Rapid Evaluation of Intestinal Paracellular Permeability Using the Human Enterocytic-Like Caco-2/TC7 Cell Line

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Abstract

Paracellular permeability of the intestinal epithelium is a feature of the intestinal barrier, which plays an important role in the physiology of gut and the whole organism. Intestinal paracellular permeability is controlled by complex processes and is involved in the passage of ions and fluids (called pore pathway) and macromolecules (called leak pathway) through tight junctions, which seal the intercellular space. Impairment of intestinal paracellular permeability is associated with several diseases. The identification of a defect in intestinal paracellular permeability may help to understand the implication of gut barrier as a cause or a consequence in human pathology. Here we describe two complementary methods to evaluate alteration of paracellular permeability in cell culture, using the human intestinal cell line Caco-2 and its clone Caco-2/TC7.

1 Introduction

The intestinal epithelium is a cell monolayer mainly composed of enterocytes, which is at the interface between gut lumen and internal environment. The intestinal epithelium was defined in physiological situation as a “leaky” epithelium as compared to “tight” epithelia such as skin or urinary bladder [1-3]. It forms a selective barrier allowing the transfer of essential dietary nutrients, electrolytes and water from the lumen into the circulation while preventing the entry of foreign antigens, microorganisms and their toxins [4,5]. Two major routes are involved in this selective permeability: the transcellular and the paracellular pathways [4]. Transcellular pathway is involved in the absorption and transport of nutrients through processes predominantly mediated by specific transporters or channels located at the apical and basolateral membranes of intestinal epithelial cells [6]. Conversely, paracellular pathway corresponds to a passive but selective transport of only certain solutes and fluids through the space between adjacent cells [6]. Intestinal epithelial cells are connected by intercellular junction complexes localized all along the lateral membrane, but concentrated mainly at its upper part in what is called the “apical junctional complex”. The paracellular space is sealed at the level of tight junctions, which are the most apical cell-cell junctions and which specifically control the paracellular permeability [4,7,5,8]. Paracellular permeability is divided in two pathways: the pore and the leak pathways (Figure 1).

The pore pathway is involved in the paracellular permeability to ions and small molecules and represents a high capacity flow depending on the size and charge of the molecules. The determination of transepithelial electrical resistance (TEER), which is inversely proportional to the flux of ions across the epithelium (ionic conductance), allows assessing the pore pathway. TEER value of the epithelium is the result of ionic fluxes through both the transcellular and paracellular routes but the contribution of the latter is predominant, especially in leaky epithelia such as intestine. As a consequence the resistance at the tight junction level determines the overall resistance of the epithelium.

The leak pathway is involved in the paracellular permeability to macromolecules of bacterial or food origin and displays a much lower capacity than the pore pathway in physiological conditions [9]. Monitoring the passage of large hydrophilic tracers using radiolabeled or fluorescent molecules such as dextrans reflects the leak pathway, which may vary depending on the size of the tracer used.

It is important to note that distinct molecular mechanisms are involved in the regulation of leak and pore pathways [9]. Thus epithelial paracellular permeability to ions/small solutes (measured by TEER) versus

macromolecules (measured by the passage of large molecules such as fluorescent-dextran) does not necessary varies in parallel. Some experimental conditions can affect both pathways while other conditions may affect only one. Determination of paracellular permeability via methods analyzing pore and leak pathways are thus complementary.

To study human intestinal paracellular permeability the challenge is to find an accurate model of intestinal epithelium close to normal human epithelium. Here we describe methods using the Caco-2/TC7 clone, which represents to date, with its parental cell line Caco-2, the most powerful *in vitro* model and the most used cell line to this purpose. The Caco-2 (Cancer colon-2) cell line was established in 1974 by Jorgen Fogh (Memorial Sloan-Kettering Cancer Center, New York, USA) from a human colon carcinoma taken from a 72-year-old man treated for his pathology with 5-fluorouacil and cyclophosphamide [10]. Among the several cell lines established from intestinal tumors by J. Fogh and others, some of them can be partially differentiated by adding synthetic or biological factors to the medium. However, one of them, the Caco-2 cell line was rather unique since it had the property to differentiate spontaneously upon reaching the confluence. The demonstration of Caco-2 cells differentiation was first published in 1983 [11] by Alain Zweibaum's group (INSERM, Paris, France). They observed under standard culture conditions that Caco-2 cells spontaneously organize into a monolayer of polarized cells after reaching confluence (Figure 2). Post-confluent Caco-2 cells display a well-defined brush border at their apical pole and high levels of intestinal hydrolases activities, which are, for some of them, close to the values observed in human normal small intestine [12,13]. Since this first description of Caco-2 cell differentiation, this cell line has been the subject of numerous studies, which have shown that Caco-2 cells share a large number of properties with enterocytes of the normal human intestine. Thus post-confluent Caco-2 cells express a large number of proteins involved in several functions or metabolic pathways present in normal human enterocytes such as vitamin transport, nutrient absorption, barrier function [14,15]. Caco-2 cells have been widely used as a model of jejunum enterocytes for lipid absorption (952 PubMed hits) but it is interesting to note that these cells express also some markers that are present *in vivo* in the human duodenum such as peptide transporters [16], and in the human ileum such as bile acid transporters [17]. Caco-2 cell monolayer cultured on plastic support is also characterized by the presence of domes, which result from apical to basal fluid transport, leading to uplift of the cell monolayer. Despite these characteristics, which bring them close to normal enterocytes, and due to their colonic and cancerous origin, these cells express also

some markers of fetal colon (such as carcinoembryonic antigen) and display glucose metabolism characteristics of cancerous cells (high lactic acid production and accumulation of glycogen). In the early 1990s, the group of Ismael J Hidalgo (Absorption Systems LLC, Exton, USA) and the group of Per Artusson (Uppsala University, Sweden) identified the Caco-2 cell line as a reliable and suitable cell model to study intestinal barrier function and in particular paracellular permeability [18,19]. To date more than 700 publications reported data acquired with Caco-2 cells to study paracellular permeability.

The Caco-2/TC7 cell line was established in the 1990s following the observation by the Zweibaum group of marked change in the expression of sucrase-isomaltase activity (a brush-border hydrolase) depending on the number of Caco-2 cell culture passages from the first establishment by J. Fogh (cells at late passages expressing more this enzyme than at early ones). They decided to establish several clones from an early passage (passage 29) and a late passage (passage 198) of the parental Caco-2 cell line. By dilution cloning, Monique Rousset (from the Zweibaum lab) isolated 26 cellular clones and compared their differentiation characteristics [20]. One of them, the TC7 clone ("T" for passage "Tardif" (the French translation of "late") and C7 for the position of the well in the 96-wells plate used for cloning) was the one that displayed the higher expression of several differentiation markers (brush border hydrolases, sugar transporters) as compared to the others [20,21]. The Caco-2/TC7 cells displayed some differences with the parental Caco-2 cells, e.g. they do not form domes when cultured on plastic support. Moreover, Caco-2/TC7 cells represent a more homogeneous cell population than the parental cell line, giving better reproducibility of results. An inter-laboratory study showed that Caco-2/TC7 cells exhibit higher alkaline phosphatase activity compared to parental Caco-2 cell line [21]. Caco-2 and Caco-2/TC7 cell lines display TEER values [22-25,21], which approximate those measured *ex vivo* in the human intestine while remaining higher [24,26]. To date, among the existing intestinal cell lines, the Caco-2 cell line and particularly the Caco-2/TC7 clone represent the cell model that exhibit the morphological and functional differentiation closest to normal human enterocytes.

2 Materials

2.1 Cell Culture

1. Caco-2/TC7 cells (*see* **Note 1**).

2. Complete culture medium: high-glucose (4.5g/L) Dulbecco's modified Eagle's medium (DMEM) Glutamax supplemented with 20% heat-inactivated fetal bovine serum (FBS), 1% non essential amino acids, penicillin (100 IU/mL), and streptomycin (100 µg/mL)
3. Serum-free medium: complete culture medium w/o FBS
4. Maintenance conditions: humidified incubator under a 10% CO_2/90% air atmosphere at 37°C (*see* **Note 2**).
5. Semi-permeable filters: 24 mm Transwell® (6-wells plate), 3 µm pore size high-density (*see* **Note 3**).

2.2 Transepithelial electrical resistance (TEER)

1. 20-30 mL sterile 70% ethanol at room temperature.
2. 20-30 mL sterile 1X PBS w/o calcium & magnesium at room temperature.
3. 20-30 mL sterile serum-free medium at room temperature.
4. Volt-Ohm Meter (*see* **Note 4**)

2.3 Paracellular permeability to macromolecules

1. Sterile 96 wells Black with Clear Flat bottom plate
2. Sterile conical tubes (1.5 mL).
3. Sterile 200µL and 1000µL pipette tips
4. 40mg/mL stock solution of FITC-labeled dextran 4 kDa (FD4) in serum-free medium, stored at 4°C (*see* **Note 5**).
5. Microplate fluorometer

3 Methods

All procedures should be carried out at room temperature and at a biosafety cabinet (BSC). All mediums should be at 37˚C before use.

3.1 Cell Culture

1. Add 2.5mL of complete medium in the basal compartment of each filter of a 6-well Transwell® plate (*see* **Note 6**) and seed Caco-2/TC7 cells on semi-permeable filters (apical compartment) at a density of $0.053x10^6$ cells/cm^2 ($0,25x10^6$ cells per filter of a 6-well Transwell® plate) in 1.5 mL of complete medium. Maintain the cells in an incubator with 10% CO_2 at 37˚C.

2. Two days after seeding, change the complete medium in both compartments (2mL in basal compartment and 1mL in apical compartment) and daily thereafter for 6 days.
3. From confluence (7 days after seeding in the conditions mentioned above), cells are switched to asymmetric conditions, i.e., with complete medium (2mL) in the basal compartment and serum-free medium (1mL) in the apical compartment (*see* **Note 7**). Both culture media are renewed daily for 14 days to reach an optimal enterocyte-like cell differentiation (*see* **Note 8**) [22,27,23,28,25].
4. The day before measurement of paracellular permeability**,** renew the culture medium by adding 2.5mL of complete medium in basal compartment and 1.5mL of serum-free medium in apical compartment (*see* **Note 9*)***.

3.2 Transepithelial electrical resistance (TEER)

Transepithelial electrical resistance (TEER) can be used to follow the formation of the cell monolayer (TEER value increases from confluence) and to evaluate paracellular permeability modulations impacting the pore pathway (*see* **Note 10**).

1. 24h prior to the experiment:
    i. Sterilize the electrode by immersing it for 10 minutes in a sterile 70% ethanol solution and leave the electrode overnight immersed in sterile PBS on the biosafety cabinet. Prepare 20-30 mL of sterile serum-free medium kept at room temperature overnight.
    ii. Prepare a plate with 2 filters (with the same characteristics that those used for the experiment) without cells and fill them with 2.5mL of complete medium in the basal compartment and 1.5mL of serum-free medium in the apical compartment (= null filters). Put them into the incubator (10% CO_2 at 37°C) (*see* **Note 11**).
    iii Change the medium of the filters containing Caco-2/TC7 cells (2.5mL complete medium in the basal compartment and 1.5mL of serum-free medium in the apical compartment).
2. If the experiment is scheduled over several days and therefore the culture medium must be renewed daily, always measure the TEER before changing the medium (*see* **Note 12**).
3. 10 minutes prior to the measurements, equilibrate the electrode in the sterile serum-free medium at room temperature.
4. According to the instructions of the material used, perform a test of the Volt-Ohm Meter.

5. Take out the first plate and the plate with 2 null filters from the incubator. Leave these plates at room temperature on the biosafety cabinet for 7 minutes (use the timer) to stabilize the temperature of the culture medium (*see* **Note 13**).
6. Measure the resistance of the null filters, then the filters of the first plate of cells by placing the electrode successively in all the notches of each filter and put the plate back to the incubator.
7. Take out the second plate from the incubator and repeat the steps 5 and 6. The temperature equilibration of the second plate can be done during the measurement of the first plate etc…
8. Calculate the TEER and express the results in ohm.cm^2 as follow:
    i. The resistance value of notches of each filter must be averaged, as well as the resistance values of null filters.
    ii. Subtract the average value of the null filters from the average value of each filter in the experiment and multiply by the membrane area of the filter (4.67 cm^2 for a transwell® of a 6-Well-plate): (average Resistance $_{\text{filter}}$ - average Resistance $_{\text{null filter}}$) * 4.67 (*see* **Note 14**).

3.3 Paracellular permeability to macromolecules

Paracellular permeability across Caco-2/TC7 cell monolayer can be evaluated by measuring the accumulation during 4 h of FITC-4kDa dextran (FD4) in the basal compartment. (*see* **Note 10**)

1. Turn off the biosafety cabinet light as soon as you use the FITC-labeled dextran.
2. Prepare sufficient solution (1.5mL per well) for all filters of FITC-labeled dextran 4 kDa (FD4) solution at 1 mg /mL in serum-free medium.
3. Replace the apical medium by 1 mg /mL FD4 in serum-free medium (1.5mL per filter) (*see* **Note 15**). Do not change the basal medium (*see* **Note 16**)
4. Incubate in 10% CO_2 at 37°C for 2 hours. Be careful not to shake the plate when removing it or putting it back in the incubator.
5. Using a single-channel 200µL pipette, set to 115µL, gently insert the cone into a notch, make a go back and forth to homogenize the basal medium, take 115µL and put into the conical tube (1.5mL) corresponding to the well, with the same pipette tip put on in another notch of the same filter, make an another goes back and forth, retake 115µL and put it into the same conical tube. Be careful not to move the filter when collecting the basal medium samples. In the end, every conical

tube (1.5mL) contains 2x115µL = 230µL. Go to the next filter and repeat the procedure. These samples are the 2-hour measurements. Protect the tubes from light at 4˚C.

6. When the basal medium of all filters is collected, with a 1000µL pipette, add 230µL of complete medium warmed to 37˚C in one notch per filter. Do not go back and forth. Put back into incubator in 10% CO_2 at 37˚C for 2 hours.
7. Repeat the step 5. These samples are the 4-hour measurements (*see* **Note 17**)
8. Prepare the FD4 standard concentrations (protect it from the light): Dilute in complete medium the remaining 1mg/mL FD4 solution prepared for the experiment to obtain the following concentrations: (10 µg/mL; 7.5 µg/mL; 5 µg/mL; 2.5 µg/mL; 1 µg/mL; 0.75 µg/mL; 0.5 µg/mL; 0.25 µg/mL; 0.1 µg/mL; 0.5 µg/mL; 0.01 µg/mL). The standard concentration of 10µg/mL corresponds to 1% of the initial concentration of FD4 in the apical compartment.
9. In sterile assay plate, 96 well black with clear flat bottom:
   i. Add 120µL from the standard range (from 10 to 0.01µg/mL) in duplicate
   ii. Add 120µL from the complete medium alone, as blank to substract the basal medium background fluorescence emission.
   iii. Add 120µL from each sample collected at time 2h and 4h.
10. Determine the fluorescence values with a microplate fluorometer. Read the fluorescence at the wavelength 485 nm excitation and 535 nm emission. Set the gain on the well corresponding to the 5µg/mL standard range concentration (*see* **Note 18**).
11. Use the regression line to determine at each time (2h and 4h) the FD4 concentration in the basal compartment. Results can be expressed as the percentage of FD4 initially added in the apical compartment (*see* **Note 19*)***.

4 Notes

1. Caco-2/TC7 cell line is available under request to Véronique Carrière or Sophie Thenet who are in charge of this cell line since the retirement of Monique Rousset. All uses of Caco-2/TC7 by a non-academic entity require a license from INSERM-transfert.

2. DMEM used for Caco-2/TC7 cells containing 3.7g/L sodium bicarbonate, the cells are cultured in a 10% $C0_2$ atmosphere. However Caco-2/TC7 cells can also grow and differentiate in a 5% $C0_2$

incubator but it is worth noting that such condition impacts metabolic activities of the cells in particular glucose metabolism (personal observations).

3. Various microplate sizes (6, 12 and 24-wells) and pore sizes membranes (i.e., 3μm, 3μm high-density, 1μm, 0.4μm) can be used. The nature of filter membrane can affect the absolute values of measured paracellular permeability [29,26].
4. There are different TEER Measurement Methods, the Ohm's Law Method and the Impedance Spectroscopy. The widely used and commercially available TEER measurement system is based in the Ohm's Law Method, known as an Epithelial Volt Ohm Meter (EVOM). The TEER readings with EVOM can be realized in manual and/or automatic system.
5. The mechanisms that regulate paracellular permeability through the different pathways are complex and it may be important to explore the variations of paracellular permeability to molecules of different sizes such 10kDa-, 40kDa-, 70kDa- dextran or sulfonic acid (0.4kDa) [30-32]. Although the size limit of molecules that can cross an epithelium through the leak pathway is still debated, larger molecules such as 40kDa-dextran and over are considered by some authors to be excluded from the paracellular pathways and are commonly used to evaluate transcellular pathway. It is likely that a mixture of paracellular and transcellular pathways is evaluated using large tracers, depending on the experimental conditions (see chapter "Measure of paracellular permeability in mouse intestine using Ussing chambers" by Aguanno et al. in this book).
6. Cell seeding is more homogenous when the plates are filled with the complete medium in the basal compartment and placed in the CO_2 incubator 10 min before plating cells.
7. Asymmetric culture conditions help to reproduce the physiological environment of intestinal epithelial cells. In particular, serum-free culture medium in the apical compartment mimics the intestinal lumen, which is devoid of serum.
8. From confluence, Caco-2/TC7 cells start to differentiate and polarize. They form a cell monolayer with an efficient selective barrier. The presence of phenol red in the culture medium helps to visually estimate the integrity of the monolayer. Indeed the secretion of lactic acid at the basal pole of polarized cells decreases the pH of the basal medium and turns it orange while the apical medium remains red.

9. Increasing the volume of culture medium ensures a good immersion of the electrodes for TEER measurement. If only paracellular permeability to macromolecules is assayed, the volume of culture medium can be kept at 2mL (basal) and 1 mL (apical).
10. EGTA can be used as a positive control of increased paracellular permeability affecting both pore and leak pathways [23,25]. EGTA is a calcium chelator known to provoke the disruption of junctional protein complexes and to prevent the return of junction proteins to the membrane, resulting in the opening of the intercellular junctions [33]. Addition of cytokines in the basal compartment can be used as a positive control for increased paracellular permeability to macromolecules (leak pathway) and, hence, FD4 passage [22,34].
11. The null filters (without cells) are used to measure the intrinsic resistance of the filter.
12. It is important to note that TEER measurements are sensitive to changes in the composition of the culture medium. The renewal of the medium before the measurement of TEER can affect the results.
13. TEER measurements have been reported to be temperature dependent. The temperature should be equilibrated to room temperature before performing resistance measurements to avoid any temperature fluctuation-induced TEER changes. However, the time out of incubator could be detrimental to cell physiology and function, thus 7 minutes waiting time is the time which seems a good compromise for stabilization of the TEER values without removing the cells from the incubator for too long [26].
14. Untreated Caco-2/TC7 cells cultured for 21 days on 3µm high-density transwell® of 6-well plate display a TEER value between 600-800 $\Omega.cm^2$ [22,23,25]
15. Since fluorescence variations are very sensitive, it is better to use the same preparation of 1mg/mL FD4 solution for all wells. When different treatments are added in the apical compartments, split the FD4 solution in separate tubes and add the molecules/drugs at the appropriate final concentrations before changing the apical medium of the cells.
16. Serum in the complete medium brings growth factors that can trigger unwanted transient modification of paracellular permeability. The non-renewal of the basal medium on the day of the experiment increases the strength and the reproducibility of the results. If an experimental treatment must be supplied in the basal compartment the day of the experiment, it is better to add concentrate solution of the drug tested without renewing the basal medium.

17. Evaluation of FD4 accumulation at two different time points (2h and 4h) helps to control the integrity of cell monolayer throughout the experiment. A 1.5 fold increase in FD4 accumulation in untreated Caco-2/TC7 cells is generally observed between 2h and 4h.
18. Usually the standard concentration 5μg/mL is used for setting the gain, since FD4 passage rarely exceeds 0.5% of the initial amount. In this case do not use the 10 μg/L and 7.5μg/mL standard range concentrations to make the regression line. Otherwise a gain can be calculated taking into account all the wells.
19. For samples collected at time-point 2h, the quantity (μg) of FD4 present in the basal compartment is calculated as follow: (FD4 concentration x 2.5mL). For samples collected at time-point 4h, take into account the μg of FD4 removed in the 0.23 mL collected at time 2h. The final quantity (μg) in samples at time 4h is calculated for each transwell® as follow: (FD4 concentration in samples at 4h x 2.5mL) + (FD4 concentration of the same filter at 2h x 0.23 mL). To express the results as percentage of initial amount of FD4 placed in apical compartment, use the following formulation: (FD4 quantity in μg calculated above x 100)/1500. A typical value of FD4 passage in the basal compartment obtained at time 4h in untreated Caco-2/TC7 cells cultured for 21 days on 3μmHD transwell® is 0.03 %.

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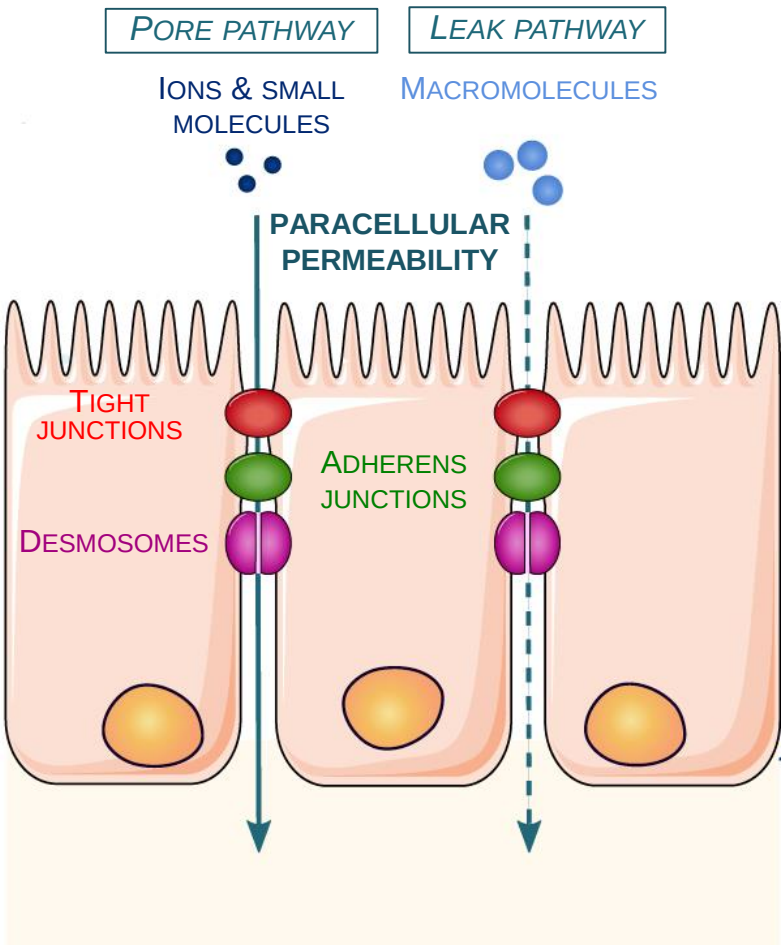
Figures

Figure 1: Paracellular permeability is controlled by tight junctions and includes two distinct routes (the pore and the leak pathways), which implicate different tight junction proteins. As adherens junctions and desmosomes indirectly regulate tight junctions, their impairment may impact paracellular permeability.

Figure 2: Post confluent Caco-2/TC7 cells form a regular monolayer of well-polarized cells with the nucleus in basal position and mature intercellular junctions as shown by immunofluorescence analysis of

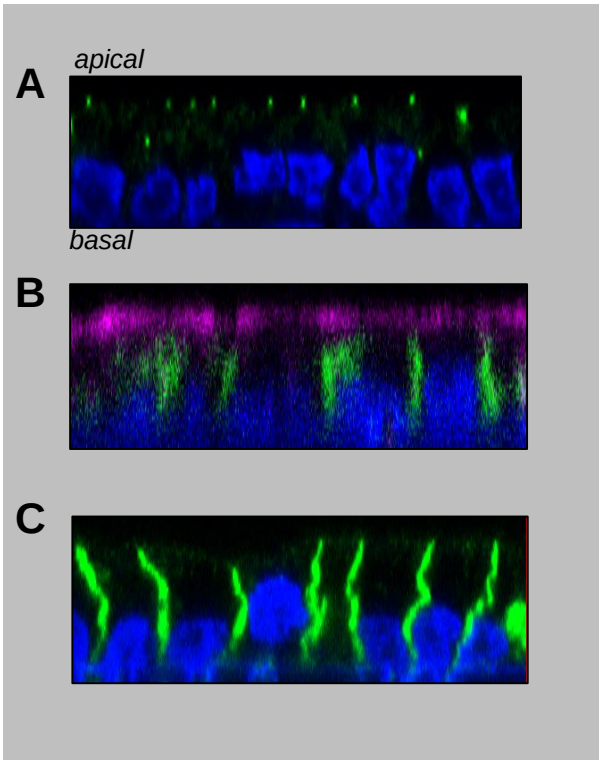
occludin, a tight junction marker (in green, panel A), E-cadherin, a marker of adherens junctions, (in green, panel B) and plakoglobin, a desmosomal protein (in green, panel C). Moreover cells display a well-formed brush border as shown by actin staining (in magenta, panel B). Nuclei were stained with DAPI (in blue, all panels) (Figure adapted from [24].

Figure 1



Postal et al. Figure 1

Figure 2



Postal et al Figure 2