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WP3 – Determination of drug transfer and infant drug exposure during lactation: generation of quantitative and translatable data

D3.3 Report on characterization in vitro human/animal mammary epithelial cell cultures models, including comparison between in vitro models

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V1.1	23/02/2021	First Draft
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Content

Publishable Summary

This report gives a comparative overview on the characterisation of the *in vitro* human and animal mammary epithelial cell culture models. Based on the findings in Deliverable 3.2 [1], it was clear that the pig is the most suitable animal model [2]. Therefore, the first *in vitro* model is based on pig mammary epithelial cells (pMECs). A protocol for the isolation and expansion of the pMECs has been developed. Furthermore, some human models were preselected for further evaluation: (i) primary human mammary epithelial cells (HMECs); (ii) Michigan Cancer Foundation 7 cell line (MCF-7); (iii) Michigan Cancer Foundation 10A cell line (MCF-10A); (iv) PMC42-LA, human breast cancer cell line. For each of these, a protocol for expansion has been developed. All the selected cell culture models show epithelial features. Only three models were able to form a tight barrier, which is essential for transepithelial transport (permeation) studies with medicines: pMECs, HMECs and MCF-7. pMECs MG8 show the best transepithelial electrical resistance and the smallest fluorescein sodium transport profile in function of time. These cells will play a crucial role for the nonclinical platform in association with the results from the animal *in vivo* studies, as they will provide mechanistic insights and scaling information. A human model is preferred as the final model to investigate the transfer of medicines into the human breast milk, however, the pig cellular model is necessary to define how much the *in vitro* results are predictive for *in vivo* ones. Therefore, we can use either the HMECs, for which further culture conditions optimization is still required, or the MCF-7 cell line in medium without beta-estradiol. HMECs represent the most biorelevant model, but primary cells are challenging, more expensive and show some variability in (functional) characteristics. Therefore, the MCF-7 cell could offer an alternative. Further characterization in terms of reproducibility, transporters expression and transporter function will allow us to make a final decision between the two human models. In line with the objective of Task 3.2, the ultimate *in vitro* model resulting from this research will prove instrumental in deriving quantitative and mechanistic information regarding the passage of medicines across the blood-milk barrier.

Methods

Based on different skills and competences WP3 participants decided to split the activities concerning this deliverable: UNIBO staff has taken care of primary cells both human and porcine: isolation, expansion and characterization, while KU Leuven staff has taken care of expansion of the human cell lines. In terms of characterization, UNIBO has taken care of drug transporters gene expression evaluation while KU Leuven has taken care of the barrier function evaluation, as well as the ongoing quantification of the transporters protein expression and function.
This division of work, performing the same analysis on different substrates in the same laboratory, assured the most accurate comparison of different aspects, between human and animal cells.

pMECs Isolation

Despite the fact that some companies claim to be able to sell porcine primary mammary epithelial cell, none was able to supply them. An attempt was made to buy cells from a company that claimed an on-demand custom production. However, although the cells were ordered, the company was unable to produce them at first due to technical problems then to the COVID pandemic emergency that finally led to the closure of the company itself. Considering that, we decided to isolate, culture and expand pMECs in house.
The *in vitro* porcine cells experiments require working in a good experimental practice, identified in three key concepts known as the "3Rs" rule: Replacement, Reduction and Refinement [3].
3Rs are implemented within the European Directive 2010/63/EU, which concerns the protection of animals used for scientific purposes [4]. In this context, pMECs *in vitro* model was based on tissues collection from local slaughterhouse in full respect of both international legislations and the 3Rs principle. The Department of Veterinary Medical Sciences (DIMEVET) works following a Quality

Management System (SGQ) and obtained the UNI EN ISO 9001:2015 certification for research and analysis activities. Considering that, we aimed to create an internal Standard Operating Procedure for the pMECs isolation protocol.

ABBREVIATIONS

DMEM/F12	Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12
DMSO	Dimethyl Sulfoxide
DPBS	Dulbecco's phosphate-buffered saline
PBS	Phosphate buffered saline
Anti-Anti	Antibiotic-Antimycotic solution
hEGF	human epidermal growth factor
BPE	Bovine pituitary extract
FBS	Fetal bovine serum
g	gravitational acceleration
P	Passage
rRNA	Ribosomal ribonucleic acid
OCT	Optimal Cutting Temperature Compound
PCR	Polymerase chain reaction
RT	Room Temperature
MECs	Mammary epithelial cell
pMECs	porcine Mammary Epithelial Cell
HMECs	Human Mammary Epithelial Cell
MG	Mammary gland
LAF	Laminar Air Flow

Tissue samples of pig mammary gland (

Figure 1) were taken from 7 sows, the first tissue sample was utilized only for the set-up of experimental approach while the other six were destined to pMECs isolation. Considering the developmental physiology of the porcine mammary gland, the abdominal breasts were selected as the best candidates for pMECs isolation. Considering that the slaughterhouse is the samples supplier, the first step was to develop a good protocol of sample disinfection based on the combination of a common cutaneous disinfectant and an alcohol-based disinfectant. Moreover, all the procedures were handled in aseptic conditions under a vertical laminar air flow (LAF) cabinet.

The strategy that was chosen to obtain the cells was based on the combination of two techniques: the mechanical tissue dissociation with the enzymatic one [5]. To easily obtain single-cell suspensions with high reproducibility an automated dissociator (GentleMACS Octo Dissociator, Miltenyi Biotec) (

Figure 1) was chosen as the best strategy. Moreover, since the mammary gland is a complex organ made up of different tissues, it was decided to use not a single enzyme, but a mixture of different enzymes designed to deconstruct the tissue in its complexity (Multi Tissue Dissociation kit, Miltenyi Biotec Company). This enzymatic kit was associated to different programs of automatic tissue dissociation to optimize a protocol that best fit with porcine mammary tissue texture. The programs tested were:

1. 37C_Multi _B
2. 37C_TDK_1
3. 37C_TDK3

All three program types used included a temperature control system at 37 ºC. In addition, each program was tested in triplicate.

For each animal, a portion of mammary tissue was also processed in parallel for histological analysis. In particular mammary gland portion of about 1 cm^3 was embedded in OCT and frozen in isopentane cooled in liquid nitrogen.

Tissue Sections (~7 µm micrometers-thick) were cut with a cryostat (CM1950 Leica, Wetzlar, Germany) and stained with haematoxylin and eosin (H&E) according to standard procedure.

Images were obtained using a Leika Aristoplan microscope equipped with a DFK 33UX264 camera (The Imaging Source Europe GmbH, Germany).

Figure 1 Fundamental initial steps of pMECs isolation

Once the cell suspension was obtained, it was necessary to define the growth conditions, to select the population of epithelial cells by disadvantaging the growth of other resident populations such as fibroblasts and adipocytes. It is nowadays well known that some cell types could grow in suspension if appropriately stimulated; in this regard, it has been shown that mammary epithelial cells possess this ability if cultured in the absence of foetal bovine serum.
Therefore, a selective medium for the epithelial cells growth was used relying on a previous publication [6]. In particular, the complete mammary epithelial cells medium for the isolation step was prepared by adding, to the Mammary Epithelial Cell Basal Medium, the Supplement Pack containing all media supplements as individual vials (PromoCell, Heidelberg, Germany), Anti-Anti solution and Gentamicin (Gibco) at the volumes and at the final concentrations listed in Table 1.
After one day without serum, to select the grow of pMECs in suspension, the spheres were collected and transferred in a multiwell, then the FBS was added to allow the sphere adhesion and consequent pMEC sprouting. When cells reached 70% of confluence were detached, counted and seeded into a T-25 primary culture flask (first passage P).
For the cryopreservation, aliquots of $1x10^6$ cells were suspended in 1 ml of freezing medium (90% FBS and 10% DMSO) and put into cryovials and transferred in a freezing container for 24 hours at -80°C. The day after cells were transferred in a liquid nitrogen biobank.

Table 1 Isolation pMECs Medium composition

Component	***Volume***	***Final concentration***
Mammary Epithelial Cell Basal Medium	*492 ml*	*/*
BPE	*1 ml*	*0.004 mg/ml*
hEGF	*500 µl*	*10 ng/ml*

Insulin	*500 µl*	*5 µg/ml*
Hydrocortisone	*500 µl*	*0.5 µg/ml*
Anti-Anti (100X)	*5 ml*	*1 %*
Gentamicin (50 mg/ml)	*500 µl*	*50 µg/ml*

The absence of mycoplasma contamination is verified through the use of EZ-PCR Mycoplasma Test Kit (Biological Industries, Kibbutz Beit Haemek - Israel) on culture medium taken from the wells of the multiwell plate in which the pMECs were cultured and expanded. Gene sequences for rRNA in prokaryotes are highly conserved and what discriminates one species from the other is the size of the space region in the rRNA operon so the kit is based on the amplification of this region. One of the components of the kit is a ready to use PCR mixture, containing the highly sensitive and specific primers, appropriate for various mycoplasma species, as well as Acholeplasma and Spiroplasma. The process consists of two main phases. The first phase concerns the PCR amplification of the conserved gene region coding for the 16S rRNA of the Mycoplasma species and the second one is the electrophoretic run carried out on the agarose gel of the amplified fragments, to verify the presence or absence of the band corresponding to the fragment of interest (270 bp). Aliquots (1 ml) of the cell culture medium were periodically collected and analysed by the EZ-PCR Mycoplasma Detection kit. After collection, the medium was centrifuged at 250 x g to bring the cellular debris to the bottom. The supernatant obtained was further centrifuged at 20.000 x g for 10 minutes. The obtained pellet was resuspended in 50 µl of Buffer Solution supplied in the kit. A first denaturation step was then carried out at 95 °C for 3 minutes. The PCR reaction was carried out using 5 µl of sample to which 35 µl of ultrapure H_2O or 1 µl of Positive Control Solution provided by the kit and 10 µl of Reaction Mix were added. In addition, a negative control reaction was set up, which contain 5 µl of ultrapure H2O. Each sample was analyzed in duplicate. The thermal cycle condition applied was: 94°C for 30 s (1 cycle); 94 for 30 s, 60°C for 120 s, 72°C for 60 s (35 cycle); 94 for 30 s, 60°C for 120 s, 72°C for 5 minutes (1 cycle). The PCR product length was 270 bp.

pMECs Expansion

In general, the use of highly selective medium is successful in isolating pure cellular populations, but this may reduce the proliferative capacity of the cells themselves [5].
A method for the pMECs expansion was developed and, after the check of its validity, the aim was to create a specific SOP that could also be used by Leuven staff to multiply cells before the testing activities. Among primary cell cultures obtained, we decided to expand three of them until tenth passage (P10). The other ones were stored in a biobank for further planned expansions

The pMECs expansion method is divided in the different steps reported below.

GENERAL
All activities are performed in the LAF hood, unless differently specified.
Always decontaminate material with 70 % ethanol before bringing it under the LAF hood.

PRELIMINARY STEP: PREPARATION AND MANAGEMENT OF THE NECESSARY MATERIAL

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DPBS, Anti-Anti, Trypsin, FBS, hEGF aliquots preparation.

- The DPBS is divided into 50 ml conical tube (40 ml per tube) and stored at room temperature.
- The trypsin and the antibiotic-antimycotic solution 100X are divided into 15 ml conical tube (10 ml per tube) and stored at -20°C.
- The FBS is divided in 50 ml conical tube (40 ml per tube) and stored at -20°C.
- The hEGF is divided in 0,5 ml tubes (25 µl per tube) and stored at -20°C.
- All aliquoted products must be parafilmed and labeled.

Preparation of pMECs Expansion Medium

- Thaw the FBS, anti-anti solution and the supplements insulin, hydrocortisone, hEGF.
- Add the amount of FBS and anti-anti solution to DMEM/F12 medium at the volumes and final concentrations listed in Table 2.
- Add the supplements insulin, hydrocortisone (Supplement Pack, PromoCell) and hEGF (Thermo Fisher) to DMEM/F12 medium at the volumes and final concentrations listed in Table 2.
- Mix well and divide the pMEC expansion medium into 50 ml conical tube (40 ml per tube). The aliquots must be sealed, labeled and stored at +4°C.

Table 2 Expansion pMEC medium composition

Component	***Volume***	***Final concentration***
DMEM/F12	*444 ml*	*/*
FBS (100X)	*50 ml*	*10 %*
hEGF (0.1 mg/ml)	*25 µl*	*5.0 ng/ml*
Insulin	*500.0 µl*	*5 µg/ml*
Hydrocortisone	*500.0 µl*	*0.5 µg/ml*
Anti-Anti (100X)	*5.0 ml*	*1 %*

Preparation of the freezing medium

- Thaw the FBS and add the DMSO at 10% concentration.
- Mix well and divide it in 15 ml conical tube (10 ml per tube).
- All aliquots must be sealed, labeled and stored at -20°C.

In general, for reference volumes of work for the different types of flasks see the Table 3.

Table 3 Volumes of DPBS, trypsin-EDTA and medium in flasks

Flask	DPBS	Trypsin 0,25%-EDTA (1X)	Medium
T25	5 ml	3 ml	5 ml
T75	10 ml	7 ml	15 ml

pMECs THAWING

- Pre-warm pMEC expansion medium in water bath at 37°C.
- Take the cryovial containing the cells from the liquid nitrogen bank.
- Place the cryovial in water bath at 37°C for 3 minutes.
- After having thoroughly cleaned the cryovial with 70% ethanol transfer it under the LAF hood.
- Pick up the cells in 1ml of freezing medium and transfer in a 15 ml conical tube.
- Add 4 ml of pMEC expansion medium (the first ml must be added very slowly) and gently resuspend.
- Centrifuge at 500 x g for 10 min at room temperature, soft stop.
- During centrifugation prepare the flask: put 4 ml of pMEC expansion medium in a T25 primary culture flask (Corning PRIMARIA 353824).
- Discharge the supernatant by inversion.
- Resuspend the cell pellet with 1 ml of medium.
- Transfer the 1 ml of cell suspension in a T-25 primary culture flask, previously prepared, mix gently to homogeneously distribute the cells.
- Mark the flask (name and passage) and observe cells under an inverted microscope, cells must be bright with intact membranes (Figure 2).

- Transfer the flask in incubator at 38.5°C, 5% CO_2.

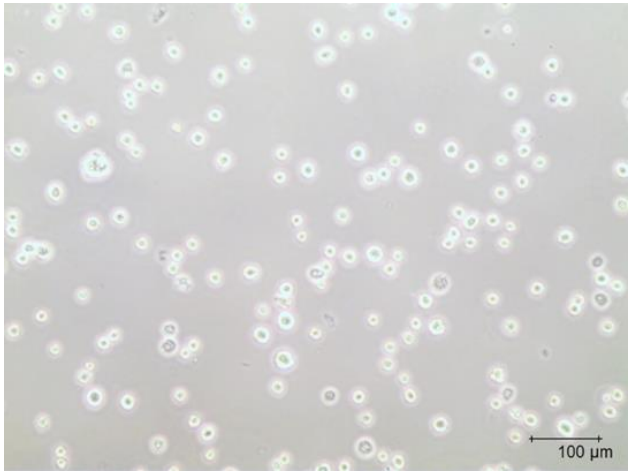


Figure 2 Representative image of pMECs immediately after the thawing (Scale bar = 100 µm)

pMECs HEALTH CHECK THE DAY AFTER THAWING

- Pre-warm the pMECs expansion medium and sterile DPBS in water bath at 37°C.
- Observe cells under inverted microscope: the day after the cells adhere to plastic and grow in small clusters (*Figure 3*) (many dead cells may be present in suspension).

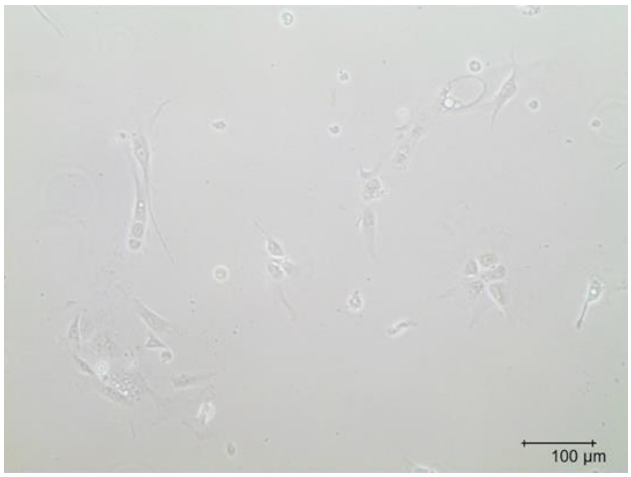


Figure 3 Representative image of pMECs (P9) the day after thawing, cells grow in small cluster (Scale bar = 100 µm)

- Aspirate and eliminate the culture medium.
- Add 5 ml DPBS to rinse, rock gently and then aspirate off.
- Add 5 ml of fresh medium.
- Transfer the flask in incubator at 38.5°C, 5% CO_2.

DAILY MANAGEMENT OF pMECs

- Check the cell growth daily under the inverted microscope, if necessary (e.g. if the colour of the medium turns to yellow or in presence of cellular debris) change the medium.
- Pre-warm the pMECs expansion medium and sterile DPBS in water bath at 37°C.
- Add 5 ml DPBS to rinse, rock gently and then aspirate off.
- Add 5 ml of pMECs expansion medium.
- Transfer the flask in incubator at 38.5°C, 5% CO_2.

- When the cells reach the confluence (75-80%) (Figure 4) in T25 flask (generally after 3/4 days around 1,5 x 10^6 cells), split them.

SPLITTING CELLS

- Pre-warm the pMECs expansion medium, DPBS, trypsin and FBS in water bath at 37°C.
- Aspirate and eliminate the existing culture medium.
- Wash with 5 ml DPBS, rock gently and aspirate off.
- Add 3 ml trypsin into a T-25 flask.
- Transfer in incubator for 7 min.
- Observe the cells under the inverted microscope and if necessary, gently beat the flask.
- Inhibit the trypsin by adding the same volume of FBS (3 ml).
- Transfer the cell suspension (6 ml) in a 15 ml conical tube.
- Wash the flask with DPBS (5 ml) to collect any remaining cell clusters and add them to the cell suspension (final volume 11 ml).
- Centrifuge at 500 x g for 10 min at room temperature, soft stop.
- Discharge the supernatant by inversion and resuspend the cell pellet with fresh medium (1 ml).
- Count cells in a counting chamber.
- Split pMECs 1:3, seed the cells in 3 T25 flasks (0,5 x 10^6 cells/flask) by adding first 4 ml of medium in each T25 and then transfer 1 ml of cell suspension) or in a T75 flask (1,5 x 10^6 cells/flask) by adding 10 ml of medium in T75 and transfer then 5 ml of cell suspension.
- Mix gently to homogeneously distribute the cells.
- Mark the flask and observe under the inverted microscope.
- Transfer the flask in incubator at 38.5°C, 5% CO_2.

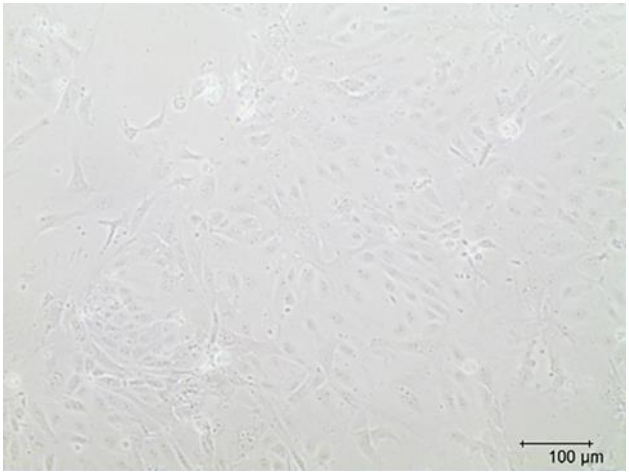


Figure 4 Representative image of pMECs (P9) at 75-80 % confluence (Scale bar = 100 µm)

CRYOPRESERVATION

- Pre-warm the pMECs expansion medium, DPBS, trypsin and FBS in water bath at 37°C, while keep the freezing medium at room temperature.
- Trypsinize cells as previously described in the paragraph “SPLITTING CELLS”.
- Count cells in the counting chamber.
- Centrifuge at 500 x g for 10 min at room temperature, soft stop.
- Resuspend the cell pellet at 1x10^6/ml freezing medium.

- Transfer 1 ml of cell suspension in the cryovial and mark it with cell type, animal, passage, n° of cells, date and operator.
- Transfer the cryovial in a freezing container and place it at -80°C overnight.
- The day after transfer the cells in a liquid nitrogen bank.

HMECs Expansion

Primary human mammary epithelial cells are produced by different companies, among these, Life Tech was chosen being a leader company in the field of cells and cellular products. Besides, this company purchased and guaranteed the best cell characterization, in fact approved lots of cells are tested by immunocytochemistry for cytokeratins 5/6,8 and E-cadherin. Therefore, four aliquots (from the same batch) of human primary cells, HMECs (A10565 Life Tec, Lot # 2098293), were bought.
With the aim to create a specific SOP that could be used by Leuven staff before the functional tests, a method for the HMECs expansion was developed.
The method is divided in different step and was reported below.

GENERAL
All activities are performed in the LAF hood, unless specified differently.
Always decontaminate material with 70 % ethanol before bringing it under the LAF hood.

PRELIMINARY STEP: PREPARATION AND MANAGEMENT OF THE NECESSARY MATERIAL

Preparation of DPBS, Anti-Anti, Trypsin, FBS aliquots
The DPBS is divided into 50 ml conical tube (40 ml per tube) and stored at room temperature.
The trypsin and the anti-anti solution 100X are divided into 15 ml conical tube (10 ml per tube) and stored at -20°C.
The FBS is divided in 50 ml conical tube (40 ml per tube) and stored at -20°C.
All aliquoted products must be sealed and labeled.

Preparation of the HMEC growth medium
Thaw the anti-anti solution and the Supplement Mix vial (PromoCell).
Add the amount of anti-anti solution and Supplement Mix contained in a vial at the volume and final concentrations listed in Table 4 to the Mammary Epithelial Cell Basal Medium (PromoCell).
Mix well and divide the medium solution into 50 ml conical tube (40 ml per tube).
The aliquots must be sealed, labeled and stored at +4°C.

Table 4 HMECs growth medium composition

Component	***Volume***	***Final concentration***
Mammary epithelial cell basal medium	*482,5 ml*	*/*
Supplement Mix	*12.5 ml*	*/*
BPE	*/*	*0.004 ml/ml*
hEGF	*/*	*10 ng/ml*
Insulin	*/*	*5 µg/ml*
hydrocortisone	*/*	*0.5 µg/ml*
Anti-Anti (100X)	*5.0 ml*	*1 %*

Preparation of the freezing medium
Thaw the FBS and add the DMSO at 10% concentration. Mix well and divide it in 15 ml conical tube (10 ml per tube). All aliquots must be sealed, labeled and stored at -20°C.

In general, for reference volumes of work for the different types of flasks see the Table 5 below.

Table 5 Volumes of DPBS, trypsin-EDTA and medium in flasks

Flask	DPBS	Trypsin 0,25%-EDTA (1X)	Medium
T25	5 ml	3 ml	5 ml
T75	10 ml	7 ml	15 ml

HMECs THAWING

- Pre-warm HMEC growth medium in water bath at 37°C.
- Take the cryovial containing the cells (5x10^5 cell aliquots in 1 ml freezing medium) from the liquid nitrogen bank.
- Place the cryovial in water bath at 37°C for 3 minutes.
- After having thoroughly cleaned the cryovial with ethanol 70% transfer under a flux laminar hood.
- Pick up the cells in 1ml of freezing medium and transfer in a 15 ml conical tube.
- Add 29 ml of complete HMEC medium (the first 5 ml must be added very slowly) and gently resuspend.
- Seed HMECs in 2 T75 primary flask (2,5 x 10^5 cells/T75 Corning PRIMARIA 353824).
- Mark the flask (name and passage) and observe cells under an inverted microscope (10 or 20 X), cells must be bright with intact membranes (
- Figure 5).

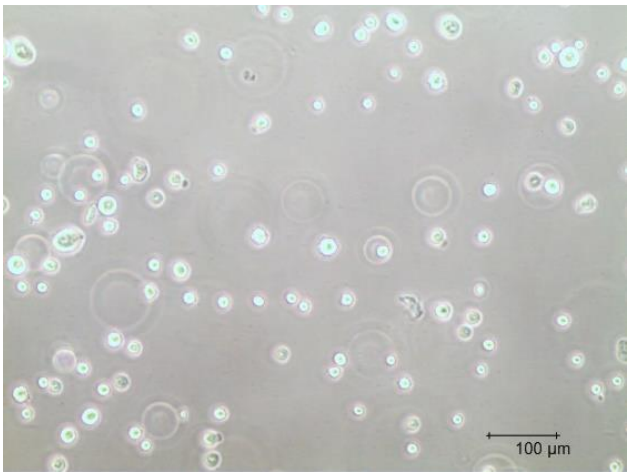


Figure 5 Representative image of HMECs, immediately after thawing (Scale bar 100 µm)

- Transfer the flask in incubator at 37°C, 5% CO2 and unscrew the flask cup by half a turn. Do not disturb cells for 18 hours.

HMECs HEALTH CHECK THE DAY AFTER THAWING

- Observe cells under the inverted microscope: the day after cells adhere to plastic and are present in single cell or small cluster of epithelial cells (
-
- Figure 6).

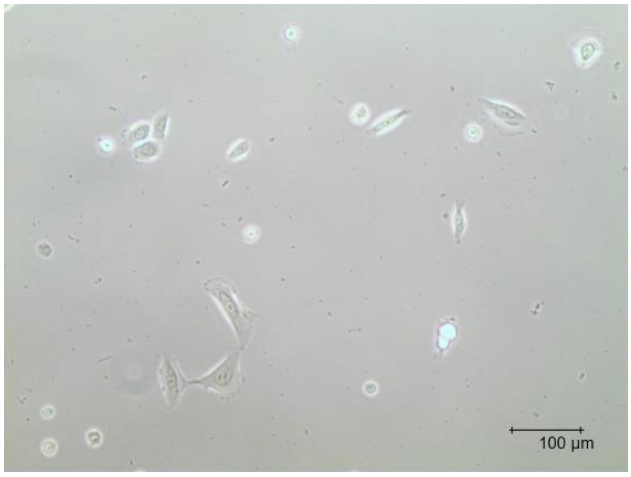


Figure 6 Representative image of HMECs (P9) the day after thawing (scale bare = 100 µm)

- Aspirate and eliminate the previous culture medium (HMECs are very sensitive and suffer when are without medium so change the medium one flask at a time and rapidly).
- Wash with 15 ml DPBS if the flask contains many dead cells, otherwise it is not necessary.
- Add 15 ml HMECs growth medium.
- Transfer the flask in incubator at 37°C, 5% CO_2 and unscrew the flask cup by half a turn.

DAILY MANAGEMENT of HMECs

- Check the cell growth daily under a microscope, if necessary (e.g., if the medium is turbid or in presence of cellular debris) change the medium.
- In general, change the culture medium every day, until the culture is approximately 50% confluent.
- Once the culture reaches 50% confluence, change the medium every day until the culture is approximately 80-90% confluent (after 5-7 days).
- When the cells reach 80-90% confluence (Figure 7), after about 5-7 days, split them (5 x 10^3 cells/cm2, 1,25 x 10^5 cells/T-25 or 3,75 x 10^5 cells/T-75).
- Pre-warm the HMECs growth medium, DPBS, trypsin and FBS in water bath at 37°C.
- Aspirate and eliminate the existing culture medium.
- Add 3 ml trypsin into a T-25 flask (or 10 ml in T-75).
- Incubate the flask at 37°C for 4-5 min (unscrew the flask cup by half a turn).
- Observe the cells under the inverted microscope and if necessary, gently beat the flask.
- Inhibit the trypsin by adding the same volume of FBS (3 ml or 10 ml).
- Transfer the cell suspension (6 ml or 20 ml) in a 15 ml conical tube.
- Wash the flask with DPBS (5 ml or 10 ml) to collect any remaining cell clusters and add them to the cell suspension (final volume 11 ml or 30 ml).
- Centrifuge at 500 x g for 7 min at room temperature, soft stop.
- Discharge the supernatant by inversion and resuspend the cell pellet with the medium (2-3 ml).
- Count cells in a counter chamber.
- Seed new subculture HMECs at a density 5x10^3 cells/cm^2: 1,25x10^5 cells/T25 or 3,75x10^5 cells/T75.
- Mix gently the flask to homogeneously distribute the cells.
- Mark the flask and observe under a microscope.

- Transfer the flask in incubator at 37°C, 5% CO_2.
- Calculate the doubling time.

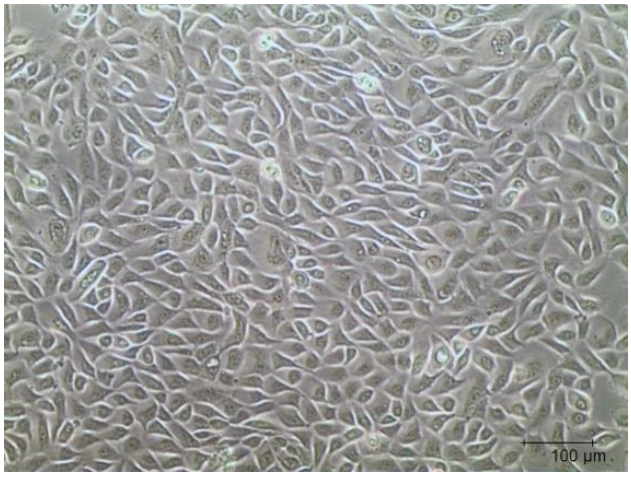


Figure 7 Representative image of HMECs (P9) at confluence (scale bar = 100 µm)

CRYOPRESERVATION

- Pre-warm the HMEC growth medium, DPBS, trypsin and FBS in water bath at 37°C, while keeping the freezing medium (FBS+10% DMSO) at room temperature.
- Trypsinize cells as previously described in the paragraph above.
- Count cells in a counting chamber.
- Centrifuge at 500 x g for 7 min at room temperature, soft stop.
- Resuspend the cell pellet at $5x10^5$ cells/ml freezing medium.
- Transfer 1 ml of cell suspension in the cryovial and mark it with: cell type, passage, n° of cells, date and operator.
- Transfer the cryovial in a freezing container and place it at -80°C overnight.
- The day after transfer the cells in liquid nitrogen bank.

MCF-7 expansion

MCF-7 cells were a kind gift from prof. Fendt from the Laboratory of cellular metabolism and metabolic regulation (VIB-KU Leuven). The cells were originally bought from ATCC. The protocol for expansion was based on the methods from the Laboratory of cellular metabolism and metabolic regulation.

GENERAL
All activities are performed in the LAF hood, unless specified differently.
Always decontaminate material with 70 % ethanol before bringing it under the LAF hood.

ABBREVIATIONS

DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl Sulfoxide
DPBS	Dulbecco's phosphate-buffered saline (buffer solution)
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
g	gravitational acceleration
LAF	Laminar Air Flow
MCF	Michigan Cancer Foundation-7 (breast cancer cell-line)
P/S	Penicillin and Streptomycin (antibiotic against infections)
RT	Room Temperature

PREPARATION OF COMPLETE GROWTH MEDIUM

- Turn the LAF hood and water bath on.
- Clean the LAF hood.
- Prepare or thaw β-estradiol (Sigma-Aldrich, E2758) stock solution (20 µg/ml). Preparation: Add 1 ml DMSO (Acros Organics 167850010) to 1 mg β-estradiol (outside the LAF hood). Gently swirl to dissolve. Bring the solution in the LAF hood and add 49 ml sterile medium (DMEM). Filter with a 2.0 µm filter, as DMSO is not sterile. Aliquot and store at -20°C.
- Thaw FBS (Lonza, BO 04-007-1A) and P/S (Lonza DE17-602E/12) in the water bath.
- Obtain 1 bottle of DMEM (Lonza, BE12-707F) (500 ml) from storage.
- Remove 55 ml from the DMEM bottle with pipette and pipetteboy.
- Add 50 ml FBS with pipette and pipetteboy.
- Add 5 ml P/S with pipette and pipetteboy.
- Add 68 µl β-estradiol stock solution (20 µg/ml).
- Mix and filter in a sterile bottle.
- Label the bottle.
- Clean the LAF hood.
- Turn water bath and LAF hood off.

MCF-7 cells were first expanded in medium containing ß-estradiol. However, the barrier experiments showed that ß-estradiol interfered with the formation of a tight monolayer (see Results section). Therefore, cells were further expanded for some passages in the same culture medium, but without ß-estradiol.

INITIATION OF CULTURE

- Put the LAF hood and water bath on.
- Clean the LAF hood.
- Swab down the flask with 70 % ethanol.
- Warm the complete growth medium.
- Swab the complete growth medium with 70 % ethanol.
- Obtain 1 vial MCF-7 cells from liquid nitrogen. Thaw by gentle agitation in the water bath (37 °C). Swab with 70 % ethanol.
- Slowly add 1 ml medium to the vial and transfer the content of the vial to the 50 ml Falcon tube.
- Rinse the vial with 1 ml medium and add to the Falcon conical tube.
- Add 5 ml medium to the conical Falcon tube.
- Gently resuspend by pipetting up and down.
- Centrifuge 10 min at 125 x g at room temperature.

- Aspirate the medium, leaving the pellet undisturbed.
- Resuspend the pellet in 1 ml fresh medium. Suspend by gently pipetting up and down (25 times). Make sure that no clumps remain.
- Count the cells. Take 3 Eppendorf microtubes add 400 µl medium to each tube. Resuspend the cell suspension and add 50 µl cell suspension to each tube. Add 50 µl trypan blue to the Eppendorf tube. Take 15 µl to count the cells. Calculate the cell yield and viability.
  Cell yield = L/40 (million cells/ml)
  Viability = L/(L+D) * 100 (%)
  Abbreviations: L: living cells; D: death cells
- Add the required volume of medium to seed at $4*10^4$ cells/cm^2 and seed in the flask. Remark: Each vial contains about 1 million cells, thus seed 1 vial in a T-25 flask. Label flask with the (a) name of cell line, (b) passage number, (c) date, (d) initials

MAINTENANCE
Change medium every 2-3 days.

- Put the LAF hood and water bath on.
- Put medium in the water bath.
- Optional: Take a picture of the cells under the inverted microscope.
- Optional: Calculate the confluence of the cells with ImageJ. Open the picture in ImageJ. First calculate the total pixel number via analyze → measure. Then select all the cells and measure again.
  Confluence = (cell pixels)/(total pixels)*100 %
- Change the medium if the cells are less than 50 % confluent.
- Clean the LAF hood.
- Swab down the flask and the growth medium with 70 % ethanol.
- Aspirate growth medium at the side of the flask opposite the cells.
  Add the required volume of new growth medium to the side of the flask opposite the cells
- Table 6and incubate the flask at 37 °C.
- Clean the LAF hood.
- Turn the water bath and the LAF hood off.

SUBCULTURE

- Put the LAF hood and water bath on.
- Put the trypsin (Sigma-Aldrich T3824) and medium in the water bath.
- Optional: Take a picture of the cells under the inverted microscope.

Optional: Calculate the confluence of the cells with ImageJ. (SOP26) Open the picture in ImageJ. First calculate the total pixel number via analyze → measure. Then select all the cells and measure again.
Confluence = (cell pixels)/(total pixels)*100 %)Subculture when the cell confluence are greater than 50 %

- Clean the LAF cabinet.
- Swab down the flask with 70 % ethanol.
- Aspirate the growth medium from the flask.
- To the attached cells remaining in the flask, add appropriate volumes of sterile Ca^{2+}- and Mg^{2+}-free DPBS to the side of the flask opposite the cells to avoid dislodging the cells (
- Table 6). Rinse the cells with DPBS (using a gently rocking motion) and aspirate. Repeat two times.

Table 6 Volumes of rinse buffer and trypsin-EDTA

Flask	DPBS	Trypsin-EDTA 0.05 %	Medium
T25	3 ml	1 ml	1.5 ml
T75	9 ml	3 ml	4.5 ml
T175	21 ml	7 ml	10.5 ml

- Add appropriate volume of 0.05 % (w/v) Trypsin-EDTA solution to the side of the flask opposite the cells (
  Table 6
- Table 6). Make sure that the entire surface is covered by a small layer of trypsin-EDTA solution.
- Incubate the flask at 37 °C until the cells round up (about 10 min). The attached cells should slide down the surface. Note: Do not leave trypsin-EDTA on the cells any longer than necessary as clumping may result.
- Set the parameters of the centrifuge at 125 g, 10 min and RT (22 °C).
- Neutralize the trypsin-EDTA cell suspension by adding complete growth medium (
- Table 6) to each flask. Disperse the cells by pipetting, gently, over the surface of the monolayer. Pipette the cell suspension (+/- 25 times) up and down with the tip of the pipette resting on the bottom corner or edge until a single cell suspension is obtained. Be careful to avoid the creation of foam.
- Transfer the cells to a 50 ml conical tube. Rinse the flask with medium (
- Table 6) and transfer to the same conical tube.
- Centrifuge at 125 x g for 10 min at RT (22 °C).
- Carefully aspirate and discard the medium, leaving the pellet undisturbed.
- Resuspend pellet in 1 ml complete growth medium by gently tapping the bottom of the tube, and, if necessary, pipetting up and down (+/- 25 times). Make sure that no clumps remain.
- Count the cells. Take 3 microtubes. Add 400 µl medium to each tube. Add 50µl cell suspension to each tube (resuspend in between). Add 50 µl trypan blue to 1 Eppendorf tube and count the cells in 15 µl. Repeat for the other two microtubes.
- Calculate the cell yield and viability.
  Cell yield = L/40 (million cells/ml)
  Viability = L/(L+D) * 100 (%)
  Abbreviations: L: living cells; D: death cells
- Calculate the number of flasks needed to seed $4*10^4$ cells/cm^2.
- Add the required volumes of full growth medium (
- Table 6).
- Transfer cell suspension into new flasks at a seeding density of about 4×10^4 viable cells/cm^2. Resuspend one time before seeding each flask. Label all new flasks with the (a) name of cell line, (b) passage number, (c) date, (d) initials.
- Clean the LAF hood.
- Turn the water bath and the LAF hood off.

CRYOPRESERVATION

- One day in advance: Check the isopropanol level. Add if necessary. Place the container in the fridge at 2 to 8 °C.
- Put the LAF hood and water bath on.
- Put the trypsin and medium in the water bath.

- Optional: Take a picture of the cells under the inverted microscope.
- Optional: Calculate the confluence of the cells with ImageJ (SOP26). Open the picture in ImageJ. First calculate the total pixel number via analyze → measure. Then select all the cells and measure again.
  Confluence = (cell pixels)/(total pixels)*100 %)
- Subculture or cryopreserve when the cells are 50-80 % confluent.
- Clean the LAF cabinet.
- Prepare cryopreservation medium: 200 µl FBS, 200 µl complete growth medium, 100 µl DMSO per vial. Filter the medium.
- Aspirate the growth medium from the flask.
- To the attached cells remaining in the flask, add appropriate volumes of sterile Ca^{2+}- and Mg^{2+}-free DPBS to the side of the flask opposite the cells to avoid dislodging the cells. Rinse the cells with DPBS (using a gently rocking motion) and aspirate. Repeat two times.
- Add appropriate volume of 0.25 % (w/v) Trypsin-0.53 mM EDTA solution to the flask.
- Incubate the flask at 37 °C ± 1 °C until the cells round up (10 min) The attached cells should slide down the surface. Note: Do not leave trypsin-EDTA on the cells any longer than necessary as clumping may result.
- Set the parameters of the centrifuge at 125 g, 10 min and RT (22 °C).
- Neutralize the trypsin-EDTA cell suspension by adding complete growth medium to each flask to neutralize the trypsin. Disperse the cells by pipetting, gently, over the surface of the monolayer. Pipette the cell suspension (+/- 25 times) up and down with the tip of the pipette resting on the bottom corner or edge until a single cell suspension is obtained. Be careful to avoid the creation of foam.
- Transfer the cells to a 50 ml conical tube. Rinse the flask with medium. (Transfer all cells to the same conical tube)
- Centrifuge at 125 g for 10 min at RT (22 °C).
- Carefully aspirate and discard the medium, leaving the pellet undisturbed.
- Resuspend pellet in 1 ml complete growth medium by gently tapping the bottom of the tube and pipetting up and down. Make sure that no clumps remain.
- Count the cells. Take 3 Eppendorf tubes. Add 400 µl medium to each tube. Add 50 µl cell suspension to each tube (resuspend in between). Add 50 µl trypan blue to 1 Eppendorf tube and count the cells in 15 µl. Repeat for the other two Eppendorf tubes.
- Calculate the cell yield and viability.
  Cell yield = L/40 (million cells/mLl)
  Viability = L/(L+D) * 100 (%)
  Abbreviations: L: living cells; D: death cells
- Calculate the number of vials needed to freeze at 1-2 million cells/1 ml cryovial.
- Label the cryovials to include information on the (a) name of cell line, (b) passage number, (c) date.
- Take medium to have 0.5 ml complete growth medium per vial. (0.5 ml per 1-2 million cells).
- Add dropwise 0.5 ml cryopreservation medium per vial. Resuspend.
- Dispense 1 ml of cell suspension, using a 5 ml or 10 ml pipette, into each 1 ml cryovial.
- Insert the cryovials with the cell suspension in appropriate slots in the container.
- Transfer the container to -70 °C to -90 °C freezer and store overnight.
- Clean the LAF hood.
- Turn the water bath and the LAF hood off.
- Next day, transfer cryovials to the vapor phase of liquid nitrogen freezer.

MCF-10A expansion

MCF 10A (ATCC® CRL10317™) cells were a kind gift from prof. Fendt from the Laboratory of cellular metabolism and metabolic regulation (VIB-KU Leuven). The protocol for expansion was based on the protocol from the BRUGGE lab, department of cell biology, Harvard medical school [7].

GENERAL
All actions should be performed in the LAF hood, unless specified otherwise.
Full growth medium contains cholera toxin. Cholera toxin is fatal if swallowed or in contact with skin.

ABBREVIATIONS

EGF	Epidermal Growth Factor
P/S	Penicillin and Streptomycin (antibiotic against infections)
DMEM/F12	Dulbecco's Modified Eagle Medium/ nutrient mixture F12
DMSO	Dimethyl Sulfoxide
DPBS	Dulbecco's phosphate-buffered saline (buffer solution)
DMSO	Dimethyl Sulfoxide
EDTA	Ethylenediaminetetraacetic acid
g	gravitational acceleration
LAF	Laminar Air Flow
MCF-10A	Michigan Cancer Foundation 10A
RT	Room Temperature

PREPARATION OF HORSE SERUM STOCK
- Thaw a bottle of horse serum (Life Technologies 16050122).
- Put the bottle 1h in a water bath on 50°C for heat inactivation.
- Prepare aliquots of 25 ml heat inactivated horse serum in conical tubes of 50 ml.
- Label the aliquots.
- Store aliquots at -20°C.

PREPARATION OF EGF STOCK SOLUTION
- Filter dH_2O through a 0.2 µm filter.
- Resuspend EGF (Peprotech AF-100-15) at 100 µg/ml filtered dH2O.
- Prepare aliquots of 100 µl in microtubes.
- Label the aliquots.
- Store aliquots at -20°C.

PREPARATION OF HYDROCORTISONE STOCK SOLUTION
- Resuspend hydrocortisone (Sigma-Aldrich, H0888), at 1 mg/ml in absolute ethanol.
- Prepare aliquots of 250 µL in Eppendorf tubes.
- Label the aliquots.
- Store aliquots at -20°C.

PREPARATION OF CHOLERA TOXIN STOCK SOLUTION
- Filter dH_2O through a 0.2 µm filter.
- Resuspend cholera toxin (Sigma-Aldrich) at 1 mg/ml in filtered dH20.
- Allow to reconstitute for about 10 minutes.
- Prepare aliquots of 50 µl in microtubes.
- Label the aliquots.
- Store aliquots at 4°C.

PREPARATION OF FULL GROWTH MEDIUM

Premix the required volumes of the additives: horse serum, EGF, hydrocortisone, cholera toxin, insulin (Sigma I9278) and P/S (Westburg, DE17-602E/12) (Table 7). Rinse all conical tubes/ microtubes with ethanol.

- Option 1: Sterile filter the additives through a 0.2 µm filter. Skip step 5 if this step is done. Alternatively, skip this step and filter the complete medium.
- Remove 30.9 mLl from the DMEM/F12 (Life Technologies 31330038) medium bottle.
- Add the additives to the DMEM/F12 medium bottle and mix.
- Option 2: Filter the complete medium with a Nalgene filter. Skip step 2 if this step is done. Alternatively, skip this step and filter the additives before adding to the medium.
- Label the bottle.
- Store the bottle at 4°C.

Table 7 Full growth medium

Component	***Volume***	***Final concentration***
DMEM/F12	*469.1 ml*	*/*
Heat inactivated horse serum	*25,0 mL*	*5 %*
EGF(100 µg/ml)	*100 µl*	*20 ng/ml*
Hydrocortisone (1 mg/ml)	*250 µl*	*0.5 µg/ml*
Cholera toxin (1 mg/ml)	*50 µl*	*100 ng/ml*
Insulin (10 mg/ml)	*500 µl*	*10 µg/ml*
P/S	*5.0 mL*	*1 %*

INITIATION OF CULTURE

- Warm complete growth medium in the water bath (37°C).
- Obtain 1 MCF-10A vial from liquid nitrogen.
- Thaw the vial by gentle agitation in the water bath (37°C).
- Transfer the content of the vial to a 50 ml conical tube.
- Rinse the vial with medium and add to the 50 ml conical tube.
- Gently resuspend by pipetting up and down.
- Centrifuge 5 min at 150g at room temperature.
- Aspirate the medium, leaving the pellet undisturbed.
- Add 1 ml medium. Gently resuspend. If necessary, pipet up and down (10 times) to resuspend.
- Count the cells (see cell counting).
- Add the required volume of medium to seed in a density of 15000 cells/cm^2.
- Seed in the appropriate flask (Table 8).
- Label the flask.
- Transfer the flask to the incubator.

Table 8 Medium volume and seeding density per flask

Flask	***Area***	***Volume medium***	***Cells for seeding***
T-25	*25 cm^2*	*7 ml*	*375 000*
T-75	*75 cm^2*	*21 ml*	*1 125 000*
T-175	*175 cm^2*	*49 ml*	*2 625 000*

CELL COUNTING

- Take 50 µl of cell suspension in a microtube.
- Add 400 µl of medium to the microtube.
- Take the microtube out of the LAF hood and add 50 µl trypan blue (VWR, LONZ17-942E).
- Gently mix and take 15 µl of the suspension to count.
- Count all death and living cells.

- Repeat step 1-4 one or two times.
- Calculate the mean for death and living cell number.
- Calculate cell yield by dividing the mean living cell number by 40. This is the cell yield in million cells per ml.
- Calculate viability by dividing the mean living cell number by the mean total cell number (living + death). Multiply by 100 to obtain the percentage viability.

MAINTENANCE
Change medium every 2-3 days.

- If needed, take a picture under the inverted microscope and calculate confluence with ImageJ.
- Warm complete growth medium in the water bath (37°C).
- Aspirate growth medium.
- Add the required volume of medium.
- Transfer the flask to the incubator.

SUBCULTURE
Subculture when the cells are 75-85% confluent.

- If needed, take a picture under the inverted microscope and calculate confluence with ImageJ.
- Warm the trypsin (Sigma-Aldrich T3824) and complete growth medium in the water bath (37°C).
- Aspirate growth medium from the flask.

Rinse the cells two or three times with DPBS (Westburg LO BE17-516F/12) (Table 9) using a gentle rocking motion and aspirate.

- Add appropriate volume of 0.05% (w/v) trypsin-EDTA solution to the flask (Table 9). Make sure that the entire surface is covered by a small layer of trypsin-EDTA solution.
- Transfer the flask to the incubator (37°C) until the cells round up (observe under microscope). This takes about 20 minutes. The cells should slide down the surface. Do not leave trypsin-EDTA on the cells any longer than necessary.
- Add at least an equal volume of complete growth medium to the flask to neutralize the trypsin-EDTA cell suspension. Disperse the cells by gentle pipetting (10 times) until a single cell suspension is obtained. Be careful to avoid the creation of foam.
- Transfer the cells to a 50 ml conical tube.
- Rinse the plate with medium and add to the same 50 ml conical tube.
- Centrifuge 5 min at 150g at room temperature.
- Aspirate the medium, leaving the pellet undisturbed. Resuspend in 1 ml growth medium by gentle tapping the bottom of the tube, and if necessary, pipetting up and down (10 times). Make sure that no clumps remain.
- Count the cells (see cell counting).
- Add the required volumes of full growth medium to seed in a density of 15 000 cells/cm2 (Table 8).
- Transfer the cell suspension into new flasks. Resuspend each time before seeding a flask.
- Label the flask.
- Transfer the flask to the incubator.

Table 9 DPBS and trypsin-EDTA volumes for subculture

Flask	***DPBS***	***0.05% Trypsin-EDTA***
T-25	*3 ml*	*1 ml*
T-75	*9 ml*	*3 ml*
T-175	*21 ml*	*21 ml*

CRYOPRESERVATION
One day in advance, check the isopropanol level. Add isopropanol if necessary. Place the container

in the fridge at 2-8°C.
Cryopreserve when the cells are 75-85% confluent.

- If needed, take a picture under the microscope and calculate confluence with ImageJ.
- Warm trypsin and complete growth medium in the water bath (37°C).
- Aspirate growth medium from the flask.
- Rinse the cells two or three times with DPBS (Table 9) using a gentle rocking motion and aspirate.
- Add appropriate volume of 0.05% (w/v) trypsin-EDTA solution to the flask. Make sure that the entire surface is covered by a small layer of trypsin-EDTA solution.
- Transfer the flask to the incubator (37°C) until the cells round up (observe under microscope). This take about 20 minutes. The cells should slide down the surface. Do not leave trypsin-EDTA on the cells any longer than necessary.
- Add at least an equal volume of complete growth medium to the flask to neutralize the trypsin-EDTA cell suspension. Disperse the cells by gentle pipetting (10 times) until a single cell suspension is obtained. Be careful to avoid the creation of foam.
- Transfer the cells to a 50 ml conical tube.
- Rinse the plate with medium and add to the same 50 ml conical tube.
- Centrifuge 5 min at 150 g at room temperature.
- Aspirate the medium, leaving the pellet undisturbed.
- Resuspend in 1 ml growth medium by gentle tapping the bottom of the tube, and if necessary pipetting up and down (10 times). Make sure that no clumps remain.
- Count the cells (see cell counting).
- Calculate the number of vials for cryopreservation in a density of 1.5 million cells per vial.
- Calculate the required volumes of full growth medium, DMSO (Acros Organics 167850010) and heat inactivated horse serum for cryopreservation based on the number of vials (Table 10).
- Add the required volume of full growth medium (remind that you already added 1 ml of full growth medium)
- Mix the required volume (additional excess of about 10 % for filtration on top of the volumes in Table 10) of DMSO and heat inactivated horse serum.
- Filter the mix of DMSO and heat inactivated horse serum through a 0.2 µm filter.
- Add the required volume of the filtered DMSO and heat inactivated horse serum (Table 10).
- Pipet 1 ml of the suspension per vial.
- Label the vials.
- Transfer the vials to the isopropranol box.
- Put the isopropranol box at -80°C.

One day later, put the vials in the liquid nitrogen.

Table 10 Cryopreservation medium

Component	***Percentage***	***Volume per vial*** *(10% excess for loss by pipetting etc.)*
Full growth medium	*/*	*495 µl*
Heat inactivated horse serum	*50 %*	*550 µl*
DMSO	*5 %*	*55 µl*

PMC42-LA expansion

PMC42-LA cells were bought from Sigma-Aldrich (SCC139). The lot number was Q2920330.

GENERAL

All actions should be performed in the LAF, unless specified otherwise.

ABBREVIATIONS

RPMI-1640	Roswell Park Memorial Institute culture medium
DMSO	Dimethyl Sulfoxide
DPBS	Dulbecco's phosphate-buffered saline (buffer solution)
EDTA	Ethylenediaminetetraacetic acid
FBS	Fetal bovine serum
g	gravitational acceleration
LAF	Laminar Air Flow
PMC42-LA	Human breast cancer cell line
P/S	Penicillin and Streptomycin (antibiotic against infections)
RT	Room Temperature

PREPARATION OF FULL GROWTH MEDIUM

- Add 55.56 ml FBS (Lonza, BO 04-007-1A) and 5.5 ml P/S (Lonza DE17-602E/12 to a bottle of 500 ml RPMI-1640 (Sigma-Aldrich R8758) and mix. Filter.
- Label the bottle.
- Store the bottle at 4°C.

INITIATION OF CULTURE

- Warm complete growth medium in the water bath (37°C).
- Obtain 1 PMC42-LA vial from liquid nitrogen.
- Thaw the vial by gentle agitation in the water bath (37°C).
  Remark: Cell viability is dependent on the rapid and complete thawing of frozen cells.
- Transfer the content of the vial to a 50 ml conical tube.
- Rinse the vial with 1 ml medium and add dropwise to the 50 ml conical tube.
- Add 8 ml medium dropwise to the conical tube.
  Remark: do not add the entire volume of medium at once, as this might result in decreased cell viability due to osmotic shock.
- Gently resuspend by pipetting up and down twice.
- Centrifuge 3 min at 300 g at RT.
- Aspirate the medium, leaving the pellet undisturbed.
- Add 1 ml medium. Gently resuspend. If necessary, pipet up and down to resuspend.
- Count the cells (see cell counting).
- Add the required volume of medium (to obtain 15 ml in total for a T75).
- Seed in the suspension in a T-75 flask.
- Label the flask.
- Transfer the flask to the incubator.
- The next day, change the medium with 15 ml prewarmed medium.

Table 11 Medium volume and seeding density per flask

Flask	***Area***	***Volume medium***	***Cells for seeding***
T-25	*25 cm^2*	*5 ml*	*333 333*
T-75	*75 cm^2*	*15 ml*	*1 000 000*
T-175	*175 cm^2*	*35 ml*	*2 333 333*

CELL COUNTING

1. Take 50 µl of cell suspension in an Eppendorf tube.
2. Add 400 µl of medium to the Eppendorf tube.
3. Take the Eppendorf tube out of the LAF and add 50 µl trypan blue (VWR, LONZ17-942E).
4. Gently mix and take 15 µl of the suspension to count.

5. Count all death and living cells.
6. Repeat step 1-4 one or two times.
7. Calculate the mean for death and living cell number.
8. Calculate cell yield by dividing the mean living cell number by 40. This is the cell yield in million cells per ml.
9. Calculate viability by dividing the mean living cell number by the mean total cell number (living + death). Multiply by 100 to obtain the percentage viability.

MAINTENANCE
Change medium every 2-3 days.

1. If needed, take a picture under the microscope and calculate confluence with ImageJ.
2. Warm complete growth medium in the water bath (37°C).
3. Aspirate growth medium.
4. Add the required volume of medium.
5. Transfer the flask to the incubator.

SUBCULTURE
Subculture when the cells are confluent.

1. If needed, take a picture under the microscope and calculate confluence with ImageJ.
2. Warm trypsin (Sigma-Aldrich T3824) and complete growth medium in the water bath (37°C).
3. Aspirate growth medium from the flask.
4. Rinse the cells two or three times with D-PBS (Westburg LO BE17-516F/12) (Table 12) using a gentle rocking motion and aspirate.
5. Add appropriate volume of 0.25% (w/v) trypsin-EDTA solution to the flask (Table 12). Make sure that the entire surface is covered by a small layer of trypsin-EDTA solution.
6. Transfer the flask to the incubator (37°C) until the cells round up (observe under microscope). This take about 3-5 minutes. The cells should slide down the surface. Do not leave trypsin-EDTA on the cells any longer than necessary.
7. Ensure complete detachment of cells by gently tapping the side of the flask with the palm of your hand.
8. Add at least an equal volume of complete growth medium to the flask to neutralize the trypsin-EDTA cell suspension.
9. Transfer the cells to a 50 ml conical tube.
10. Rinse the plate with medium and add to the same 50 ml conical tube.
11. Centrifuge 3-5 min at 300 g at room temperature.
12. Aspirate the medium, leaving the pellet undisturbed.
13. Resuspend in 1 ml growth medium by gentle tapping the bottom of the tube, and if necessary, pipetting up and down. Make sure that no clumps remain.
14. Count the cells (see cell counting).
15. Add the required volumes of full growth medium to seed in the desired density of cells/cm^2 (Table 11). Split ratio is typically 1:8 to 1:10
16. Transfer the cell suspension into new flasks. Resuspend each time before seeding a flask.
17. Label the flask.
18. Transfer the flask to the incubator.

Table 12 D-PBS and trypsin-EDTA volumes for subculture

Flask	***DPBS***	***0.05% Trypsin-EDTA***
T-25	*3 ml*	*1 ml*
T-75	*9 ml*	*3 ml*
T-175	*21 ml*	*21 ml*

CRYOPRESERVATION
One day in advance, check the isopropranol level. Add isopropranol if necessary. Place the container in the fridge at 2-8°C.

Cryopreserve when the cells are confluent. Up to 18 vials can be cryopreserved in one time.

1. If needed, take a picture under the microscope and calculate confluence with ImageJ (see SOP 26).
2. Warm trypsin and complete growth medium in the water bath (37°C).
3. Aspirate growth medium from the flask.
4. Rinse the cells two or three times with DPBS (Table 12) using a gentle rocking motion and aspirate.
5. Add appropriate volume of 0.25% (w/v) trypsin-EDTA solution to the flask (Table 12). Make sure that the entire surface is covered by a small layer of trypsin-EDTA solution.
6. Transfer the flask to the incubator (37°C) until the cells round up (observe under microscope). This take about 3-5 minutes. The cells should slide down the surface. Do not leave trypsin-EDTA on the cells any longer than necessary.
7. Ensure complete detachment of cells by gently tapping the side of the flask with the palm of your hand.
8. Add at least an equal volume of complete growth medium to the flask to neutralize the trypsin-EDTA cell suspension.
9. Transfer the cells to a 50 ml conical tube.
10. Rinse the plate with medium and add to the same 50 ml conical tube.
11. Centrifuge 3-5 min at 300 g at room temperature.
12. Aspirate the medium, leaving the pellet undisturbed.
13. Resuspend in 1 ml growth medium by gentle tapping the bottom of the tube, and if necessary, pipetting up and down. Make sure that no clumps remain.
14. Count the cells (see cell counting).
15. Calculate the number of vials for cryopreservation in a density of 1 10^6 cells per vial.
16. Calculate the required volumes full growth medium, and DMSO for cryopreservation based on the number of vials (Table 13).
17. Mix the required volume (additional excess of about 10 % for filtration on top of the volumes in Table 13) of DMSO and growth medium (remember that you already added 1 ml of growth medium).
18. Filter the mix of DMSO and medium through a 0.2 µm filter.
19. Add the required volume of the filtered DMSO and medium (Table 13).
20. Pipet 1 ml of the suspension per vial.
21. Label the vials.
22. Transfer the vials to the isopropranol box.
23. Put the isopropranol box at -80°C.

One day later, put the vials in the liquid nitrogen.

Table 13 Cryopreservation medium

Component	***Percentage***	***Volume per vial*** *(10% excess for loss by pipetting etc.)*
Full growth medium	*/*	*900 µl*
DMSO	*10 %*	*100 µl*

Morphology

Cell Morphology was routinely checked using inverted optical microscope equipped with a digital camera (Eclipse600, Nikon, Japan) at the university of Bologna or an Olympus IX70 microscope with either VisiCam 3.0, VWR International or ToupCam, Touptek Photonics. Representative images were captured at each notable stage of isolation and subsequent expansion of pMECs, HMECs or human cell lines (MCF-10A, MCF-7 and PMC42-LA). Furthermore, typical morphology of the human cell lines can be found in the manufacturer's certificates (ATCC for MCF-7 and MCF-10A or Sigma-Aldrich for PMC42-LA).

Doubling time and cell cycle

pMECs and HMECs doubling time (DT) among passages was calculated by an internal protocol for primary cell [8]. Briefly, for each passage as DT=h/CD, where h was the culture time (in hours) between two passages and CD was the cell doubling calculated as $CD=(\log_{10}N-\log_{10}N_0)/\log_{10}2$, where N is the number of cells at 80–90% confluency and N_0 is the number of cells seeded lines.
pMECs cell cycle analysis was evaluated by an internal protocol previously described [9]. Briefly, cells were harvested and counted. Aliquots of 1×10^6 cells were washed twice in 5 ml of PBS Ca^{2+} and Mg^{2+} free (Gibco- Life Technologies) and fixed overnight in 70% ice-cold ethanol (1 ml) added drop-by-drop with continuous vortexing. Then, the cells were washed with 10 ml of PBS and cellular pellet was incubated with 1 ml of staining solution containing 50 µg/ml of PI (Miltenyi Biotec, Bergisch Gladbach, Germany) and 100 µg/ml RNaseA/T1 (Thermo Sci- entific) in PBS for 30 min in the dark at room temperature (RT). Cell distribution in cell cycle phases was analyzed by MACSQuant® Analyzer10 and Flowlogic software (Miltenyi Biotec). Cellular events were discriminated from debris using forward (FSC-A) and side scatter (SSC-A). Doublets have been excluded for analysis by FSC-area versus width (FSC-A/FSC-W). Fox Synchronous Model was used to determine the percentage of the cell population in different phases of the cell cycle.
Population doubling time for the cell lines was taken from the manufacturer's documentation.

Immunophenotyping

To confirm the epithelial origin of pMECs Flow Cytometric analysis of epithelial-cadherin and immunofluorescence of tight junction's proteins Zonula Occludens-1 (ZO-1) and Occludin (OCL) were made.
For HMECs at the end of expansion phase, the expression of E-Cadherin was checked by Flow Cytometric analysis.
The antibodies used were reported in the Table 14, for each one the appropriate dilution was set in preliminary experiments.

Table 14 Antibodies used for the immunofluorescence and Flow Cytometric analysis

Antibody	P. Number	Species	Supplier
Anti ZO-1	61-7300	Rabbit	Thermo Fisher
Anti OCL	H-279	Rabbit	Santa Cruz Biotechnology
Anti Rabbit IgG-Alexa Fluor 488	A11034	Goat	Thermo Fisher
Anti Mouse IgG-Alexa Fluor 594	A11032	Goat	Thermo Fisher
Brilliant Violet 421™ anti-E-cadherin	147319	Rat monoclonal	BioLegend

For immunofluorescence analysis, cells were cultured on slide chambers, fixed and permeabilized following an internal protocol as previously described [10].

For Flow Cytometry analysis an appropriate protocol previously optimized for other cells were followed with some modifications [11]. In particular: 3 x 10^5 pMECs and HMECs were fixed in 4% paraformaldehyde for 20 min at RT (300 µl) and permeabilized in absolute methanol, pre-cooled at -20°C, for 20 min at 4°C (500 µl). Then cells were washed in DPBS, suspended in 100 µl DPBS and incubated overnight at 4°C in the dark with anti-E-cadherin antibody (Table 14). Negative controls, to

evaluate inherent background or auto fluorescence, were obtained omitting primary antibodies. After incubation, cells were washed once and suspended in 300 µl of DPBS then acquired with MacsQuant Analyzer10 (Milteyi Biotec)

Characteristics for the human cell lines were taken from the manufacturer's documentation.

Barrier functional studies

The barrier function was evaluated via transepithelial electrical resistance (TEER) and fluorescein sodium transport. TEER is a well-established method to evaluate the integrity of epithelial monolayers via measurement of the ionic conductance of the paracellular pathway [12], whereas fluorescein sodium is used to measure the passive paracellular transport [13]. Paracellular transport implies the movement of solutes (including for instance ions and small hydrophilic medicines) via the aqueous channels that are present between adjacent cells that form a monolayers. Cells were seeded on permeable supports. The transwells were incubated at 37°C (hMECs and human cell lines) or 38.5 °C (pMECs), 5% CO_2. The barrier function was evaluated measuring TEER in function of time and sodium fluorescein transport every other day.
Resistance (R) was measured in medium or transport buffer at 37°C or 38.5 °C, using an Epithelial Volt/Ohm Meter 2 (EVOM2, World Precision Instruments). Transport buffer consisted of HBSS (Lonza) (with addition of 10 mM HEPES (VWR International) and 25 mM glucose (Thermo Fisher Scientific). TEER was calculated using equation 1, where R_{total} is the measured resistance, $R_{mean\ blank}$ is the mean resistance of the blank insert for each day and M_{area} is the surface area of the membrane.

$$TEER\wedge(\Omega * cm^2) = (R_{total}(\Omega) - R_{mean\ blank}(\Omega)) * M_{area}(cm^2) \text{ (Eq. 1)}$$

Sodium fluorescein leakage was determined. 0.5 ml sodium fluorescein (2.66 mM) and 1.2 ml of transport buffer, respectively, were added to the apical and basal compartment. After 1 h incubation at 37 °C under gentle shaking, samples were taken from the basal compartment. The concentration of sodium fluorescein in the samples was measured based on relative intensities determined via fluorescence spectrometry (λ=490/524 nm) with a Tecan Infinite M200 plate reader (Tecan Group Ltd., Männedorf, Austria).
The percentage of sodium fluorescein transport was calculated using equation 2, where Csample is the concentration of sodium fluorescein in the sample, C0 is the donor concentration of sodium fluorescein, Vb is the volume of the basal compartment and Va is the volume of the apical compartment.

$$Transport\ (\%) = \frac{C_{sample}}{C_0} * \frac{V_b}{V_a} * 100 \text{ (Eq. 2)}$$

The barrier function was tested for different parameters, including:

- Medium composition (e.g. serum concentration; addition/removal of compounds like beta-estradiol, cholera toxin or prolactin)
- Seeding density
- Passage number
- Time between medium change
- Technique for medium change
- Permeable support material (PET or PC)
- Permeable support pore size

- Permeable support area

Transporter mRNA expression

The characterization of RNA levels of transporter genes in the mammary epithelial cells may identify a potential list of candidate transporters involved in the transepithelial transport of medicines and xenobiotics in the lactating mammary gland. To do this, we utilize for primary culture of pMECs MG2, MG3 and MG8 (passage 10) and HMEC (passage 10), a RT^2 Profiler PCR Array approach. As the aim of the study was to evaluate a comparison of transporters between pMECs and HMEC, we cultured the pMEC cells either in their specific expansion media and in media used for HMEC to evaluate possible medium interference.

RT^2 Profiler PCR Arrays (96 well format, Qiagen, Hilden, Germany) are designed to analyze simultaneously a panel of genes related to pathological or physiological pathway. RT^2 Profiler PCR plates contain primer assays for 84 genes and 5 housekeeping genes; in addition, one well contains a genomic DNA control, reverse transcription controls and positive PCR controls (

Figure 8).

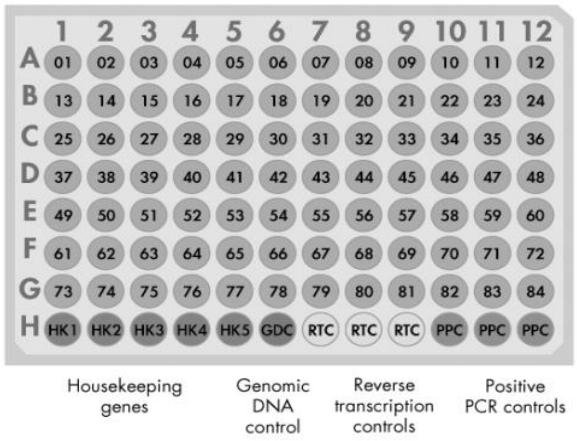


Figure 8 RT2 Profiler PCR Array Format: wells A1-G12 contain a real-time PCR assay for a pathway related gene. Well H1 to H5 contain housekeeping genes panel to normalize array data (HK1-5). Well H6 contain a genomic DNA control (GDC). Wells H7 to H9 contain replicate reverse-transcription controls (RTC). Wells H10-to H12 contain replicate positive PCR controls (PPC)

For the human species a panel for transporters was also available (RT² Profiler™ PCR Array Human Drug Transporters, Cod. PAHS-070Z, Qiagen) while, for the same panel for the swine species, it was necessary to request its preparation and now it is commercially available (RT² Profiler™ PCR Array Pig Drug Transporters, Cod PASS-070Z, Qiagen).
The list of gene analyzed and the array layout for pig (

Figure 9) and human (

Figure 10) were respectively:

Gene List Pig Drug Transporters:

ABC Transporters
ABCA1, ABCA13, ABCA3, ABCA4, ABCA5, ABCA9, ABCB1 (MDR1), ABCB11, ABCB4, ABCB6, ABCB7, ABCC1, ABCC10, ABCC12, ABCC2, ABCC3, ABCD1, ABCD2, ABCE1, ABCF1, ABCG2, ABCG8, LOC100154911 (ABCD4), LOC100512445 (ABCA12), LOC100513626 (ABCC5, MRP5), LOC100738425 (ABCC4), SLC47A2.

SLC Transporters
LOC100049683 (SLC7A11), LOC100513513 (SLC16A2), LOC100620829 (SLCO1B3), SLC15A1, SLC15A2, SLC16A1, SLC16A3, SLC17A3, SLC18A1, SLC19A1, SLC19A2, SLC19A3, SLC22A1, SLC22A11, SLC22A2, SLC22A3, SLC22A5, SLC22A6, SLC22A7, SLC22A8, SLC25A13, SLC28A2, SLC29A1, SLC29A2, SLC2A1, SLC2A2, SLC2A3, SLC31A2, SLC38A2, SLC38A5, SLC3A1, SLC3A2, SLC5A1, SLC5A4, SLC7A1, SLC7A10, SLC7A2, SLC7A3, SLC7A4, SLC7A7, SLC7A8, SLC7A9, SLCO1A2, SLCO2A1, SLCO2B1, SLCO3A1.

Other Transporters
Aquaporins AQP1, AQP7, AQP9.
Copper Pumps ATP7A, ATP7B, CTR1, LOC100622399 (SLC31A1).
Major Vault Protein MVP.
Vacuolar H+-ATPases ATP6V0C.
Voltage-Dependent Anion Channels VDAC1, VDAC2.

Array Layout Pig Transporters:

	1	2	3	4	5	6	7	8	9	10	11	12
A	ABCA1	ABCA13	ABCA3	ABCA4	ABCA5	ABCA9	ABCB1	ABCB11	ABCB4	ABCB6	ABCB7	ABCC1
B	ABCC10	ABCC12	ABCC2	ABCC3	ABCD1	ABCD2	ABCE1	ABCF1	ABCG2	ABCG8	AQP1	AQP7
C	AQP9	ATP6V0C	ATP7A	ATP7B	CTR1	LOC100049683	LOC100154911	LOC100512445	LOC100513513	LOC100513626	LOC100620829	LOC100622399
D	LOC100738425	MVP	SLC15A1	SLC15A2	SLC16A1	SLC16A3	SLC17A3	SLC18A1	SLC19A1	SLC19A2	SLC19A3	SLC22A1
E	SLC22A11	SLC22A2	SLC22A3	SLC22A5	SLC22A6	SLC22A7	SLC22A8	SLC25A13	SLC28A2	SLC29A1	SLC29A2	SLC2A1
F	SLC2A2	SLC2A3	SLC31A2	SLC38A2	SLC38A5	SLC3A1	SLC3A2	SLC47A2	SLC5A1	SLC5A4	SLC7A1	SLC7A10
G	SLC7A2	SLC7A3	SLC7A4	SLC7A7	SLC7A8	SLC7A9	SLCO1A2	SLCO2A1	SLCO2B1	SLCO3A1	VDAC1	VDAC2
H	ACTB	B2M	GAPDH	HPRT1	RPL13A	SGDC	RTC	RTC	RTC	PPC	PPC	PPC

Figure 9 Array Layout Pig Transporters

Gene List Human Drug Transporters

ABC Transporters
ABCA1, ABCA13, ABCA3, ABCA4, ABCA5, ABCA9, ABCB1 (MDR1), ABCB11, ABCB4, ABCB6, ABCB7, ABCC1, ABCC10, ABCC12, ABCC2, ABCC3, ABCD1, ABCD2, ABCE1, ABCF1, ABCG2, ABCG8, LOC100154911 (ABCD4), LOC100512445 (ABCA12), LOC100513626 (ABCC5, MRP5), LOC100738425 (ABCC4), SLC47A2.

SLC Transporters
LOC100049683 (SLC7A11), LOC100513513 (SLC16A2), LOC100620829 (SLCO1B3), SLC15A1, SLC15A2, SLC16A1, SLC16A3, SLC17A3, SLC18A1, SLC19A1, SLC19A2, SLC19A3, SLC22A1, SLC22A11, SLC22A2, SLC22A3, SLC22A5, SLC22A6, SLC22A7, SLC22A8, SLC25A13, SLC28A2, SLC29A1, SLC29A2, SLC2A1, SLC2A2, SLC2A3, SLC31A2, SLC38A2, SLC38A5, SLC3A1, SLC3A2, SLC5A1, SLC5A4, SLC7A1, SLC7A10, SLC7A2, SLC7A3, SLC7A4, SLC7A7, SLC7A8, SLC7A9, SLCO1A2, SLCO2A1, SLCO2B1, SLCO3A1.

Other Transporters
Aquaporins AQP1, AQP7, AQP9.
Copper Pumps ATP7A, ATP7B, CTR1, LOC100622399 (SLC31A1).
Major Vault Protein MVP.

Vacuolar H+-ATPasesATP6V0C.
Voltage-Dependent Anion ChannelsVDAC1, VDAC2.

Array Layout Human Drug Transporters:

	1	2	3	4	5	6	7	8	9	10	11	12
A	ABCA1	ABCA12	ABCA13	ABCA2	ABCA3	ABCA4	ABCA5	ABCA9	ABCB1	ABCB11	ABCB4	ABCB5
B	ABCB6	ABCC1	ABCC10	ABCC11	ABCC12	ABCC2	ABCC3	ABCC4	ABCC5	ABCD1	ABCD3	ABCD4
C	ABCF1	ABCG2	ABCG8	AQP1	AQP7	AQP9	ATP6V0C	ATP7A	ATP7B	MVP	SLC10A1	SLC10A2
D	SLC15A1	SLC15A2	SLC16A1	SLC16A2	SLC16A3	SLC19A1	SLC19A2	SLC19A3	SLC22A1	SLC22A2	SLC22A3	SLC22A6
E	SLC22A7	SLC22A8	SLC22A9	SLC25A13	SLC28A1	SLC28A2	SLC28A3	SLC29A1	SLC29A2	SLC2A1	SLC2A2	SLC2A3
F	SLC31A1	SLC38A2	SLC38A5	SLC3A1	SLC3A2	SLC5A1	SLC5A4	SLC7A11	SLC7A5	SLC7A6	SLC7A7	SLC7A8
G	SLC7A9	SLCO1A2	SLCO1B1	SLCO1B3	SLCO2A1	SLCO2B1	SLCO3A1	SLCO4A1	TAP1	TAP2	VDAC1	VDAC2
H	ACTB	B2M	GAPDH	HPRT1	RPLP0	HGDC	RTC	RTC	RTC	PPC	PPC	PPC

Figure 10 Array Layout Human Drug Transporters

A total RNA extraction from the three pMECs lines, cultured in two different media (media for pMECs or media for HMEC) and from the HMEC (P10) was performed.
Cells (0.5 x 10^6) were harvested and lysed using 1 ml TRIzol® Reagent (Fisher Molecular Biology, Trevose, USA) and, after incubation for 10 min at RT a volume of 200 µl of chloroform was added to the suspension and mixed well.
After incubation at RT (10 min), samples were centrifuged (12000 × *g* for 10 min) and the aqueous phase was recovered.
An equal volume of ethanol (70%) was added, and the resulting solution was applied to a NucleoSpin RNA Column (NucleoSpin®, Macherey-Nagel GmbH & Co, Düren, Deutschland) and RNA was purified according to the manufacturer's instructions and eluted in 40µl of nuclease free H_2O.
After nanospectrophotometric quantification (A260), the RNA integrity was confirmed by electrophoresis run on agarose gel (1%, buffer TBE1X). Each RNA sample (0,5 µg) was then added with genomic DNA elimination mix, then, the first strand cDNA synthesis and mastermixes containing Sybr Green (Qiagen) were prepared.
The mastermix, was then displaced into RT2 Profiler PCR array and real time PCR was run (PCR: 10' at 95°C; 15'' at 95 °C and 1' at 60°C, 40 cycles) and melting protocol was applied (1' at 95°C; 65°C to 95°C increasing temperature 0.5°C/min) into CFX96 Touch Real-Time PCR Detection System (Bio-Rad Laboratories Inc. Hercules, California, USA).

Results

pMECs Isolation

The development of the pMECs isolation protocol gave rise to the production of SOP BSBT BCM 023. Among the three protocols used for the automatic dissociation only one (*Figure 11*), the 37C_Multi _B program, permitted to isolate single or clustered viable cells (*Figure 11*A, *Figure 11*A' and *Figure 11*A'') while for the other two programs tested only collagen fibers (*Figure 11*B) or cell debris (*Figure 11*C) were present into the obtained suspensions.

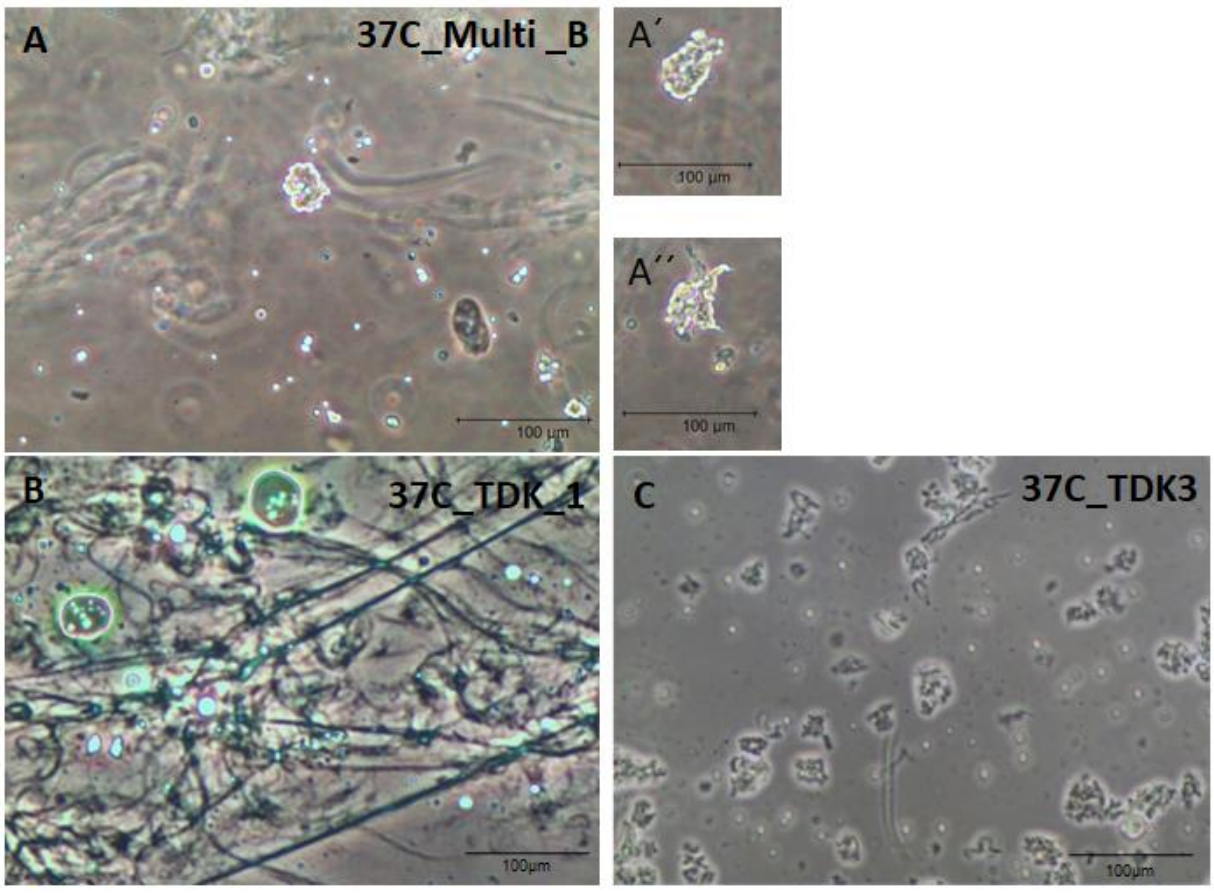


Figure 11 Representative images of the different obtained cellular suspensions after the three automated dissociation protocols (Scale bar = 100 µm)

The protocol developed through the combination of mechanical dissociation and the enzymatic degradation allowed to obtain cells from 5 tissue samples out of six processed. None showed evidence of bacterial or yeast contamination. Moreover, PCR results indicated that the cells are also free from mycoplasma contamination (Figure 12). Overall, this means that the developed tissue disinfection protocol has proven to be effective.

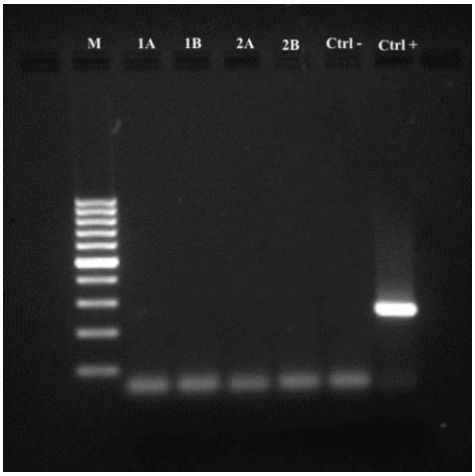


Figure 12 Representative image of the electrophoresis gel performed on PCR products. Lanes 1A-1B-2A-2B correspond to the PCR samples of culture medium of the pMECs; lane M: GeneRuler DNA 100 bp ladder (Thermo Scientific); lane Ctrl-: the negative control, lane Ctrl+: the positive control (270 bp). The same analysis was also carried out for all the other pMECs culture medium samples.

The number of cells isolated varied considerably from sample to sample (Table 15).

Table 15 Number of cells isolated from 1 g of mammary tissue

Sample	Number Cells/gr Tissue
MG2	1.1×10^5
MG3	$2,1 \times 10^5$
MG5	-
MG6	0.4×10^5
MG7	$0,3 \times 10^5$
MG8	$1,1 \times 10^5$

MG3 provided the best yield while MG6 and MG7 showed the lowest ones. MG5 did not provide any cell, on the other hand the histological pattern of MG5 revealed that the adipose tissue component was predominant while the glandular component was essentially absent (Figure 13), in comparison with MG-3, for example, in which glandular, connective and adipose components were present in the expected relative quantities.

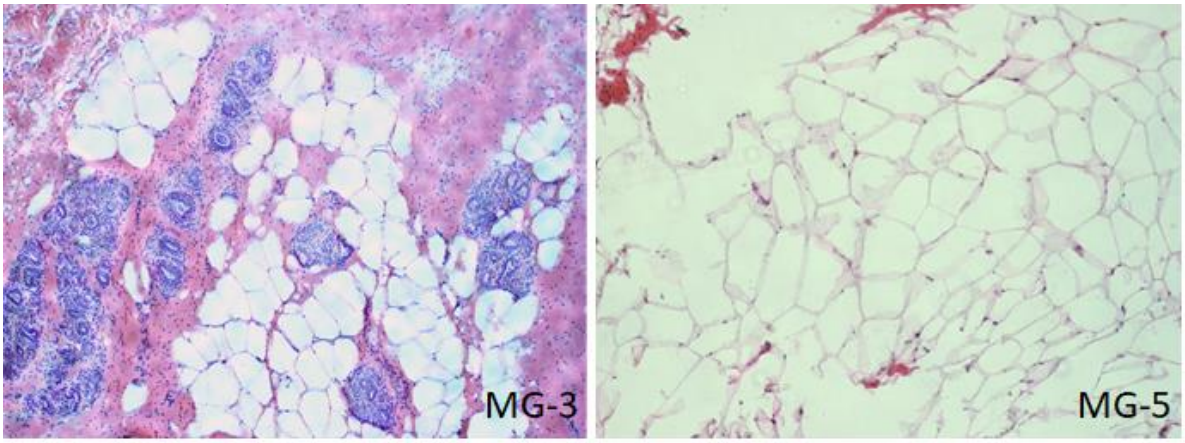


Figure 13 Representative image of mammary tissue histological pattern (10X magnification)

pMECs Expansion

The development of the pMECs isolation protocol gave rise to the production of SOP BSBT BCM 024. Thanks to this protocol, three primary cell cultures were successfully expanded till the tenth passage. The total aliquots produced were 100. No evidence of bacterial/yeast or mycoplasma contamination are evidenced at the end of the expansion cycle.

HMECs Expansion

The development of the HMECs isolation protocol gave rise to the production of SOP BSBT BCM 025.
Thanks to this protocol, primary culture of HMECs was successfully expanded till the tenth passage. The total aliquots produced was 79. No evidence of bacterial/yeast or mycoplasma contamination are evidenced at the end of the expansion cycle.

MCF-7 Expansion

A protocol was developed for the expansion of MCF-7 cells. The passage number of the original vial was not known. Therefore, the first passage of the expansion was referred to as passage 1. MCF-7 cells were expanded in the presence of beta-estradiol for 11 passages. Afterwards, MCF-7 cells were further expanded for at least three passages without beta-estradiol before they were used for experiments.

MCF-10A expansion

A protocol was developed for the expansion of MCF-10A cells. The original MCF-10A vial contained $3*10^6$ cells at P10. The vial was further expanded until P14.

PMC42-LA expansion

A protocol was developed for the expansion of PMC42-LA cells. The vial contained $\geq 1X10^6$ cells. PMC42-LA cells were expanded for 6 passages.

Morphology

All five isolated pMECs cell populations at the end of the first stage of culture showed typical epithelial morphology (

Figure 14).

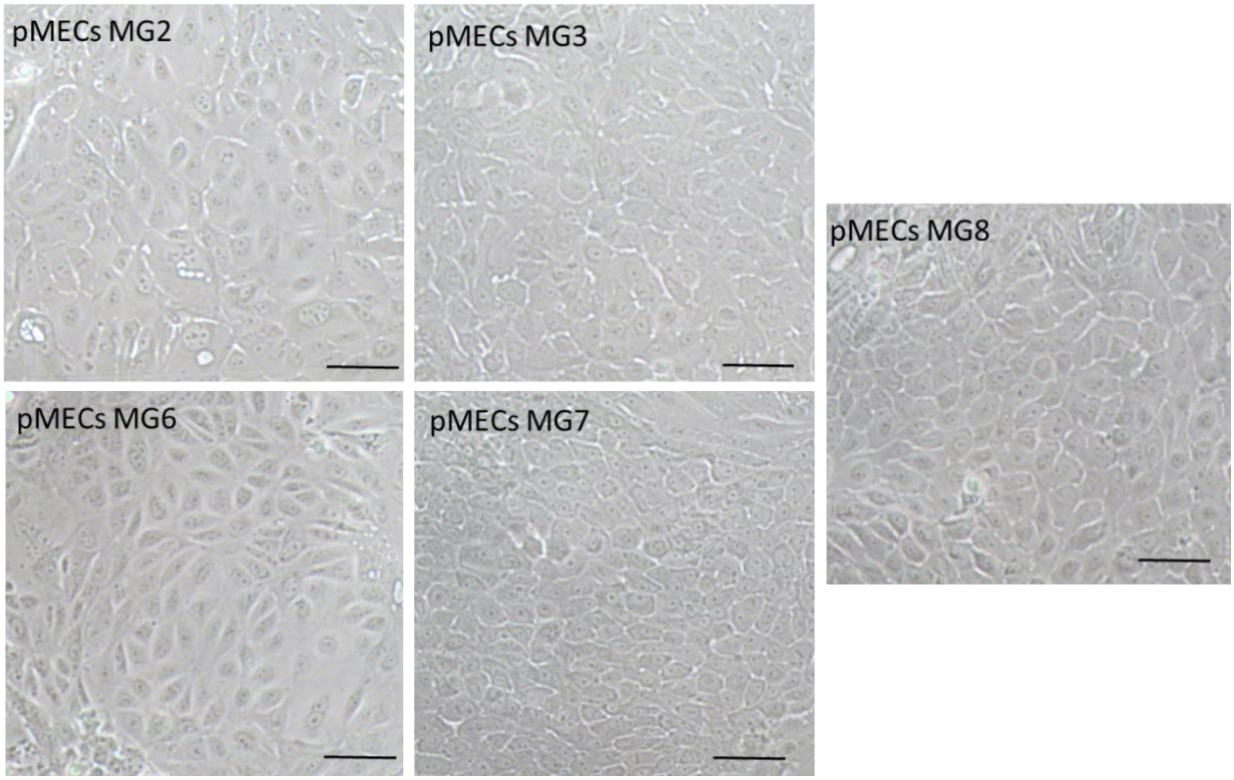


Figure 14 pMECs morphology after the isolation step (Scale bar = 100 µm)

For the expansion phase, three cellular cultures were chosen: pMECs MG2, pMECs MG3 and pMECs MG8. Ten cycles of expansion were successfully completed for all the three cellular cultures, that they showed a typical epithelial morphology (

Figure 15)

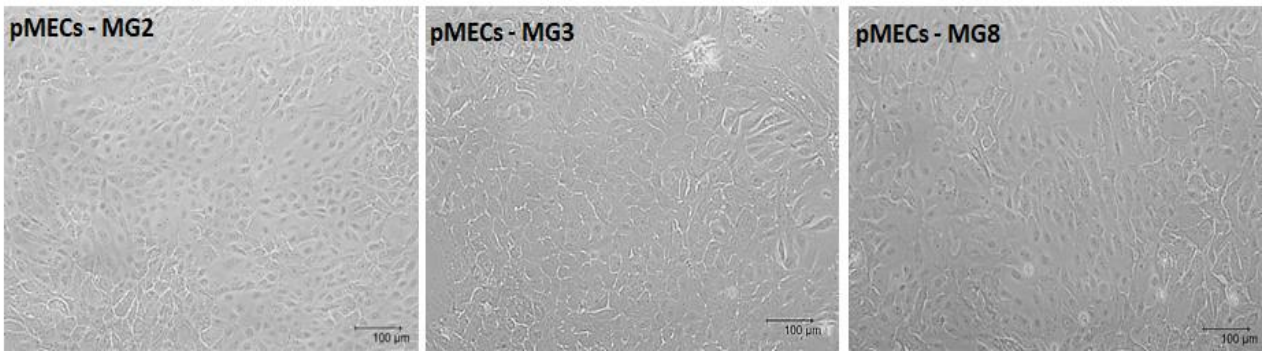


Figure 15 pMECs morphology after the expansion step (Scale bar = 100 µm)

The quality certificates provided by the company that produced HMECs stated that these cells presented a typical epithelial morphology. After the expansion phase, HMECs maintained this characteristic epithelial morphology: they grow as a compact monolayer (

Figure 16).

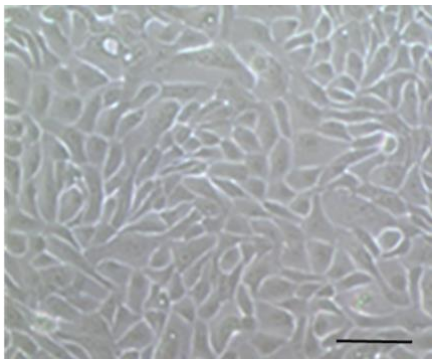


Figure 16 HMECs morphology after the expansion step (Scale bar = 100 µm)

MCF7 is a commonly used breast cancer cell line, with an epithelial-like morphology (Figure 17). MCF-7 is derived from metastatic site: pleural effusion. Figure 18Figure 18 shows the morphology of MCF-7 cells cultured in the presence of beta-estradiol. Figure 19 shows the morphology of the cells after a few passages in medium without beta-estradiol.

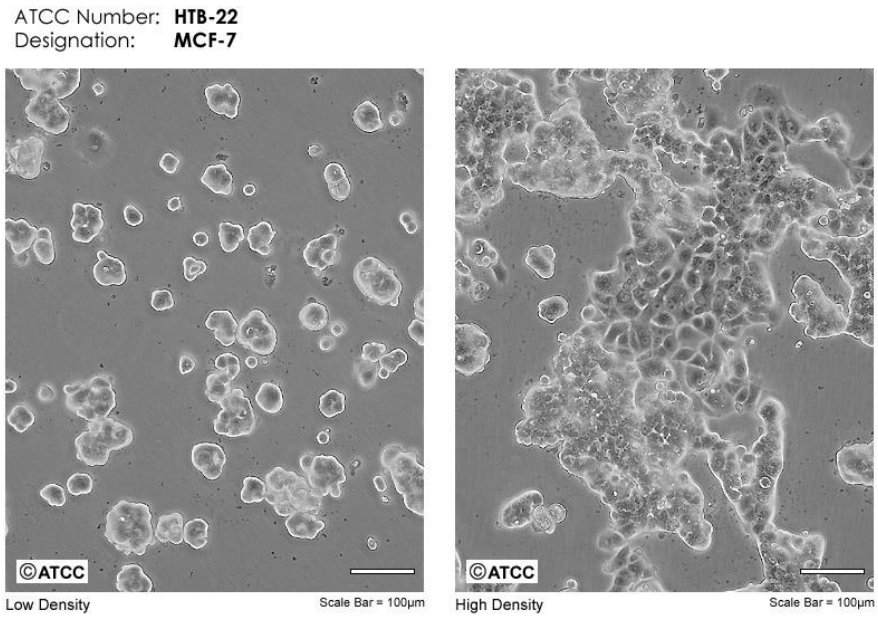


Figure 17 MCF-7 morphology (ATCC)

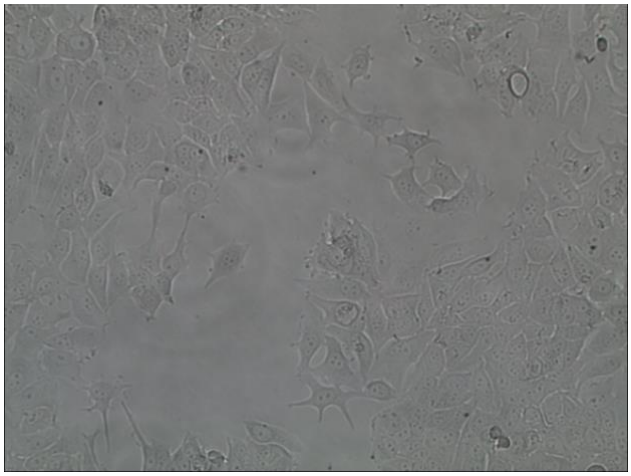


Figure 18 MCF-7 morphology after expansion in the presence of beta-estradiol (magnification 10X)

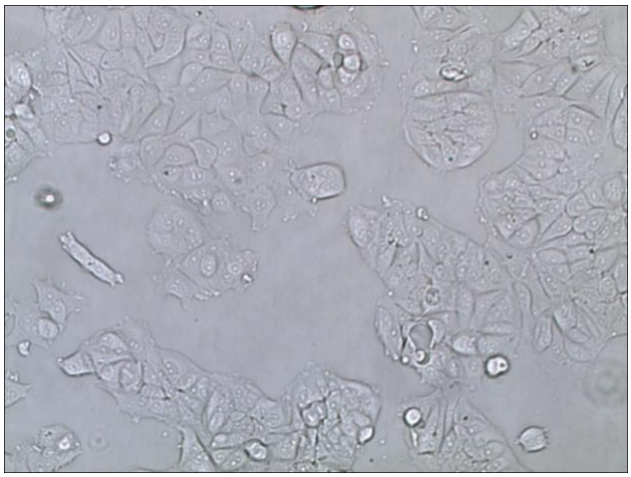


Figure 19 MCF-7 morphology after expansion in the absence of beta-estradiol (magnification 10X)

MCF10A cell line is a non-tumorigenic epithelial cell line. Pictures from ATCC (Figure 20), as well as pictures after expansion (Figure 21) show the cuboidal, epithelial morphology.

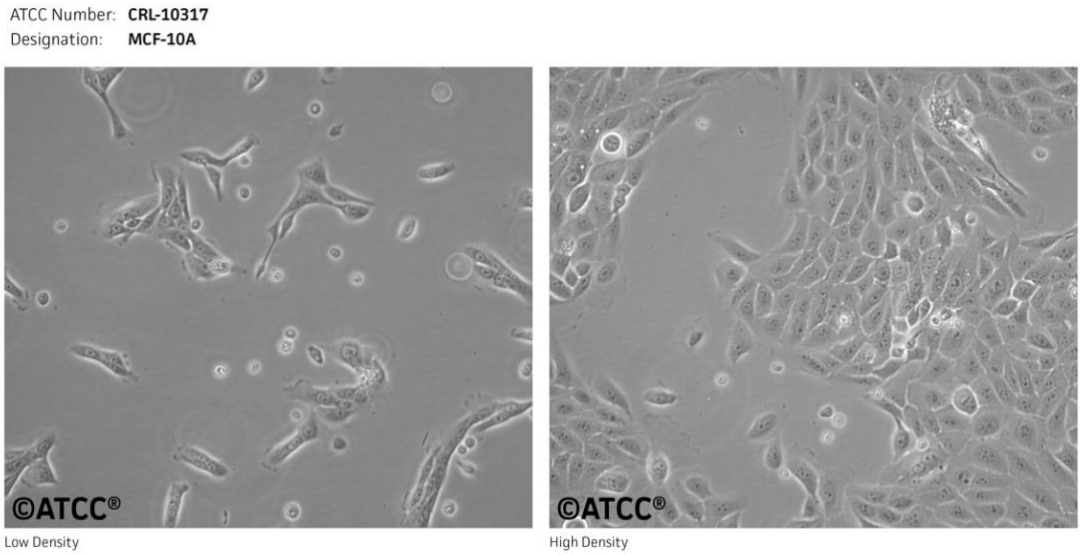


Figure 20 MCF-10A morphology (ATCC)

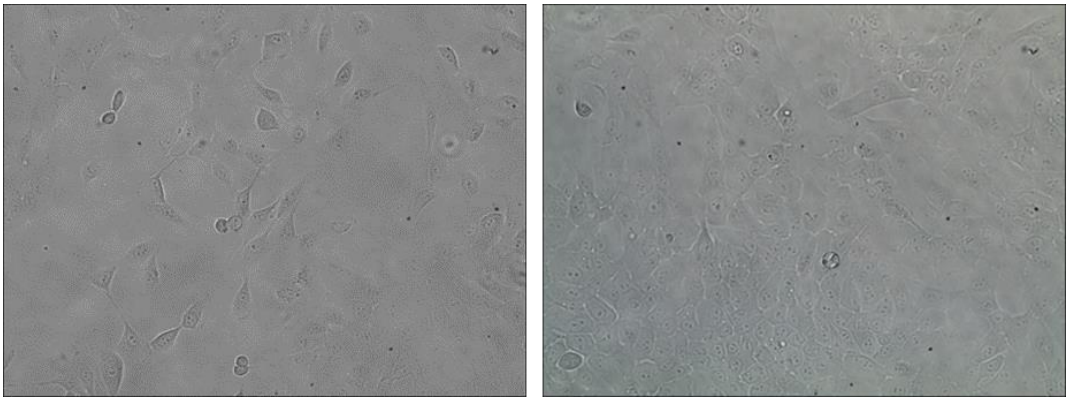


Figure 21 MCF-10A morphology after expansion (magnification 10X)

The certificate from the PMC42-LA cell line states that PMC42-LA is a stable epithelial variant of the mesenchyme-like parental PMC42/PMC42-ET cell line. The certificate also states that stimulation with epithelial growth factor can lead to an epithelial-mesenchymal transition. However, we did not stimulate the cells with EGF. Without EGF stimulation, only 5-10 % of the cells express vimentin. Figure 22 contains pictures from the manufacturer, showing the typical epithelial morphology. The same epithelial morphology is also visible at the beginning and end of the expansion in

Figure 23.

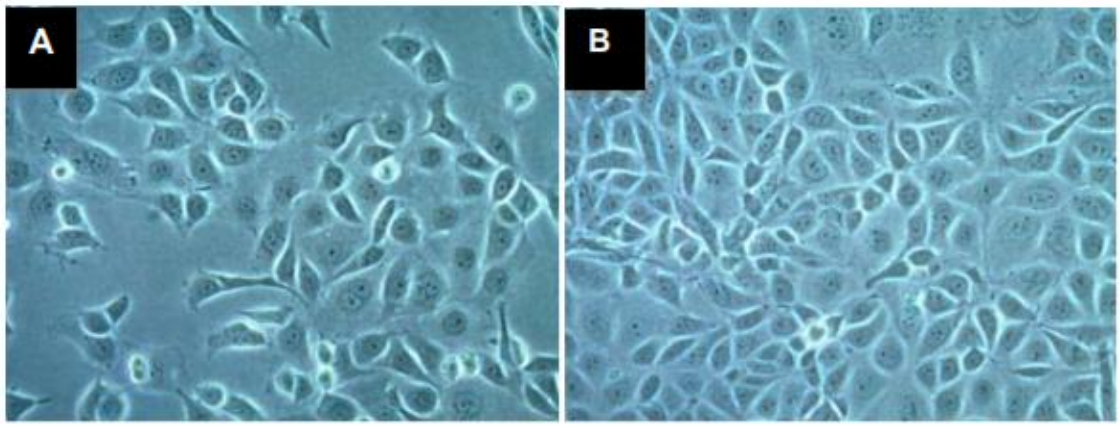


Figure 22 PMC42-LA morphology (Sigma-Aldrich)

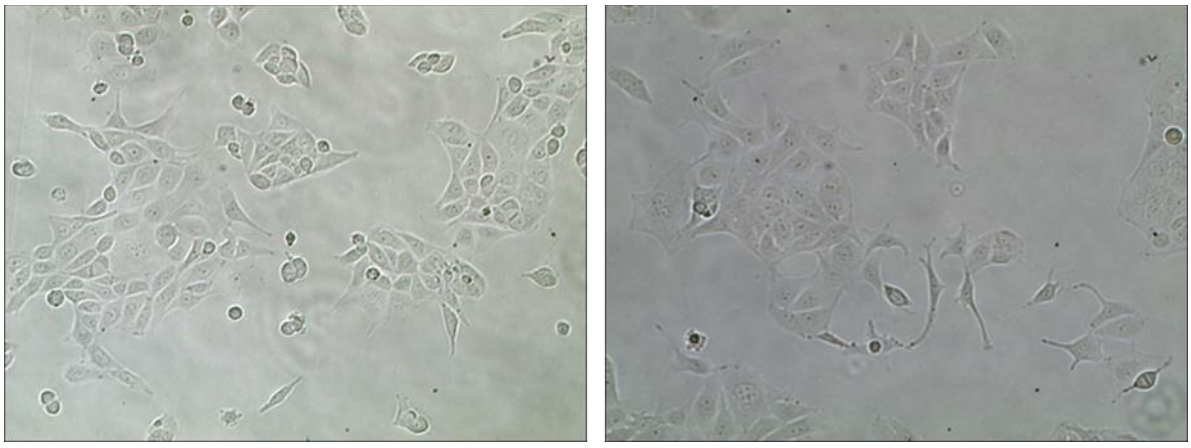


Figure 23 PMC42-LA morphology during expansion (magnification 10X)

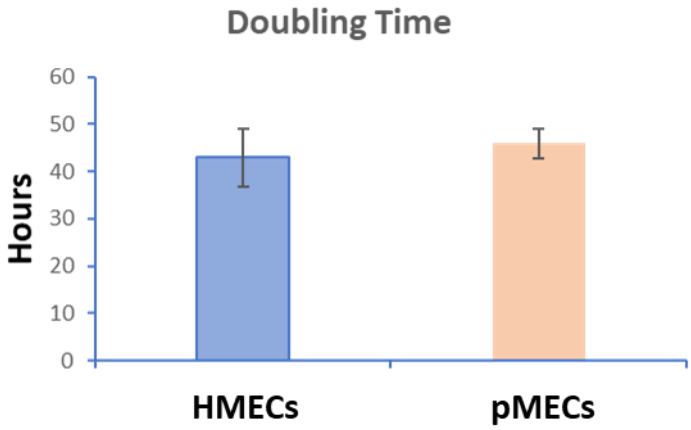
Doubling time and cell cycle

No significative differences exist among the doubling time (DT) of the three pMECs cultures expanded (Table 16).

Table 16 Doubling time (h) for the three pMECs cell cultures. No significant differences were evidenced

pMECs	doubling time
MG2	43 ± 13
MG3	47 ± 14
MG8	49 ± 16

The average DT of HMECs (from 6 to 9 passages) is 43 ± 6 h. The mean of doubling time of the three pMECs cultures expanded did not show significant differences compared to that measured for the



expanded HMECs (
Figure 24). The population doubling time for MCF-7 on the other hand is 29h according to ATCC. Population doubling times for MCF-10A and PMC42-LA were not determined, as further results indicate that they are not suitable for transport experiments (see barrier functional studies).

Doubling Time
Hours
60
50
40
30
20
10
0
HMECs
pMECs

Figure 24 Comparison between HMECs and pMECs doubling time

Cell cycle analysis of pMECs showed the three distinct phases, G0/G1, S and G2/M, that could be recognized in a normal proliferating cell population (

Figure 25).

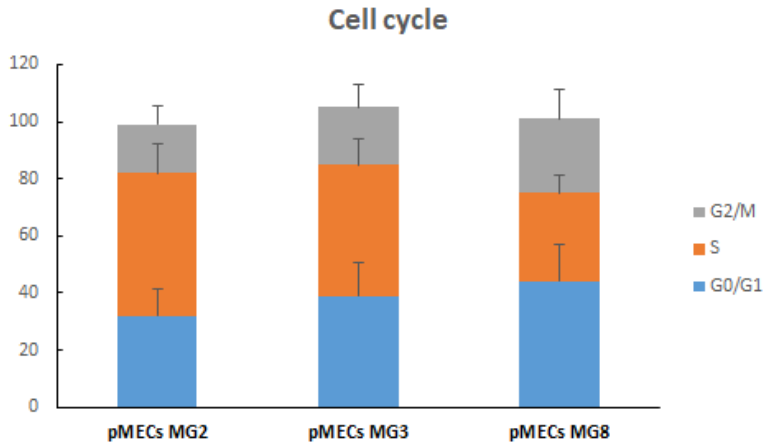


Figure 25 pMECs distribution across cell cycle phases

The quality certificates provided by the company that produced HMECs, stated that these cells are normal, not transformed mammary epithelial cells. MCF-10A is a non-tumorigenic epithelial cell line. MCF-7 is a breast cancer cell line. PMC42-LA is the stable epithelial variant of the mesenchyme-like breast carcinoma cell line PMC42/PMC42-ET.

Immunophenotyping

All the three pMECs cellular lines expressed epithelial markers E-Cadherin confirming their epithelial origin, as well as HMECs showed E-Cadherin expression after the expansion phase. (Figure 26).

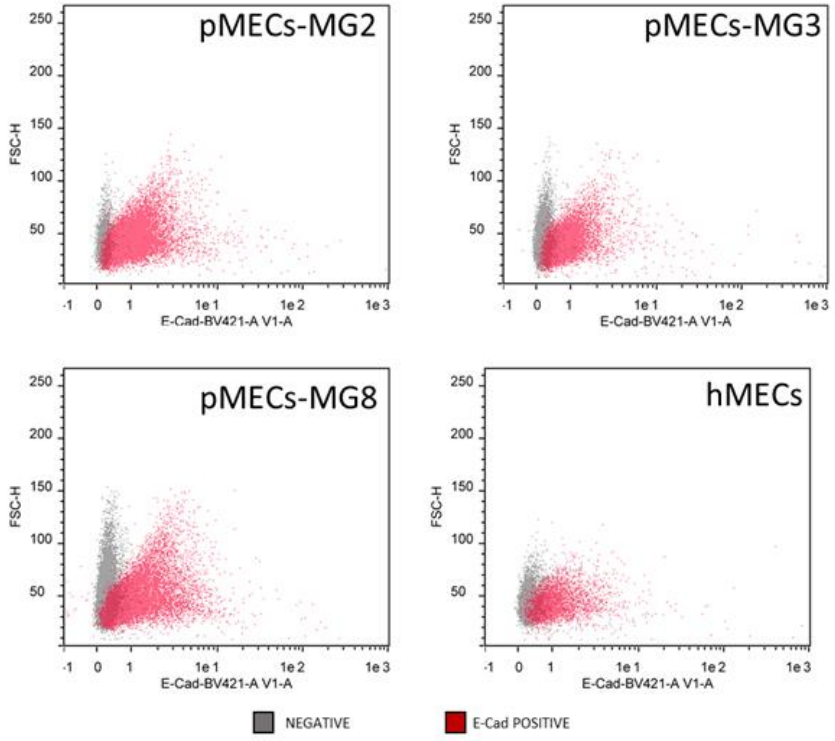


Figure 26 Flow Cytometric analysis of epithelial marker E-cadherin in pMECs and hMECs at the end of the expansion cycle.

To better validate the isolation protocol developed for the pMECs, in addition to the E-Cadherin marker, it was also evaluated the expression of two key molecules constituting the tight junctions. All the three pMECs cellular lines express components of tight junctions: ZO-1 and ccludin with a correct membrane localization (

Figure 27).

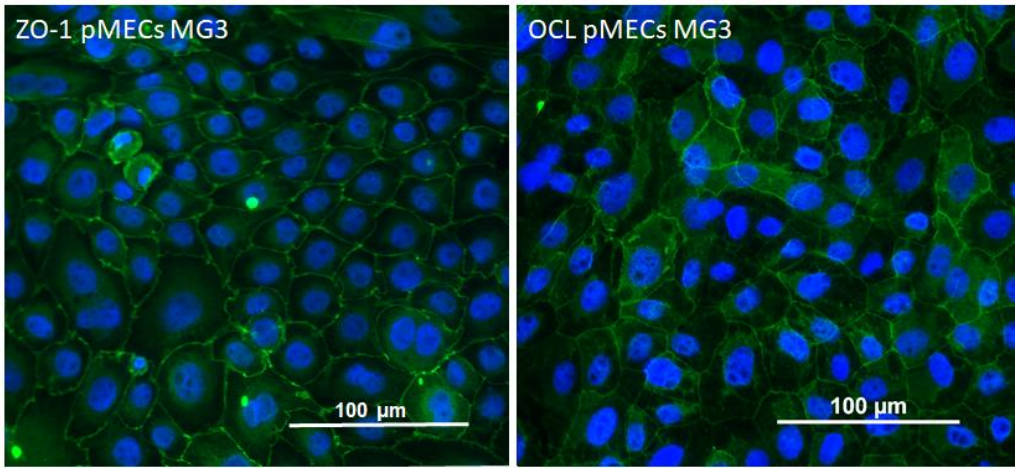


Figure 27 Representative images of ZO-1 and Occludin expression in pMECs (MG3) (Scale bar = 100 µm)

The supplier information of PMC42-LA states that PMC42-LA congregate into pavement epithelial sheets, in which the cell-cell borders contain E-cadherin and βcatenin. Only 5-10 % of the cells express vimentin. They also mention that vimentin can be upregulated after stimulation with EGF. However, we did not use EGF in the culture of PMC42-LA (

Figure 28).

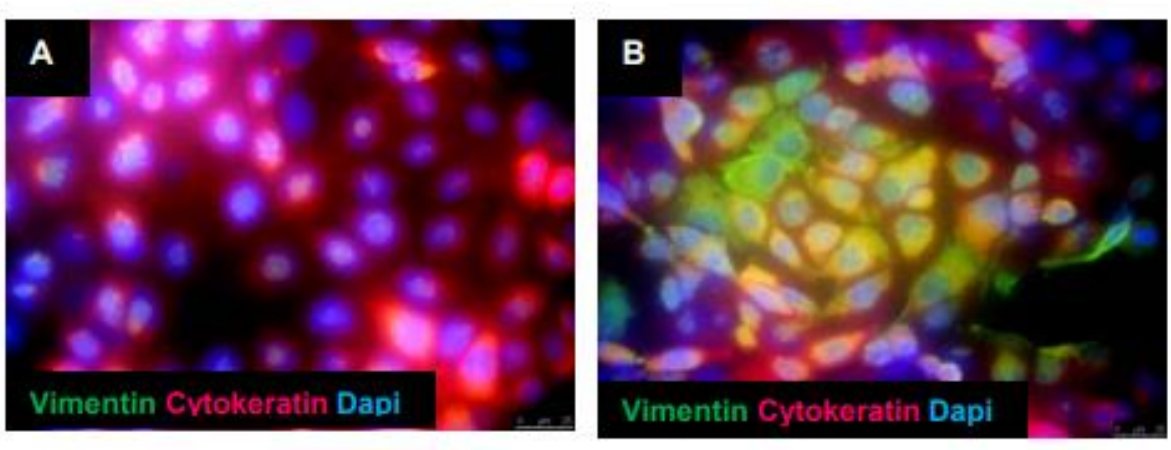


Figure 28 Double staining by the supplier with pan-cytokeratin antibody (red) and vimentin (green) indicate that without EGF treatment (A) cells were keratin positive and vimentin negative. After EGF treatment (B) the cells were vimentin and keratin positive (Sigma-Aldrich)

ATCC mentions that the MCF-7 cell line has several characteristics of the differentiated mammary epithelia. For example, the ability to respond to estradiol and the capability to form domes. The cells also express the oncogene WNT7B.

MCF-10A is a non-tumorigenic epithelial cell line. According to ATCC, they are positive for epithelial sialomucins, cytokeratins and milk fat globule antigen. They are also capable to form domes.

Barrier functional studies

Whether MCF-7 cells form a tight monolayer is not clear from literature. Some reports mention TEER values in the range of 400-500 Ωcm^2, representing a tight barrier [14,15]. Others report much lower TEER values, in the range of 20-40 Ωcm^2 [16,17]. These values do not represent a tight barrier. Therefore, we investigated multiple conditions with the MCF-7 cell line, including: (i) seeding density (4*10^4 cells/cm^2 to 6*10^5 cells/cm^2); (ii) permeable support material (PET vs PC); (iii) permeable support pore size (0.4 µm vs 3.0 µm); (iv) permeable support area (3.0 cm^2, 0.3 cm^2 or 1.12 cm^2); (v) medium change via aspiration or decantation; and (vi) removal of beta-estradiol. Our results indicate that the removal of beta-estradiol from the culture medium was critical to obtain high TEER values. Moreover, multiple passages in medium without beta-estradiol were required in order to increase the TEER values. Maximum TEER values of 479 ± 138 Ωcm^2 could be obtained at passage 13 on day 7 (Figure 29). Fluorescein sodium transport TEER was not measured at the maximum TEER but was 0.08 % for a TEER values of about 300 Ωcm^2.

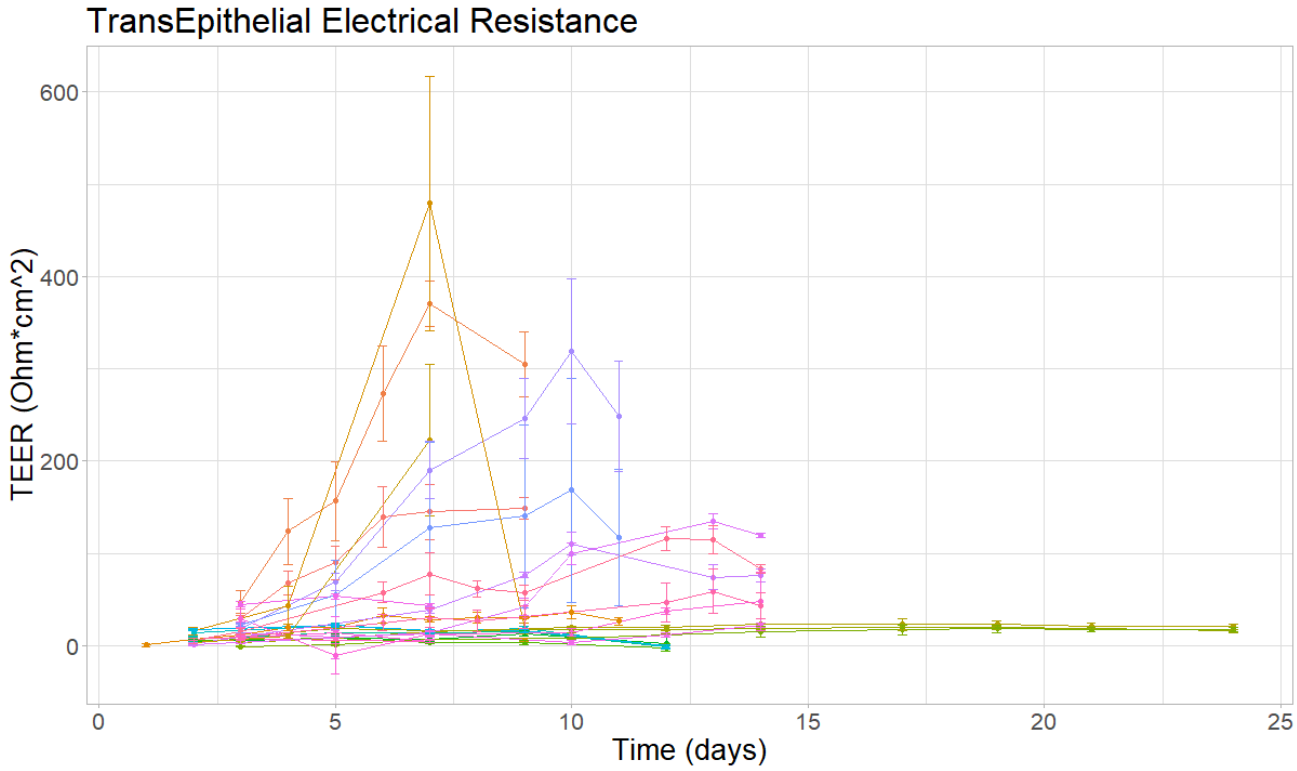


Figure 29 TEER values for MCF-7. The different colors represent different culture conditions. TEER values of 100 or more could only be obtained in medium without beta-estradiol. The number of passages in this medium improved TEER values, to a value of 479 ± 138 Ωcm^2 on day 7 at passage 13.

Marshall et al. (2009) measured the barrier formation in MCF-10A based on TEER and inulin flux [18]. They found an increase in TEER, which was preceded by a decrease in inulin flux. However, no other articles mention the formation of a tight monolayer by MCF-10A. Some reports even describe the absence of tight junctions in MCF-10A cells [19,20]. Marshall et al. (2009) explain this by the fact that they omitted cholera toxin from the medium [18].
Different conditions were explored, including: (i) seeding density (between 15000 cells/cm^2 and 200 000 cells/cm^2); (ii) time between medium change (24 h vs 2-3 days); (iii) pore size (3.0 vs 0.4 micron); (iv) permeable support area (3.0 vs 0.3 cm^2); (v) removal of cholera toxin; (vi) serum concentration (1 %, 5 % or 10 %), (vii) different serum lot numbers; and (viii) serum free medium. Only small differences in TEER values were found for these conditions, and none of the conditions (including removal of cholera toxin) resulted in the formation of a tight monolayer (Figure 30). The maximum TEER value was 33 ± 3 Ωcm^2.

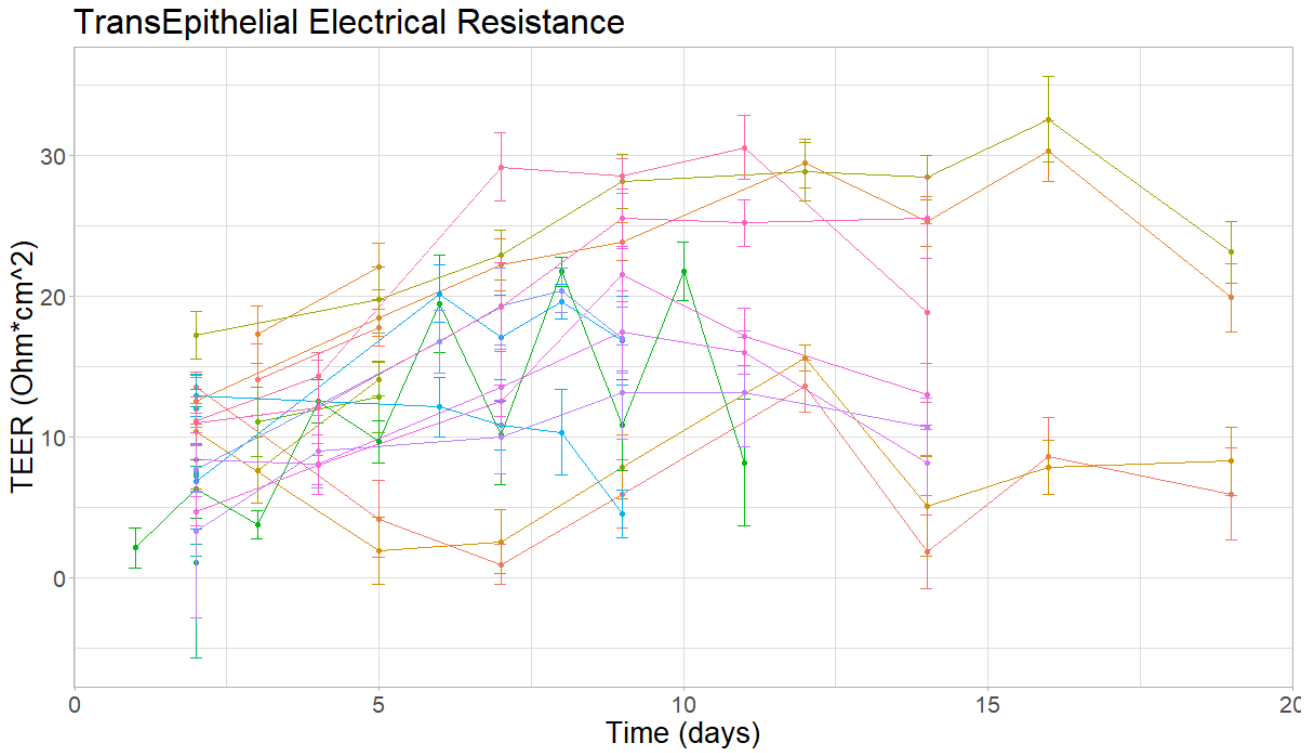


Figure 30 TEER values for MCF-10A. The different colors represent different culture conditions. TEER values significant for transport experiments were not obtained with any of the conditions.

No information about TEER or fluorescein sodium transport was found for PMC42-LA. Different conditions were explored, including: (i) seeding density ($5*10^4$ - $2*10^5$ cells/cm^2); (ii) serum concentration (5 % or 10 %); and (iii) Matrigel coating. However, these conditions did not result in the formation of a tight barrier, with maximum TEER values of 34 ± 1.5 Ωcm^2 (Figure 31). Freestone et al. described the differentiation of PMC42-LA into a lactating state in response to prolactin [21,22].

Therefore, future experiments will investigate the effect of prolactin on the barrier function. In addition, the effect of alternative coatings (e.g. collagen or Biocoated inserts) can be evaluated.

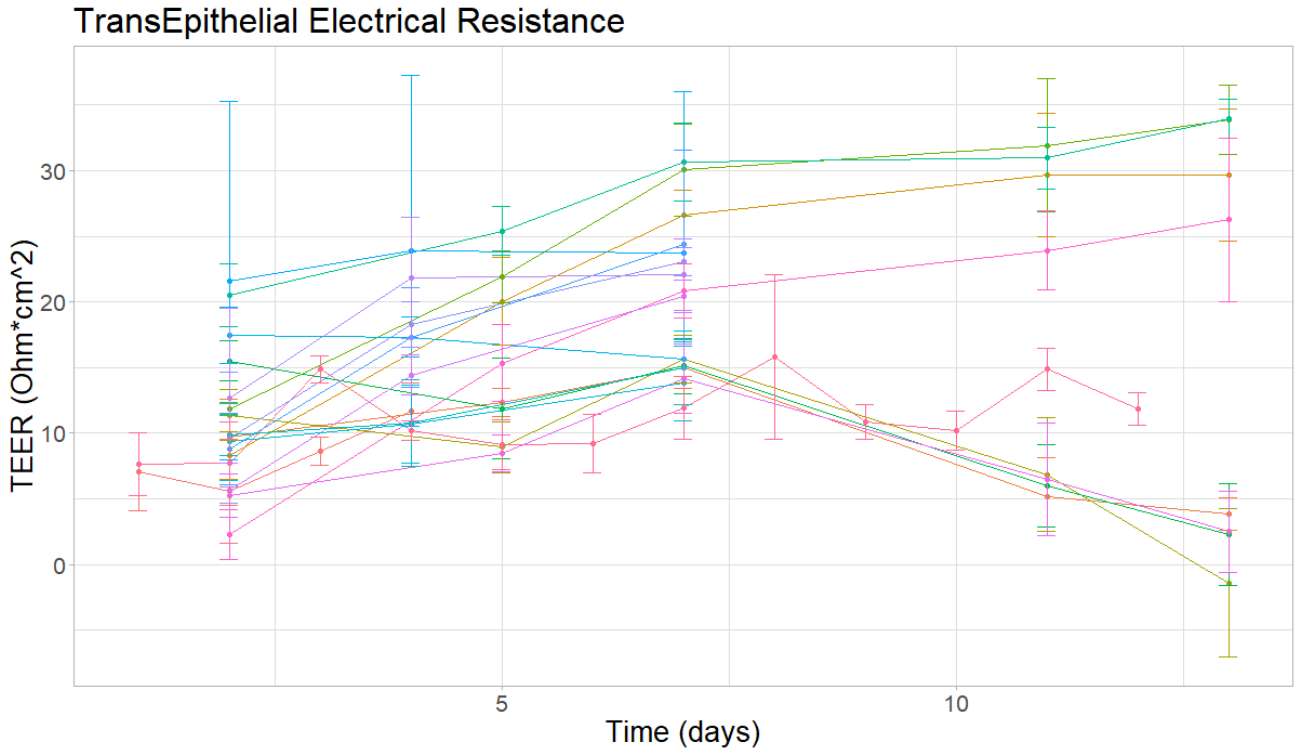


Figure 31 TEER values for PMC42-LA. The different colors represent different culture conditions. TEER values significant for transport experiments were not obtained with any of the conditions.

Kimura et al. (2006) were first to describe the use of HMEC monolayer to evaluate medicine transport during lactation [23]. They used a trypsin-resistant cell population [24]. HMEC were cultured until they reached 80% confluence. Cells were then treated with trypsin/EDTA, until only 20% of the cells remained attached. Attached cells were further cultured on the same flask, whereas detached cells were seeded on permeable supports. The trypsin/EDTA treatment was repeated 3 times. Detached cells from the different trypsin/EDTA treatments were referred to as 1-t-HMECs; 2-t-HMECs and 3-t-HMECs respectively. HMECs were seeded at a density of 20 000 cells/cm^2 on FALCON permeable supports (0.32 cm^2, pore size 1.0 µm, Becton Dickinson Inc.) coated with Matrigel by Kimura et al. (2006) [23]. They used human mammary epithelial growth medium supplemented with insulin, hydrocortisone, amphotericin B, gentamicin, bovine pituitary extract and prolactin. They found that the maximal TEER significantly increased with the number of trypsin/EDTA treatments, with a maximum value of 227 ± 48 Ωcm^2 for the 3-t-HMECs.

As described before, we did not use Matrigel in our protocol for expansion. Therefore, we also measured TEER without Matrigel coating. Two trypsin/EDTA treatments were performed on HMECs P9. The detached cells were referred to as 1-t-HMECs and 2-t-HMECs respectively. The cells were seeded on polyester permeable supports (pore size 0.4 µm; membrane area 0.3 cm^2) at a density of 3,3 x 10^5 cells/cm^2. Medium was changed every 48 h via decantation of the inserts. Medium was either full growth medium or differentiation medium. Differentiation medium is the full growth medium, with removal of epithelial growth factor, and addition of prolactin (200 ng/ml). Prolactin treatment did not seem to have a positive effect on the maximal TEER values. The number of trypsin/EDTA treatments on the other hand had a positive effect on the maximal TEER values (Figure 32 and Figure 33). The maximal TEER value was 81 ± 7 Ωcm^2 for the 2-t-HMECs at day 15. Future experiments will further investigate the effects of: (i) the number trypsin/EDTA treatments; (ii) prolactin stimulation at different concentrations; and (iii) the effect of Matrigel coating. In addition, as an alternative to Matrigel, also collagen or Biocoated inserts could be evaluated.

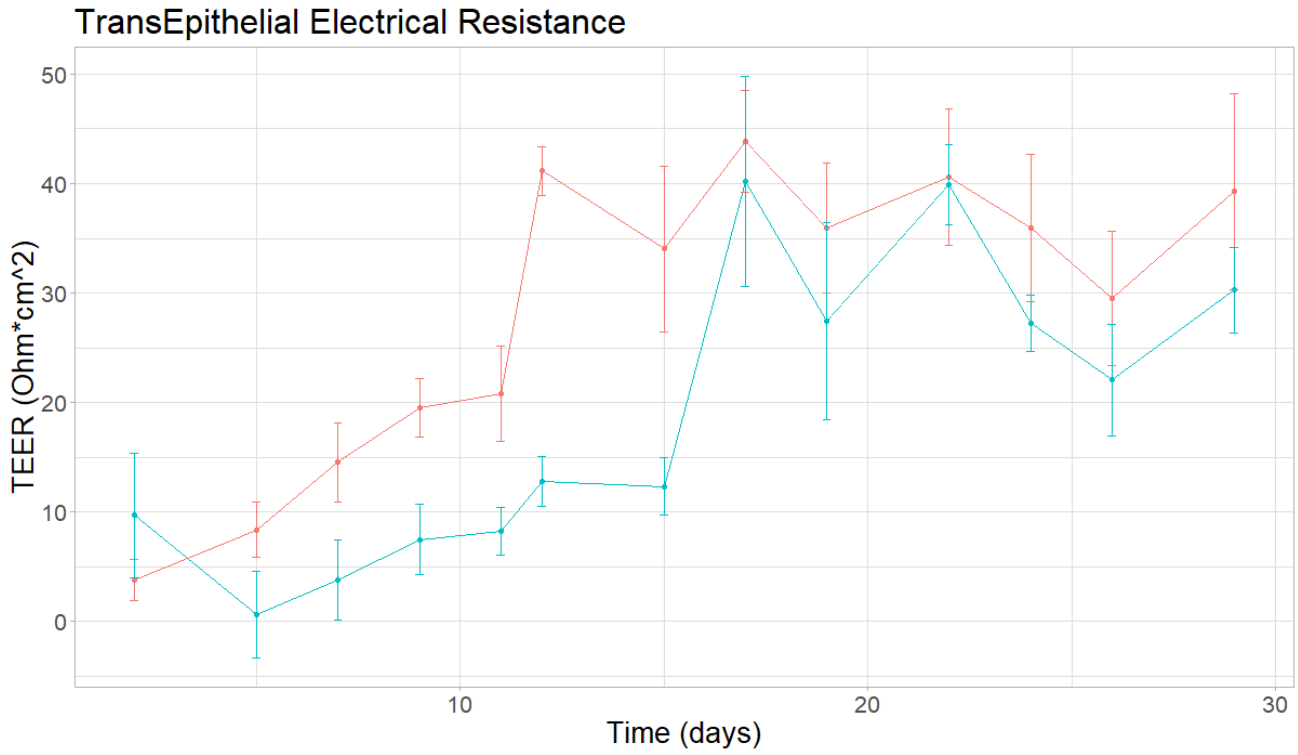


Figure 32 TEER values for 1-t-HMECs. The red line represents the TEER values in full growth medium. The blue line represents the TEER values in differentiation medium (with prolactin).

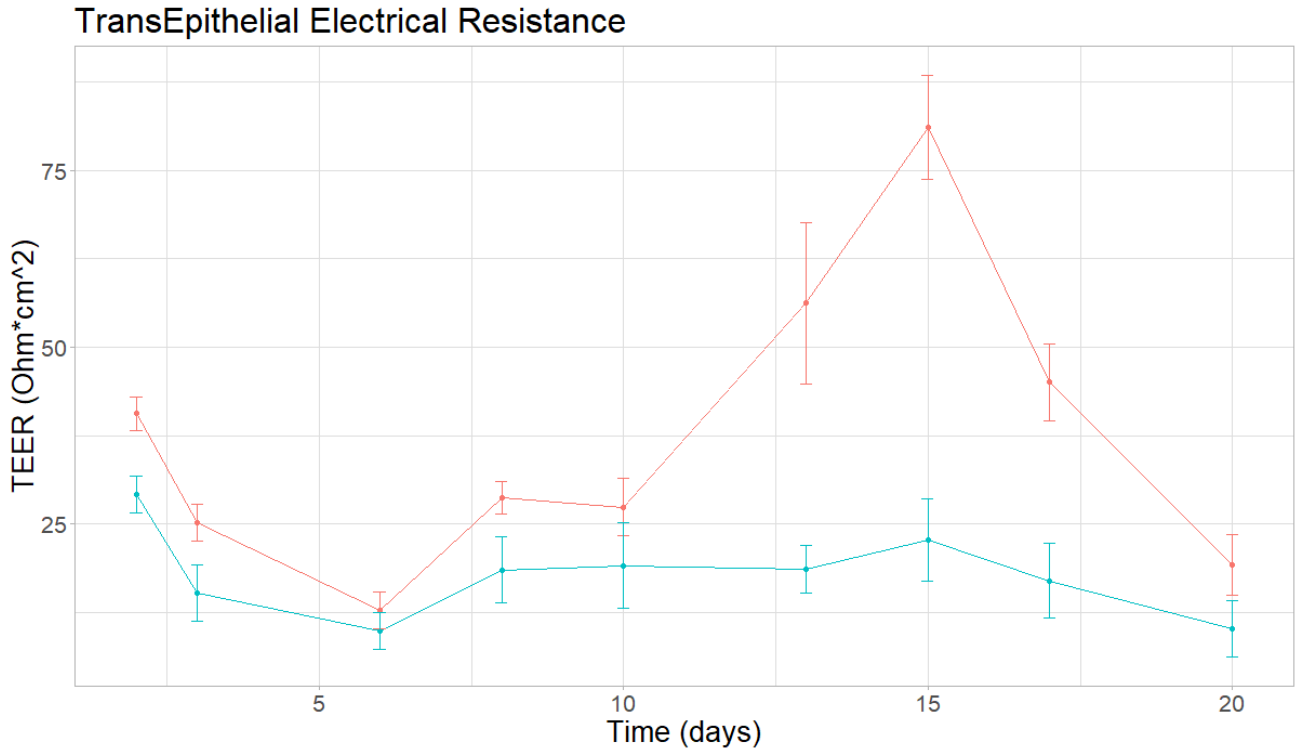


Figure 33 TEER values for 2-t-HMECs. The red line represents the TEER values in full growth medium. The blue line represents the TEER values in differentiation medium (with prolactin).

pMECs MG2 P10 were seeded on polyester permeable supports (pore size 0.4 µm; membrane area 0.3 cm^2) at a density of 3,3 x 10^5 cells/cm^2. Full growth medium was changed every 48h via

decantation of the inserts. pMEC MG2 reached a maximum TEER value of 368 ± 48 Ωcm^2 and a minimal fluorescein sodium transport of 0.06 ± 0.003 at day 2-3 (Figure 34).

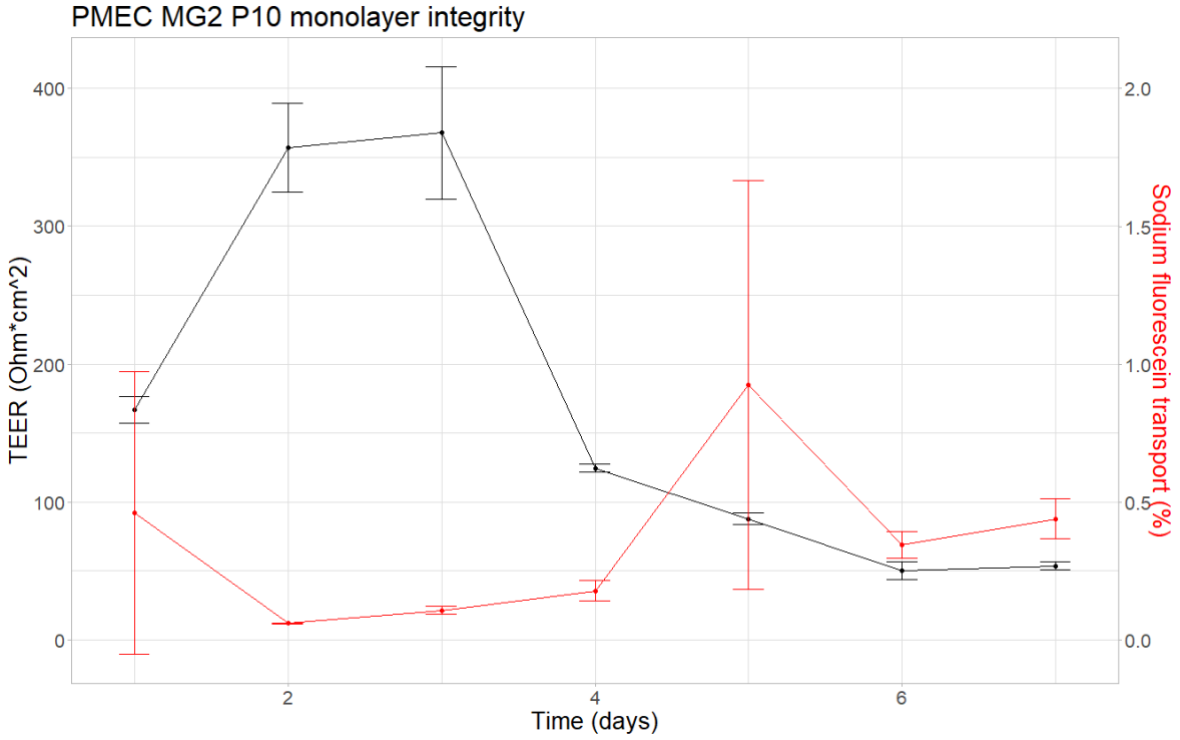


Figure 34 TEER (black) and fluorescein sodium transport (red) values for pMEC MG2 P10.

pMECs MG3 P10 were seeded on polyester permeable supports (pore size 0.4 µm; membrane area 0.3 cm^2) at a density of 3,3 x 10^5 cells/cm^2. Full growth medium was changed every 48 h via decantation of the inserts. pMEC MG3 showed a similar pattern as pMEC MG2. pMEC MG3 had a maximum TEER value of 393 ± 56 Ωcm^2 and a minimal fluorescein sodium transport of 0.07 ± 0.03 at day 2-3 (Figure 35).

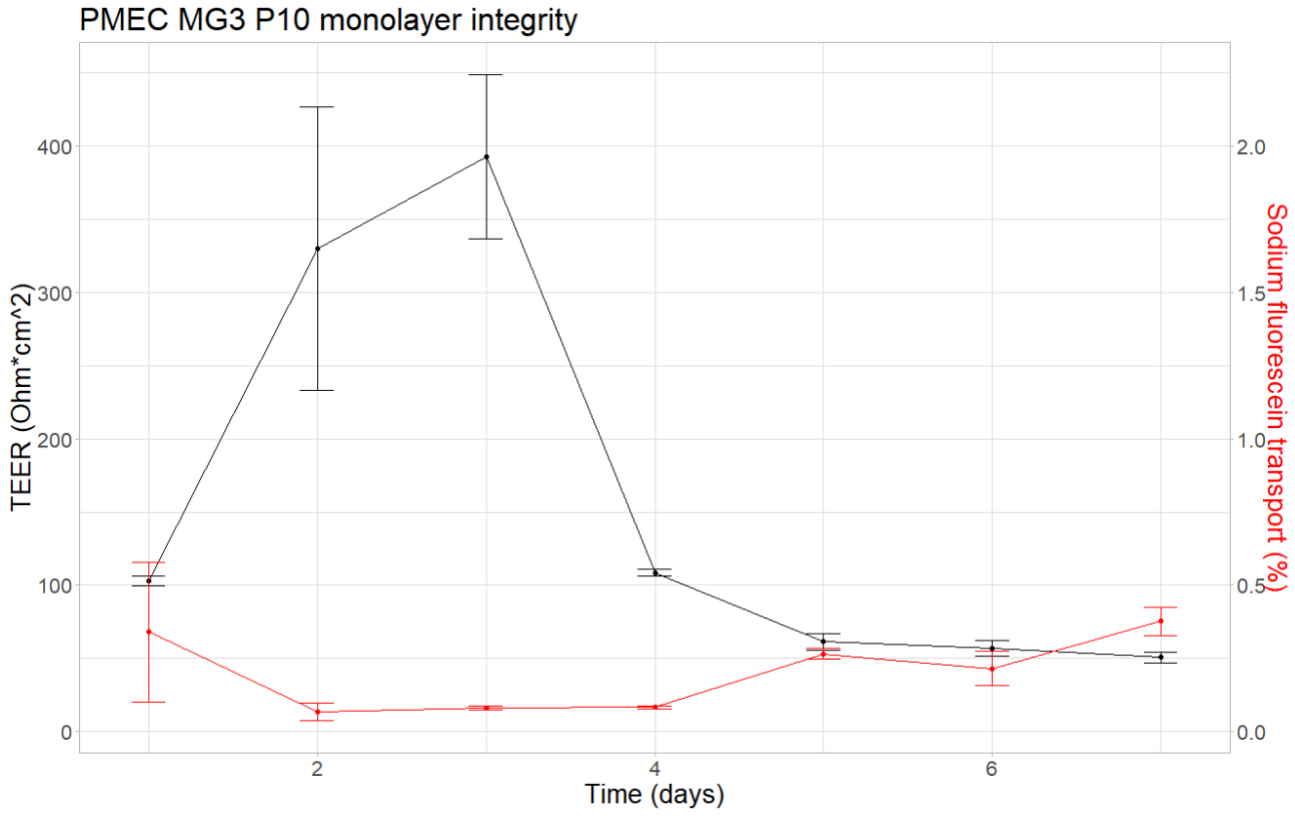


Figure 35 TEER (black) and fluorescein sodium transport (red) values for pMEC MG3 P10.

pMECs MG8 P10 were seeded on polyester permeable supports (pore size 0.4 µm; membrane area 0.3 cm^2) at a density of 3,3 x 10^5 cells/cm^2. Full growth medium was changed every 48h via decantation of the inserts. pMEC MG8 results were different from pMEC MG2 and pMEC MG3. pMEC MG8 had a maximum TEER value of 829 ± 31 Ωcm^2 and a minimal fluorescein sodium transport of 0.03 ± 0.007 at day 5-6 (Figure 36). Other conditions tested for pMECs MG8 include: (i) seeding density; (ii) passage number; and (iii) pore size. The effect of stimulation with prolactin still needs to

be investigated.

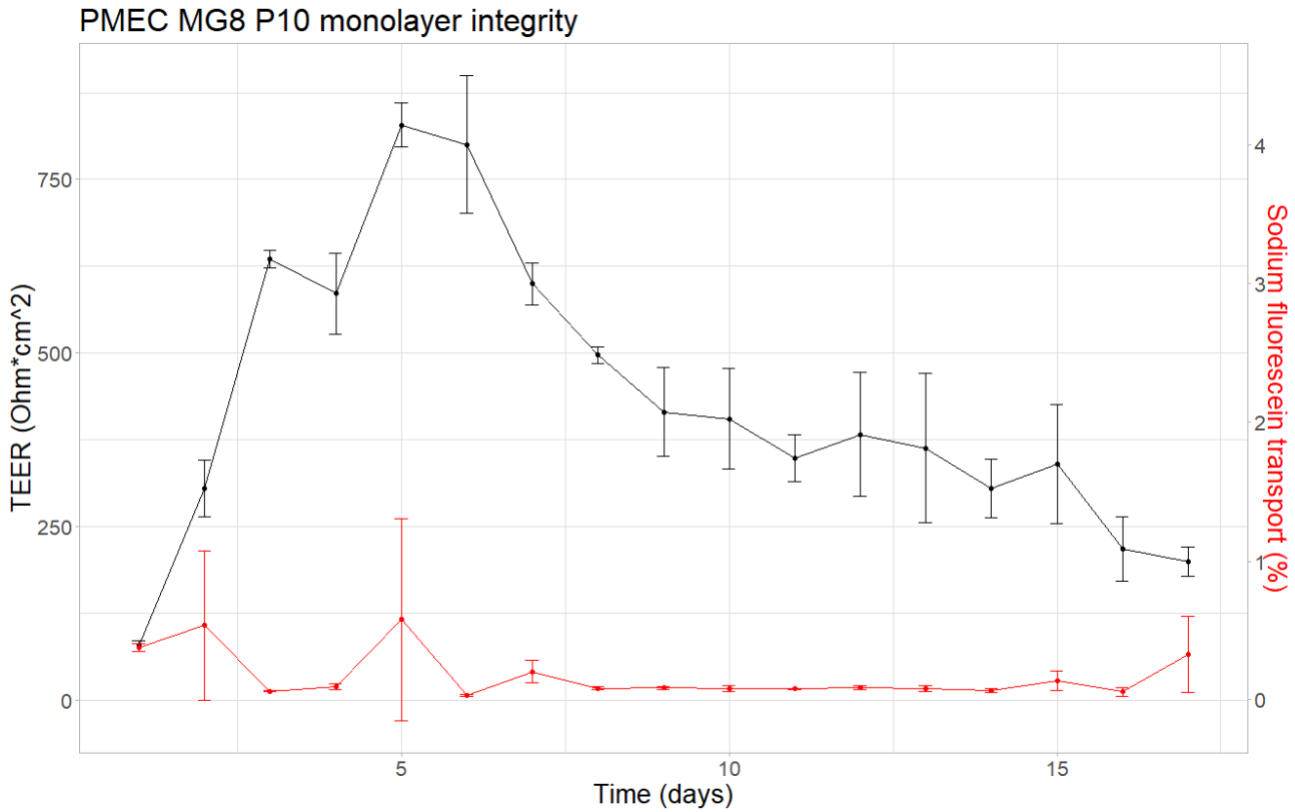


Figure 36 TEER (black) and fluorescein sodium transport (red) values for pMEC MG8 P10.

Transporter genes mRNA expression

To avoid possible medium effects on pMEC and HMEC transporter genes expression comparison, we cultured the pMECs both in pMEC as well as hMECs medium. The transcriptional profile of drug transporters was represented by ΔCt method that showed the difference in threshold cycle between the mean of reference genes and the Ct of target genes (ΔCt= Ct mean of reference genes – Ct gene of interest). When we utilize this kind of normalization, a lower ΔCt value corresponds to a lower mRNA expression.

The array analysis in pMECs showed, in both culture media, level of drug transporters gene expression ranging from ΔCt values very negative (lower expression) to ΔCt values less negative (higher expression). Among the 84 analyzed genes only 3 were not detectable (ABCC12; SLC22A3; SLC22A8), in pMECs cultured in both media (

Figure 37 and

Figure 38).

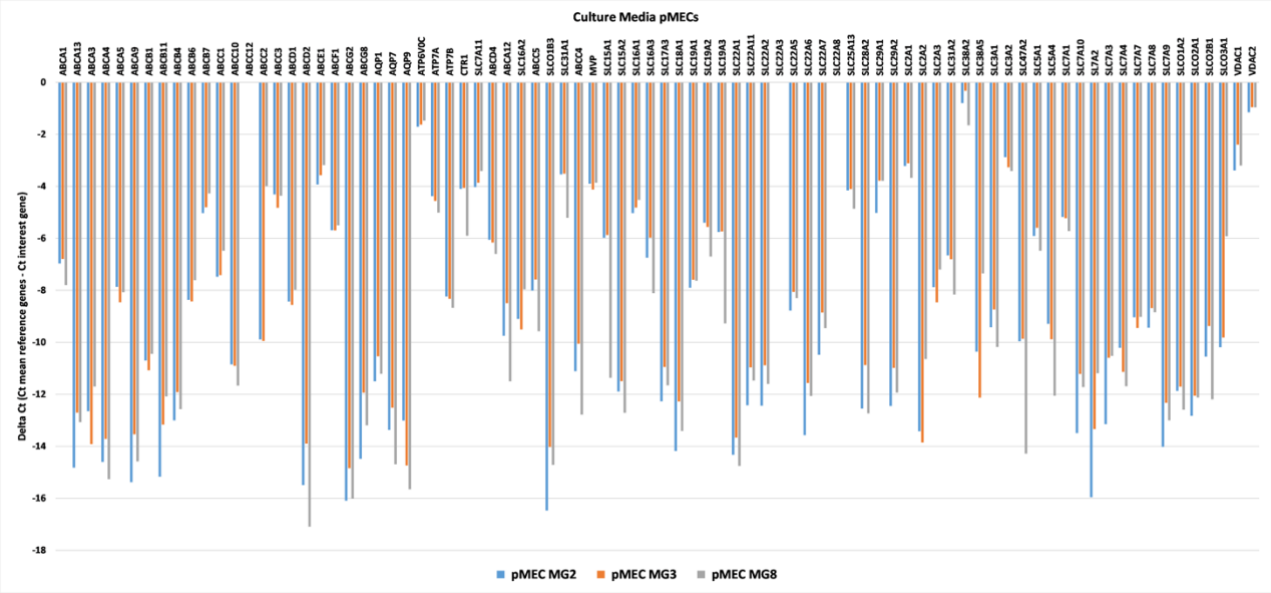


Figure 37 Transcriptional profile of the drug transporters in pMECs-MG2, pMECs-MG3 and pMECs-MG8 cultured in pMECs medium. The relative expression was calculated as ΔCt. A higher value (less negative) corresponds to higher gene expression.

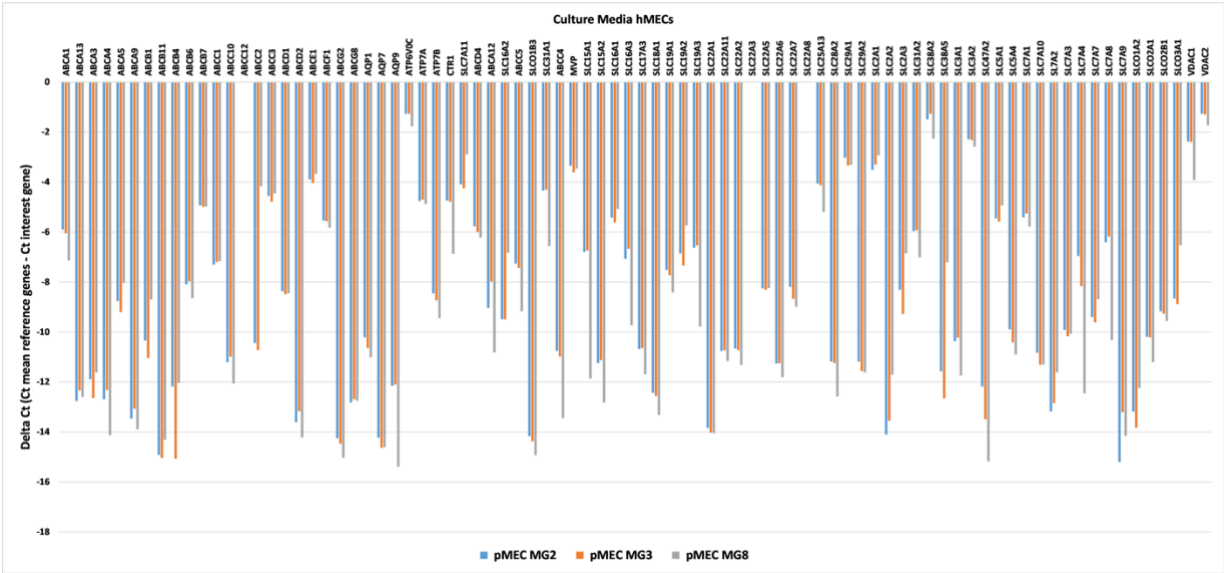


Figure 38 Transcriptional profile of the drug transporters in pMECs-MG2, pMECs-MG3 and pMECs-MG8 cultured in HMECs medium. The relative expression was calculated as ΔCt. A higher value (less negative) corresponds to higher gene expression.

To better evidence the effect of the media on pMECs drug transporters expression, the relative mRNA expression (fold of change) of the tested genes in pMECs cultured in HMECs medium was calculated in relation to the pMECs cultured in pMECs medium (considered as the control) using the $2^{-\Delta\Delta Ct}$ method [25]. The comparison between pMECs cultured in the two media used showed that the medium did not influence essentially the drug transporters expression profile. Only SLC3A2 and SLCO3A1 genes decreased their expression and VDAC2 genes increased it ($p<0.05$, Student t test) (

Figure 39,

Figure 40 and

Figure 41).

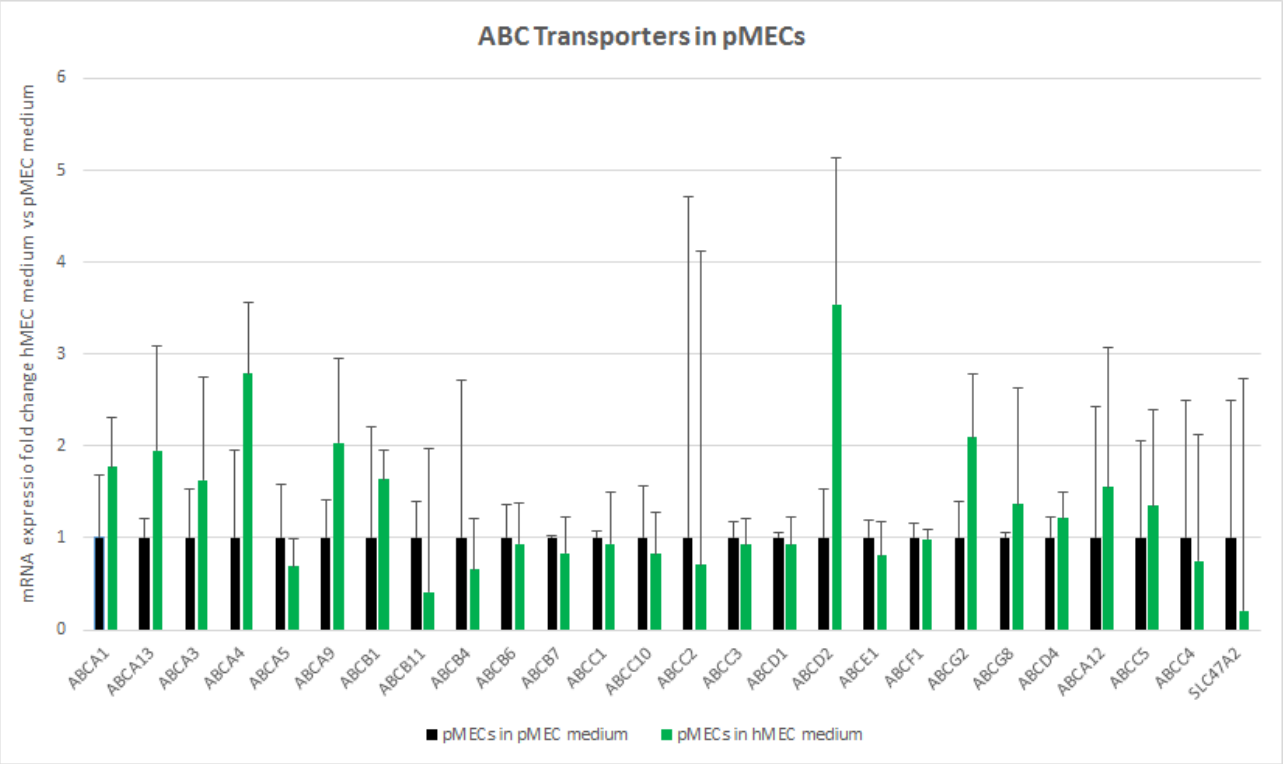


Figure 39 Transcriptional profile of the ABC drug transporters in pMECs (n=3) cultured in pMECs medium or in HMECs medium. The $2^{-\Delta\Delta Ct}$ method was used to analyze the relative genes expression (fold change) in the HMECs medium in relation to the pMECs medium (considered as control, value =1). The data are represented as $2^{-\Delta\Delta Ct}$ value + SD. No statistically significant differences were observed (Student's t-test, p<0.5)

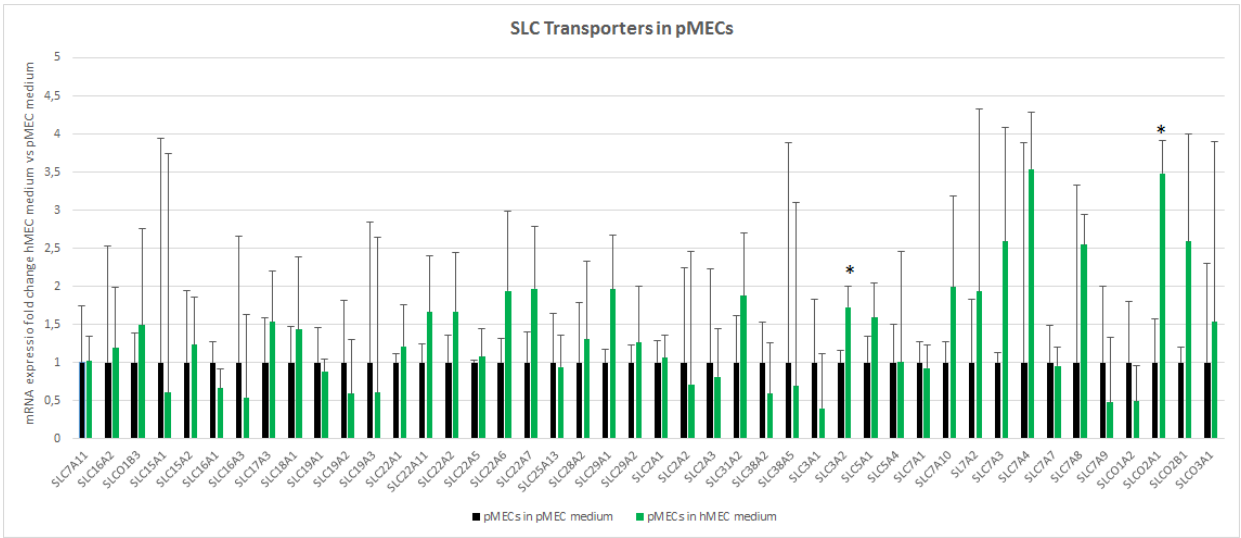


Figure 40 Transcriptional profile of the SLC drug transporters in pMECs (n=3) cultured in pMECs medium or in HMECs medium. The $2^{-\Delta\Delta Ct}$ method was used to analyze the relative genes expression (fold change) in the HMECs medium in relation to the pMECs medium (considered as control, value =1). The data are represented as $2^{-\Delta\Delta Ct}$ value + SD. A

statistically significant increase of expression in HMECs medium was observed only inSLC3A2 and SLCO2A1 genes (indicates p<0.5, Student's t-test).*

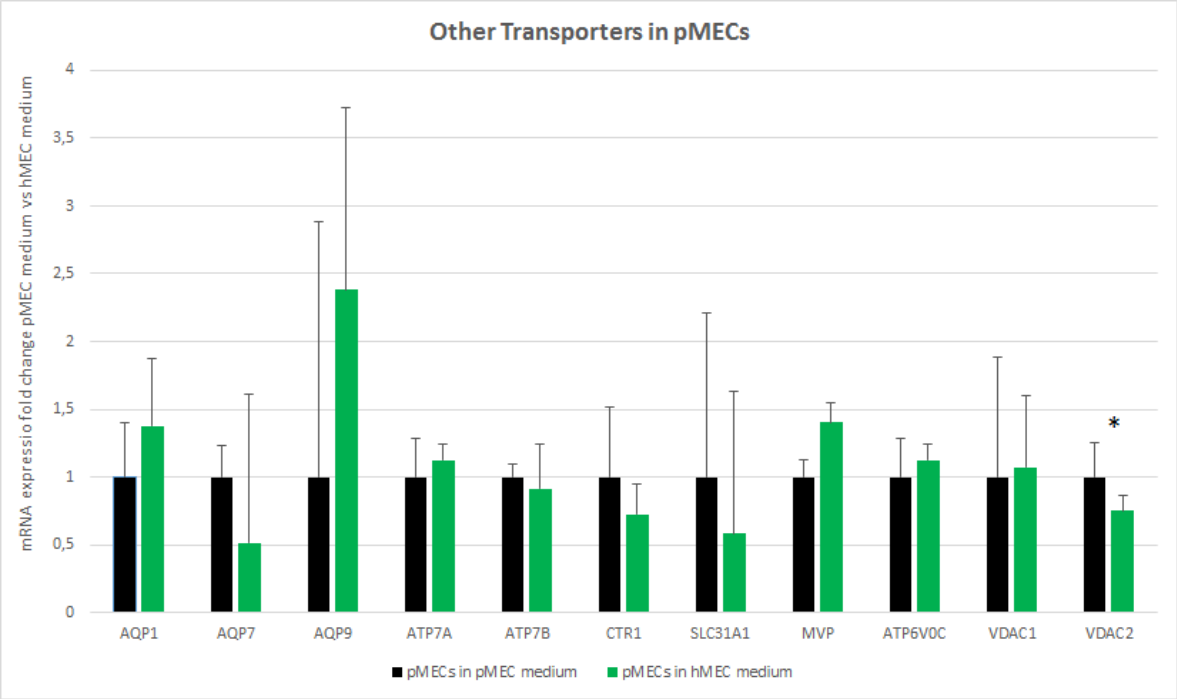


Figure 41 Transcriptional profile of other drug transporters in pMECs (n=3) cultured in pMECs medium or in HMECs medium. The $2^{-\Delta\Delta Ct}$ method was used to analyze the relative genes expression (fold change) in the HMECs medium in relation to the pMECs medium (considered as control, value =1). The data are represented as $2^{-\Delta\Delta Ct}$ value + SD. A statistically significant decrease of expression in pMECs medium was observed only VDAC2 genes (indicates p<0.5, Student's t-test).*

The array analysis of HMECs showed the gene expression of transporters with a differential level of expression ranging from ΔCt values very negative (lower expression) to ΔCt values less negative (higher expression). In HMECs, SLC2A9 and ATP6V0C genes were undetectable (

Figure 42).

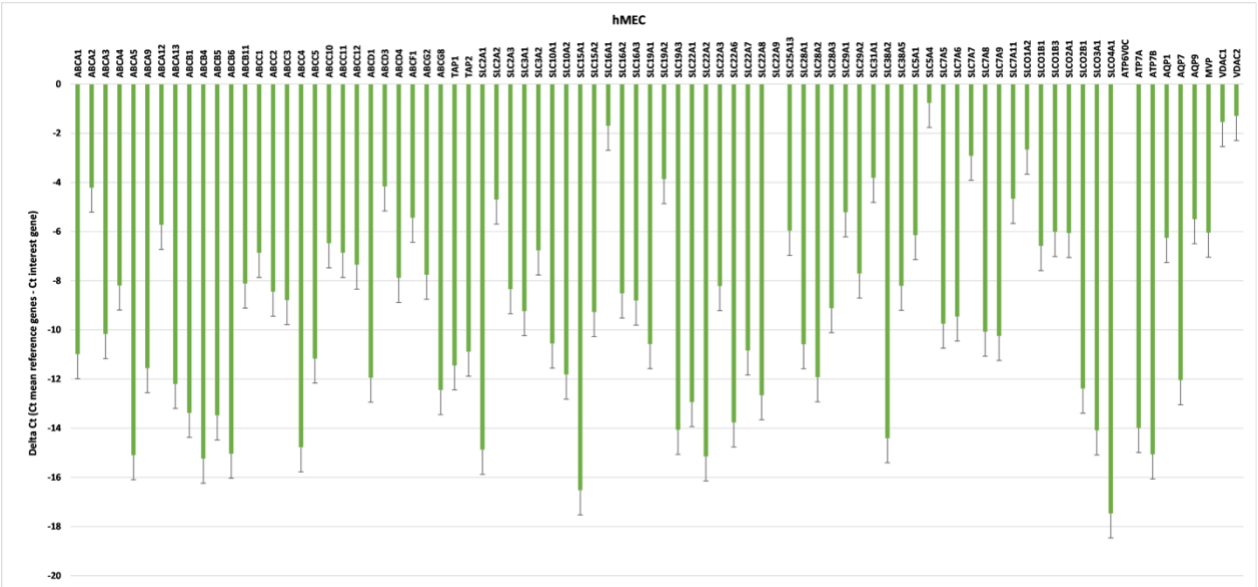


Figure 42 Transcriptional profile of the drug transporters in HMECs, (cultured in HMECs medium). Relative expression was calculated as ΔCt. At higher values (less negative) correspond higher gene expression. The data are represented as mean of ΔCt +SD (n=3 independent experiments).

A comparison between pMECs and HMECs of the common transporter genes present in both arrays was reported in Table 17. This preliminary study showed a huge variation among genes expression between the pMEC and HMECs. Among the analized genes, the expression of 31 was higher in pMECs than in HMECs (fold of change >1), with 15 of them more than 10-fold; on the other hand, the expression of 33 genes was lower in pMECs than in HMECs with 16 of them more than 10-fold. (Table 17).

Table 17 Transcriptional profile of drugs transporters in pMECs, cultured in HMECs medium. The relative mRNA expression (fold change) of the tested genes was calculated in relation to the HMECs using the 2-ΔΔCt method. The fold change data > 1 or < 1 indicate respectively an increase or decrease in gene expression in pMECs in relation to the HMECs (control, value =1)

Gene	Fold change	Gene	Fold change	Gene	Fold change
ABCA1	24,60	**SLC2A1**	3163,56	**SLC38A5**	0,20
ABCA13	0,77	**SLC2A2**	0,001	**SLC7A7**	0,01
ABCA3	0,27	**SLC2A3**	1,15	**SLC7A8**	5,40
ABCA4	0,03	**SLC3A1**	0,34	**SLC7A9**	0,06
ABCA5	86,06	**SLC3A2**	20,64	**SLCO1A2**	0,00073
ABCA9	0,26	**SLC15A1**	266,38	**SLCO2B1**	8,28
ABCB4	4,40	**SLC15A2**	0,18	**SLCO3A1**	66,748
ABCB1	10,18	**SLC16A1**	0,08	**SLC16A2**	0,93
ABCB6	111,22	**SLC16A3**	1,97	**SLC5A1**	1,76
ABCB11	0,01	**SLC19A1**	6,44	**SLC5A4**	0,0012
ABCC1	0,78	**SLC19A2**	0,15	**SLCO1B3**	0,0028
ABCC2	1,00	**SLC19A3**	85,27	**SLCO2A1**	0,044
ABCC3	18,13	**SLC22A1**	0,49	**AQP1**	0,048
ABCC10	0,03	**SLC22A2**	18,90	**AQP7**	0,18
ABCG2	0,01	**SLC22A6**	5,01	**AQP9**	0,0047
ABCF1	0,86	**SLC22A7**	4,65	**MVP**	5,94
ABCG8	0,81	**SLC25A13**	2,83	**ATP6V0C**	0,36
ABCD1	11,37	**SLC28A2**	1,20	**ATP7A**	589,76
ABCA12	0,09	**SLC29A1**	3,97	**ATP7B**	72,70
ABCC4	8,25	**SLC29A2**	0,07	**VDAC1**	0,39
ABCC5	9,24	**SLC31A1**	0,42	**VDAC2**	0,90
ABCD4	3,70	**SLC38A2**	6781,24		

The expression of transporters in MCF-7 (either mRNA or protein expression) was summarized previously [26]. Expression of transporters in MCF-10A or PMC42-LA was not further investigated, as the results from the barrier functional studies show that these models are not suitable for transport studies.

Discussion

The aim of the present deliverable was the characterization of *in vitro* human/animal mammary epithelial cell culture models, including their comparison.
To achieve this, two fundamental aspects were considered: the choice of the most suitable cellular model and the creation of the model itself.
Starting from the choice of which were the "right" cells, WP3 participants investigated many different possibilities, keeping in mind that *in vitro* cultured cells provide a living system that could become a powerful research tool if properly chosen and characterized [27,28]. In fact, *in vitro* cell models are successfully used in many research fields as the study of cellular metabolism, gene function, pharmacokinetics, drug development, vaccine and antibody production [29–31].
In vitro cultured cells can be divided into two well defined categories: primary cells and immortalized cell lines [32].
Primary cells are taken directly from the tissue, therefore they most closely represent the tissue of origin and generally exhibit a normal phenotype. For this reason, they provide excellent model systems for studying the normal physiology (e.g., metabolic studies, aging, signaling studies), and the effects of drugs and toxic compounds on the cells. On the other hand they have a limited lifespan and will stop dividing after a certain number of cell divisions, so frequent isolations must be performed. Some features could be related to the different donors so, biological replicates together with the technical ones are needed. This could represent a problematic obstacle for the experimental repeatability and reproducibility [32].
Keeping this in mind, many researchers prefer to work with immortalized cell lines that have minor effective cost, are easy to use and provide an unlimited supply of material. Being immortal cell line a pure population of cells, it's easier to reproduce results at the intra- and inter-laboratory level. Despite being a powerful tool, one must be careful when using cell lines in place of primary cells. Since cell lines are genetically manipulated this may alter their phenotype, native functions and their responsiveness to stimuli. Serial passages of cell lines can further cause genotypic and phenotypic variations over an extended period and genetic drift can also cause heterogeneity in cultures. As a result, the immortalized cell lines construct and predictive validity could be seriously affected and they may not often adequately represent the *in vivo* tissue of origin providing confounding results [33].
Therefore, great care should be taken when using cell lines and experiments where key findings are confirmed in primary cultures should always be included.
Considering this necessary premise WP3 participants chose to work with primary cell lines, without, however, excluding the possibility of using immortalized lines where obvious problems were highlighted in the use of human primary cells.
Regarding the availability of mammary epithelial cells (MECs), as we previously reported, MECs have been isolated both in humans (HMECs) and in many animal species including pigs (pMECs). As for the human ones, there are both primary cultures and immortalized lines that can be bought on the market while for the porcine species only primary lines are described but, unfortunately, actually no companies are able to provide them.
Therefore, in the present deliverable we presented at first the results obtained defining a reliable protocol to produce primary cultures of porcine mammary cells.
In full respect of international legislations and the 3Rs ethical principles [3], the protocol set up was based on mammary tissue collected from a local slaughterhouse.
The technical strategy developed to dissociate mammary tissue and the culture condition set up was resulted satisfying, in fact we obtained primary cell cultures from 5 out of 6 different animals, albeit with different quantitative percentage, due to the tissue variability, as indicated by histological analysis. Moreover, the expansion protocol developed, allowed us to obtain three primary cell lines grown till the 10th passage with a total of 100 cellular aliquots stored in the biobank, which gives us the possibility to plan many future experiments.

The results obtained from the careful characterisation of isolated cells showed that the three primary cell lines are compatible with a normal diploid proliferating population. All the three pMECs populations grow in monolayer, exhibited typical epithelial-like morphology and expressed epithelial markers, in agreement with those previously obtained by other authors [6,34].
Overall, these results indicate that the present pMECs isolation method allow to efficiently obtain a pure population of mammary epithelial cells.
For human mammary epithelial cells WP3 participants decided to buy them from a leader company supplying cells able to guarantee a number of aliquots sufficient to permit a good expansion and the full set of experiments.
The protocol set for the expansion of human primary cells allowed to obtain a sufficient number of cellular aliquots to guarantee the planification of functional test. Moreover, the main characteristics of epithelial morphology and epithelial marker expression were respected after the expansion time. Comparing pMECs and HMECs with respect to morphology, growth rate and the ability to correctly express an epithelial marker such as E-cadherin, the choice made with primary cells seemed to provide a solid basis for continuing the functional characterization.

At the same time, immortalized human cell lines are available: MCF-7 is one of the most used cells in the field of breast cancer research. The cells are epithelial, and retain some of the characteristics of the differentiated mammary epithelium. However, as discussed before, as this cell line is derived from breast cancer, it's utility for in vitro recapitulation of the blood-milk barrier might be lower. Therefore, a logical choice would be the MCF-10A cell line. MCF-10A is a non-tumorigenic cell line. According to ATCC, MCF-10A displays characteristics of luminal ductal cells, but not of myoepithelial cells. However, caution is required as there is some discussion about the use of these cells as a model for normal breast tissue.[35]. PMC42-LA on the other hand is a less known cell line. This cell line was chosen since it has been shown that this cell line is able to differentiate into a lactating cell line in response to prolactin.
Once chosen the right cells and still leaving open a wide margin of work the second step was the set up of the experimental condition model for a correct study of functional mammary epithelial barrier.
The Transepithelial/transendothelial electrical resistance (TEER) is a widely accepted quantitative technique to measure the integrity of tight junction dynamics in cell culture models [12]. TEER values are strong indicators of the integrity of the cellular barriers before they are evaluated for transport of medicines, endogenous compounds, nutrients and/or chemicals. TEER measurements can be performed in real time without cell damage and generally are based on measuring ohmic resistance or measuring impedance across a wide spectrum of frequencies.
The highest TEER values were obtained in the pMECs. Interestingly, a different TEER and fluorescein sodium transport was seen in pMECs MG8 compared to pMECs MG2 and pMECs MG3, pMEC MG8 is not only capable of reaching higher TEER values, but the plateau phase is also retained much longer. Therefore, pMECs MG8 form the most suitable model for further transport studies. Further investigations crossing data from gene and protein expression analysis will be necessary to better clarify why pMECs MG8 shows this peculiar features. For HMECs, we were not able to reach significant TEER values for transport studies. However, it has been shown that repeated trypsin-EDTA treatments and prolactin stimulation improve TEER values. Preliminary results seem to confirm that trypsin-EDTA treatments improve TEER values. However, our results seem to indicate that prolactin stimulation does not have a positive effect on TEER values. Further experiments need to clarify if this could be related to the Matrigel coating used by Kimura et al. (2006) [23]. Furthermore, they did not specify the prolactin concentration that has been used. Based on other reports, the prolactin stimulation has been used in combination with animal cells, a concentration of 200 ng/ml was used. However, it could be useful to test other concentrations. Finally, 2 trypsin-EDTA treatments did seem to improve TEER values compared to 1 trypsin-EDTA treatment, but we still need to confirm that this increase is also visible after 3 treatment.
Except from MCF-7, we did not succeed in forming a tight monolayer with the human cell lines.

Interestingly, we could find conflicting information in literature for both MCF-7 and MCF-10A. This could be either due to genetic/phenotypic drift, as also discussed above, or due to the differences in culture conditions. However, for MCF-10A, we were not able to replicate the results from Marshall et al. (2009 [18]. For MCF-7, our hypothesis is that the culture in medium without beta-estradiol for several passages is the main factor in reaching high TEER values. Tests with another lot should confirm this hypothesis in the near future.
A functional barrier depends on the expression of specific transporters molecules [36]. Indeed, detailed reviews on the expression of transporters in the epithelial cells of mammary gland have been published [37–41].
Detection of the drug transporters gene expression in pMECs allowed us to better characterize these cells, and to identify the candidate transporter protein involved in drugs and xenobiotics transfer across the mammary epithelium. As no data for pig species are available from the literature, we utilize a RT^2 profiler PCR approach to investigate the expression of 84 transporters belonging to the different transporter families.
The same approach was used in HMECs. The two arrays are not identical but most of the included genes were the same. Our results indicate that pMECs express all the studied genes, except the genes ABCC12 (coding for the Multidrug resistance-associated protein 9), and the two genes SLC22A3 and SLC22A8 (coding for ions transporter proteins). On the contrary, these three genes were detectable in HMECs. Among the genes with higher expression in pMECs, some were differently expressed: ABCC2, more expressed in pMECs-MG8 than in MG2 and MG3 while SLC15A1, SLC19A3, SLC31A1 and CTR1 less expressed in pMECs-MG8 than in MG2 and MG3. This difference may be due to the biological variability among the status of mammary gland isolated from the three animals and could also be related with the different attitude of pMECs MG8 to form a tight barrier. The gene ABCC2, that codes for the Multidrug resistance-associated protein 2 (MRP2), was the only one with a high variation of expression among the three biologicals replication of pMECs showing a statistically significant higher expression in pMECs MG8, possibly in relation with biological variability among donors. Is interesting to underline that the mean value of ABCC2 in pMECs is exactly the same obtained for HMECs (fold of expression = 1).
In general our results indicated HMECs express all the studied genes, except the SLC22A9 and ATP6VOC, the first gene is not present in the pig array while the second one is one of the most expressed in pMECs, together with ABCD2, and SLC38A2. 34 genes out of the 65 commonly expressed, exhibit a range of fold of expression <10 times (in both directions).
Large differences exist in the expression level of some transporter genes between porcine and human MECs. The functional effects of these differences need to be investigated both in relation to lactogenic stimulation and to transport of specific compounds

Conclusion

In the present report we described an efficient method to obtain pure primary mammary epithelial cells cultures from slaughtered swine as a model to study epithelial barrier function *in vitro*. Porcine cells could also overcome the limitation related to the biological random differences, due to the facility to collect mammary tissues at slaughterhouses; pMECs could be utilized in pre-screening studies leading to the Reduction of animals utilized to study the lactational drug transfer.
Considering the validity of the protocol developed, the next step will be the production of primary cell lines from minipig. The possibility to produce primary cultures of pMECs derived from minipigs could improve the reliability of the method allowing for a better-defined genetic background easier to be replicated in different laboratories across the world. As *in vivo* studies in minipigs are also planned in the context of WP3, the availability of minipig derived pMECs will be important to support future *in vitro* to *in vivo* extrapolation algorithms for transport of medicines across the blood-milk barrier.

Based on the work performed so far pMECs MG8 are the preferred animal *in vitro* model. Further

experiments will clarify the ideal conditions for HMECs. Furthermore, MCF-7 cells cultures in medium without beta-estradiol can be used as a human cell line model. However, further characterization of the transporters in terms of protein expression and function (based on probe substrates), as studying the reproducibility of the models will allow to make a final decision between the two remaining human models. The first model that can be used are the pMECs MG8. These cells are crucial in combination with the data from the in vivo animal studies. Combining these data will give us insights in the underlying mechanisms and scaling. This information will be important to interpret the data generated with the human in vitro models. HMECs are the most relevant model, but further tests are required to find the optimal culture condition. However, as working with primary cells is quite challenging, MCF-7 in medium without beta-estradiol is another human alternative. An MCF-7 based model may be an option for determining the transepithelial transport of medicines that cross the blood-milk barrier primarily by passive permeation. Ongoing experiments focusing on the transporter expression, transporter function and reproducibility of these models will give us more insight in the benefits and limitations of each of these human models.

Repository for primary data

The primary data will be stored on the ConcePTION website member area.

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