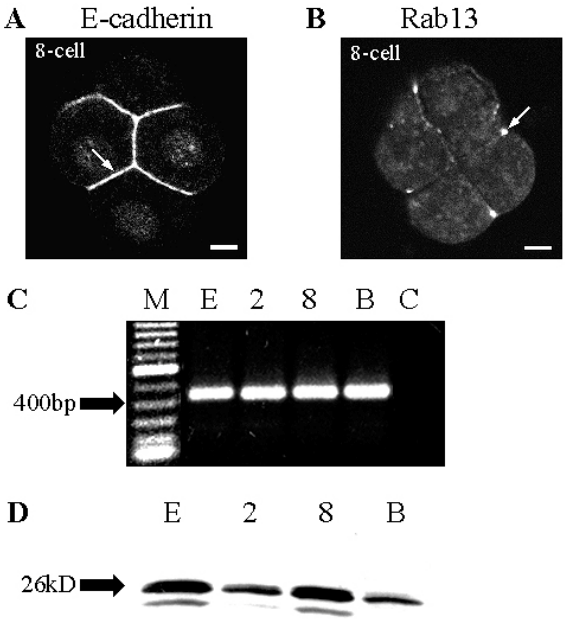

Figure 1. A, B. Compact 8-cell mouse embryos viewed in mid-plane by confocal microscopy immunolabelled for E-cadherin (A) and rab13 (B). E-cadherin is present along all regions of cell contact (A, arrow) while Rab13 is localised at the apicolateral region of cell contact (B, arrow). Bar = 10 μm. **C.** Detection of rab13 mRNA by RT-PCR in single mouse eggs (E), and in embryos at the 2-cell (2), 8-cell (8) and blastocyst (B) stages; M = marker lane, C = negative control. **D.** Detection of rab13 protein by Western blotting in mouse eggs (E), and embryos at the 2-cell (2), 8-cell (8) and blastocyst (B) stages (all lanes loaded with equal numbers of eggs/embryos).

contribution to blastocyst morphogenesis. Throughout, the mouse is used as the primary experimental model but reference is made to other species, including the human, where possible.

3. ACTIVATING CELL ADHESION DURING CLEAVAGE

In the mouse, adhesion between blastomeres occurs for the first time at the 8-cell stage in a process known as compaction, some two cell cycles before blastocyst formation. At compaction, cells flatten against each other over a relatively short time-frame so that their outlines become indistinct. Adhesion is brought about by of proteins characterises the epithelial zonula adherens (ZA) junction which forms a belt-like region of membrane contact in the apicolateral domain between epithelial cells and is also present at lower levels along their lateral surfaces (12, 13; Figure 1A). E-cadherin transmembrane protein binds homotypically extracellularly to engage in adhesion and cytoplasmically with catenin proteins for linkage to the actin cytoskeleton (14). Biochemical and immunocytochemical analyses indicate that the proteins of the E-cadherin/catenin complex are expressed and assemble at membranes throughout cleavage and so activation of adhesion at the 8-cell stage must be initiated post-translationally (15-18). Indeed, use of inhibitors of transcription and translation have shown that synthetic events required for compaction take place well in advance of this time (19, 20). At compaction, E-cadherin redistributes from all membrane surfaces to those associated with intercellular contact (15, 21) and a distinct apicolateral ZA junctional complex becomes recognisable ultrastructurally (22-24). Similarly, beta-catenin relocalises to contact sites at this time (25).

Several studies have indicated a role for protein kinase C (PKC) in activation of compaction. Activation of PKC with phorbol ester or treatment with diacylglycerides caused 4-cell embryos to compact ahead of normal timing whilst treatment with the PKC inhibitor sphingosine inhibited both induced and normal compaction (26). PKC alpha isotype redistributes from the cytoplasm to the cell surface at compaction (27). Although E-cadherin has been shown to become phosphorylated during the 8-cell stage (17), the timing of this modification is insensitive to kinase-directed treatments that either induce premature compaction or inhibit compaction (28), suggesting it is not a substrate of PKC activity. However, it has been shown that beta-catenin in the embryo becomes phosphorylated at serine and threonine sites at the time of compaction and in response to phorbol ester-induced premature compaction (27). Thus, post-translational modification of beta-catenin may occur downstream of PKC signalling to activate E-cadherin-mediated cell adhesion. There is also evidence for the involvement of other kinase/phosphatase systems in the activation of compaction but data is not conclusive. Thus, changes in intracellular free calcium levels and calmodulin localisation have been shown to accompany modulation of compaction in embryos (21). In addition, the serine-threonine kinase inhibitor, dimethylaminopurine, can induce premature compaction at the 4-cell stage, without advancing the timing of E-cadherin phosphorylation (29). Further, major tyrosine dephosphorylation of beta-catenin occurs at the time of compaction (30). However, phosphotyrosine kinase inhibition in embryos by genistein or torphostin, or tyrosine phosphatase inhibition by vanadate treatment, do not appear to affect the timing of compaction (25, 31). A role for myosin light chain kinase activity has also been proposed in embryo compaction (32).

When E-cadherin adhesion occurs during the 8-cell stage, all blastomeres have a surface on the outside of the embryo and reorganise into a polarised phenotype with an outer apical membrane rich in microvilli and basolateral contact surfaces devoid of microvilli (33, 34). In addition, polarisation of the cytoplasm with respect to the actin and microtubule cytoskeletons (35, 36), endocytic organelles (37-39), and the positioning of the nucleus, occur. These phenotypic changes coincide with preferential uptake of endocytosed tracers from the apical rather than basolateral surfaces (38). Collectively, the reorganisation of blastomeres at compaction is into a proto-epithelial phenotype, representing the first stage in trophectoderm epithelial differentiation. E-cadherin intercellular adhesion contributes to the epithelial differentiation process by defining the axis along which blastomeres polarise and catalysing the timing of this reorganisation (16, 34). When

compaction occurs, the rho family of GTPases play a central role in the reorganisation of the actin system which therefore may provide a link between adhesion and polarisation (40). This contribution of E-cadherin adhesion to trophectoderm differentiation is wide-ranging such that its inhibition will disturb later maturation of trophectoderm basolateral membranes in terms of junctional organisation (see below) and vectorial transport capacity (41). Moreover, E-cadherin and alpha-catenin null embryos fail to generate a normal trophectoderm and die before implantation (1-4).

Compaction appears to occur in all mammalian embryos but at different cell cycles. In the pig, compaction and cell polarisation occur more gradually than in the mouse and coincide with relocation of E-cadherin to contact sites, the process not completing long before blastocyst formation (42). Bovine embryos also engage in compaction somewhat later than in the mouse (43). E-cadherin expression and membrane localisation have also been identified in human eggs and embryos (44, 45) and compaction and polarisation occur on day 4 when approximately 16 cells are present (46).

4. MATURATION OF ADHESION DURING TROPHECTODERM DIFFERENTIATION

From the time of compaction in the mouse embryo (8-cell) up until the time the blastocyst forms (32-cell), the lateral membrane domain of blastomeres in the outer trophectoderm lineage continues to mature with assembly of further proteins, particularly of those belonging to the tight junction (TJ). The TJ, like the ZA junction, is a belt-like complex located in the apicolateral contact region between epithelial cells (47, 48). It is positioned just apical to the ZA junction, the two components functioning together in many respects. The TJ is a site of very close intercellular adhesion including partial membrane fusion, forming a seal or barrier against paracellular diffusion and contributing to the transepithelial electrical resistance characteristic of epithelia (47, 48). The TJ comprises several proteins, both transmembrane and cytoplasmic, that interact with the actin cytoskeleton and extracellularly with the next cell to form the paracellular barrier. Three transmembrane protein families, occludin (49), claudins (50, 51) and junction adhesion molecule (JAM; 52) have been identified at the TJ. Cytoplamic TJ proteins associate with the transmembrane proteins and include a family of zonula occludens (ZO) proteins with signalling characteristics, comprising ZO-1 (53, 54), ZO-2 (55) and ZO-3 (56). The ZO-1 protein is expressed in two alternatively spliced isoforms either with or without an alpha domain in its C-terminus (57). In addition, cingulin (58) may contribute to TJ linkage to the cytoskeleton. The epithelial apicolateral junctional complex also includes rab proteins which have a role in intracellular transport processes (59). Rab13 has been shown to co-localise with ZO-1 in the epithelial junction complex (60).

The molecular maturation of blastomere lateral membranes following compaction is temporally regulated with each cell cycle displaying new additions of proteins. Membrane assembly of these TJ proteins occurs at the apicolateral contact site where the E-cadherin ZA junction is focused. The first wave of membrane assembly occurs within 1-2 hours of compaction and includes the proteins ZO-1 (alpha minus variant only; 61, 62) and rab13 (Figure 1B), both proteins are co-localised and assemble together as a complex (24). Expression of ZO-1 alpha minus and rab13 at mRNA and protein levels is detectable during earlier cleavage (Figure 1C,D), it is the activation of E-cadherin adhesion that regulates their membrane assembly. Thus, if E-cadherin adhesion at compaction is inhibited, ZO-1 alpha minus membrane assembly is delayed and is no longer polarised to the apicolateral contact site but rather occurs randomly on the cell surface (61). Moreover, normal TJ membrane assembly has been shown not to occur in E-cadherin null embryos (63). The early assembly of rab13 at the apicolateral junctional complex indicates it may have a role in targeting constituents to this site (24).

The next phase of molecular maturation of the apicolateral complex takes place some 12 hours later during the 16-cell stage when cingulin protein assembles and co-localises with ZO-1 alpha minus and rab13 (64). The level of expression of this protein and its stability from turnover are significantly enhanced by compaction in a reversible manner, indicating cytoskeletal anchorage of cingulin and a further role for E-cadherin adhesion in maturation of the TJ (65). A final phase in apicolateral membrane maturation occurs 12 hours later again during the early 32-cell stage when the TJ transmembrane protein, occludin, and the cytoplasmic plaque protein, ZO-1 alpha plus isoform, assemble (62, 66). ZO-1 alpha plus is transcribed and translated for the first time just prior to assembly and this appears critical in controlling occludin assembly. Occludin is detectable throughout cleavage at mRNA and protein levels but does not engage in membrane assembly until ZO-1 alpha plus is expressed, the two proteins co-localising in the cytoplasm before assembling together at the membrane (66; Figure 2A,B for ZO-1). Four forms of occludin are detectable in the embryo during cleavage and only one form, band 2 at 65-67 kDa, assembles at the membrane during which time it becomes phosphorylated and enters the detergent insoluble pool (66).

This final phase in membrane maturation in the trophectoderm is critical since it coincides with the embryo generating a permeability seal, prohibiting paracellular transport and switching from a morula to a nascent blastocyst (24). It also coincides with the apicolateral membrane complex segregating for the first time into two elements with an outer TJ clearly distinct from an internal ZA junction (24). The timing of ZO-1 alpha plus expression in the late morula therefore appears to be developmentally regulated to activate trophectoderm vectorial transport and blastocyst formation. This mechanism, of delayed expression of a single key component to control the function of a multiprotein complex, also appears to play a role in desmosome junction formation. These spot-like junctions between epithelial cells are first formed in trophectoderm from the time of cavitation (67) and may maintain the integrity of the epithelium during blastocyst expansion. Thus, constituent

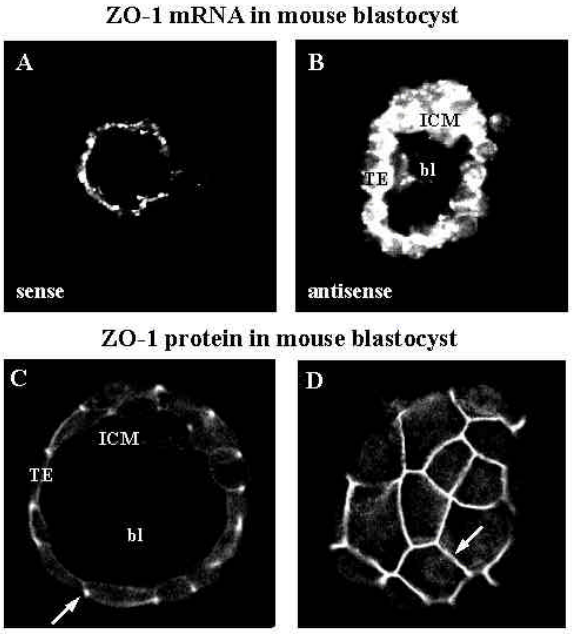

Figure 2. A, B. *In situ* hybridisation of whole mount mouse blastocysts showing the localisation of ZO-1 mRNA in both trophectoderm (TE) and ICM lineages in confocal microscopy images of z-series optical sections, A control sense probe, B antisense probe; bl = blastocoel. **C, D.** ZO-1 protein localisation in mouse blastocyst by immuno-confocal microscopy viewed in midplane (C) and tangential (D) optical section; ZO-1 occurs at the site of cell contact between trophectoderm (TE) cells (C, D arrows) but is essentially absent from the ICM; bl = blastocoel.

proteins of desmosomes (plakoglobin, desmoplakin) have been detected during earlier cleavage but de novo transcription and translation of the membrane glycoprotein, desmocollin 2 (DSC2) is delayed until just before these junctions form for the first time (68).

5. CELL ADHESION AND BLASTOCYST MORPHOGENESIS

When E-cadherin adhesion is activated at compaction, the resulting stable polarisation of blastomeres permits the initiation of phenotypic divergence within the embryo. A variable number of polarised 8-cell blastomeres divide differentiatively, along an orientation orthogonal to the axis of polarity, such that apical and basal regions become segregated into daughter blastomeres (69). Inheritance of apical polarised membrane and underlying cytocortex is permissive for blastomeres to maintain a polarised phenotype at the 16-cell stage and remain within the trophectoderm lineage, while those failing to inherit apical cytocortex, become non-polar in phenotype and allocate to the ICM lineage (69-71). The incidence of differentiative cytokinesis is regulated by the timing of adhesion occurring at compaction and the relative size of apical and basolateral membrane surfaces generated (72). The differential localisation of E-cadherin, present along basolateral surfaces but absent from apical membrane (15), also ensures spatial segregation of 16-cell polar and non-polar blastomeres in outer and inner regions of the embryo, respectively (73). The continuance of adhesion during the 16-cell stage further controls the final allocation of blastomeres into trophectoderm and ICM lineages. Thus, if E-cadherin adhesion is completely neutralised by culture in inhibiting antibody, all blastomeres may eventually enter the trophectoderm lineage (74, 75). In the context of morphogenesis, when the embryo cleaves from 16- to 32-cell stages, E-cadherin adhesion, most likely through its influence on cell shape, once again regulates the orientation of cytokinesis and thereby the proportion of trophectoderm and ICM cells present as the blastocyst forms (76, 77). As indicated in the Introduction, phenotypic divergence has implications for the long-term viability of the embryo.

Cell adhesion mediated by E-cadherin is present equally within both trophectoderm and ICM lineages of the blastocyst while the later steps in membrane maturation including TJ and desmosome formation occur exclusively within the trophectoderm (24, 62, 66, 68, 78; Figure 2C,D). This distinction reflects positional information obtained by cell contact patterns in blastomeres that acts to up- or down-regulate expression of junctional constituents in a lineage-dependent manner. Thus, in the trophectoderm lineage, the combination of an outer, contact-free domain and a basolateral contact domain (ie, contact asymmetry) is stimulatory for epithelial TJ and desmosome expression while, in fully internalised ICM cells, the presence of universal cell contacts (ie, contact symmetry) is inhibitory. Cellular processes derived from trophectoderm cells at the polar-mural boundary cover the blastocoelic face of the ICM and ensure contact symmetry during blastocyst expansion (79).

For the TJ constituents, ZO-1 (both isoforms), rab13 and occludin, cell contact symmetry acts to suppress translation rather than transcription since mRNAs for these genes are readily detectable within the ICM (62, 66, 68; Figure 2A,B). Indeed, evidence for significantly reduced translation of cingulin protein in the ICM compared with trophectoderm has been obtained (65). The stability of TJ proteins within the ICM is likewise decreased since allocation of cells during cell division to the ICM coincides with rapid degradation of inherited ZO-1 (78). The influence of contact patterns on junction protein expression is reversible since after immunosurgery and isolation of ICMs from early blastocysts, the outer ICM cells rapidly develop an apicolateral junctional complex with membrane assembly of ZO-1 that is independent of new transcription but dependent upon protein synthesis (78). In the case of desmosome junctions, contact symmetry may also act to suppress expression at the transcriptional level since DSC2 transcripts were detectable in single trophectoderm cells but usually not in ICM cells disaggregated from early blastocysts (68).

6. CONCLUSIONS

Cell adhesion has been shown to have a profound influence on early preimplantation development for the differentiation of the trophectoderm and the morphogenesis of the blastocyst. The contribution of adhesion can be seen

not only in terms of spatial organisation of the embryo but also in timing of cellular interactions, most important in morula to blastocyst transition and in phenotype divergence. However, since components of adhesion complexes are also involved in signal transduction and regulation of proliferation (47, 48, 80), this aspect of their role during blastocyst morphogenesis needs to be further explored.

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