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Elucidation of the impact of cell culture conditions of Caco-2 cell monolayer on barrier integrity and intestinal permeability

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1. Introduction

Since many decades, the prediction of oral drug absorption in humans is performed through the use of the Caco-2 model [1]. The human colon carcinoma cell line Caco-2, when seeded on permeable supports is able to form a monolayer of polarized epithelial cells, presenting the phenotype of the small intestine. This model provides a good correlation with the fraction of drug absorbed by the human intestine [2]. Although technical protocols have already been supplied [3, 4], many lab-to-lab variability in Caco-2 permeability assays have been highlighted. Lee *et al.* have shown quantitative difference in Caco-2 permeability results from several laboratories and explained how these results can influence the prediction of intestinal absorption of drugs [5]. As reported also by Sambuy *et al.*, cell culture conditions can influence Caco-2 cells behaviours and, ultimately, permeability results [1]. Therefore, the cell type, the culture medium as well as the cell seeding density have an impact on the morphology, the integrity and the transport through the monolayer. Behrens *et al.* demonstrated that the time in culture, the membrane support and the seeding density strongly influence the transepithelial electrical resistance (TEER) and consequently, the permeability of a paracellular marker drug [6]. However, the impact of membrane pore size and density of pores on Caco-2 monolayer integrity and permeability has never been performed. In this study, it was investigated the influence of different pore size and pore density while the same permeable support of polycarbonate membrane was used. Transwell™ supports with pore size of 1 µm or 3 µm in normal and high density were employed. Moreover, it is known that the type of Caco-2 cell lines has different properties and therefore, can modify the permeability of drugs [7]. Multiple clones have been isolated from Caco-2 regular cells to obtain a more homogeneous population of cells in terms of brush border structure, transport or biotransformation activities. Thereby, the difference between regular Caco-2 cells and the clone type C2BBe1 was also considered being both used in this study. This clone developed by Peterson *et al.* has the property to form a homogeneous polarized monolayer with an apical brush border morphologically comparable to that of the

human colon [8]. The objective of this study is to characterize the integrity and the permeability of Caco-2 monolayers in function of different cell culture conditions as the membrane pore sizes, the density of pores and the Caco-2 cell lines.

2. Materials and methods

2.1 **Materials**

Human colon carcinoma Caco-2 and Caco-2 clone type C2BBe1 cell lines were obtained from the American Type Culture Collection (ATCC, USA). Dulbecco's Modified Eagle Medium (DMEM), Fetal Bovine Serum (FBS), non-essential amino acids, penicillin and streptomycin, trypsin–EDTA and Hank's Balanced Salt Solution (HBSS) were purchased from Invitrogen Corporation (Life Technologies, S.A., Madrid, Spain). Triton X-100, DAPI (40,6-diamidino-2-phenylindole) and 4 kDa fluorescein isothiocyanate-dextran molecule were purchased from Sigma-Aldrich (St. Louis, MO, USA). Paraformaldehyde (PFA) was purchased from Merck Millipore (Billerica, MA, USA). Alexa Fluor® 546 Phalloidin and Goat anti-rabbit Alexa-Fluor 488® secondary antibodies were purchased from Molecular Probes® (Life Technologies S.A., Madrid, Spain). Fluorescence mounting medium was provided by Dako (Peterborough, UK).

2.2 **Cell culture**

Caco-2 regular (passage 28–40) and Caco-2 clone type C2BBe1 (passage 57-68) cells have grown separately in tissue culture flasks (Orange Scientific, Belgium) in a complete medium, consisting of DMEM supplemented with 10% (v/v) inactivated FBS, 1% (v/v) l-glutamine, 1% (v/v) non-essential amino acids and 1% (v/v) antibiotic–antimitotic mixture (final concentration of 100 U/ml Penicillin and 100 U/ml Streptomycin). Cells were maintained in an incubator (CellCulture® CO2 Incubator, ESCO GB Ltd., UK) at 37 °C temperature and 5% CO_2 in a water saturated atmosphere.

2.3 **In vitro cell model**

Monocultures of Caco-2 cells have grown in 12-well Transwell™ (polycarbonate membrane) plates with different pore size and pore density. Transwell™ with pore size diameter of 3 µm and normal pore density (8 x 10^5 pores/cm^2, area 0.9 cm^2) and Transwell™ with same pore size but a higher density (2 x 10^6 pores/cm^2, area 0.9 cm^2) were used (Corning, Madrid, Spain). Moreover, Transwell™ with pore size diameter of 1 µm (area 1.1 cm^2) were also used (Merck Millipore, Overijse, Belgium). Caco-2 cells were seeded on the apical chamber of Transwell™ inserts, to a final density of 1 × 10^5 cells/cm^2 in each insert. Cells were allowed to grow for 21 days with medium changes every two days based on our previous experiments [9, 10]. The TEER values of monolayers were measured every time that medium was refreshed to monitor the evolution of confluence, using an EVOM epithelial voltohmmeter equipped with chopstick electrodes (World Precision Instruments, Sarasota, FL, USA).

2.4 **Confocal laser scanning microscopy**

The confluence of the monolayers was analysed with confocal microscopy after 21 days of growth. Cells on Transwell™ were fixed with 2% (w/v) PFA for 30 min and permeabilized by incubating for 7 min with 0.2% (v/v) Triton X-100 in PBS. The blocking was done with PBST (PBS (1×) containing 0.05% (v/v) Tween-20) with 10% (v/v) FBS for 30 min. Actin was then labelled with Alexa Fluor® 488 Phalloidin (1:50) for 2 hours in the dark and then Transwell™ were washed twice with PBS. Cell nucleus were counterstained with DAPI (1:1000). The membrane was washed three times with PBS, cut and mounted on a glass slide with fluorescence mounting medium.

2.5 **Transmission electron microscopy**

Monolayers were analysed by transmission electron microscopy (TEM) after 21 days of growth. Transwell™ membranes were washed and cells were fixed with 2.5% glutaraldehyde for 20 min

at room temperature. Then, membranes were washed twice with sodium cacodylate buffer (NaCac) for 3 min. Afterwards, the cells were post-fixed with 1% osmium tetroxide in 0.1 M NaCac buffer (pH 7.4) and then dehydrated and embedded in epoxy resin. Ultrathin sections (60 nm) were cut perpendicular to the insert, post-stained with uranyl acetate and examined with TEM using an acceleration voltage of 120 kV.

2.6 **Dextran-FITC permeability studies**

Permeability studies were performed using dextran-FITC as a marker of paracellular pathway [11]. For that, DMEM was removed from both chambers and the Transwell™ membrane was washed twice with pre-warmed HBSS, then replaced by new HBSS and allowed to equilibrate for 30 min at 37 °C. This experiment was performed at 37 °C during 2 h with 0.5 mL of 200 µg/mL dextran-FITC prepared in HBSS in the apical chamber and 1.5 mL of HBSS in the basolateral chamber. Aliquots of 200 µL were taken from the basolateral chamber after 5, 10, 15, 30, 60, 120 and 180 min and then replaced with fresh HBSS. The cell monolayer integrity was monitored by TEER measurement each time point and dextran-FITC content was quantified by fluorescence spectrophotometry. The permeability results are expressed in percentage of permeability.

2.7 **Statistical analysis**

All the results are represented as mean ± standard deviation (SD). One-way ANOVA, together with Tukey's post-test, was used to compare different groups of data using GraphPad Prism® software (Prism 6 for Windows, CA, USA). $p<0.05$ is denoted as (*), $p<0.01$ as (**) and $p<0.001$ as (***).

3. Results

3.1 Monolayer integrity in function of Caco-2 cell types and membrane supports

In order to study the impact of the Caco-2 cell line and the different characteristics of the membrane support on monolayer integrity, Caco-2 regular and the Caco-2 clone cells were seeded at the same initial density. The integrity of tight junction dynamics in epithelial monolayers was followed through the measurement of the TEER during the growth until 21 days [12]. Figure 1.A showed that in function of time, the evolution of the TEER between culture conditions was strictly different. Regardless the cell line, the TEER values obtained with Transwell^{TM} of 1 µm increased fast and reached a plateau after 12 days. However, when the Caco-2 clone cell line was seeded on Transwell^{TM} of 3 µm with high pore density (HD), the TEER values were stabilized sooner, thus the final TEER value was lower. The same behaviour was observed for the two cell lines on Transwell^{TM} of 3 µm with normal pore size density (N). Surprisingly, when the Caco-2 regular cell line was seeded on Transwell^{TM} of 3 µm N, the TEER value after 12 days was not stabilized but decreased from 1500 Ω.cm² to 500 Ω.cm², traducing a decrease of cohesion between cells.

Then, it was compared in detail the final TEER values after 21 days in culture (Figure 1.B). Significant differences were found between Caco-2 regular and Caco-2 clone cells on Transwell^{TM} inserts of 1 µm with TEER values around 1500 and 2200 Ω.cm², respectively. Moreover, the final TEER values were compared in function of the membrane supports used (Figure 1.C). Regarding the two cell lines, high difference between pore size of 1 µm and 3 µm were highlighted.

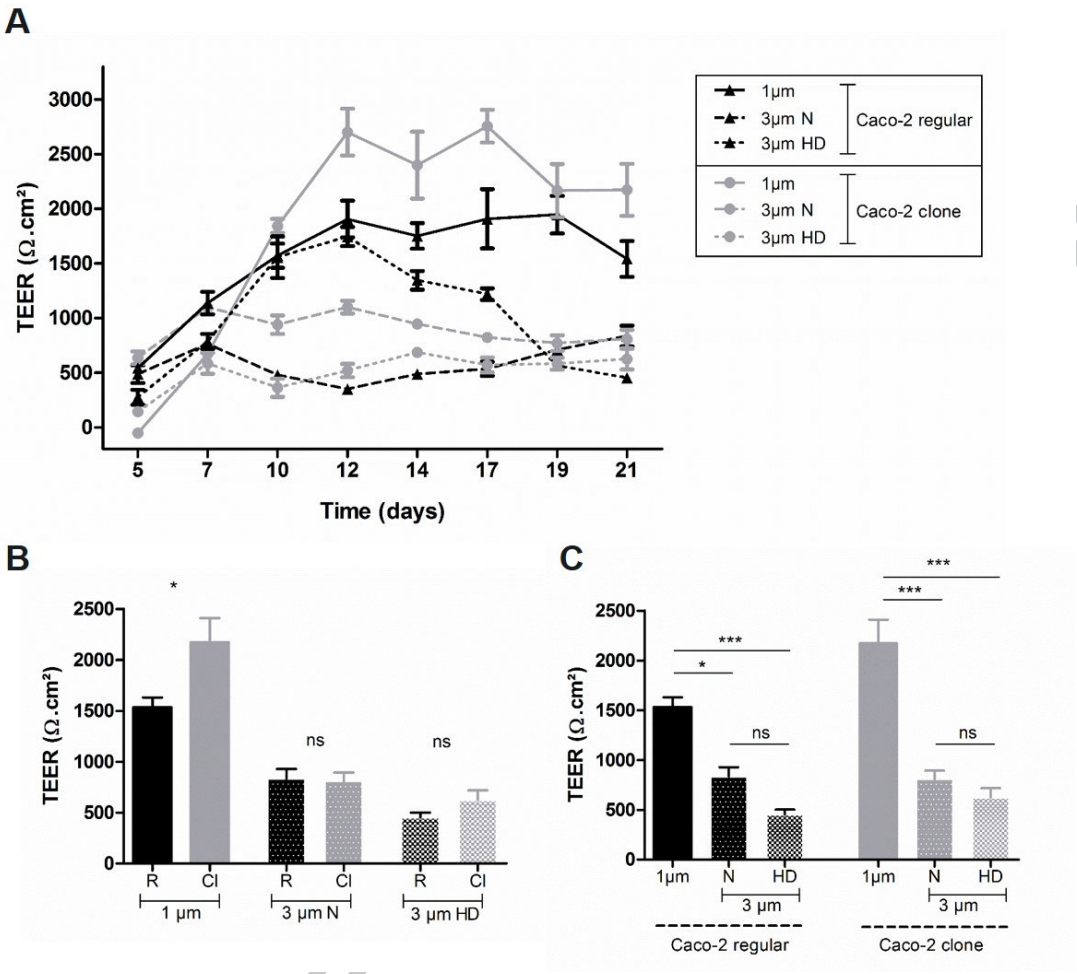

Figure. 1. TEER values ($\Omega.cm^2$) in function of cell culture conditions. (A) TEER monitored in function of time during the 21 days of Caco-2 regular and Caco-2 clone cell lines on different Transwell™ membranes. (B) Comparison of the TEER between the Caco-2 regular (R) and Caco-2 clone (Cl) cells after 21 days of culture on the same Transwell™ membrane. (C) Comparison of the TEER between the three different Transwell™ membranes after 21 days of culture for the two different cell lines (n=6).

Except for Caco-2 regular cells seeded on Transwell™ of 3 µm N, all monolayers were confluent since TEER measurements were stable. Moreover, the strength of tight-junctions between cells seeded on membrane support with pore size of 1 µm seems to be higher to those seeded on 3

µm since the TEER values were higher. The choice of the membrane support pore size influences the robustness of Caco-2 monolayer and could therefore, impact the permeability of drugs which cross the barrier by paracellular pathway.

Subsequently, staining of each type of membrane was performed after 21 days in culture to analyse the morphology of the monolayer (Figure 2).

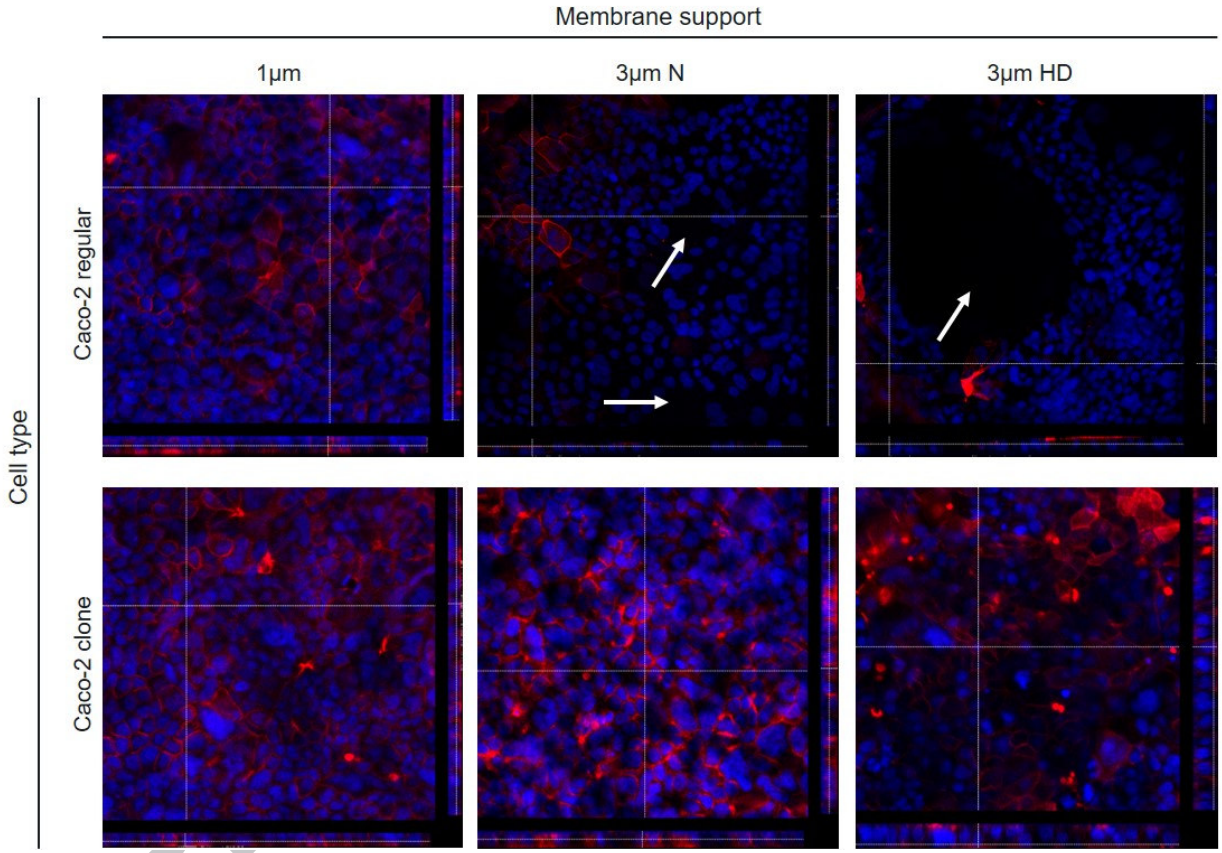

Figure. 2. Immunofluorescence showing the morphology of the monolayer in function of cell type and membrane support after 21 days in culture. FITC-phalloidin staining for actin is represented in red and nucleus were counter-stained with DAPI in blue. The blank arrows show gap into the monolayer. Images were obtained at a higher magnification (x63 in immersion oil).

The monolayers obtained with Caco-2 clone cell line were all confluent independently of the membrane support. The same observation can be done for the Caco-2 regular cell line on 1 µm. Moreover, cross-sections showed that cells were well-organized in a monolayer with actin filaments mainly present in the apical side of cells, traducing microvillosity. The presence of tight-junctions and microvillosity were also showed with TEM (Figure 3).

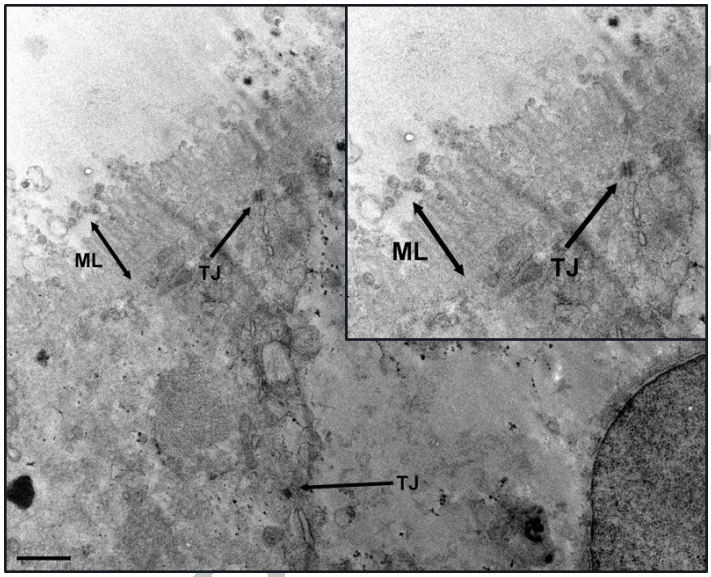

Figure. 3. Transmission electron microscopy (TEM) images of Caco-2 clone seeded on Transwell^{TM} of 3 µm with normal pore density, after 21 days in culture (TJ: tight-junctions; ML: microvillosity).

However, gaps in the monolayer were found when Caco-2 regular cells were seeded on Transwell^{TM} of 3 µm N and HD. Those results were consistent to TEER observations and can be explained by the passage of this cell line across pore of 3 µm. This hypothesis was proved with confocal and TEM since it was observed cells in the apical but also in the basolateral sides of the Transwell^{TM}. Figure 4.A showed the basolateral view of Transwell^{TM} 3 µm HD with Caco-2 regular seeded above. The same observations were found on Figure 4.B obtained with TEM.

Therefore, the Caco-2 regular cell line on 3 µm pore size membrane was not selected for further experiment since cells can cross the membrane.

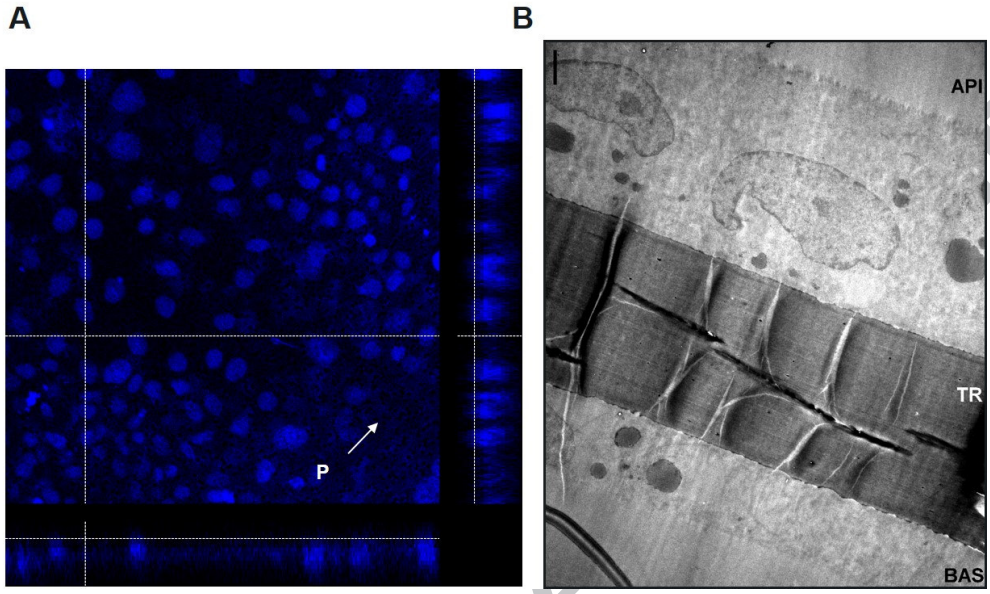

Figure. 4. (A) Immunofluorescence showing the basolateral view of Caco-2 regular cells seeded on Transwell™ of 3 µm with high pore density, after 21 days in culture. Nucleus were counter-stained with DAPI in blue. Images were obtained at a higher magnification (x63 in immersion oil). (B) Transmission electron microscopy (TEM) images of Caco-2 regular cells seeded on Transwell™ of 3 µm with high pore density, after 21 days in culture (API: apical side; TR: Transwell™ membrane; BAS: basolateral side).

3.2 Dextran-FITC permeability

The permeability of dextran-FITC was monitored on the monolayers selected in previous section in order to prove their barrier integrity. First, this study was performed on each membrane support without cell seeding to confirm that the drug can cross each pore size. As depicted in Figure 5.A, the permeability of dextran-FITC after 180 min was close to 80% for each Transwell™ without any difference between them. Figure 5.B shows the permeability (%) of the

drug across all Caco-2 monolayers in relation with the TEER measurements (%). It can be observed that the permeability of dextran-FITC was close to zero when membrane supports of 1 µm were used, independently of the Caco-2 cell line. However, the percentage of drug which crossed the monolayer was 9.24% and 4.24% for Caco-2 clone cells seeded on membrane support of 3 µm N and HD, respectively. As can be seen in Figure 5.C, these results were explained by significant differences of the TEER values, as showed previously. Indeed, the high TEER values obtained with Caco-2 cells seeded on membrane with pore size of 1 µm prevented the paracellular transport of the drug. However, the models with lower TEER values allowed the diffusion of a small fraction of drug.

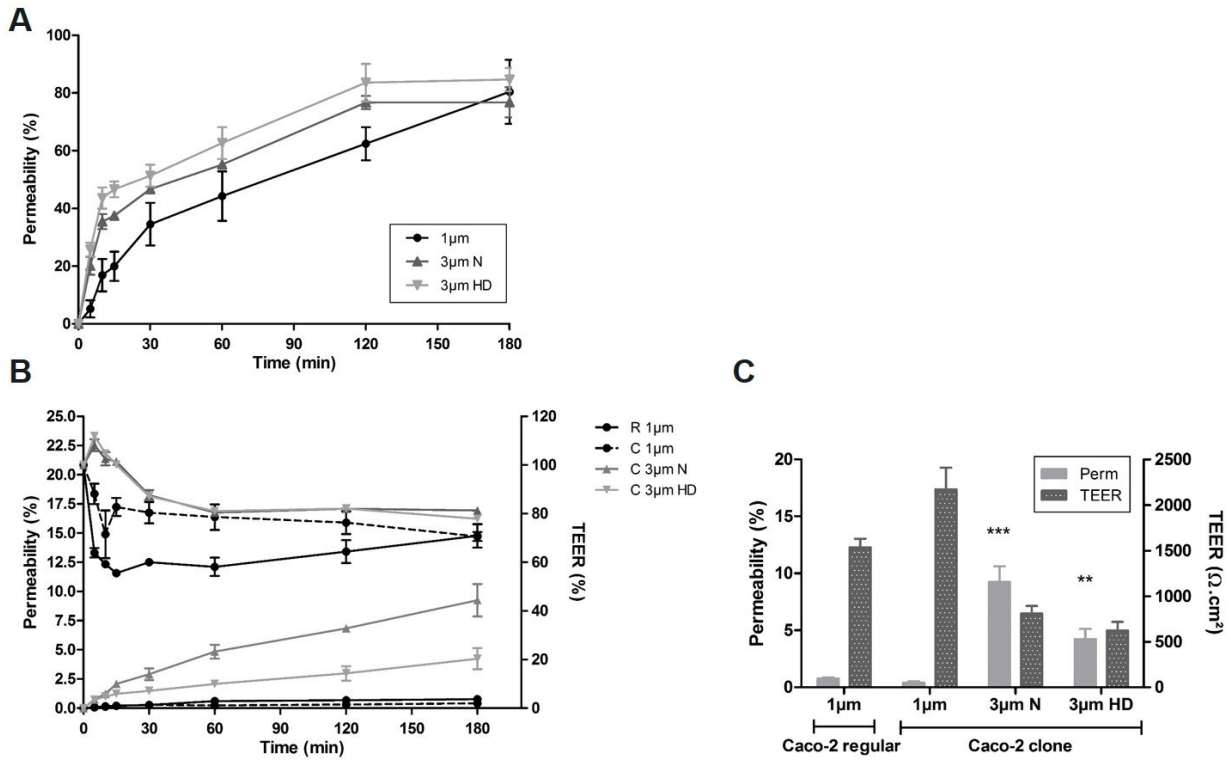

Figure. 5. TEER and permeability (%) for the paracellular marker dextran-FITC across Caco-2 regular and Caco-2 clone monoculture models after 21 days in culture. (A) Permeability (%) of dextran-FITC across membrane with pore size of 1 µm (1 µm) and pore size of 3 µm with normal (3 µm N) or high pore density (3 µm HD) without cell seeding. (B) TEER and Permeability (%)of

Dextran-FITC across Caco-2 regular monolayer seeded on membrane with pore size of 1 µm (R 1 µm) and Caco-2 clone monolayer seeded on membrane with pore size of 1 µm (C 1 µm) and pore size of 3 µm with normal (C 3 µm N) or high density (C 3 µm HD). (C) Permeability (%) of dextran-FITC after 180 min and correspondent TEER values ($\Omega.cm^2$) of each monolayer tested. Results are the mean of replicates and bars represent the standard deviation (n=3).

In this study, it was demonstrated that culture conditions can strongly influence the integrity of Caco-2 monolayer, as well as the permeability of drugs. We could recommend that the choice of the membrane pore size has to be done in function of the cell type used in order to achieve a valid model to perform permeability studies.

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1. Sambuy, Y., et al., *The Caco-2 cell lines as a model of the intestinal barrier: influence of cell and culture-related factors on Caco-2 cell functional characteristics.* Cell Biology and Toxicology, 2005. **21**: p. 1-26.
2. Artursson, P. and G. Karlsson, *Correlation between oral drug absorption in humans and apparent drug permeability coefficients in human intestinal epithelial (Caco-2) cells.* Biochemical and Biophysical Research Communications, 1991. **175**(3): p. 880-885.
3. Hubatsch, I., E.G. Ragnarsson, and P. Artursson, *Determination of drug permeability and prediction of drug absorption in Caco-2 monolayers.* Nat Protoc, 2007. **2**(9): p. 2111-9.
4. Pereira, C., et al., *Cell-based in vitro models for intestinal permeability studies.* 2016: p. 57-81.
5. Lee, J.B., et al., *Quantitative analysis of lab-to-lab variability in Caco-2 permeability assays.* Eur J Pharm Biopharm, 2017. **114**: p. 38-42.
6. Behrens, I. and T. Kissel, *Do cell culture conditions influence the carrier-mediated transport of peptides in Caco-2 cell monolayers?* European Journal of Pharmaceutical Sciences, 2003. **19**(5): p. 433-442.
7. Behrens, I., et al., *Variation of peptide transporter (PepT1 and HPT1) expression in Caco-2 cells as a function of cell origin.* J Pharm Sci, 2004. **93**(7): p. 1743-54.
8. Peterson, M. and M. Mooseker, *Characterization of the enterocyte-like brush border cytoskeieton of the C2BBe clones of the human intestinal cell line, Caco-2.* Journal of Cell Science, 1992. **102**: p. 581-600.
9. Antunes, F., et al., *Establishment of a triple co-culture in vitro cell models to study intestinal absorption of peptide drugs.* Eur J Pharm Biopharm, 2013. **83**(3): p. 427-35.
10. Araujo, F. and B. Sarmento, *Towards the characterization of an in vitro triple co-culture intestine cell model for permeability studies.* Int J Pharm, 2013. **458**(1): p. 128-34.
11. Pereira, C., et al., *Dissecting stromal-epithelial interactions in a 3D in vitro cellularized intestinal model for permeability studies.* Biomaterials, 2015. **56**: p. 36-45.
12. Srinivasan, B., et al., *TEER measurement techniques for in vitro barrier model systems.* J Lab Autom, 2015. **20**(2): p. 107-26.

Graphical abstract