

Chapter 3

Methods to Study Tight Junctions

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Abstract A cell in the ocean exchanges with a constant reservoir, that is not exhausted of nutrients consumed by the cell nor polluted by the wastes it excretes. On the contrary, when a cell belongs to a metazoan, the situation is completely different, as the ocean is now replaced by an extracellular milieu less than one micron thick, that would be quickly exhausted and spoiled, were it not by a circulatory apparatus that continuously carries nutrients and wastes to and from enormous areas of epithelia, where the exchange with the extracellular environment actually takes place. Thanks to this continuous purification and stability of the internal milieu performed mainly by “*transporting epithelia*”, metazoan cells can enormously simplify their housekeeping efforts, and engage instead in differentiation and multiple forms of organization (tissues, organs, systems) that enable them produce an astonishing diversity of higher organisms. Metazoan exist thanks to transporting epithelia.

This chapter summarizes the main methods to study the structure and function of the tight junctions, with natural epithelia, as well as monolayers of cell lines grown *in vitro* on permeable supports.

Keywords Tight junctions • Epithelia • Transporting epithelial phenotype • Polarity • Claudins • Freeze-fracture • TER (transepithelial electrical potential) • Calcium switch

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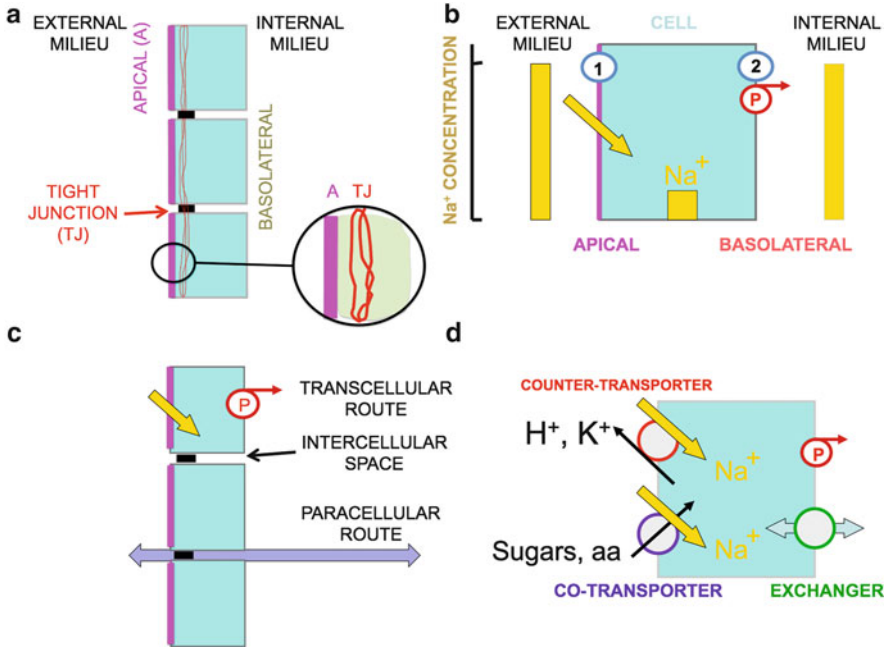


Fig. 3.1 The “transporting epithelial phenotype”. (a) Epithelial cells form layers with an apical (magenta) and a basolateral side (gray). The intercellular space is closed on its outermost end by tight junctions (TJs) observed in freeze fracture replicas as a continuous belt of anastomosing intramembrane fibrils (red), and in transmission electron microscopy as a fusion of the outer leaflet of the plasma membrane, that together with the submembrane cytoskeleton and specific molecules form an obscure, osmium stained spot (black) (see also Fig. 3.2a, b). (b) The model of Koeffoed-Johnson and Ussing (KJU) proposes a first step “1” of penetration of Na⁺ (yellow), down its gradient from the outer milieu into the cytoplasm, followed by its extrusion “2” towards the internal milieu. This step is operated by the Na⁺,K⁺-ATPase (P red). (c) Permeating substances traverse the epithelium through a transcellular route, as proposed by the KJU model, plus a paracellular route that crosses the TJ and proceeds through the intercellular space. (d) As long as the pump keeps the concentration of Na at a low level, this ion is continuously entering the cytoplasm through the apical membrane. Nature uses this Na-gradient to drive counter-transporters and co-transporters

3.1 Introduction

Transporting epithelia are vast areas of cells (e.g. 90 m² of lung, and 270 m² of intestinal mucosa in humans) that have two basic differentiated features: (1) **Tight junctions** (TJs), that form a continuous belt of intramembrane fibers that seals the outermost end of the lateral intercellular space, completely surrounds the cells, and transforms the layer of epithelial cells into an effective permeability barrier (Fig. 3.1a, red). And (2) **Polarity** of the cells, consisting in having an apical domain (Fig. 3.1a, magenta) in contact with the external milieu, and a basolateral domain (Fig. 3.1a, gray) in contact with blood through the interstitial fluid. The apical and basolateral domains have drastically different anatomical, molecular and physiological properties, that constitute the

structural basis that allow cells to transport substances vectorially, i.e. in a net amount towards the outer or the internal milieu (Cerejido et al. 1988).

Given that 90% of the cancers in mature humans start or compromise an epithelium, we expect a strong correlation between cancers and altered TJs (Chen et al. 2006; Martin et al. 2011; Cerejido et al. 2000, 2007; Escudero-Esparza et al. 2011). Several claudins, proteins of the tight junction have been evaluated in primary human tumors to examine their expression levels and cancer progression. For example, claudin-1 exhibits a consistent elevation in colon carcinoma (Miwa et al. 2001; Dhawan et al. 2005). Yet claudin-3 and -4 are frequently overexpressed in ovarian, breast, pancreatic, and prostate cancers (Morin 2005; Hewitt et al. 2006). Down regulation of claudins could contribute to epithelial transformation by increasing the paracellular permeability of nutrients and growth factors to cancerous cells. For example, claudin-11 decreases the invasiveness of bladder cancer cell (Awsare et al. 2011), Claudin-7 decreases or disappears in breast cancer, and head and neck squamous cell carcinoma (Kominsky et al. 2003; Al Moustafa et al. 2002). On the other hand, the barrier function of neoplastic cells could also be altered. At this moment, it is not easy to predict whether this correlation will involve causality, i.e. a cancer would interfere with the normal expression of a TJ or, conversely, alterations of the TJ would cause or favor the development of a cancer, or interfere with pharmacologic treatment of a cancer. This may be the case of a cancer of the nervous system, when it cannot be reached by pharmaceutical agents because of the blood/brain barrier. It is in this case that one would wish that knowledge of the modulation of TJs would afford a way of circumvent the hermeticity of this structure.

In this chapter we will focus on TJs, and the main technical procedures and protocols to study them.

3.2 The Tight Junction (TJ) in Retrospect

It takes a good microscope to –scarcely- see a TJs, that is why this structure only started to be mentioned in biomedical publications in the second half of the nineteenth century, under a wide variety of names, all of them pejorative, as cell contacts were regarded as little more than neutral bolts and fasteners that secured the framework of the tissue, lest it would disintegrate on deformation. Even a century later the TJ continued to be disregarded, and was not even represented in the seminal model put forward by Koefoed-Johnson and Ussing (1958) that served as fertile blueprint for all transporting epithelia (Fig. 3.1b) (Koefoed-Johnsen and Ussing 1958). This disregard was due to the fact that in those days it was taken for granted that substances only cross an epithelium through the so called “transcellular route”, in two successive steps: a first one to penetrate from the external milieu into the cytoplasm of epithelial cells, and a second one to exit from the cytoplasm towards the internal milieu.

Later on, several circumstances brought TJs to the forefront: (1) epithelia with an exclusive transcellular route (“tight epithelia”) are an extreme case, as most natural epithelia have also a “paracellular route” that does not cross through the

cytoplasm of the cells (Fig. 3.1c, *lilac*). (2) the structure that determines whether the paracellular route is important or negligible is, precisely, the TJ. (3) the overall permeability (transcellular *plus* paracellular) is reflected by the transepithelial electrical resistance (TER) of the epithelium, so this parameter is widely used, as it is also an easy to measure one (*see below*). Epithelial TER ranges from a mere 8–10 $\Omega\cdot\text{cm}^2$ as in the proximal tube of the nephron, to hundreds of thousands as in epithelia like the urinary bladder. (4) A given TJ does not have a constant structure nor TER, as these are adjusted to physiological conditions, hormones and pharmacological challenge. When required, the TJ can relax its hermeticity enough to allow the passage of an entire cell, as it is the case of macrophages en route to a spot invaded by microorganism. (5) The TJ is formed by more than 50 different species of proteins, most of them so specific that are used as markers of this cell contact. Some of them are membrane proteins exposed to the intercellular space and contact with analogous proteins belonging to a neighboring cell (e.g. occludin, claudins, JAMs). There is a group of TJ-proteins that form the submembrane scaffold (e.g. ZO-1, ZO-2, ZO-3, ZONAB). There is another a group of proteins that relate the TJ to the cytoskeleton (e.g. cingulin); (6) some of these peptides exist in several chemical states, because they undergo a variety of phosphorylations, and are governed by several routes of intracellular signaling, a scenario that seems far too complex to fulfill the humble role of neutral bolt that secures the epithelial framework (Turksen and Troy 2004). (7) There is an ever growing body of evidence that the TJ is involved in some grave human diseases; in particular those associated to auto-immunity. Thus one century ago, sages of the stature of Paul Ehrlich regarded the intestinal flora as a nuisance that intoxicated the organism, to the point that some enthusiastic followers resorted to abdominal surgery to remove large segments of gross intestine, hoping to favor a longer and healthier life. Today the intestinal fauna is considered instead a quasi-organ, whose cells can even transiently penetrate into the epithelial mucosa to participate in a now reassessed collaboration. (8) The intestinal fauna exchanges substances and signals with the rest of the organism. (9) Yet if for some abnormal reason (e.g. a molecular defect in a molecule constituting the TJ) some of the peptides produced by the flora gain access to the blood, the immune system may generate antibodies that also attack normal proteins in the thyroid gland, the brain, pancreas, etc. thereby triggering terrible diseases such as Hashimoto's thyroiditis, multiple sclerosis, diabetes, Crohn disease, etc. (Cerejido et al. 2007).

3.3 The Ability of the TJ to Adjust Its Hermeticity to Physiological Needs

The fluid just filtered in the renal glomerulus has virtually the same composition of water, amino acids, glucose, urea, and ions than plasma; therefore the chemical gradient across the walls of the proximal tubule is not sharp, and the hermeticity of the epithelial wall is indeed very low (some 8–10 $\Omega\cdot\text{cm}^2$). Yet as the fluid in the

lumen flows towards more distal parts of the nephron, the active removal of chemical components (e.g. sugars, vitamins, amino acids) and the addition of some others (e.g. urea, urobilin, certain ions such as potassium, hydrogen ions), make the tubular fluid more and more different from plasma, requiring tighter TJs to withstand the now sharp gradients between the lumen and the interstitial fluid. Accordingly, the value of TER across the wall of the urinary tract increases, reaching $1,000 \Omega \cdot \text{cm}^2$ in the distal nephron, $2,000$ in the collecting tube, and $60,000\text{--}100,000 \Omega \cdot \text{cm}^2$ in the urinary bladder.

While this is teleologically sound, we still ignore the mechanisms that sense the asymmetry of composition and adjust TER accordingly. This information would be very valuable, as it may help to develop molecular tools to correct the leakiness of the TJs (Flores-Benitez et al. 2009; Contreras et al. 2006) that, as mentioned above, is deemed responsible for many autoimmune diseases.

3.3.1 *Specific Techniques and Approaches to Study TJs*

This description will be based on the rationale as well as use of a given technique, not in the detailed protocol to really use it in a given experiment. These are provided in the Methods section of the references to a publication that uses it.

3.3.1.1 Transmission Electron Microscopy (TEM)

The religious tradition of body/soul, as well as the nineteenth century idea that life consists in structures that function, are misleading and unsuited to understand life at the atomic, molecular and organelle levels, as function comports a change in structure, and *vice versa*: changes of structure have functional consequences. Therefore, in order to “see” a TJ its function must be suddenly stopped with extremely low temperature and quickly penetrating fixatives that crosslink its proteins, so that in spite of being now dead, its structure resembles one of the configurations it had when it was alive. In spite of the fact that water constitutes up to 70–90% of the substance of a cell, it must be totally removed while preserving the micro-anatomy of the epithelium. Then another problem is in line: an epithelium is too thick to permit the passage of the electron beam of the ultramicroscope. Therefore the preparation has to go to several steps of dehydration, while it is infiltrated by embedding resins, again, without distorting the ultramicroanatomy of the TJ, so that the epithelium becomes rock-hard and can be cut with a diamond knife in ultrathin layers that will allow the passage of the electron beam. A third difficulty arises from the fact that the plasma membrane offers almost no resistance to the passage of the electron beam, is invisible, and therefore one cannot see how does it form a TJ. Nevertheless a treatment with OsO_4 binds Os to the two lipid leaflets of the plasma membrane, that now can be seen as a double line resembling the track of a railroad (Fig. 3.2b). Therefore when the plasma membrane of neighboring cells approach each other,

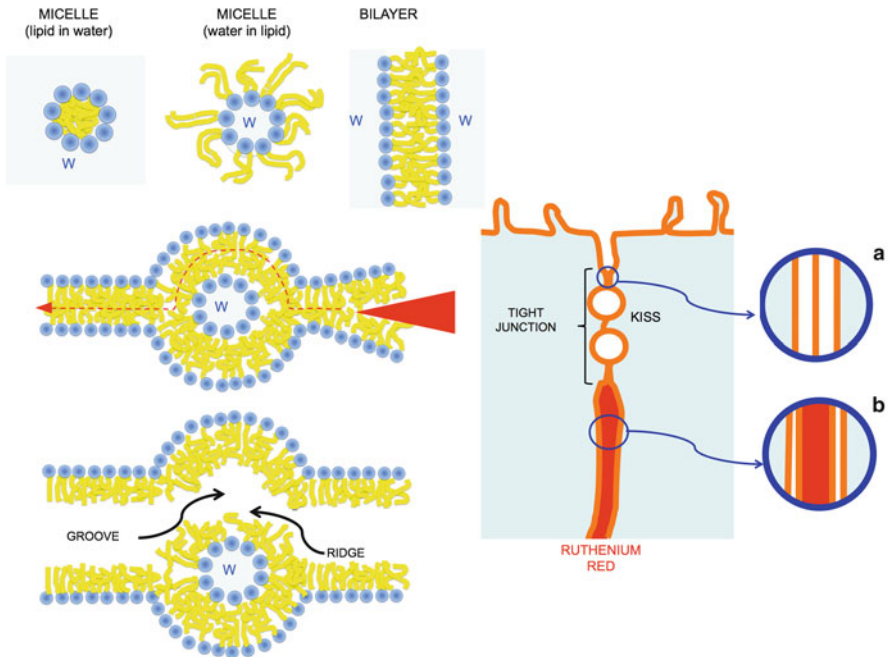


Fig. 3.2 The ultrastructure of the TJ: freeze fracture and transmission electron microscopy.

Above: a micelle is a group of lipid molecules suspended in water (*W* light blue). When water abounds polar groups (*blue spheres*) hide the hydrophobic chain of the lipids in the interior of the micelle. When water is instead relatively scarce the micelle arranges in just the opposite way. Instead of spherical micelles they can also form cylindrical structures perpendicular to the plane of the page. Lipids of a plasma membrane cannot hide their hydrophobic chains in micelles nor cylinders, and the energetic balance of forces favors the formation of bilayers. *Middle:* a TJ is a long cylindrical micelle perpendicular to the page, which is sandwiched between the two leaflets of the plasma membrane. Once the epithelium is deep frozen, it can be split with a special knife (*red*), and the plane of cleavage separates two leaflets, one with ridges and the other with grooves. These halves can be treated with several techniques, such as shadowing with vapors of platinum and carbon, antibodies against TJ proteins containing an electron dense gold particle, etc. *Right:* A TJ is formed at the outermost end of the intercellular space, when the outer leaflets of the plasma membranes approach and fuse (**a**). This occurs successively several times in punctuated points called “kisses”. Ruthenium red added from the basolateral side (*below*) can only penetrate to this point (**b**)

one can see four dark lines but, at the TJ itself, their outermost dark lines fuse, so the TJ appears as trilaminar dark tracks (3 dark lines instead of the $2+2=4$, Fig. 3.2a). But after this trilaminar contact the plasma membranes separate and join again, repeating this separation/fusion several times in a short distance, conferring to the TJ a “punctate” appearance (these contacts are often called “kisses”). When an optically dense substance such as ferritin, horse radish peroxidase, or lanthanum is added to one side of the epithelium, it penetrates in between the cells, but is stopped at the TJ, revealing that this is the structure responsible for restricting the diffusion of substances through the paracellular route (Fig. 3.2b).

3.3.1.2 Freeze-Fracture Electron Microscopy (FF)

A typical micelle is a small sphere formed by amphiphilic lipids (loving both, water and oil, albeit at different parts of the molecule) with their polar groups (Fig. 3.2 *blue*) in contact with the surrounding water (*W blue*) and hydrophobic chains hiding at the center (Fig. 3.2 *yellow*). Yet, when the water is too scarce, the micelle can be inside-out, water at the center and hydrophobic chains pointing outwards. When lipid molecule contains more than one hydrophobic chain and, in particular, when these are long and bulky, the micelle configuration cannot hide them from water. This forces lipid molecules to form instead bilayers, in contact with water on both sides and hydrophobic chains hiding between the two leaflets. This is, precisely, the typical situation at the plasma membrane. Yet crystallographic studies have shown that the spherical micelle and the bilayer are two extremes configurations, but there are other arrangements that depend on the structure of the lipid molecule, the nature of the polar group, whether the hydrophobic chains are saturated or unsaturated, the nature of mobile ions present in the water phase and, above all, when there is a diversity of lipid species. Micelles do not need to be spherical, but can also be elongated forming cylinders with polar heads toward surrounding water, or inward when the cylindrical micelles are surrounded by lipids, as indicated in Fig. 3.2 (*center*). Furthermore, these studies show that these lipid/water structures can coexist. These cylinders establish a belt-like mesh sandwiched between the outer and the inner lipid leaflets of the plasma membrane, that surrounds epithelial cells at the apical/lateral junction.

The monolayer of epithelial cells to be studied by FF should be rapidly frozen at -196°C . This requirement stems from the fact that the molecular assembly of the TJ is extremely weak, as it depends on hydrogen and hydrophobic bonds, as well as electric forces and salt linkages that are irreversibly distorted by even mild temperature vibrations. Actually one cannot freeze the monolayer directly in liquid nitrogen (-186°C) because the thermal conductivity of liquid nitrogen is not fast enough. Thermal conductivity is fast in Freon 22, but the temperature of this liquefied gas is not cold enough (-70°C). These problems are circumvented by cooling Freon to -196°C with liquid nitrogen. When this rock-solid piece of frozen epithelium is broken with a knife (Fig. 3.2 *red*), the plane of cleavage passes through the weakest points that happens to be in between the end of the tails of the hydrophobic chains of the lipids forming the plasma membrane. Since this plasma membrane is a bilayer, it splits into an external leaflet that is in contact with the extracellular space, and an inner one that is in contact with the cytoplasm. At this time these leaflets may be handled in several ways, such as a fine spray with platinum/carbon from above, digested with acid or sodium dodecylsulphate, treatment with antibodies prepared against proteins belonging to the TJ that carry a ultra-thin sphere of gold whose position with respect to the junctional strands can be then studied with a scanning electron microscope, etc. (Andersson Forsman and Pinto da Silva P 1988; Dinchuk et al. 1987; Gruijters et al. 1987).

3.3.1.3 Flux of Dextran

A flux is the amount of a given substance that crosses an epithelium per unit time and unit area, and is usually expressed as $\mu\text{mole}\cdot\text{h}^{-1}\cdot\text{cm}^{-2}$. It can be measured under a mindboggling variety of conditions: e.g. as a function of the concentration of the substance, in the presence of competing analog molecules, in epithelia treated with all sort of inhibitors, etc. One usually starts with the measurement of a small inert molecule that would serve as comparison for the flux of other substances, or the flux of the same substance but in a variety of epithelia (e.g. ileum mucosa, gallbladder epithelium), or the same epithelium but from different animal species. Although we will illustrate the measurement with Dextran, it can be substituted by any other substance that would not damage the cells.

Dextran is a complex, branched polysaccharide made of many glucose molecules, that are available in chains of the desired length and molecular weight. The permeability of electrically neutral molecules across the TJ is measured with dextran of some 3 kDa, that it is too big to cross through the transcellular route, a circumstance that makes it ideal to study the paracellular one. To make its flux easy to measure, Dextran is commonly bound to fluorescent tags, e.g. FITC-Dextran. A freshly prepared solution with 10 $\mu\text{g}/\text{ml}$ of FITC-Dextran is dissolved in P-buffer, and placed in the chamber in contact with the apical compartment. The one in contact with the basolateral side only contains P-buffer with or without the drug whose effect on TJs is investigated (e.g. 10 nM ouabain). After 1 h incubation at 37°C, the basal medium is collected, and the fluorescence of the transported FITC-Dextran is measured with a fluorescence spectrometer at 492 nm (excitation) and 520 nm (emission). The quantity of FITC is calculated by comparing with a standard curve. The unidirectional flux of Dextran from the apical to the basolateral direction (J_{DEX} in $\text{ng}\cdot\text{cm}^{-2}\cdot\text{h}^{-1}$) is calculated by dividing the fluorescence intensity of a given sample of the bottom solution by the corresponding value of the upper solution conveniently diluted.

3.3.1.4 Transepithelial Electrical Resistance (TER)

The degree of sealing of TJs can be assessed by measuring the TER of the monolayer as depicted in Fig. 3.3. A small current ($20\ \mu\text{A}\cdot\text{cm}^{-2}$) causes a voltage deflection which is measured with a voltohmmeter (EVOM) and an EndOhm-6 systems (World Precision Instruments, Sarasota, FL). Ohm's Law is then used to calculate an overall TER, and the component due to the resistance of monolayer itself can be obtained by subtracting the resistance of the bathing solution and the empty support. Results are expressed as $\Omega\ \text{cm}^2$. The specific conductance of the TJ to a given ion can be studied by substituting control ions (usually Na^+ , K^+ , or Cl^- by other univalent cations and anions (Larre et al. 2010, 2011; Cereijido et al. 1978)).

Frömter and coworkers have used a more refined procedure based on impedance measurement (see (Kottra et al. 1989, 1996)).

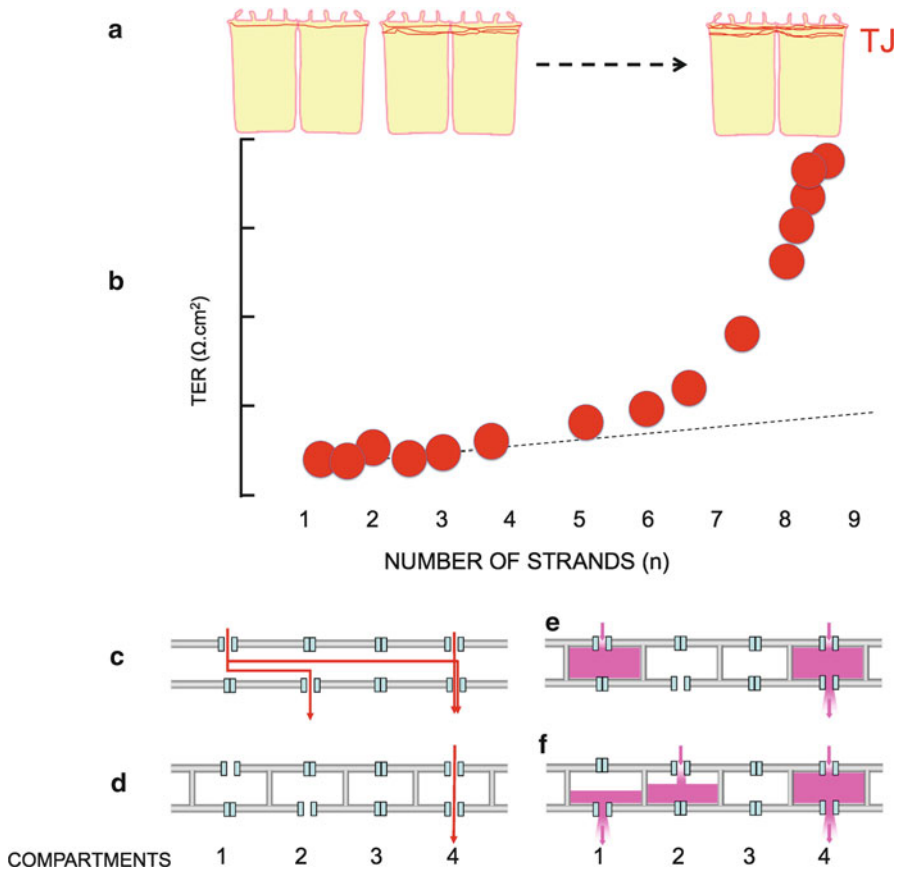


Fig. 3.3 The relationship between junctional structure, TER and permeability of the TJ. (a) TJs (red) vary in the number of strands (n). (b) If strands were simple ohmic resistors, then TER would increase with n in a linear manner (dashed black line). Yet experimental measurements in a wide variety of epithelia, show that TER increases exponentially with n (red circles). (c) Flickering channels (1, 2, 3 and 4) that alternatively switch from an open to a closed state would not explain the discrepancy between TER and permeation studies though, because ions may cross a given strand (red lines) by any channel that happens to be open at the time. (d) Yet strands have frequent anastomoses and trabeculae that compartmentalizes the TJ, so that for electric current to flow across the whole TJ it is necessary that channels in the upper and lower strands be open simultaneously. Only compartment 4 would be conducting in this example. (e) Permeation would instead obey a different set of rules. Thus a diffusing substance like mannitol or dextran (pink) may flow into compartments 1 and 2 in spite of the lower channel being open (1) or closed (4). (f) When channels in the lower strand open, the solute would leave the compartment regardless of whether the channel in the above strand remains open or closed. Therefore, in a segment of TJ with compartments imposed by the presence of trabeculae, only those compartments with channels in the open state in the upper **and** the inner strand will let the electric current through (d # 4, and f # 4). On the contrary, in a study of permeation to a given substance, this substance will enter the compartment whenever the channel in the upper strand is open, and will proceed its movement towards the basal side of the epithelium whenever the channel in the lower strand is in the open state. In summary, the difference between electrical measurements and solute permeability studies is that, while the first requires that all channels in the series should be simultaneously in the open state, solute permeation does not

3.3.1.5 Electric Current Paths Through an Epithelium

When an electric current is applied through an epithelium it can follow several paths. If the epithelium is a very tight one, current will only successively flow through the apical and the basolateral domain of the plasma membrane of the cells. But it can in principle flow through spurious paths. The most common one would be *edge damage*, i.e. the points where the pressure of the rim of the Lucite chamber would destroy cells at the perimeter of the exposed area (Fig. 3.3f). Yet today these nuisances are avoided by a suitable design (e.g. rubber O-rings), greasing the halves of the chamber, etc. The other potential source of artifacts would be damaged or dying cells. Yet the adherence of cells to a solid support entails a very competitive process; as cells continuously strive to adhere to a larger area of support, including the formation of an actin ring that effectively deprives the unhealthy cell of attachment to the support, as well as interrupts the access of nutrients coming from the organism through the basolateral side (Peralta Soler et al. 1996). Eventually, unhealthy cells are forced to abandon the monolayer and are discarded towards the lumen. In a natural tissue, this is the usual destiny of aging cells.

Apart from these spurious paths due to technical inefficiencies, in epithelia or monolayers with low TER (usually below $2,000 \Omega \cdot \text{cm}^2$) more than 98% of the applied current follows the paracellular route, that is one of the reasons that TER is routinely used to gauge the hermeticity of the TJ (Cereijido et al. 1984).

Frömter and Diamond (1972; Frömter 1972), and Steffani and Cereijido (1983) have developed *ad hoc* procedures in which a glass microelectrode is used to scan the surface of an epithelium, to detect the points where current flows through the epithelium of the gall bladder. Invariably these coincide with the intercellular space.

3.3.1.6 “Discrepancies” Between Permeability and TER

The electrical resistance of an epithelium measures how easily an ion can cross it. Yet at times resistance measurements indicate that an ion crossing the epithelium meets a relatively high degree of difficulty, while its permeability, measured through the flux of a tracer (e.g. a radioisotope) is nevertheless high. This puzzling result entails no discrepancy and, in fact, is quite common. Imagine this analogy: five persons open the front door, enter into the living room, close the door behind them, and proceed likewise to the dining room, the kitchen, the back room: they would “flow” through the entire house, i.e. the house is “permeable” to people. This compares to the flux of a substance that penetrates a route of compartments in series, each one limited by flickering channels (Fig. 3.4e, f). On the contrary, the measurement of the electrical conductance/resistance requires instead that doors be simultaneously open. Solving this “discrepancies” between conductance (or resistance) and flux measurements may afford information on the TJ.

Since each TJ strand is a resistor, one would expect that TER would increase linearly with the number of strands (Fig. 3.4a, b). Yet in natural epithelia TER increases exponentially with the number of strands, as indicated in Fig. 3.4a, b. This situation is also explained by the existence of trabeculae that compartmentalize the TJ.

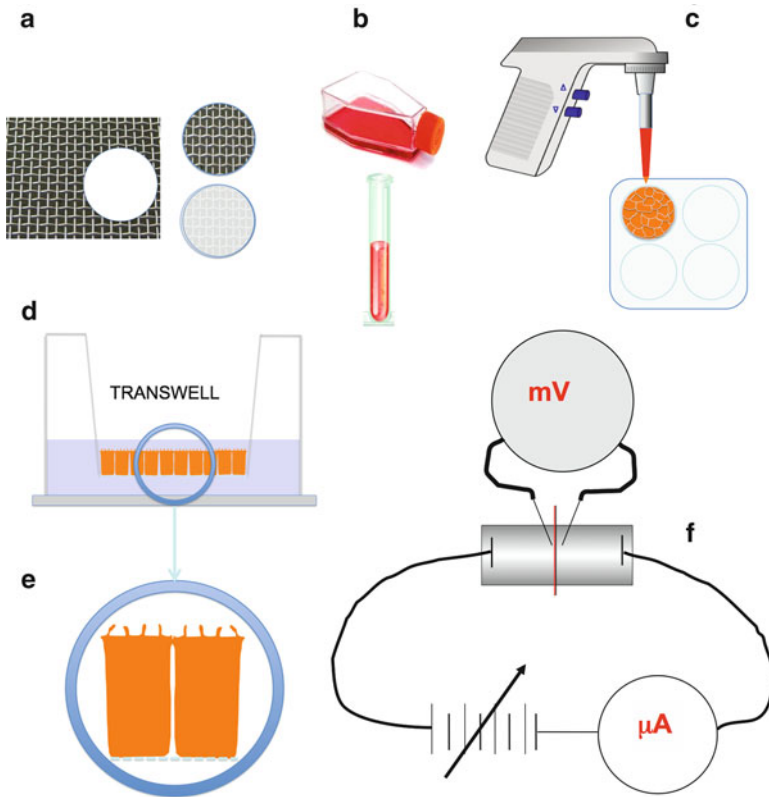


Fig. 3.4 An epithelial model system: Epithelial cells can be cultured as monolayers that resemble natural epithelia and offer a series of experimental advantages. (a) Disks are cut out of nylon cloth with square windows of $100\ \mu\text{m}$ on the side, which is coated with collagen and sterilized. (b) MDCK cells (epithelial from dog kidney) are mass-cultured in plastic bottles, harvested with trypsin-EDTA, and (c) plated at saturating density on the collagen coated disc. (d, e) Alternatively, monolayers can be prepared in a Transwell assembly. (f) The disk with the monolayer is mounted as a flat sheet between two Lucite chambers with Ringer solution, a current of $20\ \text{A}\cdot\text{cm}^{-2}$ is passed and the voltage deflection measured. These parameters are used to calculate TER with Ohm's Law

3.3.1.7 Monolayers of Epithelial Cell Lines Cultured on a Permeable Support

By the end of the decade of 1960 TJs and polarity were sufficiently characterized, and their basic properties as well as physiological role were reasonably understood, and the next step was to study when, how and why these structures are synthesized, assembled and become ready to function (Cereijido et al. 2008). It was not possible to learn about the mechanisms involved using natural epithelia as model systems, because these have TJs and polarity already established. In this respect, a great breakthrough was made by using epithelial cell lines cultured on permeable supports (Cereijido et al. 1978, 2004). These cell lines can be mass cultured in bottles, harvested with trypsin-EDTA and plated at confluence on a Nitex cloth

coated with collagen, or on Millipore filters (Fig. 3.3d). This harvesting is so harsh that makes cell lose their TJs and polarity. Yet upon re-seeding, cells re-establish these features under culturing conditions amenable for experimental control, e.g. in the presence of inhibitors of the synthesis of proteins, DNA, RNA, glycosylation, and assembly of microtubules and microfilaments. There are finer procedures to block protein synthesis more specifically, resorting to RNA interference (RNAi), or force the cell to express an engineered version of a given molecule, block phosphorylation, or mutate a given amino acid suspected to be crucial in the making of a TJ or during the process of polarization. Monolayers prepared in this way can be then inspected by a variety of electron microscope techniques, epifluorescence with tagged molecules, etc.

Cells can be plated at low density, followed by waiting until they proliferate and achieve confluence. This protocol has the disadvantage of mixing cells that are still engaged in proliferation, with those that in the meanwhile have recovered from trypsin, contacted several neighboring cells and are ready to synthesize and assemble TJs. Most often instead, cells are plated at saturating density and allowed to attach for 20–30 min, followed by a change to a bathing medium without cells. This protocol has the advantage of removing cells that were unable to find room in the support, and those that did attach would not waste time in proliferation.

3.3.1.8 The Calcium Switch

The procedure described in the previous paragraph can be perfected in still another way, based on the observation that removal and restoration of Ca^{2+} opens and reseal TJs (Cereiido et al. 1978). Thus Fig. 3.5 (*left*) shows two sort of monolayers; the first is basically the one described in the previous paragraph (*open circles*), but in the second the assembly of TJs as well as polarization are arrested by the removal of Ca^{2+} 30 min after cell attachment to the support (*filled circles*). If this ion is re-admitted at the 20th hour (*blue arrow*), TER is observed to increase with a much faster kinetics, because cells are already recovered from trypsination and had adjusted their borders to each other. This assembly and sealing can be so abrupt, that a fraction of Na^+, K^+ -ATPase, that in mature monolayers of MDCK cells only occupy the basolateral side can be trapped on the apical (wrong) side as well. Even the observation of the fate of these misplaced subpopulation of the enzyme provides valuable knowledge on the removal and relocation of membrane molecules during polarization. (Contreras et al. 1989; Shoshani et al. 2005)

Besides of being a useful technical procedure to observe the kinetics of TJs assembly and polarization, this technique provided a way to study some intricacies of the mechanism triggered by Ca^{2+} , that results in the development of the “epithelial transporting phenotype”. Cells incubated overnight in the absence of Ca^{2+} lose this ion, that achieves a very low concentration in the cytoplasm, and markedly increase their specific Ca^{2+} -permeability. Hence 20 h later, when this ion is restored to the bathing fluid, it rapidly penetrates into the cells and increases its

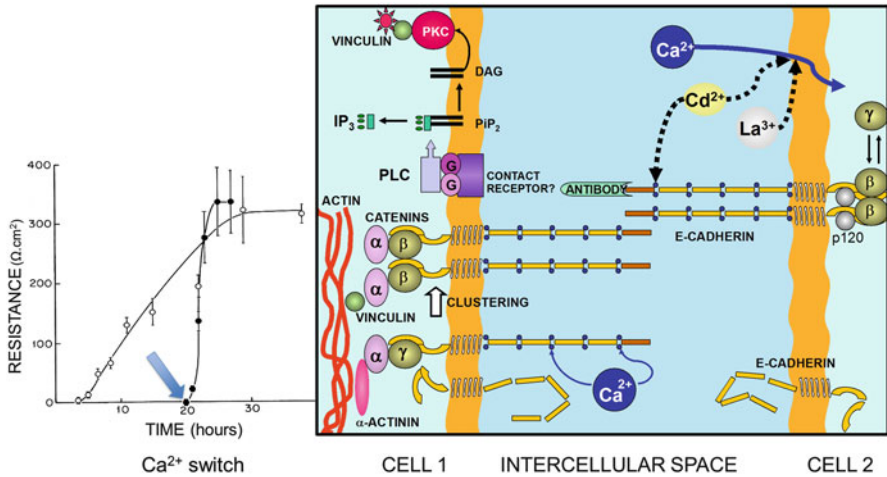


Fig. 3.5 (left) Monolayers of MDCK cells progressively assemble and seal TJs, a process that can be followed by the value of TER (open circles). Monolayers of cells plated in the presence of Ca²⁺, are allowed to attach for 20–40 min, switched to a medium without this ion and incubated overnight, have a negligible TER at the 20th hour. At this time the restoration of Ca²⁺ triggers a fast assembly and sealing of TJs (filled circles). This maneuver can be performed under a variety of experimental conditions, such as the presence of inhibitors of RNA or protein synthesis, glycosylation, the presence of competing cations such as Cd²⁺ and La³⁺. (right) Two neighboring epithelial cells showing that Ca²⁺ acts primarily on the extracellular segment of E-cadherin, making this segment straight so that it can interact with other E-cadherin molecules located on the same cell membrane. Once in this position, extracellular segments can interact through van der Waals' forces, that decay with the 5th power of distance. Since these events are occurring in the neighboring cell as well, straightened molecules of E-cadherin can reach those on the other side of the intercellular space and establish a firm cell-cell adhesion (see text)

concentration in the cytoplasm. For a while this penetration misled research workers that investigated the role of *intracellular* Ca²⁺, and resorted to prevent the increase concentration in the cytoplasm by, for instance, using Ca-buffers. On the contrary, Contreras et al. (1992) and González-Mariscal et al. (1990) demonstrated that it is instead the *extracellular* Ca²⁺ that triggers junction formation and polarization. The demonstration is as follows: (1) the concentration of Ca²⁺ necessary to promote TJ formation and polarization is so low, that it may trigger these processes without modifying the concentration of calcium in the cytoplasm (Nigam et al. 1992). (2) The penetration of Ca²⁺ can be blocked by Cd²⁺ or La³⁺ that are nevertheless unable to trigger differentiation. Put in other words, Ca²⁺ triggers the making of TJs and polarization from outside of the cytoplasm. (3) In spite of their affinity for the mechanisms used by Ca²⁺ to penetrate, Cd²⁺ and La³⁺ cannot substitute calcium in triggering TJ formation and polarization. (4) Later on it was found that extracellular Ca²⁺ acts on the extracellular domain of E-cadherin, at the joins between the five repeats in the extracellular segment of this molecule (Fig. 3.5 right) (Ringwald et al. 1987). This causes this molecular segment to straighten, and attach to similar segments in neighbor

E-cadherin molecules in *cis* position, i.e. in molecules in the plasma membrane of the same cell. This enables the group of E-cadherins to bind to those present in the *trans* position in the plasma membrane of a cell placed on the opposite side of the extracellular space. These processes in the extracellular segment of E-cadherin cause the cytoplasmic end of the molecule to release the γ -subunit of catenins, and replace it with β -ones. This exchange leads the α -subunit to bind vinculin and trigger the formation of a scaffold of actin. Once the two neighboring cells approach to a suitable distance, contact receptors convey the information of the attachment through two different isoforms of G-proteins, causing PLC to split PIP_2 , and release DAG, that activates PKC, a reaction that starts a lightening of phosphorylations among molecules that belong specifically to the TJ (*see* (Balda et al. 1991)).

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