European Journal of Pharmaceutics and Biopharmaceutics 144 (2019) 2–10

Contents lists available at ScienceDirect

European Journal of Pharmaceutics and Biopharmaceutics

journal homepage: www.elsevier.com/locate/ejpb

Assessment of the feasibility to develop a fast and easy reproducible 3D bronchial model growing at the air-liquid interface: Which critical culture parameters must be controlled?

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1. Introduction

The lack of effective new lung therapeutics is partially due to the absence of *in vitro* accurate models which reproduce the architecture and the physiology of bronchial mucosa. Usually, the effectiveness and the cytotoxicity of pulmonary drugs is evaluated through petri dish cell cultures or with *in vivo* mouse models [1]. Mice provide useful models but they are often associated to poor correlation between expected and observed results [2]. Those models are also facing ethical considerations and are expensive and time consuming. Otherwise, two-dimensional (2D)-cell cultures and even more, 3D models are proposed to fill the gap between monolayer cultures and *in vivo* studies [3,4]. In the context of bronchial barrier, different cells have been used as primary cells (normal human bronchial epithelial (NHBE)) or cell lines (Calu-3, 16HBE14o) [5]. As recently reported by Bosquillon *et al.* who have compared the permeability of seven compounds with different log P values, these three types of cells allow the evaluation of drug permeability whereas Calu-3 have the advantages of being available, robust and easy to use for routine assays [6].

Previous studies about Calu-3 based model development using a Transwell™ system showed that the growing of Calu-3 cells in an air–liquid interface (ALI) configuration is more appropriated than liquid-covered culture (LCC) [7]. Fiegel et al. explained that ALI culture of Calu-3 leads to a significant lower Transepithelial electrical resistance (TEER) since after 8 days in culture the TEER for LLC and ALI were respectively of 2250 and 750 $\Omega\,cm^2$. Moreover, through mucus staining, they have highlighted the mucus secretion only for ALI model which protect the underlying epithelium [8]. The production of mucus is of major importance when mucosal cell-based models are considered since it drastically influences the diffusion and consequently the permeability of drugs or encapsulated drugs [9,10]. Despite the fact that the bronchial model composed of Calu-3 has been largely studied, we figured out that researchers reported different culture methods (i.e. cell densities, culture medium or culture time) which leads to misunderstandings. As a result, a few studies on permeability or toxicology characterize properly the properties of the *in vitro* model leading to misinterpretation of results [8,11]. There is an urgent need to provide a standard experimental method to develop an *in vitro* cell based model, mimicking the bronchial mucosa for permeability studies.

At a higher level of complexity, three-dimensional (3D) cell culture models have gained increasing interest because they closely mimic *in vivo* cell environments partially due to interactions of epithelial cells with the extracellular matrix (ECM). 3D-cell based models are expected to provide better prediction in drug discovery [12,13]. In practice, although these models are attractive, they are even more restrictive due to multiple culture parameters, which must be under control. Using a Transwell™ system, these models consist of epithelial cells seeded on the top of a reconstructed ECM, which is composed of collagen and fibroblasts. Fibroblasts must survive and proliferate within the collagen matrix and the epithelial cells growth have to be optimal. Therefore, the concentrations of collagen, fibroblasts [14] and epithelial cells as well as the time of culture are some examples of critical parameters to test in order to provide an ideal 3D-model without shrinking of the gel. There are few studies about the elaboration of 3D bronchial epithelial models [15,16] but none of them use Calu-3 cells and WI-38 as fibroblasts cells in ALI configuration.

Therefore, the first objective of our study was to investigate important culture parameters of the 2D model using Calu-3 cells in order to give some guidelines for the development of an easy-to-use, simple and reproducible bronchial model for permeability or cytotoxicity studies. Then, we have used these optimized conditions to develop a 3D-bronchial cell culture model. To the best of our knowledge, this work is the first attempts to investigate the feasibility of building a 3D bronchial model with Calu-3 cells laid over an ECM made of collagen and fibroblasts at the air-liquid interface. We have selected some critical parameters, such as the origin of the collagen and its concentration as well as the ratio between fibroblasts and the gel and evaluated the integrity and the morphology of the model. Such experimental method

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https://doi.org/10.1016/j.ejpb.2019.09.001
Received 25 June 2019; Received in revised form 22 August 2019; Accepted 3 September 2019
Available online 04 September 2019
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information could be advantageous in the development of epithelial barriers and subsequently may influence drug discovery.

2. Materials and methods

2.1. Materials

Human airway epithelial Calu-3 cells (HTB-55) and human normal lung fibroblasts WI-38 (CCL-75) were obtained from the American Type Culture Collection (ATCC, USA). MEM (Minimum Essential medium), advanced MEM (Minimum Essential medium), Fetal Bovine Serum (FBS), penicillin and streptomycin, glutaMAX, trypsin–EDTA and Hank's Balanced Salt Solution (HBSS) were purchased from Invitrogen Corporation (Life Technologies, S.A., Madrid, Spain). 4 kDa fluorescein isothiocyanate-dextran molecule was purchased from Sigma-Aldrich (St. Louis, MO, USA). Paraformaldehyde (PFA) was purchased from Merck Millipore (Billerica, MA, USA). Hoechst 33,342 Solution (20 mM) was ordered in Thermo Fisher Scientific and ProLong® Diamond Antifade Mountant was provided by Dako Molecular Probes® (Life Technologies S.A., Madrid, Spain). The primary antibody Zonula occludens-1 (ZO-1) and the secondary anti-rabbit Alexa555 antibody were provided by Invitrogen (Life Technologies S.A., Madrid, Spain) and by Abcam (Cambridge, MA), respectively. Type I collagen from Calf skin and Type I collagen from Bovine Purecol were purchased from MP Biomedicals (Bruxelles, Belgium) and from Advanced Biomatrix (Carlsbad, CA, USA), respectively.

2.2. Cell culture

Calu-3 cells (passage 19–40) were maintained in tissue culture flasks (Orange Scientific, Belgium) in two different complete media, Minimum Essential Medium (MEM) or Advanced Minimum Essential Medium (MEMa). As explained by Gibco, MEM is the most commonly medium used of all cell culture media and can be used for a variety of mammalian cells. On the other hand, MEMa is different from other media due to direct addition of the following ingredients: ethanolamine, glutathione, ascorbic acid, insulin, transferrin, AlbuMAX® I lipid-rich bovine serum albumin for cell culture, and the trace elements sodium selenite, ammonium metavanadate, cupric sulfate, and manganous chloride.

Moreover, both media were supplemented with 1% (v/v) of non-essential amino-acids, 1% (v/v) of sodium pyruvate and are supplemented with 10% (v/v) inactivated FBS, 1% (v/v) GlutaMAX, and 1% (v/v) antibiotic–antimitotic mixture (final concentration of 100 U/ml Penicillin and 100 U/ml Streptomycin).

Wi-38 (passage 25–35) cells were cultivated in MEMc supplemented with 1% (v/v) of non-essential amino-acids, 1% (v/v) of sodium pyruvate and are supplemented with 10% (v/v) inactivated FBS, 1% (v/v) GlutaMAX, and 1% (v/v) antibiotic–antimitotic mixture (final concentration of 100 U/ml Penicillin and 100 U/ml Streptomycin).

All cell lines were grown in an incubator (ICO150, Memmert, Eeklo, Belgium) at 37 °C temperature and 5% CO_2 in a water saturated atmosphere.

2.3. In vitro cell models development

2.3.1. 2D cell-based model

Calu-3 cells were seeded in 12-well Transwell™ (polycarbonate membrane, Merck Millipore, Overijse, Belgium) plates with pore size of 0.4 μm or 1 μm (area 1.12 cm^2). Calu-3 cells were seeded to three final densities of 1.5×10^5 cells/cm^2, 3×10^5 cells/cm^2 and 6×10^5 cells/cm^2 on the apical side of Transwell™ inserts in 0.5 ml of culture medium. 1 ml of medium was added in the basolateral side Transwell™. After two days, medium was removed from both compartments and 1 ml of fresh medium was added only in the basolateral side in order to let cells grow at the air–liquid interface (ALI). Afterwards, medium in the basolateral side was replaced each 2 days and cells were allowed to grow in total for 7, 14 or 21 days.

2.3.2. 3D cell-based model

In comparison to the 2D bronchial model, the 3D model encompassed an ECM equivalently made with collagen and fibroblasts. MP Biomedicals Type I collagen from Calf skin was prepared according to manufacturer instructions. Acid acetic was added to obtain a solution of 5 mg/ml and conserved at 4 °C. Advanced Biomatrix Purecol 3 mg/ml Type I collagen from Bovine was ordered directly in solution. Before the mixing with fibroblasts WI-38 (1.10^4 cells/cm^2) [17], additives were added to the collagen to obtain a final volume of 150 μL per Transwell™ (pore size of 0.4 μm). First, 10% of HANKS buffer and 20% of acetic acid were added and the solution was mixed slowly to prevent the presence of bubbles. Then, sodium hydroxide was added drop by drop until obtaining a slightly yellow solution due to pH indicator in the HANKS buffer. In parallel, fibroblasts were trypsinized and resuspended in medium calculated according to the ratio used. Three different ratios between medium containing fibroblasts and the collagen were used, 1:3, 1:4 and 1:5 (v/v). For all ratios, quantities of each components were adapted in order to pipette 150 μL in the apical side of each insert. Then Transwell™ were placed in the incubator during 20 min to allow the gelification of collagen. 0.5 ml of MEMa was added slowly on the top of the dermis equivalent during 24 h. After this incubation time, 3.10^5 Calu-3 cells were added in 0.5 ml MEMa and allowed to grow in ALI configuration as previously explained (Section 2.3.1).

2.4. Assessment of layer integrity

2.4.1. Transepithelial electrical resistance (TEER)

The TEER values of *in vitro* models were measured every two days using an EVOM epithelial voltohmmeter equipped with chopstick electrodes (World Precision Instruments, Sarasota, FL, USA). The medium in the basolateral side (1 ml) was refreshed and 0.5 ml was added slowly in the apical side. Then cells were incubated during 20 min to allow the medium equilibration and TEER were measured. Right after, medium in the apical compartment was removed and cells were incubated.

2.4.2. Dextran-FITC permeability

Dextran-FITC (200 μg/ml) was used as a marker of paracellular pathway. A tight layer of cells must prevent the permeability of this hydrophilic molecule [18]. After the desired incubation time of *in vitro* models, culture medium was removed from basolateral 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 4 h with 0.5 ml of dextran-FITC diluted in HBSS in the apical chamber and 1 ml of HBSS in the basolateral compartment. Aliquots of 200 μL were taken from the basolateral chamber after 5, 10, 15, 30, 60, 120, 180 and 240 min and then replaced with an equal volume of fresh HBSS. The cell monolayer integrity was monitored by TEER measurement at each time point and dextran-FITC content in the basolateral chamber was quantified by fluorescence spectrophotometry (495–521 nm, SpectraMax® i3x, Molecular Device, San Jose, CA). The permeability results are expressed in percentage of permeability in function of time.

2.5. Assessment of cells morphology

2.5.1. Confocal laser scanning microscopy

The confluence and the expression of tight junctions of the 2D *in vitro* model were analysed with confocal microscopy after 7, 14 and 21 days of growth. Cells on Transwell™ membrane were washed two times with PBS, fixed with 2% (w/v) PFA for 1 h and permeabilized by incubating for 7 min with 0.2% (v/v) Triton X-100 in PBS. The blocking was done with PBST (PBS (1X) containing 0.05% (v/v) Tween-20) with

10% (v/v) FBS for 30 min. Zonula occludens-1 (ZO-1) was then labelled with primary rabbit antibody (1:50) for 2 h at room temperature and then Transwell™ were washed twice with PBS. The secondary anti-rabbit Alexa555 antibody (1/200) was incubated during 1 h at room temperature in the dark prior to washing step. Cell nucleus were counterstained with Hoechst (1/2000) during 1 h at room temperature in the dark. The membrane was washed three times with PBS, cut and mounted on a glass slide with fluorescent mounting medium.

2.5.2. Transmission electron microscopy and scanning electron microscopy

Morphology of cells composing the 2D *in vitro* models were also analysed by transmission electron microscopy (TEM) and scanning electron microscopy (SEM) after 7, 14 and 21 days of growth. Transwell™ membranes were washed and cells were fixed with in a 1.6% glutaraldehyde solution in 0.1 M sodium phosphate buffer (pH 7.4) at room temperature and then stored at 4 °C. After three times rinsing in 0.1 M cacodylate buffer (pH 7.4, 15 min each), membranes were postfixed in a 1% osmium tetroxide and 1% potassium ferrocyanide solution in 0.1 M cacodylate buffer for 1 h at room temperature. Samples were subsequently rinsed in DDW and dehydrated in a series of ethanol baths (96%, 100% three times, 15 min each). Samples were then split for SEM and TEM observations. For SEM, after a final bath in hexamethyldisilazane (HMDS, 5 min), samples were left to dry overnight. Samples were coated with platinum (3 nm) prior to observations. SEM observations were performed with a Jeol JSM-6700F SEM at an accelerating voltage of 3 kV. For TEM, samples were progressively embedded in Epon 812 (Fluka) resin (ethanol/resin 1:1, 100% resin two times, 2 h for each bath). Resin blocs were finally left to harden at 60 °C in an oven for 2 days. Ultrathin sections (70 nm) were cut perpendicular to the insert and obtained with a Reichert Ultracut S ultramicrotome equipped with a Drukker International diamond knife and collected on copper slot grids with a formvar support film. Sections were stained with lead citrate and uranyl acetate. TEM observations were performed with a JEOL JEM-1400 transmission electron microscope, equipped with a Morada camera, at a 100 kV acceleration voltage.

2.5.3. Immunohistochemistry

The morphology of the 2D and 3D model was investigated by histology and immunohistochemistry after 7, 14 or 21 days of cell incubation. Transwell™ membranes were washed twice with PBS, fixed in 4% paraformaldehyde and routinely processed for paraffin embedding. Five µm sections were stained with hematoxylin-eosin (H&E), or by Alcian Blue and Periodic Acid Schiff (PAS) for histological examination. The staining of Alcian Blue and PAS were done with a kit ready to use and provided by Merck Chemicals, Darmstadt, Germany.

Moreover, immunostaining was performed on 5 µm sections with antibodies against KI-67 for the detection of cells in proliferation (Confirm anti-KI-67(30–9) rabbit monoclonal antibody- Roche n° 790–4286, Ventana Medical System, Inc, Tucson, USA) or with antibodies against the vimentin protein (Confirm anti-Vimentin (V9) primary antibody- Roche no 790–2917, Ventana Medical System, Inc), as a marker of dermal fibroblasts.

2.6. Statistical analysis

Data were expressed as the mean ± standard deviation. Statistical analysis was performed using GraphPad Prism. A p value < 0.05 was considered as significant. Unpaired *t* test was performed comparing the TEER value of the 2D and the 3D models in function of time.

In the manuscript, the term n = is related to independent experiments.

3. Results and discussion

3.1. Selection of culture medium, membrane pore size and cell density for 2D cell-based model in air–liquid interface (ALI) configuration

As proved by the number of research papers, the cell line Calu-3 is the most commonly used to establish a bronchial epithelial cell-based model. First, this cell line is easier to use and less time consuming than primary cells. Secondly, Calu-3 own valuable behaviours like mucus production and polarized monolayer formation with tight-junctions equivalent to *in vivo* characteristics [19]. Despite these advantages, the culture of Calu-3 is not as easy as other cell lines used for 2D *in vitro* model development like Caco-2 [18].

Calu-3 cells (ATTC) growth is not fast especially after the thawing. Moreover, they grow in clusters and therefore never reach confluence. To be able to manage this cell line and built an easy reproducible 2D model, the impact of three cell culture parameters on the integrity of cell layer in function of time was evaluated. As explained by Kreft et al., Calu-3 may growth on polyethylene terephthalate (PET) substrate whatever the manufacturer [20]. We have worked with PET membranes from Millipore with two pore sizes, 0.4 µm and 1 µm. According to multiple publications, the pore size of 0.4 µm seems the most commonly used [6,7,21]. However, in the case of nanoparticles or drugs permeability assays, we thought it would be interesting to use membrane with pores of 1 µm. Finally, we considered two types of cell culture medium (MEMc and MEMa) to address the grow time of cells seeded on Transwell™. The evolution of TEER in function of these three parameters is shown on Fig. 1 according to the time.

We found that two parameters are critical for the formation of an intact cell layer: the membrane pore size and the type of cell culture medium. Using membranes with pore sizes of 1 µm, the TEER does not rise at all until day 14 meaning that tight junctions (TJ) are not expressed or not functional before this time. On contrary, with pore sizes of 0.4 µm, cells probably join with TJ after 5–7 days leading to an increase in TEER value. If the integrity of the cell layer is maintained, the TEER is then stable which means that cells are still alive and confluent.

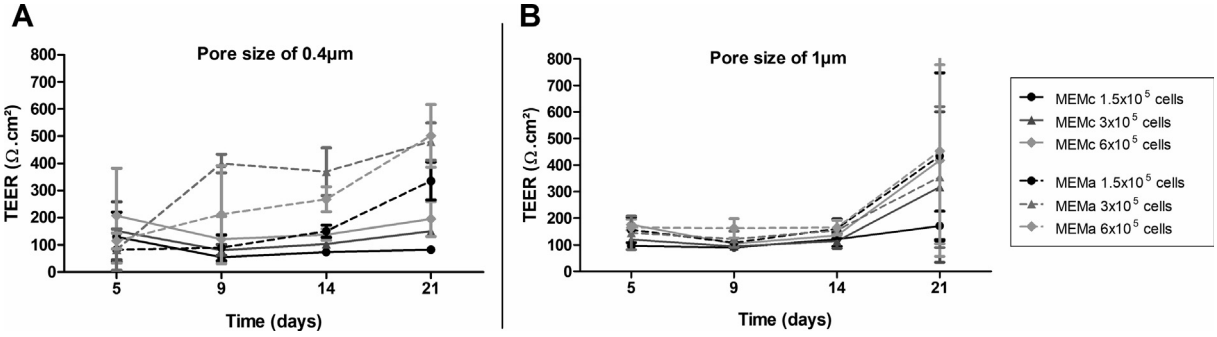


Fig. 1. TEER values (Ω cm^2) in function of cell culture conditions. Cells were cultivated in complete MEM (MEMc) or advanced complete MEM (MEMa) medium. Cells were seeded on Transwell™ membranes at three densities, 1.5×10^5, 3×10^5 and 6×10^5 cells per cm^2. The TEER was monitored in function of time during the 21 days after the seeding of Calu-3 cell lines on Transwell™ membranes having pore sizes of 0.4 µm (A) and 1 µm (B) (n = 3).

We suggest that this very slow increase of the TEER with Transwell™ of 1 μm was either due to a very slow growing speed or to a slow differentiation of cells on this membrane. In comparison, when Transwell™ with pore sizes of 0.4 μm were used, the TEER increased already after 5 days in culture when MEMa was employed. We cultivated cells in different densities with two types of cell culture media. This experiment allowed us to choose the best medium as well as the best seeding density.

If the growing of cells in flasks was similar with both MEMc or MEMa, we observed a huge difference when cells were grown in ALI configuration. Overall, cells reached confluency earlier and therefore a better TEER value was obtained with the MEMa media. This behaviour may be explained by different additives composing the MEMa media (see Section 2.2). However, with the lower cell densities of 1.5×10^5 cells/cm^2, the increase of the TEER was gradual but slow. When cell densities of 3×10^5 cells/cm^2 and 6×10^5 cells/cm^2 were used, higher TEER values could be obtained after between 5 and 9 days. As both cell densities allow to obtain similar TEER values over the time, the lower cell density was selected for further experiments. After 9 days in culture, the TEER value of Calu-3 cells seeded at a density of 3×10^5 cells/cm^2 on Transwell™ membrane (pore size of 0.4 μm) was stable until 21 days. After 21 days, the TEER was of 480.5 (± 68.52) Ω cm^2 which is consistent to literature for an ALI configuration model [20,22].

3.2. Selection of incubation time for 2D cell-based model in ALI configuration

The upper and the lower respiratory tracts are lined by a pseudostratified and ciliated columnar epithelium [23]. This bronchi epithelium is characterized as a complex structure involving goblet, ciliated, and basal cells. Therefore, the time of incubation of the cell-based model is critical. Cells need time to become confluent and to differentiate in order to form a pseudostratified epithelium, to express TJ and cilia or to produce mucus. The incubation time concerning the culture of Calu-3 cells in 2D ALI configuration is not well-defined in the literature. Incubation time varies from 14 days until 21 days depending of authors. Moreover, Haghi et al. showed that transporter (P-glycoprotein) as well as mucus were already expressed/produced at day 7 suggesting the need of a shorter culture time for optimal Calu-3 model in ALI configuration [24]. Through this study, we have used TEER measurement, Dextran-FITC permeability assay, confocal microscopy as well as TEM and SEM to deeply analyse the integrity, the morphology and the structure of the model after 7, 14 and 21 days.

All experiments were performed with Calu-3 cultivated in MEMa medium and seeded on Transwell™ membrane of pore size of 0.4 μm at a density of 3×10^5 cells/cm^2 as previously selected. Results are shown on Fig. 2.

As already mentioned previously, TEER starts to be stable after 7 days but the standard deviation at this time was very high (519.8 Ω cm^2 ± 228.0). Then, TEER reached a plateau around 400 Ω cm^2 until 21 days which indicates the growth and confluence of cells expressing TJ. Concerning the permeability of the paracellular marker across Calu-3 cells, regardless of the time, dextran-FITC did not cross the membrane at all (Fig. 2B). Moreover, TEER measurements remained stable during all the assay proving that the integrity of the membrane was maintained. On Fig. 2B, TEER measurements were shown for the membrane of Calu-3 cells having grown during 7 days but TEER results obtained after 14 and 21 days were similar. Finally, confocal microscopy pictures showed a confluent monolayer and the expression of ZO-1 already after 7 days and further. These superficial results revealed that 7 days of Calu-3 cells under ALI conformation is enough to develop a confluent 2D cell-based model for permeability studies.

The bronchi mucosa comprises other characteristics like a pseudostratified structure, the production of mucus and the expression of microvilli. These parameters are extremely important for the function of the escalator mucociliary. Therefore, we have analysed the morphology of the mucosa after 7, 14 and 21 days. First, SEM images showed that the surface of the ALI model after 7 days was flat and smooth whereas a lot of circumvolutions were observed for day 14 and 21 (Suppl. Fig. A). Furthermore, the Fig. 3 showed the presence of microvilli whatever the time of incubation although the length of them were longer when Calu-3 had grown at least 14 days suggesting a better *in vitro* differentiation. TEM pictures exhibited the presence of TJ and desmosomes for all culture conditions. However, many mucus secretory vesicles were observed after 14 and 21 days. These vesicles were located in the apical side of Calu-3 cells.

Immunohistochemistry was also performed to obtain a transversal view of each model and to highlight proliferative cells or to confirm the production of mucus. Fig. 4 shows that after 7 days, cells were cubic and organized in a monolayer. This observation may be correlated to the smooth surface observed by SEM analysis. Ki-67 staining was done to prove that cells were in proliferation whereas the Alcian Blue (AB) stains mucopolysaccharides to reveal the presence of mucus. After 7 days, cells were all in proliferation but it seems that mucus was not produced at all. Indeed, the staining AB was negative and the staining Periodic acid Schiff confirmed this information (Suppl. Fig. B). After 14 days, the structure of cells was completely different since cells were able to form a pseudostratified epithelium. Therefore, the height of the epithelium was much higher and the mucus was well-identified. As previously, mucus (highlighted in blue) was observed at the apical side of cells after 14 and 21 days.

Overall, we have seen that a culture time of 7 days seems enough to form a confluent monolayer of Calu-3 cells expressing TJ. However, longer time is crucial to let cells differentiate thus to obtain pseudostratified cells expressing mucus and microvilli. Finally, we did not observed any major difference between 14 days and 21 days of incubation which suggest that two weeks are enough to develop an *in vitro* bronchi model.

As recently reviewed, in the context of the development of drug delivery system (DDS) and of permeability studies, the presence of the mucus layer is primordial [9]. Indeed, multiple researches are ongoing worldwide to develop mucopenetrating or mucoadhesive nanocarriers to circumvent mucus layers [25–28]. Whether it is at intestinal, bronchial, buccal or cervico-vaginal level, the presence of mucus represents a determinant biological barrier and has to be considered to speed up the emergence of new medicines [10,29]. Although *in vitro* models are widely used to test the permeability of DDS, they must be as close as possible to *in vivo* situations to be useful. Poor development or bad characterization of *in vitro* models could lead to misinterpretation of results and distort overall research. In our model, even if with integrity of the model seems optimal after 7 days, we have proven that 14 days are required for a good differentiation of cells.

3.3. Critical parameters to a bronchial 3D model with Calu-3 cells

Currently, *in vitro* 3D-models are suggested as a strategy to fill the gap between 2D monolayer cultures and *in vivo* studies [30]. In comparison to 2D cell-based models built with Transwell™ system, 3D models encompass an ECM which can modulate the phenotype of surrounding epithelial cells above. The ECM is a 3D network mainly composed of fibroblasts cells which synthetize structural macromolecules like collagen. As mentioned by Pageau et al., the most abundant type of collagen in human body and in lung is the collagen-type-I. Therefore, this collagen is the most used to mimic the ECM *in vitro* for bronchial or for intestinal models [15,17].

We have figured out that practically, it is tricky to work with *in vitro* model encompassing an ECM layer. Indeed, in the context of lung barrier, while few researchers explained the influence of the ECM on the epithelial layer [14,15], the utilization of these models to test the permeability of DDS or drugs is rare or null. In this study, we have

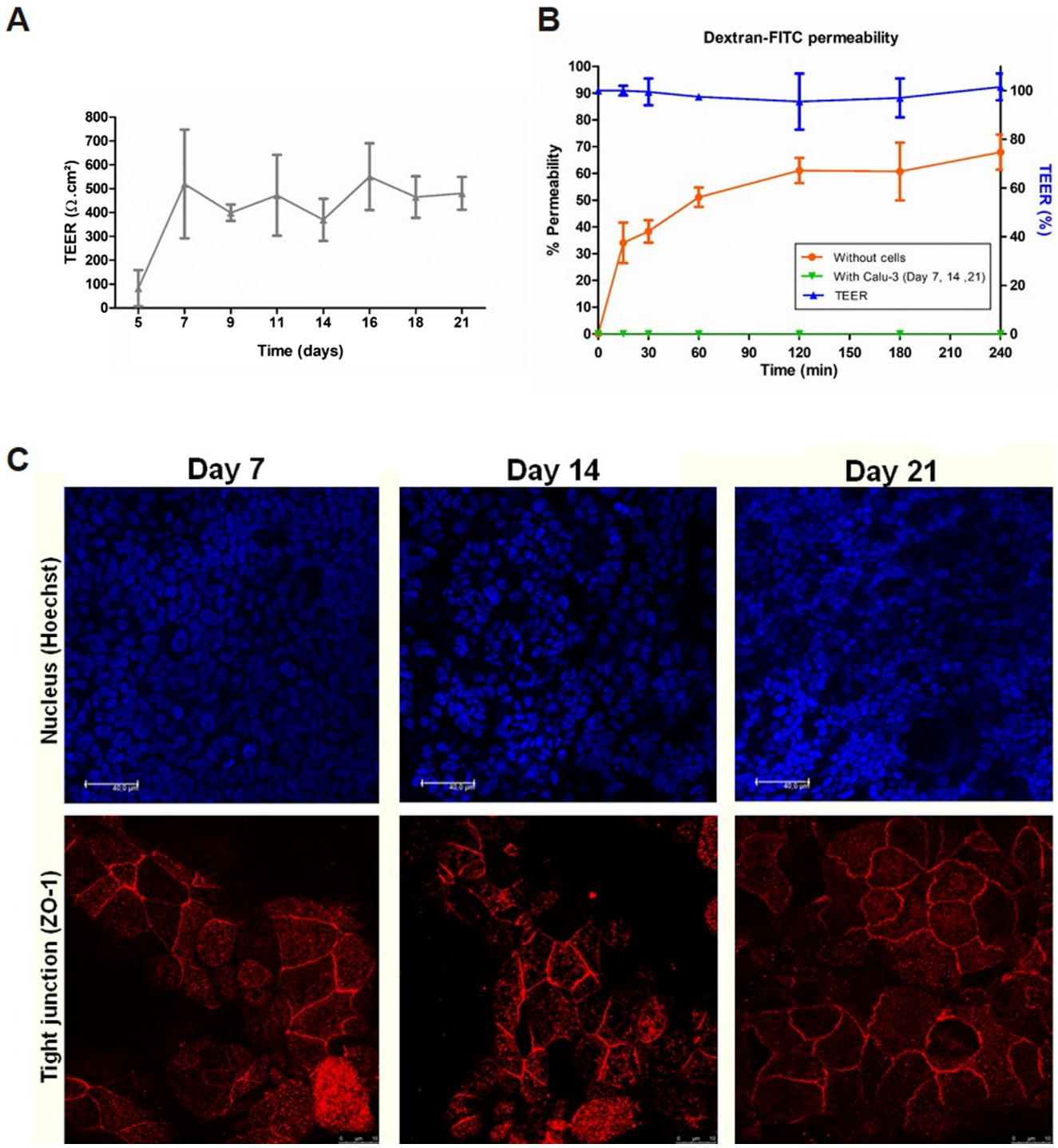


Fig. 2. Evaluation of integrity of Calu-3 cultivated in MEMa medium and seeded on Transwell™ membrane of pore size of 0.4 μm at a density of 3×10^5 cells/cm^2 in function of time. (A) TEER values (Ω cm^2) were measured in function of time. Here is represented TEER measurements for Calu-3 cells having grown during 7 days. (B) Permeability (%) of dextran-FITC (200 nM) in HBSS across Calu-3 cells grown under AIC conditions during 7 days, 14 days and 21 days. Membranes without any cells were also used as a control for each assay and the TEER was measured each time that a sample were withdrawn and replaced by fresh HBSS (200 μL). (C) Immunofluorescence showing the confluence of the cells and expression of tight-junctions (TJ). Zona occludens 1 (ZO-1) were stained in red and nucleus were counter-stained with Hoechst in blue. Images of nucleus were obtained at a higher magnification (×63 in immersion oil) and a zoom (2 times) was applied to show TJ. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

identified important and underestimated parameters to help in the development of 3D-mucosal models.

As represented in Fig. 5.A, the mix of collagen and fibroblasts may be introduced on the Transwell™ membrane 24 h before the seeding of epithelial cells. Thus, the mix must have an appropriate viscosity. Moreover, the gelification must be as fast as possible to prevent the sedimentation of fibroblasts. The viscosity and the gelification of the collagen and fibroblasts mixture are huge problems governed by multiple parameters. First, the temperature influences the viscosity since commercial collagens are liquid at 4 °C but solidify due to polymerization from 8 °C. Therefore, to keep the temperature low, the preparation of the mix has to be done on ice. As described by Bouhout

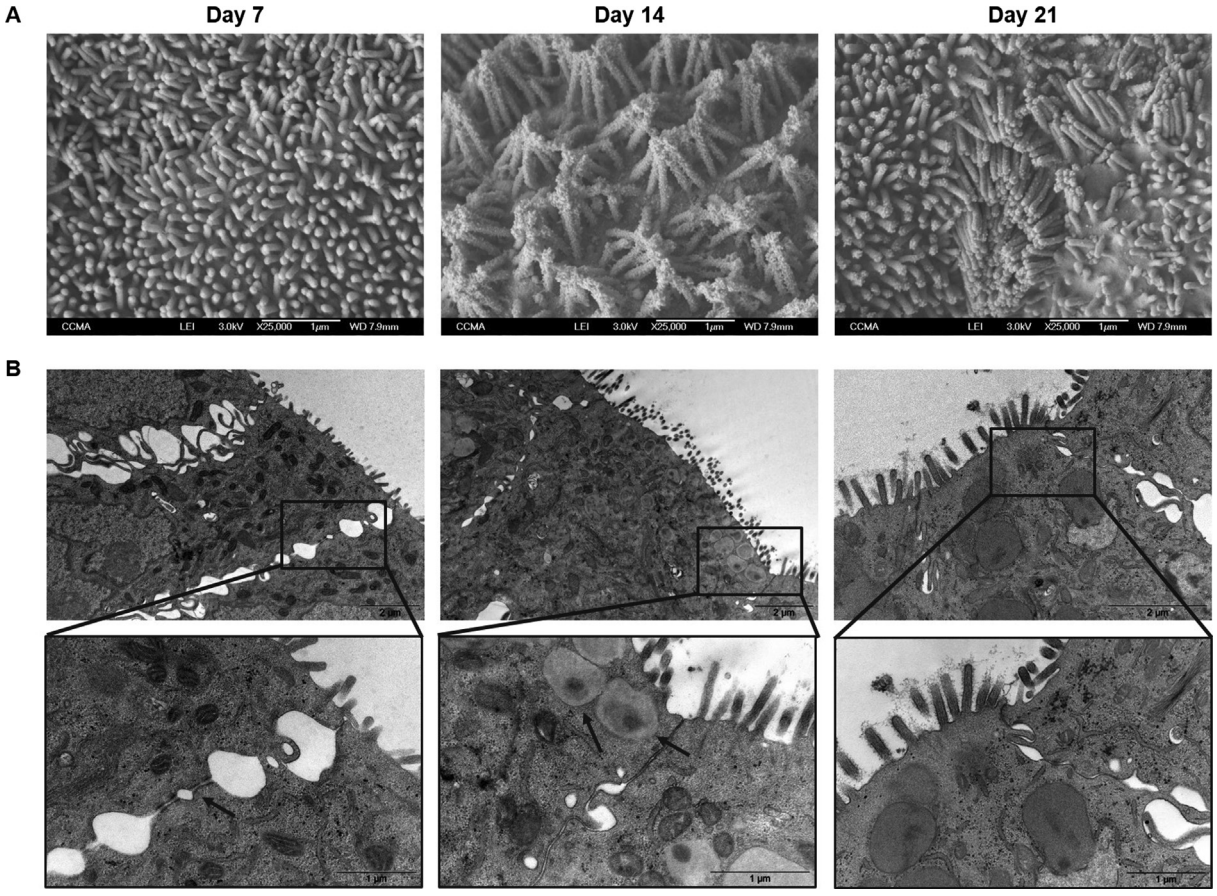


Fig. 3. Calu-3 cells cultivated on Transwell™ under ALI configuration during 7, 14 and 21 days and observed with Scanning Electron Microscope (A) and Transmission Electron Microscope (B). Arrows show TJ and secretory mucus vesicles. Scale bar: (A) 1 µm, (B) 1 or 2 µm.

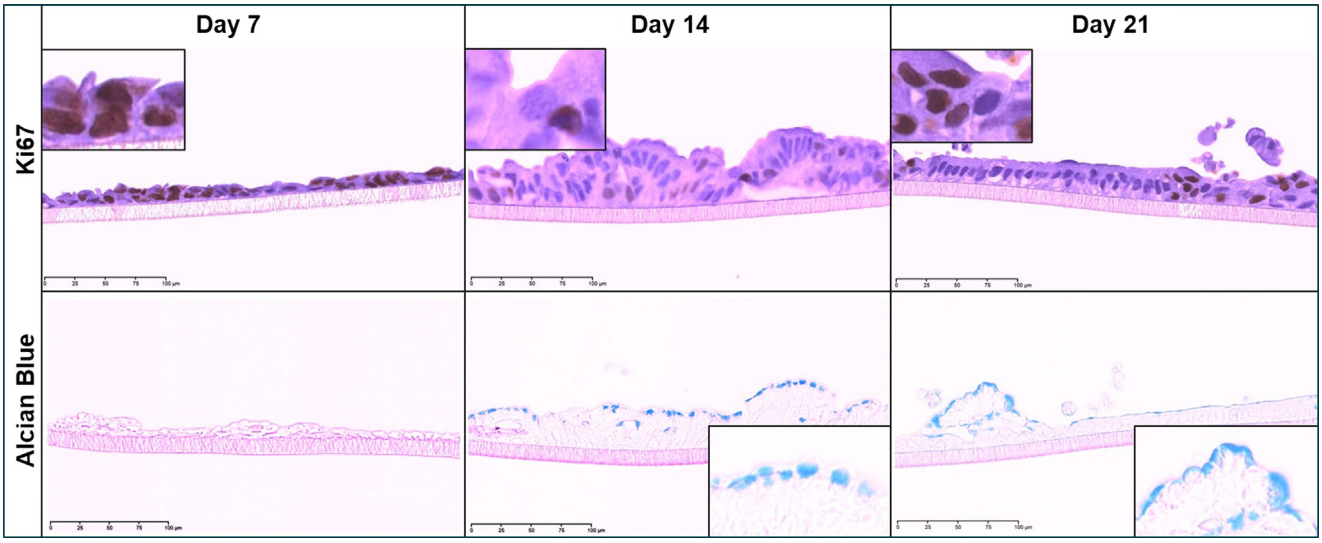
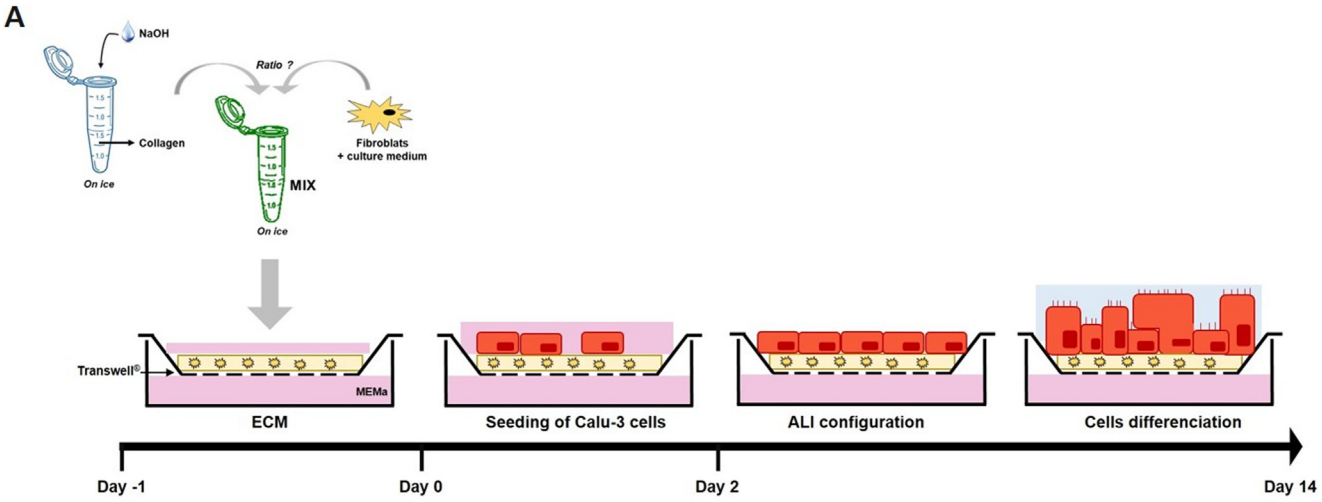


Fig. 4. Transversal optic images of Calu-3 cells cultivated on Transwell™ under ALI configuration during 7, 14 and 21 days. Alcian Blue (BA) coloration and KI-67 staining were performed on each slice. Images are representative of three different experiments and were obtained with the scanner Hamamatsu (objective 40X). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

et al., the pH also strongly influences the viscosity [31]. Commercial collagens are acidic (pH 2) and liquid but the pH must increase (with the addition of sodium hydroxide) for polymerisation and to maintain fibroblasts survival. However, if the pH is too high (> 8), the mix solidifies which makes sampling impossible. Then, the ratio fibroblasts/collagen in the culture medium is determinant. Indeed, this ratio impacts both viscosity and solidification at 37 °C. Sampling has to be easy and reproducible while gelification as to take place into the incubator. Furthermore, the origin and the concentration of collagen itself are also important. Finally, as described by Matsusari et al., the major issue of drug permeability on 3D barrier is the shrink of the collagen-fibroblasts matrix when epithelial cells are seeded above and growth



B

Shrinking ?	Collagen concentration					
	3mg/ml			5mg/ml		
Ratio	1:3	1:4	1:5	1:3	1:4	1:5
Assay 1.	No	Yes	No	No	No	No
Assay 2.	No	No	No	Yes	No	No
Assay 3.	Yes	Yes	No	Yes	Yes	Yes
Assay 4.	No	No	No	Yes	Yes	Yes

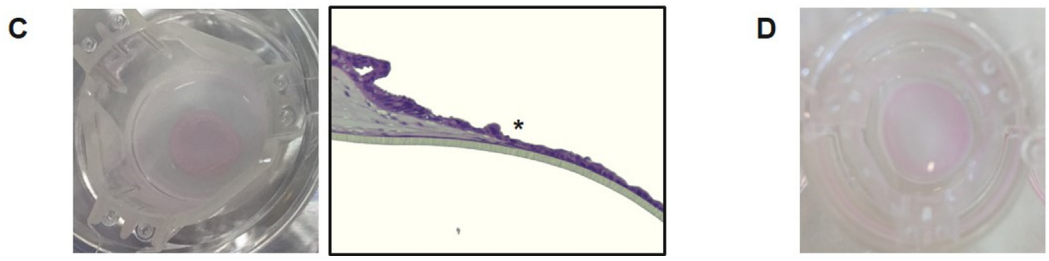


Fig. 5. (A) Schematic illustration of the 3D-bronchial model encompassing an extracellular matrix (ECM) equivalent and bronchial epithelial cells (Calu-3 cells). First, the mix between fibroblasts (WI-38) and neutral collagen is done and put onto a Transwell™ membrane. One day after the polymerisation of the gel, Calu-3 cells are seeded and let to grow during 2 days in a liquid-liquid configuration. Then, the medium in the apical compartment is removed and the model growth at an air–liquid interface (ALI) until cells differentiation (day 14). (B) Results of gel shrinking or not among four distinct experiments. Two types of collagen type I and two concentrations, 3 mg/ml (from Bovine, Purecol™, Advanced Biomatrix) and 5 mg/ml (from Calf skin, MP Biomedicals) were used. Three ratios (v/v) between the volume of medium containing fibroblasts and the volume of collagen were used. The shrinking of the gel was evaluated from the beginning of the Calu-3 seeding and until 14 days in culture. (C) Representation of gel shrinking by picture of a Transwell™ system with the gel shrink in the middle of it and by immunohistochemistry (hematoxylin eosin). This picture was obtained 5 days after the seeding of Calu-3 cells. * represents the place where the gel shrink (D) Picture a gel correctly dispersed in a Transwell™ system, 14 days after the seeding of epithelial cells. In this case, fibroblasts were mixed with collagen (3 mg/ml) at a ratio 1:5 (v/v).

[32].

In order to evaluate the impact of these parameters, we have selected two collagens-type I; one at a concentration of 5 mg/ml from Calf skin (MP Biomedicals) and the other one at 3 mg/ml from Bovine (Purecol™, Advanced Biomatrix). Different ratios fibroblasts/collagen were chosen as 1:1, 1:2, 1:3; 1:4 and 1:5. First, we have performed a screening study to know if these mixes were pipettable and if they could solidify into the incubator (37 °C) in less than 30 min. We excluded ratios of 1:1 and 1:2 due to concerns of slow gelification. We went further with ratio of 1:3, 1:4 and 1:5 and Calu-3 cells were seeded on the top of the ECM in same conditions as selected previously.

As shown on Fig. 5B, among four distinct experiments, only one condition provided reproducible results. We can see that 3D-model made with the collagen at a concentration of 5 mg/ml were more susceptible to shrink compared to 3D-models made with collagen at 3 mg/ml. As shown on Fig. 5C, Calu-3 cells were well-dispersed on Transwell™ but the gel matrix shrinked, probably due to the growth of epithelial cells. The shrinkage of the gel matrix appeared after 4 or 5 days of cells growing thus in this case, experiments were stopped. The only condition which was reproducible was obtained with fibroblasts mixed with collagen (3 mg/ml) at a ratio 1:5. This condition was selected for further characterizations.

The integrity and the structure of the 3D model selected previously were evaluated. The TEER obtained after 14 days for the 2D model and the 3D model was of 472.4 ± 169.5 Ω cm^2 and 295.2 ± 205.3 Ω cm^2, respectively (Fig. 6A). Thanks to the HE coloration, we can clearly identify each layers of the 3D model as the epithelial layer, the ECM and the Transwell™ membrane. This assay revealed a well-formed pseudostratified epithelium typical of native tissue. According to Fig. 6B, the presence of a thick ECM encompassing active fibroblasts can be highlighted. First, the staining of KI-67 showed a lot of epithelial cells in proliferation (Fig. 6B). Moreover, the formation of a mucus film

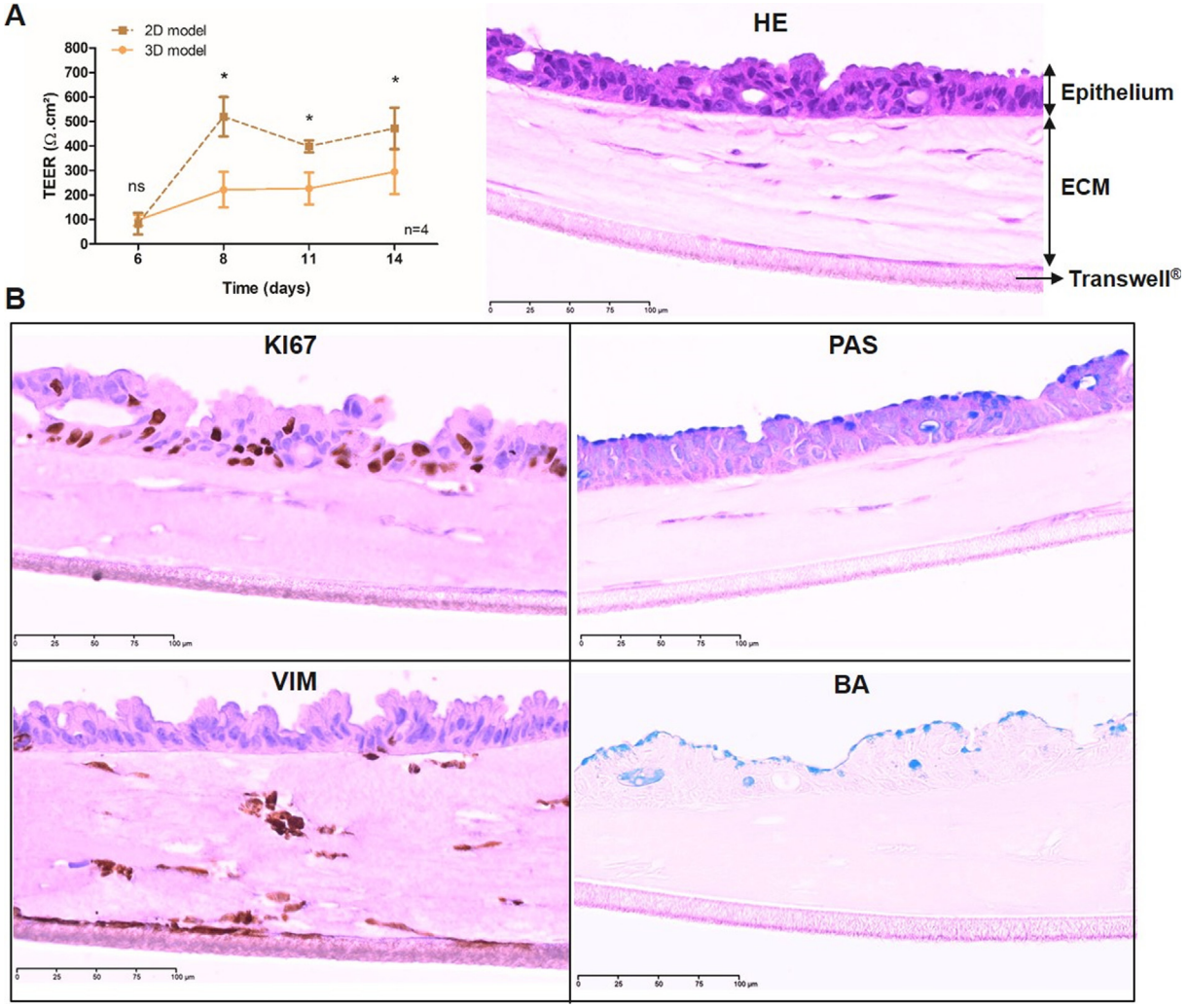


Fig. 6. Evaluation of integrity and structure of Calu-3 cells seeded under an extracellular matrix (ECM) composed of fibroblasts (WI-38) in a matrix of collagen. (A) TEER values ($\Omega.cm^2$) were measured in function of time. Unpaired *t* test, $p < 0.05$ (*). (B) Transversal optic images of the 3D model cultivated during 14 days. Hematoxylin eosin (HE) coloration shows each layers of the 3D model as the epithelial layer and the ECM. (C) KI-67 and the vimentin (VIM) were also stained and coloration of Alcian Blue (BA) and periodic acid schiff (PAS) were performed. Images are representative of three different experiments and were obtained with the scanner Hamamatsu (objective 40X). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

covering Calu-3 cells was highlighted by BA and PAS. Finally, the intrinsic activity of fibroblasts was revealed due to the expression of the intermediate filament protein vimentin (VIM) [33]. Overall, the 3D model seems reproducible and exhibits a morphology close to *in vivo* situation. This model is the basis for further investigations like the impact of the density of fibroblasts or of the volume of the mix composing the ECM. Then, permeability studies or gene expression will be performed in order to prove its benefit compared to 2D model.

4. Conclusion

Despite the fact that 2D and 3D *in vitro* models are largely described as essential tool to speed up drug discovery, it is still tricky to use them for high throughput screening of compound libraries. The level of complexity of the models increases which makes the experiments difficult to achieve or to reproduce. In this study, we were able to build a 2D-bronchial model with differentiated Calu-3 cells expressing mucus, microvilli and having a pseudostratified architecture. This model was obtained after 14 days of culture which is reasonable considering the screening of drugs. Then, we have provided some practical tips to reproduce an ECM with fibroblasts embedded in a collagen matrix. Based on these observations, we propose a base for the optimization of a 3D-bronchial model which closely mimic the *in vivo* conditions, easily performed and reproducible in laboratory. Furthermore, these practical information could be transposed to other 3D-mucosal models, as the intestinal barrier.

Acknowledgements

Authors would like to acknowledge the FRS. FNRS-Télévie for financial support. Moreover, authors would like to thank Dr. Chantal Humblet and her team for immunohistochemistry analysis (Platform Immunohistology, GIGA, University of Liege, Belgium) and François Orange from the University of Nice for TEM and SEM assays. CCMA electron microscopy equipments have been funded the Région Sud Provence-Alpes-Côte d'Azur, the Conseil Général des Alpes Maritimes, and the GIS-IBiSA. We are also thankful to Prof. Didier Cataldo for the use of the Hamamutsu scanner and to Dr. Sandra Ormenese for the utilization of confocal microscope at the GIGA-imaging platform.

Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejpb.2019.09.001.

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