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Review

Tight junction biogenesis during early development ☆

Judith J. Eckert [a,b], Tom P. Fleming [a,*]

[a] *School of Biological Sciences, University of Southampton, Bassett Crescent East, Southampton, SO16 7PX, UK*

[b] *Developmental Origins of Health and Disease Division, Institute of Developmental Sciences, Mailpoint 887, Southampton General Hospital, Tremona Road, Southampton, SO16 6YD, UK*

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Abstract

The tight junction (TJ) is an essential component of the differentiated epithelial cell required for polarised transport and intercellular integrity and signalling. Whilst much can be learnt about how the TJ is constructed and maintained and how it functions using a wide range of cellular systems, the mechanisms of TJ biogenesis within developmental models must be studied to gain insight into this process as an integral part of epithelial differentiation. Here, we review TJ biogenesis in the early mammalian embryo, mainly considering the mouse but also including the human and other species, and, briefly, within the amphibian embryo. We relate TJ biogenesis to inherent mechanisms of cell differentiation and biosynthesis occurring during cleavage of the egg and the formation of the first epithelium. We also evaluate a wide range of exogenous cues, including cell–cell interactions, protein kinase C signalling, gap junctional communication, Na^+/K^+-ATPase and cellular energy status, that may contribute to TJ biogenesis in the embryo and how these may shape the pattern of early morphogenesis.

Keywords: Tight junction; Adherens junction; Mouse embryo; Compaction; Blastocyst; Trophectoderm; Cell polarity; Cell–cell interactions; Protein kinase C; *Xenopus* embryo; Blastula

Contents

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* Corresponding author. Tel.: +44 2380 594398.
E-mail address: T.P.Fleming@soton.ac.uk (T.P. Fleming).

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1. Introduction—the value of embryos

Early embryos begin as undifferentiated, totipotent cells derived from cleavage of the egg following fertilisation. For cell biological studies, especially mammalian embryos, they have a major disadvantage of small size with scarcity of available material for analysis. The mammalian embryo before implantation is some 100 μm in diameter, comprises around 50–100 cells, and has a protein content of about 20 ng, a nightmare for biochemical investigation. However, early embryos have several advantages that compensate for this restriction and make them a valuable model for inclusion in cell biological review series such as this one, focusing on tight junctions (TJs). The first differentiated cell type formed in embryogenesis is an epithelium; in mammals this is a unique single-layered tissue called the trophectoderm (TE) which forms on the outside of the embryo during cleavage and gives rise later in pregnancy to the chorioallantoic placenta. In the amphibian early embryo, also considered in this review, the outer epithelium becomes the founding tissue for gastrulation and comprises progenitors for all three germ layers. In both cases, this initial epithelium engages in polarised transport to generate the blastocoel cavity, providing an opportunity for the embryo to regulate the composition of its internal tissues for metabolism and developmental purposes and, most importantly, providing a malleable platform upon which cell rearrangements and morphogenesis can shape the future anatomy of the developing embryo.

Why are embryos so valuable in cell biology? Because these epithelia are *real* and constitute a natural and essential step in animal development; the process of their biogenesis is controlled by an inherent developmental programme during which cells mature from an undifferentiated to a differentiated state. The overwhelming bulk of research on epithelial (and TJ) form and function utilize epithelial cell lines which are commonly manipulated in culture using artificial protocols (such as extracellular calcium switching) to 're-enact' the biogenesis process in cells, already fully mature and long-removed from the in vivo environment. Whilst cell lines provide unrivalled capacity for detailed biochemistry, we need to return to the native process of differentiation to confirm our findings on epithelial, and TJ, biogenesis. Early embryos formed during cleavage are the lead models in this respect because they can readily undergo epithelial differentiation in an accessible environment for investigation, throughout the entire period of differentiation, and, unlike most adult primary tissues, in the absence of any contaminating cells. Moreover, with the current advances in reproductive technologies, embryo epithelial differentiation is a critical step in, for example, clinical treatment for infertility, improving efficiency of domestic animal bioproduction, and the isolation and manipulation of embryonic stem cells.

In this review, we focus primarily on TJ biogenesis in the mammalian early embryo, with the mouse being the predominant species. Key steps in embryo morphogenesis associated with TJ biogenesis are considered, as well as the underlying developmental programme governing temporal expression of TJ constituents and the role of cell contact patterns and signalling in the spatial organization of the TJs. We also consider, more briefly, TJ biogenesis in the amphibian model with respect to *Xenopus* cleavage.

2. Tight junction biogenesis in the mouse early embryo

The outer epithelial TE begins to form overtly from the 8-cell stage and completes this process at the 32-cell stage, covering a period of approximately 24 h. Upon completion, the embryo is known as a blastocyst and the TE engages in vectorial transport to generate the blastocoel (Fig. 1). During the biogenesis period, asymmetric cell divisions at 8- and 16-cell stages result in an inward allocation of daughter cells which lose their epithelial characteristics and form the inner cell mass (ICM), the progenitor of the entire fetus. In the late blastocyst, upon expansion of the blastocoel to occupy the bulk of embryo volume, the ICM segregates a second epithelium, the primary endoderm on its blastocoel surface which gives rise to extra-embryonic lineages of the parietal and visceral yolk sac layers. The remaining ICM cells, the epiblast, form the embryo and fetus proper (Fig. 1).

3. Compaction, the foundation for tight junction biogenesis

Following fertilisation, sperm–oocyte mediated signalling via the phosphatidyl inositol second messenger pathway activates intracellular calcium oscillations which in turn reinitiate cell cycling, stimulate cortical granule extrusion to block polyspermy, and activate chromatin remodelling and the embryonic genome transcription programme [1,2]. Transcriptional and translational activity from the embryonic genome during the 2-cell and 4-cell stages is necessary for the first morphogenetic transition in the embryo, the activation of intercellular adhesion and cell polarity, known as compaction, which occurs during the 8-cell stage, some 48 h after fertilisation [3,4]. Compaction converts an embryo with eight clearly defined blastomeres into a ball of cells where cell outlines are not readily distinguishable and is mediated primarily by activation of E-cadherin adhesion [5], although nectin-2 and vezatin adhesion are also recognisable at this time [6–8]. E-cadherin adhesion occurs coincident with its redistribution from uniform to basolateral membrane domains and is regulated predominantly post-translationally apparently involving cell contact-mediated protein kinase C α isotype and myosin light-chain kinase signalling [9–15]. Other transmembrane proteins may also interact with E-cadherin to mediate compaction, such as epithin [16] and vezatin [6,7]. Evidence suggests that modification to β-catenin via serine–threonine phosphorylation and tyrosine dephosphorylation may result from signalling activity to provide cytoskeletal anchorage for the E-cadherin/catenin complex and intercellular adhesion [12,17].

Activation of cell adhesion at compaction coincides with cell polarisation and the emergence of distinct apical (outward-facing) and basolateral (cell contact-facing) domains on all blastomeres. Morphologically, cell polarity comprises a non-adhesive apical pole of ezrin-rich microvilli [18–20]; an adhesive, non-microvillous, basolateral surface where intercellular junctions form (see below); and reorganization of cytoplasmic

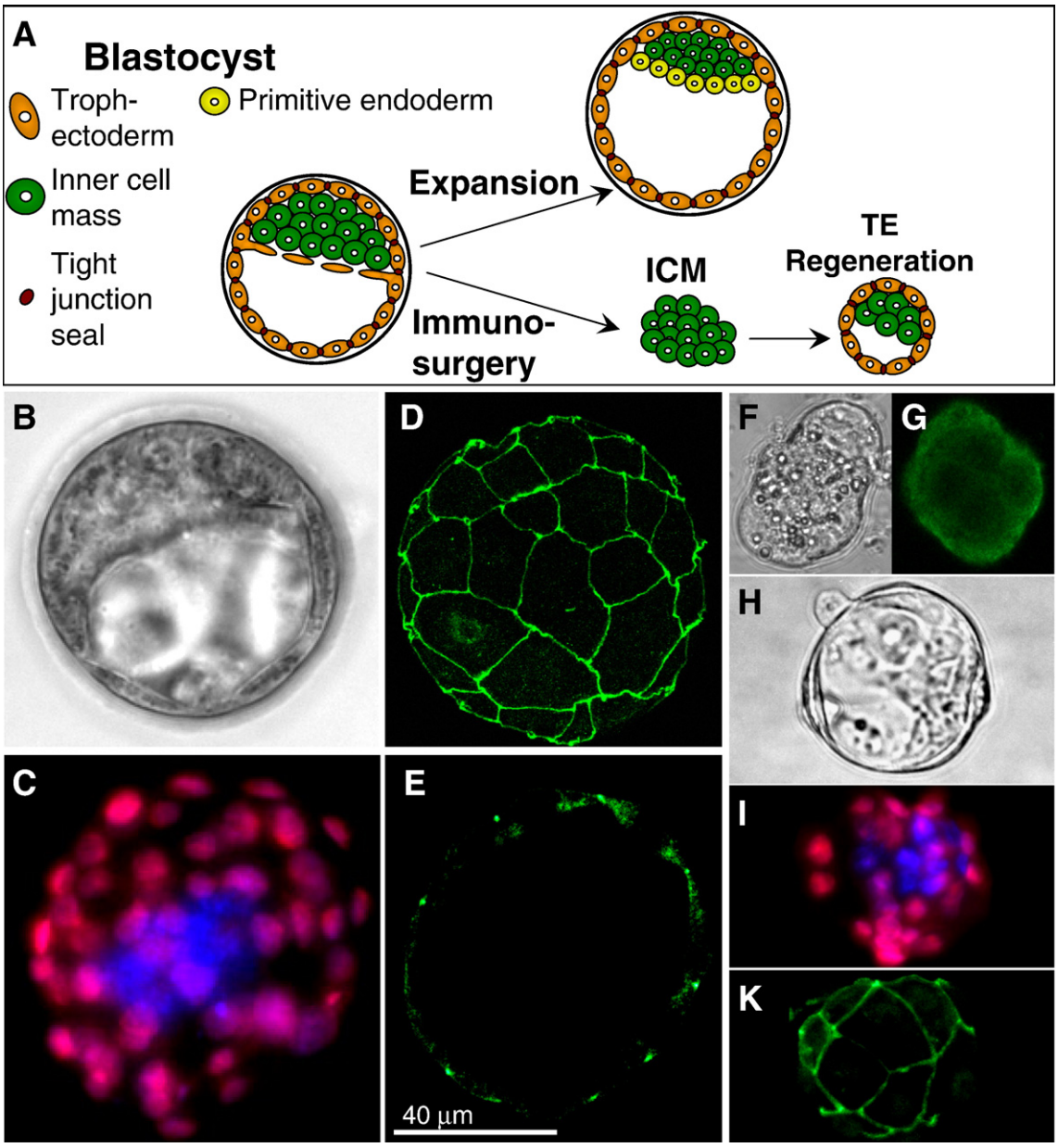

Fig. 1. Blastocyst cavitation, tight junction formation and inner/outer cell orientation. (A) Schematic depicting inner cell mass (ICM) and trophectoderm (TE) cell lineages in early blastocysts and in re-cavitated isolated ICMs upon culture as well as additional primitive endoderm lineage in expanded blastocysts. (B) Bright field photograph of nascent mouse blastocyst before and (C) after differential labelling [172] to visualise inner cells (blue) and outer cells (red) by the fluorochromes, bisbenzimide and propidium iodide, respectively. (D, E) Confocal images of mouse blastocysts fixed and stained for TJ proteins, (D) showing a 3D projection demonstrating the belt-like distribution of ZO-2 and (E) a single slice midplane image showing apicolateral localisation of ZO-1α^+ exclusively within the TE lineage. (F) Bright field picture and (G) single slice confocal image of a freshly isolated ICM fixed and stained negative for ZO-2. (H) Bright field image of a re-cavitated isolated ICM after 20 h of in vitro culture and (I) after differential labelling to visualise inner (blue) and outer (red) cells. (K) 3D projection of confocal slices taken from a re-cavitated cultured ICM fixed and stained showing the belt-like distribution of ZO-2 as an intact blastocyst.

organelle and cytoskeletal systems along the apico-basal axis involving Rho-GTPases [21–24]. Like other examples of de novo cell polarity in developmental model organisms, the initiation of polarity appears to be mediated by the Par (partitioning defective) complex proteins comprising Par-3, Par-6, atypical protein kinase C (aPKC) and cdc42 [25,26]. These may localise to the cell membrane via JAM-1 (junction adhesion molecule-1 or JAM-A) to regulate local cytoskeletal organization and cell asymmetry [27,28]. All of these proteins localise to the apical domain of blastomeres undergoing compaction and inhibition of normal aPKC and Par-3 functioning results in loss of control of asymmetric cell divisions in later cleavage [8,29,30]. However, targeted deletion of the mouse *Par-3* gene results in mid-gestation lethality [31] that may indicate redundancy amongst Par genes in epithelial polarity during earlier development.

The requirement for compaction for subsequent normal epithelial differentiation including TJ formation by the blastocyst stage has been demonstrated in E-cadherin and α-catenin null mutants [32–35] and by E-cadherin RNA interference [36]. Interestingly, genetic replacement of E-cadherin with N-

cadherin is insufficient for TE formation and TJ protein assembly during later cleavage, indicating a specific role for E-cadherin [37].

4. Tight junction assembly from compaction to blastocoel formation

Once the 8-cell embryo undergoes compaction, proteins recognised as TJ constituents then assemble at the apicolateral contact region between blastomeres. This assembly process occurs in a stepwise sequence over the 24 h period between compaction and blastocoel cavitation and can be broken down into phases of assembly during 8-cell, 16-cell and early 32-cell cycles [15,38], summarised in Fig. 2. For review on TJ constituents, see [39] and other papers in the current BBA Biomembranes series. Immediately upon compaction, the peripheral membrane scaffold protein, ZO-1, assembles together with the rab-GTPase, rab13 [40–42]. ZO-1 occurs as two principal isoforms either with or without the C-terminal α exon [43]; it is exclusively the ZO-1α minus form that assembles at compaction. JAM-1 also occurs at this domain during the 8-cell stage prior to and after its localisation at the apical microvillous pole [8]. During the 16-cell stage, the peripheral membrane proteins, cingulin and ZO-2, assemble at the apicolateral contact site for the first time [15,44] (Sheth et al., 2007 in preparation). These first two stages in TJ biogenesis are clearly dependent upon E-cadherin adhesion which, if inhibited, causes randomisation of membrane assembly and, in the case of cingulin protein, increases turnover time [15,40,44]. Lastly, during the 32-cell stage, ZO-1α$^+$ and the transmembrane proteins occludin and claudin-1/3 assemble [38,41,45]. It is only after the final assembly phase that the embryo generates a permeability seal between TE cells and the nascent blastocoel cavity thus forms [42] indicating this phase as being critical in the biogenesis process (Fig. 2).

Analysis of TJ gene and protein expression during cleavage has shown that the temporal assembly programme is dynamic and transcription becomes upregulated between the 4–8 cell stage [46,47] although mRNAs of most constituents are detectable throughout cleavage [41,42,45]. An important exception is ZO-1α$^+$ which initiates de novo transcription and translation immediately prior to the time of first assembly [41]. Critically, this transcriptional event appears regulatory for functional activity of the TJ since newly synthesised ZO-1α$^+$ co-localises with occludin, expressed at mRNA and protein levels from early cleavage, at perinuclear Golgi sites before assembling together for the first time at the junction site [45]. In structural terms, this late event also appears pivotal in the generation of discrete zonula adherens and TJ belt domains within the apicolateral junctional complex. Prior to ZO-1α$^+$ assembly, all junctional proteins, whether belonging conventionally to adherens or TJ entities, co-localise in a single junctional domain with an ultrastructure typical of the zonula adherens. After this assembly step, tight and adherens junction

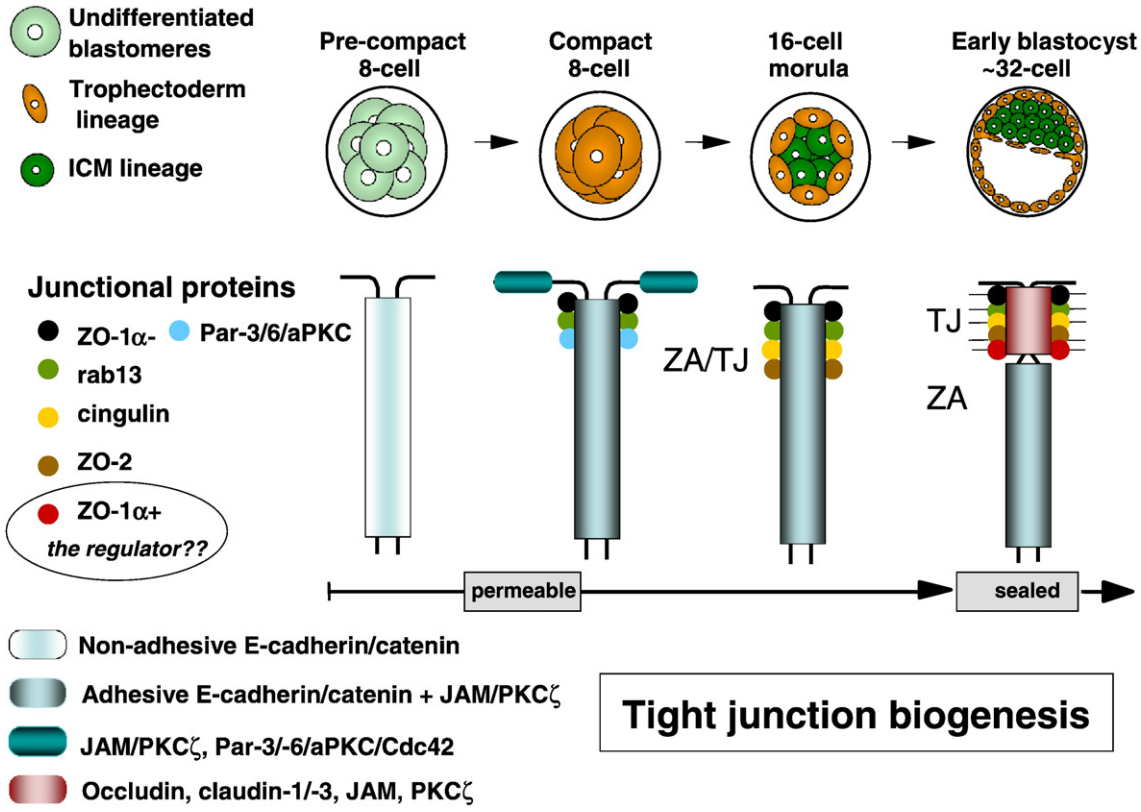

Fig. 2. Schematic overview of the major endogenous events associated with TJ biogenesis in mouse blastomeres over the 8- to 32-cell stages as they undergo differentiation into the trophectoderm epithelium. Top: embryo stages; lower: apicolateral regions of cell contact and the changing pattern of proteins assembled with respect to cell cycle. See text for further explanation and references of the maturation of the TJ domain.

proteins separate into distinct but closely aligned domains and the two junction types with classical morphology are evident ultrastructurally, coinciding with the onset of the paracellular seal [42].

The TJ biogenesis programme has clear implications for epithelial differentiation in the embryo since inhibition of individual constituent membrane assembly associates with the inhibition or slowing of morphogenesis, particularly the process of blastocoel cavitation [8,45,48]. We next consider other mechanisms by which TJ biogenesis may be regulated in the embryo mediated via exogenous cues.

5. Control of de novo tight junction formation during blastocyst morphogenesis

Timing of expression and membrane assembly of the different TJ components are highly regulated by exogenous as well as intrinsic mechanisms during blastocyst biogenesis. It has been broadly accepted that exogenous mechanisms such as cell contact patterns dictate cellular restriction of TJ formation exclusively to the outer TE cells of the blastocyst. In cell lines and tissues, re-establishment of TJ after disruption appears to be regulated at transcriptional, post-transcriptional or post-translational level, dependent upon protein and insult experienced [49–51]. However, as indicated above, most TJ mRNAs are detectable throughout preimplantation development and, hence, TJ protein presence and membrane assembly are likely to be subject to post-transcriptional and post-translational control mechanisms. Understanding these mechanisms will be of crucial importance in understanding how epithelial morphogenesis occurs.

6. Cell contact patterns and TJ biogenesis

Exogenous cell contact patterns regulate the spatial restriction of epithelial differentiation and junction maturation within the blastocyst [15,52–54]. Within the outer TE lineage, asymmetric cell contacts induce epithelial differentiation stepwise throughout cleavage whilst differentiation is suppressed by the symmetric contacts of the enclosed ICM lineage [15,55,56]. The fact that cell contact patterns induce signal transduction pathways is widely acknowledged but remains poorly understood [57]. Although it is known that very complex signalling via extracellular matrix/integrins integrates cell morphology and signal transduction in three-dimensional aspects, the intracellular signalling pathways affected are not well characterized [58,59]. Moreover, in the early embryo, it remains elusive which mechanisms are involved as the first epithelial TE develops independent of extracellular matrix/integrin components although many are beginning to be present in the preimplantation embryo [60,61].

Provision of a contact-free cell surface alone changes the gene expression programme and cellular organization in the embryo [15,52,62]. The plasticity and asynchrony of the stepwise epithelial differentiation process have made it difficult to investigate underlying pathways regulating such mechanisms during normal embryo development more closely and may have contributed to some controversy [63–67]. Fortunately, such a spatial control mechanism is reversible since provision of contact asymmetry to the ICM by immunosurgical isolation from the early blastocyst permits upregulation of TJ membrane assembly following a similar sequence as in the whole embryo but accelerated to less than one cell cycle and reformation of a blastocoel [56,68–73]. Thus, and because isolated ICMs retain their developmental potential [70,74], the ICM-model is an ideal tool suited to investigate cell contact-mediated developmental mechanisms. For example, the ICM-model has been utilized to establish the role of post-translational modification of gp330, a component of the endocytotic machinery, or of ezrin phosphorylation in microvillous formation during polarity development [72,75] as well as re-expression of TJ proteins [15,38]. Most recently, the model has been utilized to determine the role of signalling via specific isoforms of the PKC family during TJ biogenesis [56,73].

7. Protein kinase C signalling and TJ biogenesis

Since transcripts for TJ constituents are mostly detectable well in advance of membrane assembly and within both TE and ICM lineages, transcriptional regulation is unlikely to be a limiting endogenous mechanism [15]. Similarly, (post)translational mechanisms are held responsible within non-developmental epithelial models comprising already differentiated cultured cells, such as Madin–Darby canine kidney (MDCK) cells. Here, signalling systems involving heterotrimeric G-proteins, intracellular calcium levels and protein kinase Cs (PKCs), control assembly, maintenance and function of TJs [76–78]. Detailed exploration of these pathways is very complex as each of these signalling systems consists of a large number of subgroups and other overlapping players involved. Amongst the G-proteins, for example, rab13 has been identified in the mouse embryo and contributes to adherens and TJ specification and segregation [42]. Other members of the family or related proteins are present in the embryo but their detailed roles in TJ biogenesis have not yet been identified [79–83]. Furthermore, all of the 10 PKC isoforms identified so far are present in the embryo [63,84,85]. PKCs are classified according to structure and activation requirements [86–88] into conventional PKCs (cPKCs; α, βI, βII, and γ), novel PKCs (nPKCs; δ, ε, η and θ), and atypical PKCs (aPKCs; ι/λ and ζ). The biological function of PKCs is dependent upon the availability of various PKC isoforms and co-factors such as calcium, phospholipids or phorbol esters within the same cell as well as localisation to specific intracellular compartments mediated by specific anchoring proteins [89,90].The dynamic expression and localisation profiles of specific PKC isozymes during mouse preimplantation embryo development are now well characterized although not without controversy, suggesting involvement in key developmental transitions including epithelial differentiation [8,63,84,85].

Whilst some TJ proteins in certain cellular contexts may be direct phosphorylation targets of PKCs (e.g. occludin, ZO-2 [91,92]), others or in different contexts, for example claudin-1, ZO-1 or occludin, may be regulated directly or indirectly by

PKC affecting TJ RNA expression levels [93,94]. During de novo TJ biogenesis in embryo development, post-translational changes are seen in occludin and may be important in gaining competence to assemble on TE membranes and could be PKC-mediated [45]. In support to this idea, we have shown that specific PKC isoforms co-localise at least partially with the ZO-1α^+ isoform [56,63]. This may suggest some direct interaction either at the cell membrane during membrane assembly or within the cytoplasm to coordinate the assembly process. More indirect effects, for example involving internalization of Na^+/K^+-ATPase in response to PKC modulation [63] may also contribute. In addition, broad chemical activators of PKC are able to stimulate TJ formation using the ICM-model but the use of chemical inhibitors also suggested the presence of a more complex network utilizing different PKC isoforms [56]. The use of PKC isotype-specific peptide modulators [89,95] confirmed that several PKC isoforms and, dependent upon the respective TJ protein, in different combinations appear to contribute to regulate TJ formation in the ICM-model: ZO-2 membrane assembly required PKCδ and ζ activity whilst ZO-1α^+ assembly only needed PKCζ activity [73]. However, in systems that represent an established epithelial phenotype, involvement of certain PKC isotypes, particularly aPKCs, is evident for both ZO-2 and ZO-1 [39,96]. In MDCK cells the capacity of ZO-2 to function at the junctional complex is determined by the phosphorylation state of different sites and whilst an overall increase in ZO-2 phosphorylation mediated by aPKCs caused TJ degradation, cPKCs and nPKCs were thought to promote TJ reassembly. Translocation of ZO-1 during toxin-induced TJ membrane disassembly in colon epithelium was triggered by cPKCs α/β signalling [97] but, in MDCK cells, ZO-1 may also be a direct target for PKCζ [98]. Overexpression of dominant-negative aPKCs could disrupt ZO-1 membrane assembly in MDCK-II cells suggesting a stimulative role for aPKC in TJ assembly [99] whereas, in a mouse mammary epithelial cell line (HC11), a specific aPKCζII has been identified which is inhibitory for ZO-1 assembly [100]. Nevertheless, it should be noted that, in the blastocyst-model, pre-assembled TJ protein was relatively insensitive to PKC modulating agents, possibly reflecting enhanced stabilization by cytoskeletal anchorage but not excluding potential permeability changes.

8. Sodium pump and TJ formation

Coincident with the maturation of the apicolateral junctional complex during TE differentiation, the Na^+/K^+-ATPase pump becomes localised to the basolateral membrane and drives vectorial transport for blastocoel formation and exchange of ions, amino acids, energy substrates and other metabolites [101]. We cannot exclude that some of the effects observed with PKC inhibition on TJ biogenesis are due to internalization of the Na^+/K^+-ATPase [63]. It has been reported that structural organization and vectorial ion transport via adherens junctions and Na^+/K^+-ATPase together are required to establish cell polarisation and TJ and desmosome formation in epithelial Madin–Darby canine kidney cells (MDCK cells; [102]). Similarly, in the early embryo, Na^+/K^+-ATPase activity, especially β1, is required for TJ assembly [103,104]. Biological activity and function of Na^+/K^+-ATPase may also be regulated by PKC in various cellular systems, with the α1 subunit as the potential target (reviewed in [105]).

9. Gap junctions

The role of gap junctional intercellular communication (GJIC) remains controversial in early development since pharmacological inhibition and a series of connexin (Cx) knockouts, the subunits of gap junctions, show no early lethality whilst antibody-mediated inhibition did perturb compaction and blastocyst development [106,107]. However, GJIC is considered critical in growth and differentiation within developmental models [108]. Some evidence suggests that GJIC could be involved in TJ assembly control, thus regulating formation and maintenance of cell lineage divergence. Initiation of GJIC coincides with the initiation of TJ membrane assembly at compaction [109] and gap junction components were found to interact with ZO-1 and other TJ proteins [110–112]. Most importantly, in Cx knockout hepatocytes, newly expressed GJIC induced functional TJ formation, a process preventable by a chemical inhibitor of GJIC [113]. PKC signalling appears to be involved in these processes since membrane assembly and internalization of Cxs depend upon PKC- and PKA-mediated phosphorylation [114,115] and broad chemical PKC activators such as phorbol esters affect GJIC [116]. In contrast, recent evidence suggesting that the presence of intact ZO-1 plaques is critical for GJIC function and PKC-driven Cx43 localisation may place TJs upstream of PKC signalling and GJIC at least in some epithelial cell types [117]. However, we did not find any evidence to support the notion that GJIC may be involved in regulating de novo TJ biogenesis in the early embryo [63,73]. On the other hand, cell contact asymmetry in isolated ICMs may provoke a spontaneous decrease in GJIC which could be due to PKC-mediated phosphorylation of Cxs similar to previous reports in liver cells or fibroblasts [73,118,119], thus indicating that GJIC, like TJ membrane assembly, may be affected by cell contact disturbance and PKC signalling, possibly even involving TJ disturbance as the trigger [117].

10. Cellular energy status and biosynthesis

It is well established that an elevated intracellular AMP–ATP ratio stimulates AMPK activity, connecting sensing of the cellular energy status with biosynthetic processes controlled via mTor [120]. Intriguingly, recent evidence from epithelial cell lines has suggested a link between mechanisms sensing the cellular energy status (AMPK) and TJ membrane assembly: activation of AMPK, which coincided with Ca-induced TJ assembly, promoted ZO-1 membrane assembly and AMPK inhibition disrupted TJ membrane assembly, an effect that could be ameliorated by the mTor inhibitor rapamycin [121,122]. Such control processes may be bi-directional since depletion of specific cell junction complexes allows upregulation of rapamycin-sensitive mTor activity, indicating a mechanism how cell junctions may contribute to the control of biosynthetic

processes within the cytoplasm and nucleus [123]. In addition, ATP-depletion may also trigger TJ disassembly involving aPKC–PAR complex signalling, via reduction of Rac-GTPase activity and reducing the phosphorylation status of ZO-1 and Par-3 [124]. Taken together, such evidence suggests close interlinked relationships between mechanisms sensing and regulating cellular biosynthetic processes, signalling events and the function and assembly of junctional complexes.

11. Hierarchy of mechanisms

The diversity of control mechanisms that may influence TJ biogenesis within the embryo reviewed above and represented diagrammatically in Fig. 3, require a hierarchy for functional integration. Recently, we have shown that signs of active PKC signalling and de novo TJ membrane assembly in the ICM-model were dependent upon complete loss of cell contact symmetry but remained suppressed by partial remnants of outer cells. This suggested a functional hierarchy placing cell contact patterns upstream of the function of protein kinases which, in turn, may be partially responsible for biochemical modifications of TJ proteins and, hence, regulation of TJ membrane assembly [56,63,73]. During de novo TJ biogenesis, GJIC did not appear to have a crucial role, but more subtle effects of TJ function or permeability cannot be excluded. Similarly, indirect effects via Na^+/K^+-ATPase perturbation may have remained undetected. On the other hand, cell contact patterns may further sustain the change in cellular phenotype by downregulating the transcription factor Oct-4 involved in ICM and germline pluripotency [125], thereby further promoting an epithelial phenotype possibly involving TJ function [126,127]. This idea may be supported by the observed upregulation of Oct-4 and downregulation of H19 expression during blastocyst formation in response to a functional inhibitor of occludin [48].

12. Tight junction biogenesis in other mammalian species

The sequence and establishment of junction biogenesis in the early embryo appears to be well preserved across mammalian species from rodents [15,38] to livestock [128–133] up to human [134–137]. Mechanisms regulating junction biogenesis have been mostly explored using the rodent model, due to the scarcity of material from other species. However, assisted reproductive technologies including in vitro culture can severely impair junction biogenesis in livestock [130,132] or human [135–137] often without inhibiting morphological blastocyst differentiation. Such compromised junctional sealing may be brought about by reduced expression levels or mislocalisation of TJ proteins [130,138,139] or indirectly involving perturbed GJIC or Na^+/K^+-ATPase function [140–142]. Intriguingly, in

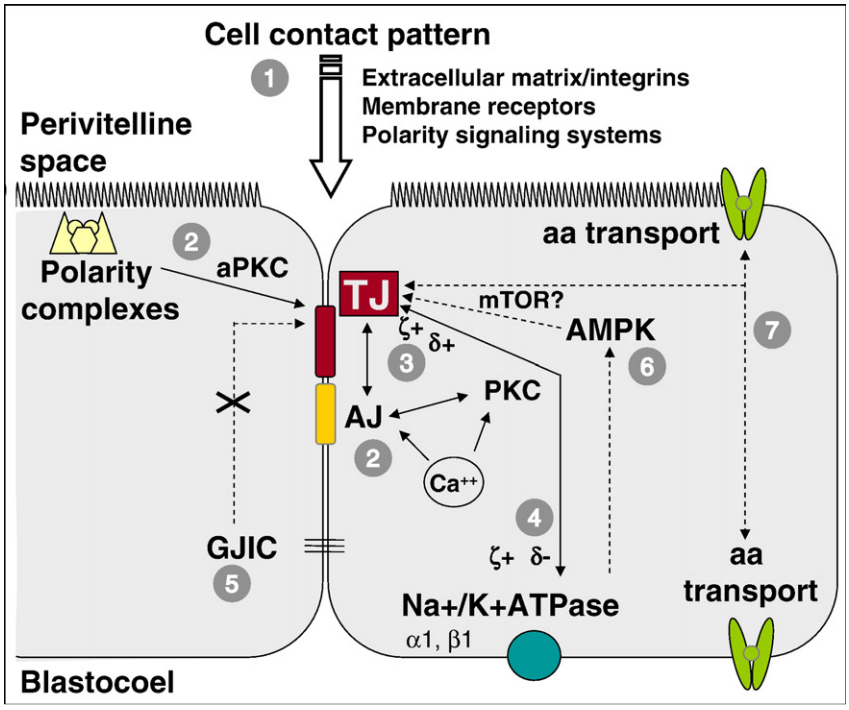

Fig. 3. Schematic overview of some mechanisms identified to contribute to the control of tight junction (TJ) biogenesis in the trophectoderm layer during early embryo development (see text for details). (1) Asymmetric cell contact patterns, possibly involving signalling via extracellular matrix, membrane receptors and polarity systems, are the major regulator permitting TJ formation. (2) Intracellular polarity complexes and adherens junctions (AJ) are critical for TJ assembly and maturation, involving atypical PKCs (aPKC) and calcium, possibly utilizing the calcium-sensitive conventional PKC (cPKC) as intermediates. (3) In addition, TJ membrane assembly is dependent upon PKC signalling (at least the novel PKCδ and aPKCζ). (4) Similarly, membrane localisation of the α1 subunit of Na^+/K^+-ATPase is sensitive to PKC signalling and its β1 subunit participates in permitting correct TJ membrane assembly. (5) Gap junction intercellular communication (GJIC) does not appear to contribute to TJ biogenesis but may itself be regulated by cell contact patterns. (6) Cellular energy status involving the ATP/ADP ratio sensor AMPK does participate in TJ membrane assembly regulation, possibly following through to cellular biosynthetic activity controlled by mTOR. (7) Amino acid transport and/or turnover are also linked to the quality of TJ membrane assembly but hierarchical details and mechanisms remain unclear.

the human, we recently established, for the first time, a connection between impaired junction biogenesis and embryo amino acid turnover [137]. Such connections are most likely to be indirect interlinking several pathways although there is evidence that individual amino acids or small polypeptides (e.g. poly-L-arginine) can trigger internalization of TJ proteins via PKC-dependent (de)phosphorylation [143]. For example, since Na^+/K^+-ATPase is one of the main energy consumers in the human [144], deficits in TJ membrane assembly could be a consequence of limited capacity to meet the increasing energy demands during blastocoel formation when Na^+/K^+-ATPase activity increases, hence involving AMPK (possibly mTOR) and Na^+/K^+-ATPase-driven mechanisms [103,122]. Alternatively, amino acid transporter function or localisation may be perturbed due to deficient TJ fence function and could allow for disturbances in balancing amino acid distribution within the blastocyst [145]. Correct TJ biogenesis is acknowledged to be critical for many processes, from spatial organization of cellular architecture including membrane domains, signalling networks up to cell cycle progression and regulation of downstream transcription [146–149] (discussed in Chapters 13, 14, 16, 19). Thus, TE deficiency in the form of suboptimal junction assembly could not only be a consequence but a cause of disturbed differentiation of the first epithelium in development as has been implied in the mouse [48].

13. Tight junction biogenesis in the amphibian early embryo

In contrast to the mammalian embryo, the amphibian early embryo develops rapidly and the emergence of the first epithelium by the blastula stage is controlled by expression of maternal rather than embryonic genes. In addition, the presence of yolk within the vegetal hemisphere of the egg restricts cytokinesis, generating asynchrony in cell divisions along the animal–vegetal axis. The rapidity and maternal control of early development in this model ensures that the apical domain of the primary epithelium formed during cleavage is relatively unchanged from that of the oocyte membrane and becomes impoverished in typical epithelial basolateral components (cadherins, Na^+/K^+-ATPase, integrins) during oocyte maturation by endocytosis [150,151].

When cleavage initiates, cell polarity is established immediately since new membranes forming at contact sites comprise cadherins (notably XB/U-cadherin and EP-cadherin) and constitute typical basolateral domains such that adherens junction formation can occur even at the 2-cell stage [151–155]. These maternally encoded cadherins form a complex with catenins and engage in homotypic cell–cell adhesion [156,157]. The old apical membrane and new basolateral membranes of polarised early blastomeres exhibit a sharp boundary morphologically [153] and such cells actually engage in polarised transport activity very early since a nascent blastocoel can be detected even at the 2-cell stage in *Xenopus* [158]. The early onset of cell polarity in the *Xenopus* embryo is consistent with the early detection of the Par/aPKC protein complex in the animal hemisphere of the maturing oocyte [159] and its regulation of the polar phenotype [160]. During later cleavage, just as in the mammalian embryo, the Par/aPKC complex, together with blastomere cell shape, appears to contribute to the generation of outer and inner cell populations [161,162].

Early cell polarity in the *Xenopus* embryo implies that TJ biogenesis also occurs rapidly and, indeed, the first evidence of nascent TJ structures can be found in the 2-cell embryo deep along the basolateral membranes; these structures subsequently locate at the apical–basolateral membrane boundary [163–167]. TJ biogenesis, although occurring earlier in cleavage than in the mammalian embryo, does so by distinct assembly stages for individual proteins such as cingulin, occludin and claudins [165,167,168]. Occludin, as in the mammalian embryo, appears to undergo post-translational modifications, notably changes in phosphorylation state, coincident with assembly [169]. Once assembly is complete, a permeability seal can be detected around the nascent blastocoel by the late 2-cell stage [164]. Similar to the mammalian embryo, the impact of TJ biogenesis on *Xenopus* morphogenesis has been demonstrated. Interestingly, overexpression of *Xenopus* claudin not only causes loss of tissue integrity but also randomisation of the left–right body axis [168].

14. Conclusions

We began by emphasising the contribution that can be made by early embryos to understanding the mechanisms of epithelial differentiation and TJ biogenesis because of their authenticity in a biological context, especially their composition of blastomeres which gradually undergo differentiation from undifferentiated precursors, and their relevance for clinical and biotechnological strategies. In this context, we have outlined the endogenous steps in cellular differentiation with respect to competence for TJ formation and identify an important role for delayed ZO-1α^+ expression in regulating the timing of TJ formation in the 32-cell stage mouse embryo. This association between an endogenous expression programme and TJ formation cannot be readily identified by the use of epithelial cell lines and so emphasises the importance of developmental models in cell biology. Interestingly, a reliance upon ZO-1 expression and TJ sealing has also recently been identified using cell lines [170]. The endogenous TJ biogenesis programme in the embryo also unfolds after compaction when epithelial cell polarisation is first evident. This is also consistent with epithelial cell line research where cell polarity and TJ formation, especially associated with ZO-1 expression, occur independently [170].

We also show that embryos combine the inherent, temporally-regulated, developmental programme with exogenous, spatially-relevant, signalling cues to coordinate TJ formation with morphogenesis. Deeper understanding of control mechanisms linking temporal and spatial inputs will come from judicious use of gene microarray screens on stage-dependent embryo samples to identify novel candidates [46,47]. We also need to understand whether and how TJ biogenesis may be affected by inherent asymmetry that may reside within the mammalian oocyte and is reported to contribute to development of embryonic axes [67]. Lastly, the relationship between mechanisms of cell polarity important in epithelial differentiation and

TJ biogenesis and the stabilization of the resulting epithelial phenotype controlled by transcription factor families [171] needs further exploration. These new areas of interface between developmental and cell biology will maintain a central role for embryo models in the foreseeable future.

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