Methods to examine tight junction physiology in cancer stem cells: TEER, paracellular permeability, and dilution potential measurements

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i. Summary/Abstract

Our understanding of the essential role played by cancer stem cells or tumor-initiating cells in epithelial cell-derived tumor types is rapidly advancing. Nevertheless, the identification and characterization of these cells pose a considerable challenge. Among changes in the epithelium in oncogenesis are changes in the permeability barrier, a phenotypic trait based on tight junction formation and function. Tight junctions regulate the movement of solutes, ions and water across the paracellular space. On a cellular level, they maintain cell polarity by limiting the lateral diffusion of membrane components. Depending on the type of epithelial tissue, the barrier characteristics with respect to electrical resistance, size and ion charge selectivity vary quite significantly. Thus, elucidating changes in expression of Claudins, an essential component of tight junctions, has become a very active area of investigation in oncogenesis. This chapter provides detailed protocols on how to quantify three aspects of tight junction physiology using in vitro cell culture systems that are particularly applicable to analysis and comparison of cancer stem cells and their normal counterparts.

ii. Key words: Cancer Stem Cells, Tight junction; cell polarity; TEER (transepithelial electrical resistance); dilution potential; paracellular transport; [^3H]- mannitol; transwell filters.

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1. Introduction

Changes in cell polarity and the permeability barrier are two of the hallmarks of alterations that occur in epithelial tissues and disease processes, including those involved in oncogenesis [1-4]. One important issue is how such changes may relate to the sub-populations of cells identified within an increasing number of solid tumors and called cancer stem cells (CSC) or tumor-initiating cells (TIC), based on their ability to differentiate, self-renew, and replicate the phenotypic diversity of the original tumor, i.e., processes responsible for the recurrence of a tumour following therapeutic treatments [5-7]. To date, CSCs have been distinguished from the bulk-tumor population by their pattern of expression of cell-surface proteins and their ability to efflux Hoechst dye or aldehyde dehydrogenase [7,8]. Amongst cell surface proteins recently noted to be aberrantly expressed in CSCs are the Claudins, a family of proteins involved in tight junction (TJ) formation [9,10]. For example, low or no claudin expression has been reported in breast cancer-derived CSCs [11-13]. Although mechanisms underlying changes in Claudin expression during normal epithelial development or oncogenesis are only beginning to be elucidated, the low or no Claudin characteristic is interesting in the context of alterations in cell polarity and in epithelium permeability that occur during tumor initiation, progression and metastasis.

Permeability barrier function is established by TJs and the TJ barrier consists of continuous rows of transmembrane proteins from adjacent cells that connect in the intercellular space. The physiological behaviour of the TJ barrier suggests that the rows and strands that fill the space between cells, on the one hand, form relatively large aqueous pores which are less discriminating of size and charge than transmembrane channels, but on the other hand, do not support completely indiscriminate passage of solutes. Two types of TJs can be found in epithelia, named “leaky” and “tight” (14,-16). Whereas the former is found in the lining epithelium of tissues such as the intestine where large volumes of iso-osmotic fluids need to be moved across the barrier, the latter predominates in tissues that require high electro-osmotic gradients such as renal distal tubules and collecting ducts [17]. With the discovery of the Claudin family, it has become evident that TJ-based barriers are formed and regulated by Claudins, a family of tetraspan transmembrane molecules comprising 27 members [18-22]. Their expression profile during development and in the adult reflects the requirements of the particular epithelial barrier analyzed, and changes in their expression and distribution has been observed in various disease states, reflecting the functional modulations occurring in the epithelial tissue [19,20, 22]. Thus, assessing changes in Claudin expression has become a

valuable tool to characterize and predict changes in barrier function within specific cell populations, including CSCs, in many disease states.

In this protocol study, we present straightforward methods to quantify three different aspects of TJ physiology, trans-epithelial resistance, size and ion selectivity across a monolayer of epithelial cells grown on tissue culture filter\membrane inserts. It is important to point out that assessment of TJ physiology described in this protocol must be evaluated in cells that are not adapted to tissue culture conditions so as to avoid giving a misleading picture regarding barrier function. Alternatively, their use in cultured cells must be used advisedly under conditions mimicking the in vivo condition, an approach that may be invaluable in characterizing subpopulation of cells that acquire stem cell-like characteristics.

2. Materials

2.1 Cell Culture

1. Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) supplemented with 10% fetal calf serum (Invitrogen). Doxycycline-free fetal calf serum (Clontech) should be used when cells express inducible gene regulation systems.
2. Solution of trypsin (0.25%) and ethylenediamine tetraacetic acid (EDTA) (1 mM) from Invitrogen
3. 12 well tissue culture plates (Becton-Dickinson)
4. Polycarbonate Transwell inserts with 0.4 μm pore size for 12 well multiwell tissue culture plates (Cat.# 3401, Corning, Lowell, MA)(*see notes 1 and 2*).
5. Millicell-ERS (Electrical Resistance System) from MILLIPORE. This device reliably measures membrane potential and resistance of epithelial cells in culture. A silver/silver chloride (Ag/AgCl) pellet on each electrode tip measures voltage. Because of the small size of the electrodes, it is optimal for the measurement of transepithelial voltage and resistance of cells grown on microporous membranes.

2.2 Buffers

1. Buffer A: 120 mM NaCl, 10 mM HEPES, pH 7.4, 5 mM KCl, 10 mM NaHCO_3 , 1.2 mM CaCl_2 , and 1 mM MgSO_4)
2. Buffer B: 60 mM NaCl, 120 mM mannitol, 10 mM HEPES, pH 7.4, 5 mM KCl, 10 mM NaHCO_3 , 1.2 mM CaCl_2 , and 1 mM MgSO_4
3. P-Buffer (1x): 10 mM HEPES, pH 7.4, 3 mM CaCl_2 , 145 mM NaCl, 1 mM Sodium Pyruvate, 10 mM D-Glucose.

3. Methods

3.1. Measuring Transepithelial Resistance

The following instructions are based on the use of a Millicell-ERS (MILLIPORE) to measure the transepithelial electrical resistance (TEER) across a monolayer of cells grown on Transwell polycarbonate membrane inserts. At the beginning of a new series of measurements the meter needs to be tested to ensure proper operation. This involves three procedures that will test the meter and electrodes and equilibrate the electrodes. We refer the reader to the manufacturer's instructions on how to perform these procedures.

1. TEER measurements are made in a sterile environment (e.g. laminar flow hood).
2. Set up a laminar flow hood with the meter, 70% ethanol solution, a screwdriver (normally provided by the meter's manufacturer) and your tissue culture plate containing the cells grown to confluence on a Transwell filter insert (*see notes 1 to 6 on how to best use Transwell filters*).
3. TEER measurements are performed at room temperature, therefore it is important to take the cells out of the 37°C incubator and leave them in the laminar flow hood for at least 30 minutes before measurements are being made.
4. While cells adjust to room temperature, sterilize the electrodes by immersing them in 70% ethanol for 15 minutes, and then allow them to air dry for 15 seconds (*See notes 7 to 9 for more details on electrodes manipulation*).
5. Rinse the electrodes in sterile tissue culture medium. They are now ready for resistance measurements.
6. On the meter, switch the MODE switch to "R" and the POWER switch to "ON" (*see also note 10*).
7. Immerse the electrodes so that the shorter electrode is in the Transwell filter insert and the longer electrode is inserted between the outer wall of the

multiwell tissue culture well and the filter insert. Make sure that the electrode tips are completely covered in solution and the shorter electrode does not touch the cells growing on the filter insert.

8. To record the resistance, press and hold the MEASURE button (not the “R” button). The reading should be stable.
9. Measure the blank resistance by immersing the electrodes into a well containing the same tissue culture medium and an identical filter insert but without cells growing on it.
10. Subtract the blank value from the value obtained when measured across cell layer.
11. Convert the blank corrected value to the Unit Area of Resistance by multiplying it by the effective surface area of the Transwell filter insert (in cm^2). Resistance of a unit area = Resistance (Ω) x Effective Membrane Area (cm^2)

3.2. Paracellular Flux assay

1. Sterilize P-Buffer by autoclaving, and store at room temperature.
2. Resuspend Texas Red-Dextran (10,000 MW) powder in P-Buffer at 2 mg/mL. If the Dextran conjugate is delivered as an aqueous solution, it needs to be dialyzed against P-Buffer over night at 4°C and then diluted to a final concentration of 2 mg/mL in P-Buffer. Store solutions of Dextran conjugates protected from light at 4°C for several weeks, with the addition of sodium azide to a final concentration of 2 mM (*see notes 11 and 12 for the preparation of Dextran solutions*).
3. Cells are grown to confluence on Transwell filter inserts (*Note 1*).
4. Start the assay by replacing the medium in the basolateral chamber with 1.8 mL pre-warmed (37°C) P-Buffer and the medium inside the filter insert with 0.5 mL P-Buffer containing 2 mg/mL Texas Red-Dextran.

5. Incubate the cell monolayers at 37°C, 5% CO₂ for 3 hours.
6. Collect the medium from the basolateral chamber and measure Texas Red-Dextran with a fluorometer with excitation and emission wavelengths set to 595 nm and 620 nm, respectively.
7. All experiments are performed in triplicates and the results expressed as average ± s.e.m.
8. Calculate the paracellular flux as “% passage of Dextran dye” whereby the value obtained for the passage across a Transwell filter insert without cell monolayer serves as the 100% passage control.

$$\% \text{ passage} = (F(i)[\text{Monolayer}]) / (F(i)[\text{Transwell}] - F(i)[\text{P}^{\text{''}}\text{-Buffer}]) \times 100$$

3.3. [³H]-mannitol permeability assay

1. Grow cells to confluence on Transwell filter inserts (*Note 1*).
2. Prepare 1 M D-mannitol (Cat # M9546, Sigma Aldrich) stock solution by resuspending 18.22 g in 100 ml distilled water and sterilize by autoclaving. Keep refrigerated.
3. Prepare Dulbecco's Modified Eagle's Medium (DMEM) (Invitrogen) containing 10% fetal calf serum (Invitrogen) and add D-mannitol stock solution to a final concentration of 5 mM.
4. Prepare enough DMEM, 10% FCS, 5 mM D-mannitol medium to be able to add 1.8 mL and 0.5 mL to basal and apical compartment, respectively, of all wells being assayed.
5. Add 1.8 mL and 0.5 mL to the basal and apical chambers, respectively and incubate for 30 minutes at 37°C, 5% CO₂.
6. Then replace medium in each top chamber with medium containing 0.25 µCi [³H] - Mannitol).
7. Incubate for 1 hour at 37°C, 5% CO₂.

8. Dispose of medium in apical (top) chamber and filter inserts in radioactive waste.
9. Mix medium in basal (bottom) chamber well and transfer a 100 μ L aliquot into scintillation tubes containing 7 mL liquid scintillation cocktail (ACS solution, Amersham/GE Healthcare).
10. Measure radioactivity (in dpm) recovered in bottom chamber in scintillation counter (Packard). Refer to manufacturer's instruction on correct settings for liquid scintillation counts of ^3H -isotopes.
11. All experiments are performed in triplicates and the results expressed as average $\pm$ s.e.m.
12. Permeability to [^3H]-Mannitol are represented as either “% passage / hour” or as apparent permeability “ P_{app} ”.

$$\% \text{ passage} = \frac{\text{radioactivity in bottom chamber} \times 60 \times 100}{\text{initial radioactivity in top chamber} \times \chi \text{ min}}$$

χ is the time of passage in minutes.

$$P_{app} = \frac{V}{[A] \times [Co]} \times \frac{\delta C}{\delta t}$$

$\delta C / \delta t$ is the flux across the cellular monolayer in $\mu\text{M}/\text{sec}$ or $\text{dpm mL}^{-1}/\text{sec}$.

V is the volume of recipient chamber.

$[A]$ is the total surface of Transwell filter membrane in cm^2 .

$[Co]$ is the initial concentration of [^3H]-mannitol in the apical chamber in μM or dpm mL^{-1}

3.4. Measuring transmonolayer dilution potentials

1. Grow cells to confluence on Transwell filter inserts.

2. Prepare enough buffer A to be able to add 1.8 mL and 0.5 mL to basal and apical compartment, respectively, of all wells being assayed. Store at room temperature.
3. Prepare enough buffer B to be able to add 0.5 mL to the apical chamber of each assayed well. Store at room temperature.
4. Prepare the Millicell-ERS meter for voltage measurements. Connect the electrodes to the instrument (power off) and soak them for at least 24 hours in electrolyte solution (100-150 mM KCl) if the electrodes have never been tested for voltage drift or have been stored dry. If electrodes had been stored in solution, a 2 hour equilibration step is sufficient. After the equilibration period is terminated, turn the MODE switch to “V” (voltage) and turn POWER on. Check the voltage reading. If it is between 5 or 10 mV, wait another 15 minutes and then adjust the “Zero V” screw until the meter reads 0.0 mV. The instrument is now ready to use. If the voltage reading is above + 15 mV, the electrodes may be dirty or contaminated. (*See also note 13 on how to maintain the electrodes*).
5. Take cells out of the 37°C incubator and store inside the laminar flow cabinet for 30 minutes to bring cells and medium to room temperature.
6. Replace the medium in the apical and basal chambers with 0.5 mL and 1.8 mL buffer A, respectively.
7. Measure dilution potential across blank filter in buffer A. This value will be subtracted from subsequent measurements across cell monolayers grown on filter membrane inserts.
8. Insert the electrodes so that the shorter electrode is inside the apical chamber (avoid touching the cells) and the longer electrode is in the outer wall.
9. Set the MODE switch to “V” and turn POWER switch on. The instrument should give a stable voltage reading in millivolts.

10. Record the voltage. This is the $P_{(A\text{-buffer})}$ value in mV. If the readout is positive, this means that the basal side of the cell monolayer is positive with respect to its apical side and when negative, its basal side is negative.
11. Replace the buffer A in the apical chamber with 0.5 mL of buffer B and immediately re-immerses electrodes.
12. Once the dilution potentials become stable (usually within seconds), measure for at least another 30 seconds and record the voltage. This is the $P_{(B\text{-buffer})}$ value in mV.
13. All experiments are performed in triplicates and the results expressed as average $\pm$ s.e.m
14. Dilution potentials are then calculated using the following formula:

$$\Delta P = (P_{[A\text{-buffer}]} - P_{[blank]}) - P_{[B\text{-buffer}]}$$

4. Notes

1. Transwell inserts are used by first adding 1.8 mL medium to the multiple 12 well plate well, then adding the Transwell insert, and then adding the cells in 0.5 mL cell culture medium to the inside compartment.
2. Cell morphology and cell densities on permeable membrane supports are influenced by pore size. Larger pore sizes may allow certain cell types to migrate through the pores.
3. Check medium level daily and top up with fresh medium if necessary. Make sure amount of medium is identical in all samples
4. Determine optimal seeding density for each cell type. Cells grown on permeable support are often sensitive to initial seeding densities for good cell attachment. Typical seeding densities range from $10^3 - 10^5$ cells/cm².

5. Optional: Preincubate polycarbonate membrane Transwell inserts in cell culture medium for overnight at 37°C before seeding to improve cell attachment and spreading.
6. Cells that require extracellular matrix coatings on plastic surfaces may also require them when grown on permeable membrane supports.
7. Avoid spills of saline solutions or culture media on the Millicell-ERS meter to prevent corrosion.
8. When sterilizing, do not leave the electrodes for more than 30 minutes in the alcohol. Continuous soaking of the electrodes in alcohol will weaken its protective coating and shorten its lifetime.
9. The electrodes can be left in the laminar hood to remain sterile. However, they should be shielded from strong visible or UV light to avoid formation of an oxide film on the electrode's surface.
10. Never press the "R" button on the powered meter since this could lead to damaging the system.
11. Dextran conjugates are generally soluble in aqueous buffers to at least 10 mg/mL. Solubility may be increased by briefly heating (40-50°C), sonicating or vortexing. Insoluble particles should be removed by centrifugation at 12,000 x *g* for 5 minutes.
12. Dextran solutions can be sterilized by filtration through a 0.2 µm pore diameter filter, if the concentration of the solution is between 1-10 mg/mL. For long-term storage, aqueous dextran solutions should be kept at -20°C. Avoid repeated freeze-thawing cycles.
13. If electrodes are suspected to be dirty or contaminated, lightly rub the surface of the electrodes with a cotton swab soaked in 70% ethanol. Alternatively, or if inconsistent measurements persist, soak the electrodes (tip only!) for 3 minutes in household bleach and rinse immediately with plenty of water after soaking. A final means to clean the electrode tips when measuring voltage is to lightly sand the silver pellet on the inner

surface near the electrode tips with 600-grade ultrafine sandpaper. Caution is required here to only remove a very thin layer of the silver pellet. Repeated sanding will eventually result in complete removal of the silver pellet. Alternatively, an ink remover can be used to clean the electrodes. When all these electrode maintenance options don't result in improving the voltage readings, the electrodes need to be replaced.

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