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An in vitro human mammary epithelial cell permeability assay to assess drug secretion into breast milk

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ABSTRACT

Determining the amount of a drug transferred into breast milk is critical for benefit-risk analysis of breastfeeding when a lactating mother takes medications. In this study, we developed a human mammary epithelial cell (MEC)-based permeability assay to assess drug permeability across the mammary epithelium. Human MEC cell MCF10F formed tight junctions when cultured on Transwells with culture medium containing insulin, hydrocortisone and epidermal growth factor (EGF). Formation of integral cell barrier and morphology of the cells were confirmed by assessing trans-epithelial electrical resistance (TEER), flux of fluorescent tracers and imaging with transmission electron microscopy (TEM). MCF10F cells showed consistent P-glycoprotein (P-gp) transporter expression when culturing on Transwell inserts versus on petri dish. A few P-gp transporter drug substrates were used to estimate the permeability from this assay. Human plasma and breast milk were used as incubation medium in basolateral and apical chambers respectively to mimic physiological conditions. The predicted milk to plasma (M/P) ratios were reasonably good. The current effort to develop the MEC-based permeability assay to facilitate M/P ratio prediction showed promising results. This assay may have a potential to be developed as a useful in vitro technique for determining the transfer of small-molecule therapeutic drugs into breast milk.

1. Introduction

Extensive epidemiological research has widely supported the benefits of breastfeeding (Jones et al., 2003; Kramer, 2010). Since approximately 90% of women take some form of medication during their first week postpartum (Matheson, 1985; Anderson, 1991), a general concern is the amount of drug transfer into milk and the toxicity due to this unnecessary exposure of infants to those drugs. Currently, there is a lack of knowledge on safety of many drugs during breastfeeding and thus maternal drug therapy is often affected. Therefore, the knowledge of drug amount in milk and drug exposure in infants are critical for risk-benefit assessment of drug use during lactation.

Determining the amount of a drug transferred into breast milk is critical in selecting safer drugs for breast feeding infants from both drug discovery and therapeutic points of view. Despite significant efforts to improve drug labeling for medication use during lactation, there remains a paucity of pharmacokinetic data in human milk. Among the drug labels approved between 2003 and 2012, only 5.2% labels contained human lactation data (Wang et al., 2017). There are many challenges in conducting a dedicated pharmacokinetic study in lactating women, such as difficulty in enrollment of lactating women and limited market value for sponsors (Wang et al., 2017). Data from animal lactation studies are not very useful due to the large inter-species variability in protein and lipid contents of milk. Several in vitro mathematical models have been developed to predict drug transfer into milk, with both strengths and limitations (Fleishaker et al., 1987; Atkinson and Begg, 1988; Atkinson and Begg, 1990; Begg and Atkinson, 1993; Larsen et al., 2003; Koshimichi et al., 2011). Different physicochemical

Abbreviations: ER, extraction ratio; FITC, fluorescein isothiocyanate; GH, growth hormone; IVIVE, in vitro to in vivo extrapolation; MEC, mammary epithelial cell; *M/P* ratio, milk to plasma ratio; P-gp, p-glycoprotein; TEER, trans-epithelial electrical resistance; TEM, transmission electron microscopy; TJ, tight junction.

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characteristics (i.e., lipophilicity, pKa, and protein binding) have been used in these models. These models can roughly estimate M/P ratios but they were derived from regression analyses and the prediction accuracy needs to be improved.

During lactation, the mammary epithelium is organized in lobes containing numerous lobules and connected to lactiferous ducts, which drain milk towards the nipple (Truchet and Honvo-Houeto, 2017). Each lobe contains numerous lobules formed by several alveoli, which are the basic secretory units producing milk (Truchet and Honvo-Houeto, 2017). The alveolus is defined by a monolayer of polarized alveolar mammary epithelial cells (MECs) arranged around a lumen, where milk is secreted. Drugs enter MECs and are secreted into milk by passive diffusion and/or active transport. Paracellular pathway is blocked during lactation because of the closure of tight junctions triggered by hormonal changes (Truchet and Honvo-Houeto, 2017). The MECs are the functional units that mediate the transport of drugs from blood to milk. Although In vitro permeation models and in vitro to in vivo extrapolation (IVIVE) have been successfully used to predict human intestinal absorption (Patel et al., 2012), hepatic clearance (Izumi et al., 2017), biliary excretion (Zou et al., 2013) and renal clearance (Kunze et al., 2014), there are very few studies of in vitro permeability models to estimate drug transport across mammary epithelium (Toddywalla et al., 1997; Kimura et al., 2006; Empey, 2007; Athavale et al., 2013). The CIT3 cell line, a subline of the Comma 1D normal mouse mammary epithelial cell, was the cell line that was mentioned in a few studies (Toddywalla et al., 1997; Empey, 2007; Athavale et al., 2013). However, due to the interspecies difference, the prediction performance may not be desirable.

In the present study, we used a human normal mammary epithelial cell line to form an integrated barrier and assessed the permeability of several P-gp substrates across the cells. The estimated milk to plasma (M/P) ratios were compared with the M/P ratios observed in lactating women.

2. Materials and methods

2.1. Cell lines and cell culture

The MCF10F (ATCC® CRL-10318™) and Caco-2 (ATCC® HTB-37™) cell lines were purchased from American Type Culture Collection (ATCC) (Manassas, Virginia). MCF10F cells were maintained in medium composed of DMEM/F12 (Corning, NY) supplemented with 5% horse serum (Fisher Scientific, Waltham, MA), 1% antibiotic-antimycotic (Corning, NY), EGF (20 ng/mL) (Corning, NY), 1 μg/mL hydrocortisone (MilliporeSigma, Burlington, MA), and 5 μg/mL insulin (MilliporeSigma, Burlington, MA), and 100 ng/mL cholera toxin (MilliporeSigma, Burlington, MA). The boosting medium used for culturing MCF10F on the Transwell plate (Corning, NY) were composed of DMEM/F12 (Corning, NY) supplemented with 10% fetal bovine serum (ATCC, Manassas, Virginia), 1% antibiotic-antimycotic (Corning, NY), EGF (20 ng/mL) (Corning, NY), 2 μg/mL hydrocortisone (MilliporeSigma, Burlington, MA) and 5 μg/mL insulin (MilliporeSigma, Burlington, MA). Caco-2 cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) high glucose with L-glutamine (without pyruvate) (Gibco/ Invitrogen, Fisher Scientific, Waltham, MA) supplemented with 10% fetal bovine serum, 1% nonessential amino acids (Gibco/Invitrogen, Fisher Scientific, Waltham, MA) and 1% Penicillin/Streptomycin (Gibco/Invitrogen, Fisher Scientific, Waltham, MA). Human growth hormone (GH) was purchased from Invitrogen (Fisher Scientific, Waltham, MA) and 17β-estradiol was purchased from Sigma (MilliporeSigma, Burlington, MA). Growth hormone at final concentration of 100 ng/mL and 17β-estradiol at final concentration of 70 nM were added to the cell culture medium for 48–72 h to examine their effect on tight junction formation.

A 12-well polyester or polycarbonate Transwell plate (pore size 0.4 μm, growth area 1.12 cm^2, membrane diameter 12 mm) (Corning, NY) was used to seed cells for Transwell cell culture, with 0.5 mL medium in the apical chamber and 1.5 mL medium in the basolateral chamber. MCF10F cells were seeded to the Transwell plate at a density of 125,000–200,000 cells per well and switched to boosting medium when culturing on the Transwell plate. The medium was changed once daily. The trans-epithelial electrical resistance (TEER) was measured daily or every other day with an Epithelial Volt/Ohm Meter (EVOM) (World Precision Instruments, Sarasota, FL) to determine if tight junctions are formed. Electrical resistance is calculated with the following equation:

$$\text{TEER}\left(\Omega^{*}\ \text{cm}^2\right) = \left[R_{\text{cells}}\ (\Omega) - R_{\text{blank}}\ (\Omega)\right] \times \text{Area}\left(\text{cm}^2\right)$$

2.2. Permeability tracer flux assay

Qualitative determination of paracellular permeability and monolayer integrity was also assessed using fluorescein isothiocyanate (FITC)-inulin (MW = approximately 5000 Da) and Lucifer yellow (MW = 442 Da) (MilliporeSigma, Burlington, MA). FITC-inulin or Lucifer Yellow tracers were dissolved in phosphate-buffered saline (PBS) and added to the apical side chamber of the Transwell plate at a concentration of 0.1 mg/mL. They were incubated for 3 h. Samples were taken from receptor chamber and fluorescence was read using a Biotek Synergy HT plate reader (Winooski, VT). The excitation and emission wavelengths were 485 nm and 520 nm, respectively for FITC-inulin, and 410 and 520 nm for lucifer yellow.

2.3. Transmission Electron microscopy (TEM) imaging

Cells cultured in Transwell with different medium conditions were used for electron microscopic analysis with a JEOL JEM-1400 Plus TEM with AMT NanoSprint12 CMOS camera (Peabody, MA). The preparation was similar to previously described (Steenbergen et al., 2018). The 2.5% glutaraldehyde (GA) sodium cacodylate buffer (Electron Microscopy Sciences, Hatfield, PA) was added to the cell culture media to fix the cells. After that, cells were washed to remove residual aldehyde from cells using 0.05 M sodium cacodylate buffer (Electron Microscopy Sciences, USA). Cells were further incubated with 1% ice-cold osmium tetroxide (OsO4, Electron Microscopy Sciences, USA) in 0.05 M sodium cacodylate buffer to fix lipid. The 1% uranyl acetate (Electron Microscopy Sciences, USA) was used to improve contrast of cell membrane and subcellular membrane. Dehydration of the samples was conducted with ascending ethanol concentration (30, 50, 70, 80, 90, 95 and 100%) solutions. After infiltration of cell membranes with Spurr's resin (Electron Microscopy Sciences, USA), several pieces of the cell culture inserts were embedded into BEEM flat embedding molds (Electron Microscopy Sciences, USA), sectioned perpendicular to the membrane surface and polymerized. A Leica EM UC7 ultramicrotome was used to section 60 nm samples, which were post-stained with uranyl acetate (2%) and Reinolds' lead citrate. Finally, sections were observed under the TEM.

2.4. Immunofluorescence

Cells were grown on a Transwell plate for two weeks and reached TEER values of over 1000 Ω·cm^2. Cells were rinsed once quickly with 37 °C DPBS and immediately fixed with 4% warm paraformaldehyde (in PBS) for 15 min. The cells were stored at 4 °C for immunostaining later. The following antibodies and fluorescent stains were used: Rabbit mAb anti-Zonula Occludens 1 (ZO-1) (Cell signaling Technology, Inc., Danvers, MA); Alexa Fluor 555, Goat anti-rabbit IgG (Invitrogen, Waltham, MA); DAPI (Cell signaling Technology, Inc., Danvers, MA). Fixed cells were incubated in blocking buffer (1× PBS / 5% normal serum / 0.3% Triton™ X-100) for an hour, then were incubated overnight at 4 °C with primary antibody diluted in antibody dilution buffer (1× PBS / 1% BSA / 0.3% Triton X-100). Afterwards, they were incubated with fluorochrome-conjugated secondary antibody diluted in antibody dilution for 2 h at room temperature. DAPI was added to a final

concentration of 1 μg/mL and incubated for 5 mins before imaging with a Life Technologies EVOS® FL cell imaging system (Thermo Fisher Scientific, Waltham, MA).

2.5. Quantitative Real-Time PCR

Cells were cultured in 100 mm tissue culture dishes or on Transwell inserts and collected. The isolation of total RNA was conducted using TRIzol reagent (Invitrogen, Thermo Fisher Scientific, Waltham, MA) according to manufacturer's protocols. Extracted RNA from the sample was reverse-transcribed into cDNA using SuperScript™ IV First-Strand Synthesis System Kit (Invitrogen, Thermo Fisher Scientific, Waltham, MA). Real-time PCR was performed in 96-well plates using a Biorad CFX Connect Real-Time PCR System (Hercules, California) using SsoAdvaced™ universal SYBR® Green Supermix kit (Bio-Rad Laboratories, Hercules, CA. The predesigned primers for human P-gp (Hs.PT.58.26805128) and house-keeping gene beta-actin (Hs.PT.39a.22214847) and glyceraldehyde 3-phosphate dehydrogenase (GADPH) (Hs.PT.39a.22214836) were purchased from Integrated DNA Technologies (Coralville, IA). The PCR was run at 50 °C for 2 min, 95 °C for 10 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Triplicate PCR reactions were performed for each sample. The setting of the threshold and baseline was performed according to the manufacturer's instructions. The data were analyzed using the default program of Biorad CFX Connect Real-Time PCR System. Quantification of the gene expression was performed using a relative standard curve method. The standard curve was generated by the mixtures of cDNA from the cells and serially diluted by a 1:5 ratio. A six-point standard curve was made for each gene. The expression of P-gp was normalized to the expression of beta-actin and GAPDH.

2.6. Transport assays

MCF10F cells grown on Transwell plates for 4 weeks were monitored for TEER with the EVOM Meter. Cells were rinsed with PBS before the assay. The transport solutions used were human breast milk at apical side and human female plasma at the basolateral side. Human breast milk was donated by 20–40 year-old nursing women, who were breastfeeding infants and did not take any medications. The process of human breast milk donation for this study was approved by Husson University Institutional Review Board (IRB) and all donors signed the informed consent before donating the breastmilk. Expressed human breastmilk was frozen after collection and milk from three donors were pooled together before running the assay. Pooled human female plasma was purchased from Valley Biomedical Products and Services, Inc. (Winchester, VA). Transport assays were conducted using 0.5 mL of apical solution and 1.5 mL of basolateral solution. Compounds were added to the donor solution (triplicate wells per concentration) and the assays were run for 2 h, with gentle shaking at 100 rpm at 37 °C. Samples (100 μL, three samples per time point) were taken at 5, 15, 25, 50, 80, 120 min from receptor side, and at the beginning (0 min) and end of the study (120 min) from donor side. Bi-directional permeability assay was conducted. The following compounds and concentrations were used for the transport assay: clarithromycin (100 and 1000 ng/mL), doxorubicin (50 and 500 ng/mL), digoxin (50 and 500 ng/mL), loratadine (100 and 1000 ng/mL), pefloxacin (1000 and 10,000 ng/mL), venlafaxine (100 and 1000 ng/mL). TEER values were measured at the end of the study to ensure the integrity of the cell barrier. All assays were run in triplicate.

The quantitation of the drug concentration in the samples was performed with an AB SCIEX QTRAP 4500 with a TurboV electrospray ionization source mass spectrometer (Applied Biosystems, Carlsbad, CA) coupled to an Agilent 1200 Series HPLC system (Agilent Technologies, Santa Clara, CA). HPLC separation was performed on a Waters XBridge® C18 3.5 μm 5 cm × 2.1 mm column (Waters Corporation, Milford, MA). Three-fold acetonitrile was added to the samples to precipitate protein before analysis with LC-MS/MS. Acetonitrile was selected as the organic solvent in the mobile phase. Mobile phase A (water containing 0.1% formic acid) was first kept at 90% for 0.5 min, and then decreased to 5% over 1 min and maintained at 5% for 2 min, and then returned to 10% mobile phase B (ACN containing 0.1% formic acid) and maintained for 3 min. The flow rate was 400 uL/min. The mass spectrometric conditions were as follows: source temperature, 400 °C; curtain gas (CUR), 30 psi; ionspray voltage (IS), 4500 V; desolvation gas temperature (TEM), 500 °C; ion source gas 1 (GS1), 60 psi; ion source gas 2 (GS2), 40 psi; collision gas (CAD), high; entrance potential (EP), 4 eV; collision energy (CE), 15 eV. Positive ion MS/MS was conducted to detect the drugs with the following MS/MS transitions: clarithromycin (748.5 > 590.5), doxorubicin (545 > 362), digoxin (798.7 > 651.5), loratadine (383.6 > 337.5), pefloxacin (334.2 > 290.2), and venlafaxine (278.4 > 260.2).

Papp (apparent permeability) values were calculated according to the following equation:

$$P_{app} = \frac{\left(\frac{dC}{dt}\right) \times V_r}{A \times C_o}$$

Where V_r is the volume of the recipient compartment, dC/dt is the slope of the cumulative concentration of the compound in the recipient chamber over time, A is the membrane surface area, C_0 is the compound initial concentration in the donor chamber.

Efflux ratio (ER) was calculated by $P_{app,\ b\text{-}a}$ /$P_{app,\ a\text{-}b}$. The M/P ratio was estimated using two approaches. In the first approach, the ER determined in MCF10F cells was the predicted M/P ratio. In the second approach, M/P ratio was calculated by incorporating three in vitro parameters ($F_{ni,7.4}$, $F_{ni,7.0}$ and ER) as shown below (Yang, Xue et al.):

$$M/P = ER^{*}Fni,7.4/Fni,7.0 \quad (1)$$

Where, $F_{ni,7.4}$ and $F_{ni,7.0}$ are nonionized fraction at pH 7.4 and pH 7.0, respectively.

2.7. Collection of M/P and in vitro parameter data

Clinically observed M/P data were collected from published literature (Yang, Xue et al.). Experimental values of fraction unbound in plasma (f_{up}) and fraction unbound in milk (f_{um}) data were collected from literature (Yang, Xue et al.). $F_{ni,7.4}$ and $F_{ni,7.0}$ were predicted by a prediction toolbox (F_{ni} module) in SimCYP (version 20, Certara USA Inc., Princeton, NJ, USA). The efflux ratios in Caco-2 cells were collected from literature (Yang, Xue et al.).

2.8. Statistical analysis

Statistical analyses were done in Microsoft Excel. Each experimental data point represents the mean ± standard deviation (SD) of at least triplicate assay results. A student's *t*-test was applied to test for statistically significant differences. A *P*-value ≤0.05 was considered significant.

3. Results

3.1. MCF10F cells growing on Transwells form intact cellular barriers

To achieve a polarized, intact and confluent epithelial cell layer, the effects of different cell culture medium supplements and hormones were evaluated. As shown in Fig. 1, the combination of different supplements in cell culture medium had different effects on tight junction formation in MCF10F cells. Maintenance medium with 5% horse serum (HS) did not seem to promote MCF10F cells to form integrated barrier, but boosting medium which contained fetal bovine serum (FBS), EGF, insulin and hydrocortisone (HC) enhanced TEER values of cells plated on Transwells steadily over time (Fig. 1A). The TEER values usually reached over 2000 Ω·cm^2 after a week and was able to reach as high as over 5000 Ω·cm^2 in some wells. Removal of each supplement resulted in

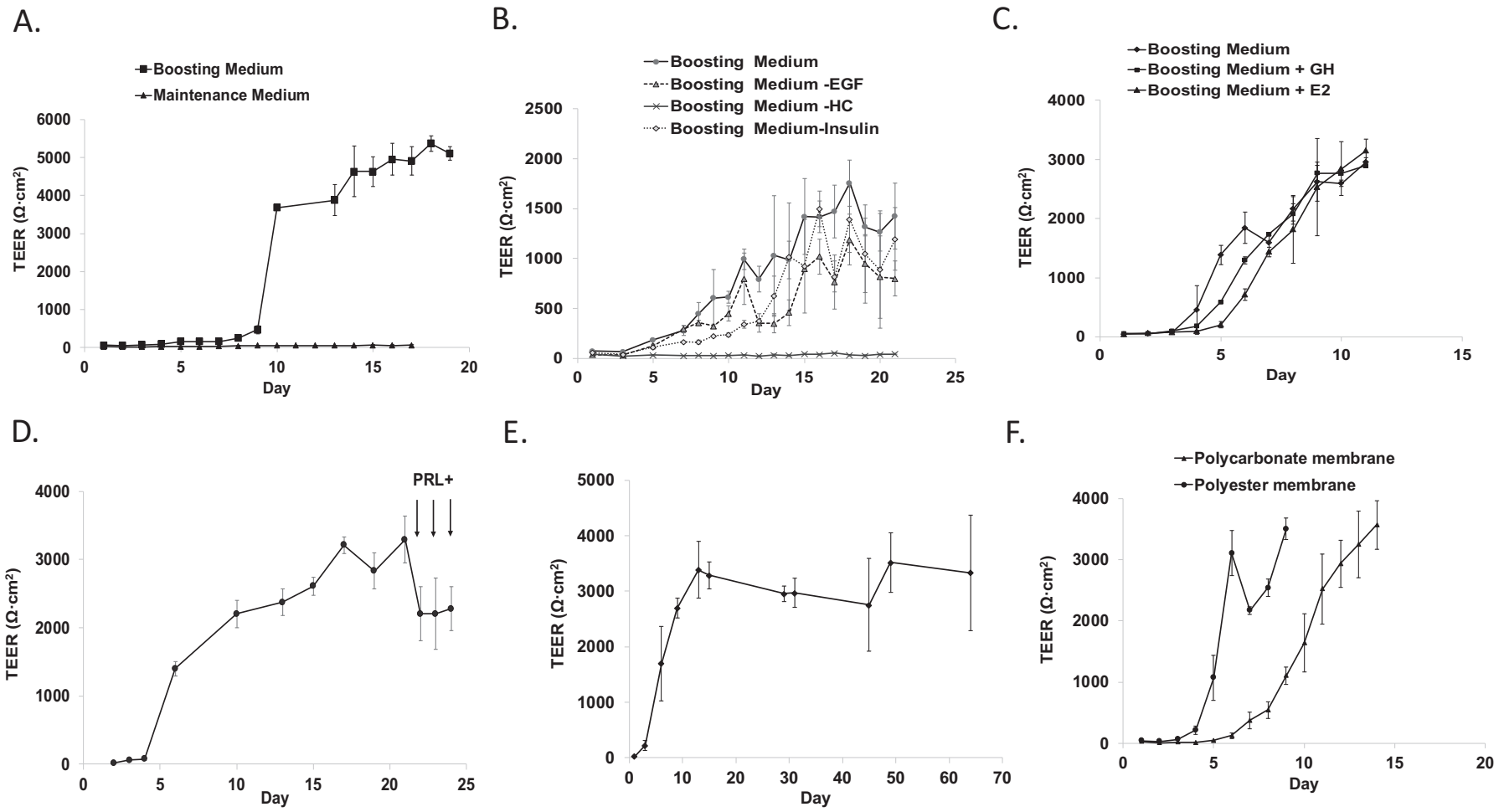

Fig. 1. Assessment of barrier integrity of MCF10F cells by TEER measurement. A. TEER values of MCF10F cells grown on Transwell inserts with boosting medium and maintenance medium. B. Effect of removal of different hormones or growth factors on TEER values. C. Effect of adding growth hormone (GH) and 17-β-Estradiol (E2) to boosting medium on TEER values. The measurement was stopped after two weeks as there was no difference observed. D. Effect of prolactin (PRL) on TJ formation. E. Two months culture of MCF10F cells on Transwell inserts. F. Comparison of polycarbonate membrane versus polyester membrane on TEER values.

TEER decrease at different magnitudes, and the influence of removing HC was the most dramatic (Fig. 1B). For the other two hormones the growth hormone (GH) and 17β-estradiol (E2) showed no effect on the formation of tight junction (Fig. 1C). Prolactin (PRL) has been reported as an important hormone that regulates alveologenesis and lactogenesis II (Borellini and Oka, 1989; Strange et al., 1991). Our results showed that prolactin negatively impacted tight junction formation and reduced TEER values by about 20–30% (Fig. 1D). However, since the addition of PRL was only for 3 days and its effect on TEER values seemed to be minor, it did not pose a risk to disrupt tight junctions.

MCF10F cells can maintain tight junction for a long time under our culture conditions. Even after two months growing on Transwell inserts, the cells still maintain intact barrier indicated by high TEER values (Fig. 1E). Because Transwell permeable supports are available as either polycarbonate or polyester membrane, we compared tight junction formation of MCF10F cells grown on these two types of membranes and found that the polyester membrane promoted tight junction formation a few days earlier than the polycarbonate membrane (Fig. 1F).

In addition to TEER measurement, the paracellular flux of fluorescent tracers across the cell barriers also reflects the permeability. The most common paracellular tracers used in in vitro models are fluorescent compounds (e.g., lucifer yellow) or fluorescently labeled compounds (e.g., FITC-dextran and FITC-inulin). Paracellular flux of fluorescent tracer FITC-inulin and Lucifer yellow were thus employed to confirm tight junction formation. Both FITC-inulin and lucifer yellow pass across cell barrier through passive paracellular diffusion. FITC-inulin has a relatively large molecular weight (MW) of ~5 kDa while lucifer yellow has a MW of 442 Da. As shown in Fig. 2, after the TEER values of MCF10F cells reached over 1000 $\Omega \cdot cm^2$, neither fluorescent markers passed through the cell barrier effectively. This further confirms the formation of tight junctions.

3.2. MCF10F cells maintain barrier integrity and produce lipid droplets

To view the morphology of MCF10F cells grown on Transwell inserts, TEM was used to take images. As shown in Fig. 3A, cells aligned closely to each other one day after being plated on the Transwell insert. At 3–4 weeks, cell to cell tight junctions were clearly observed under TEM (Fig. 3B). The formation of microvilli was also shown on the apical side of the cells, demonstrating the polarization of the cells. After the cells were incubated with prolactin, more and larger lipid droplets were observed inside the cells (Fig. 3C&D), which indicated the functional differentiation of MCF10F cells to milk-secreting mammary epithelial cells. The staining of tight junction protein Occludens 1 (ZO-1) demonstrated a continuous TJ formation (Fig. 4).

3.3. Consistent P-gp expression in MCF10F cells in Transwell plate

The estimation of permeability using Caco-2 cells for many drugs including P-gp substrates often produces a large inter-study variability (Bentz et al., 2013; Bruck et al., 2017; Lee et al., 2017). We thus compared the P-gp mRNA expression in MCF10F cells and Caco-2 cells cultured on regular cell culture petri dishes versus on Transwell permeable membranes. The expression was normalized to both beta-actin and GAPDH as house genes. Although there are some differences in normalized expression levels when using different house keeping genes, the trend is the same and the selection of reference gene doesn't affect the findings. The results showed P-gp mRNA expression in Caco-2 cells grown on Transwells was ~30–50 fold higher relative to Caco-2 cells grown on regular cell culture petri dishes (Fig. 5). The dramatic increase in P-gp expression may inevitably cause large inter-assay or inter-lab variations when Caco-2 cells are used to estimate drug permeability for P-gp substrates. In contrast, the expression of P-gp in MCF10F cells between Transwell culture and regular petri dish culture was similar. In addition, it has been reported that P-gp expression is dramatically downregulated during lactation, with ~52-fold lower mRNA expression in lactating MECs relative to nonlactating MECs (Alcorn et al., 2002). Therefore, when Caco-2 permeability is used to predict drug excretion into human milk, the overexpression of P-gp in Caco-2 cells grown on Transwell plate is likely to cause overprediction of M/P ratio.

3.4. Permeability estimation with MCF10F transport assay

We next used MCF10F cells grown on Transwell inserts to estimate drug permeability. Six P-gp transporter substrate, clarithromycin, doxorubicin, digoxin, loratadine, pefloxacin, and venlafaxine were selected as the testing drugs. Human plasma and human breast milk were added to basolateral and apical chambers as incubation media, respectively. The MCF10F permeability results are shown in Table 1. Values were reported as average of replicates ± standard deviation (SD). The ER determined in MCF10F cells was used to predict M/P ratios.

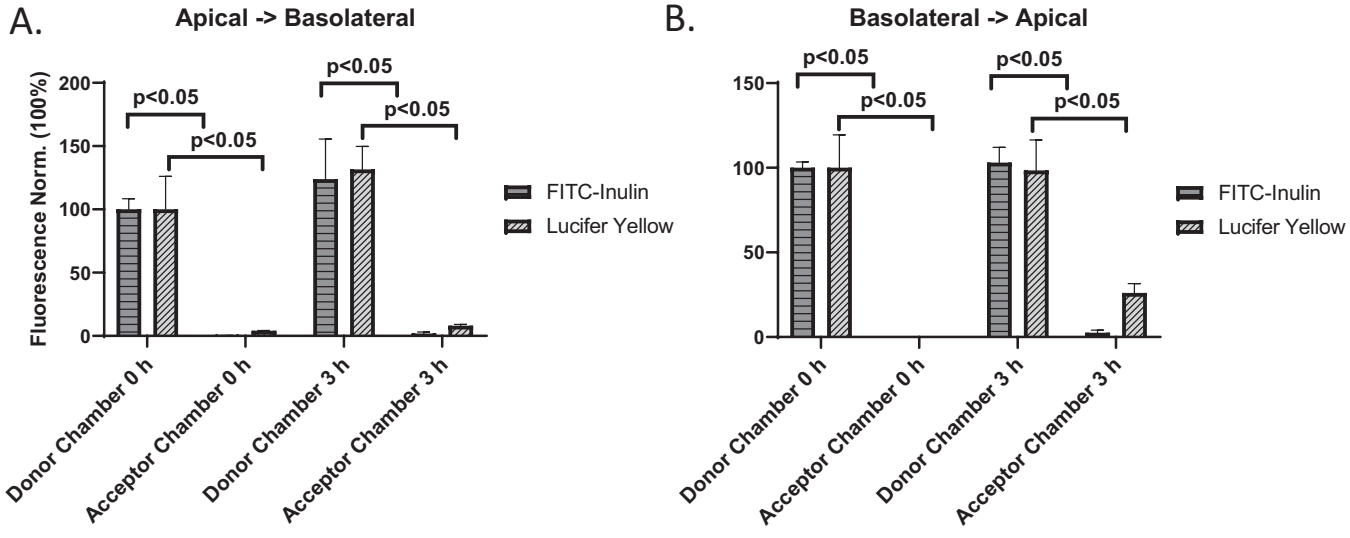

Fig. 2. Assessment of barrier integrity by FITC-Inulin and lucifer yellow. FITC-Inulin or lucifer yellow were added to the apical (A) or basolateral (B) chamber of MCF10Fs grown on Transwell inserts with tight junctions. The paracellular flux of FITC-Inulin or lucifer yellow from the apical to basolateral side and basolateral to apical side was assessed. Results are expressed as Mean ± SD from triplicates. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

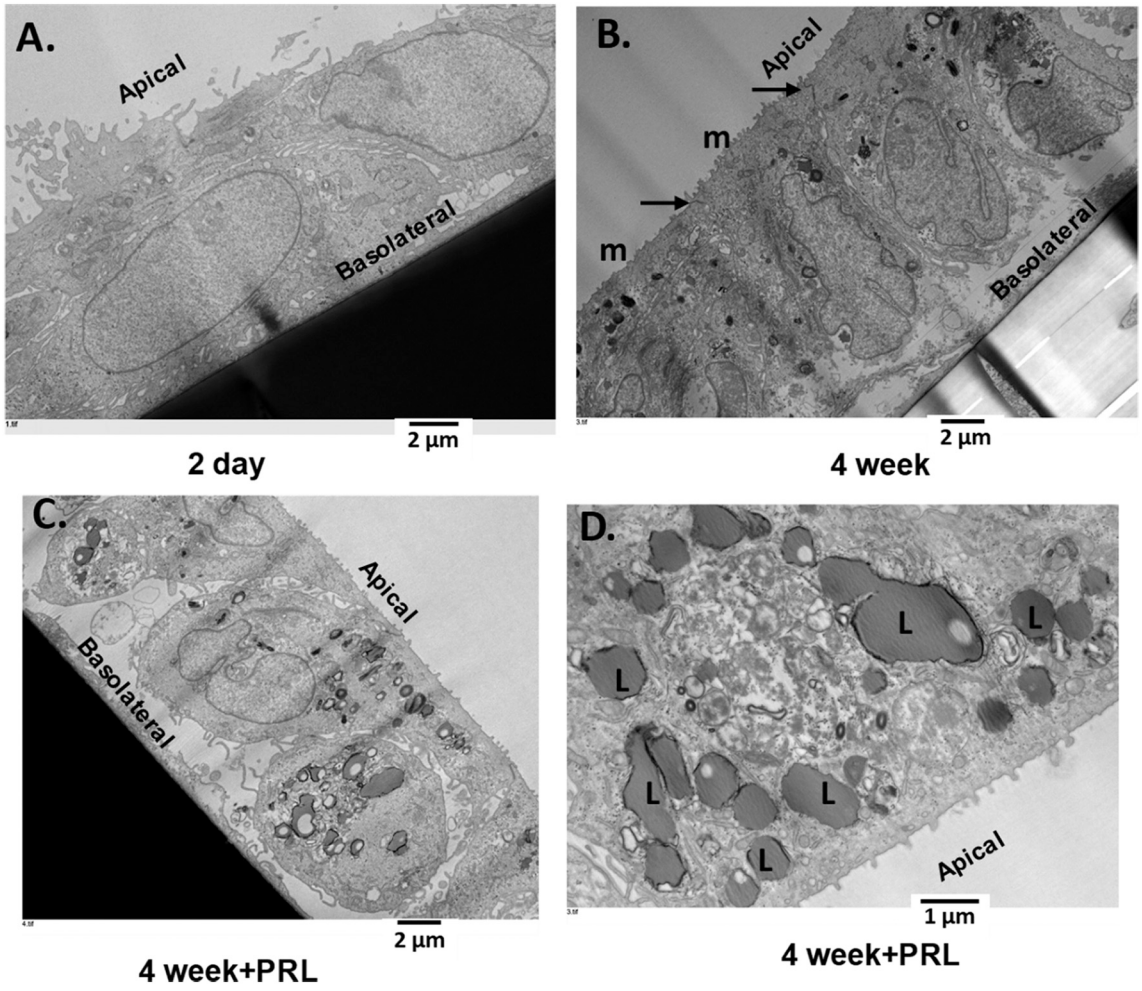

Fig. 3. TEM imaging of MCF10F cell morphology grown on Transwell inserts. A. MCF10F cells plated on Transwell inserts for one day. B. MCF10F cells grown on Transwell inserts for 4 weeks. Arrows indicate tight junction, m, microvilli. C&D. MCF10F cells grown on Transwell inserts for 4 weeks were further incubated with prolactin for three days. L indicates lipid droplets. Bar is 2 µm in A, B &C, and 1 µm in D. Shown are representative images.

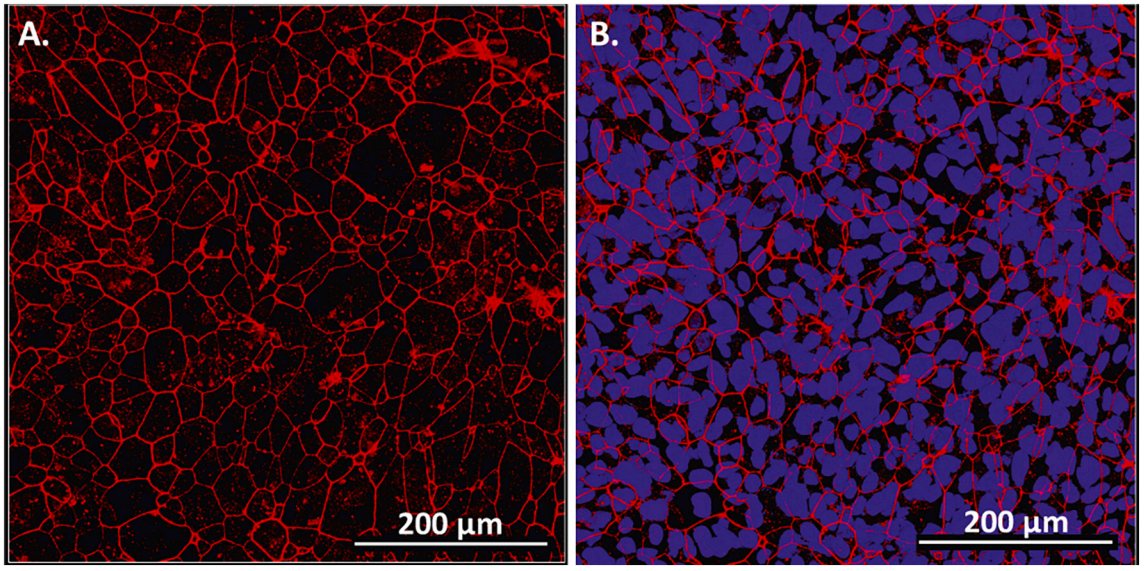

Fig. 4. Staining of the tight junction protein Zonula Occludens 1 (ZO-1) of MCF10F cells formed tight junction on Transwell inserts. MCF10F cells were grown on Transwell inserts for two weeks and the TEER values for the cells reached over 1000 $\Omega \cdot cm^2$. Cells are stained for the cell nucleus (blue) (Panel B) and tight junctions (ZO-1, red) (Panel A & B), scale bar: 200 µm. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

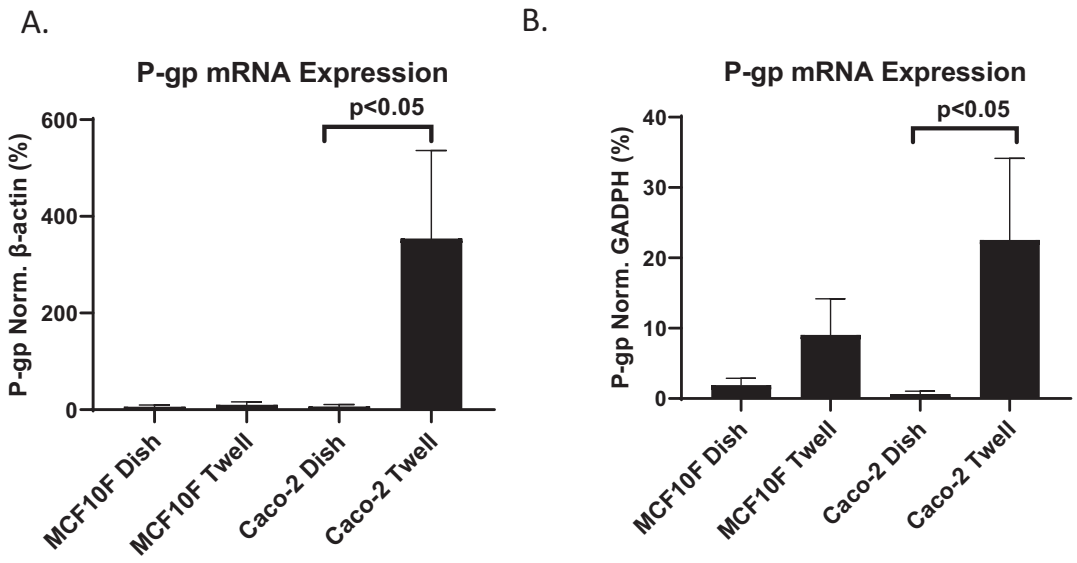

Fig. 5. Real-Time PCR quantitation of P-gp mRNA expression. Relative expression of P-gp mRNA of MCF10F cells and Caco-2 cells grown on petri-dish or Transwell was determined by RT-PCR. A. Expression normalized by beta-actin. B. Expression normalized by GADPH.

Table 1
Milk-to-plasma (M/P) ratio prediction based on permeability data.

Drug		B- > A	A- > B	Predicted M/P Using ER	Fni7.0*(Yang, Xue et al.)	Fni7.4*(Yang, Xue et al)	Predicted M/P Using ER & pH correction	Observed M/P	Ratio (Pred/Obs)	Refer.
Clarithromycin (P-gp)	MEC	5.9	18	0.327			0.812		3.69	(Togami et al., 2014)
	Caco-2	10.4	2.7	3.86	0.0101	0.0251	9.59	0.22	43.6	
Digoxin (P-gp)	MEC	13.3	2.83	4.72			2.9		2.4	(Skolnik et al., 2010)
	Caco-2	7.76	1.01	7.68	0.585	0.36	4.73	1.21	3.91	
Doxorubicin (P-gp/BCRP)	MEC	0.372	0.37	1.01			2.32		1.95	(Hugger et al., 2002)
	Caco-2	3.45	0.439	7.86	0.059	0.136	18.1	1.19	15.2	
Loratadine (P-gp)	MEC	1.1	1.27	0.87			0.87		0.725	(Crowe and Wright, 2012)
	Caco-2	39.5	39.6	0.997	0.998	0.999	0.998	1.2	0.832	
Pefloxacin (P-gp/BCRP)	MEC	4.03	1.7	2.36			1.11		1.16	(Griffiths et al., 1994)
	Caco-2	3.72	3.17	1.17	0.034	0.016	0.552	0.96	0.575	
Venlafaxine (P-gp)	MEC	11.8	6.38	1.84			4.59		1.67	(Hellinger et al., 2012)
	Caco-2	40.3	50.4	0.8	0.00397	0.0099	1.99	2.753	0.724	

* Fni7.0, Fni7.4 values were from Yang et al. (2022).

Furthermore, to represent the pH gradient between human plasma and milk, $F_{ni,7.4}$ and $F_{ni,7.0}$ were included Eq. (1) as correction factors for M/P ratio calculation. As shown in Fig. 6, compared to Caco-2 permeability data, MCF10F cell permeability data improved M/P ratio predictions for four P-gp substrates (clarithromycin, doxorubicin, digoxin and pefloxacin), particularly when pH correction factors were included. The predicted M/P ratio for loratadine and venlafaxine from MCF10F cell permeability was comparable with that derived from Caco-2 cell permeability.

4. Discussion

Although the knowledge of drug amount in milk and drug exposure in infants are critical for risk-benefit assessment of drug use during lactation, there have been only a few in vitro mammary cell permeability-based studies to evaluate drug transfer from blood to milk. All the reported studies were conducted using murine CIT3 cell line (Toddywalla et al., 1997; Kimura et al., 2006; Empey, 2007; Athavale et al., 2013), except only one study using primary MECs (Kimura et al., 2006). We herein use a human normal MEC line to form tight junctions and estimate drug permeability. The easy accessibility and low maintenance cost of human normal MEC line provide a better option compared to primary human MECs. In addition, the human primary MECs plated on Matrigel® coated inserts had a maximum TEER values of 227 $\Omega \bullet cm^2$ (Kimura et al., 2006), which seemed to be low compared to the TEER values of other commonly used cell lines. In our lab, we obtained human primary mammary epithelial cells from American Type Culture Collection (ATCC) and followed the same procedure of triple trypsin approach but failed to produce cells that can form tight junctions (Data not shown).

Formation of intact tight junctions by MECs at the start of lactation is important to establish lactation (Truchet and Honvo-Houeto, 2017). Some hormones, such as estrogen, progesterone, human placental lactogen (hPL), follicle stimulating hormone (FSH) play a role from early to late pregnancy, but rapidly withdraw a few days after birth before mature milk is produced (Truchet and Honvo-Houeto, 2017).

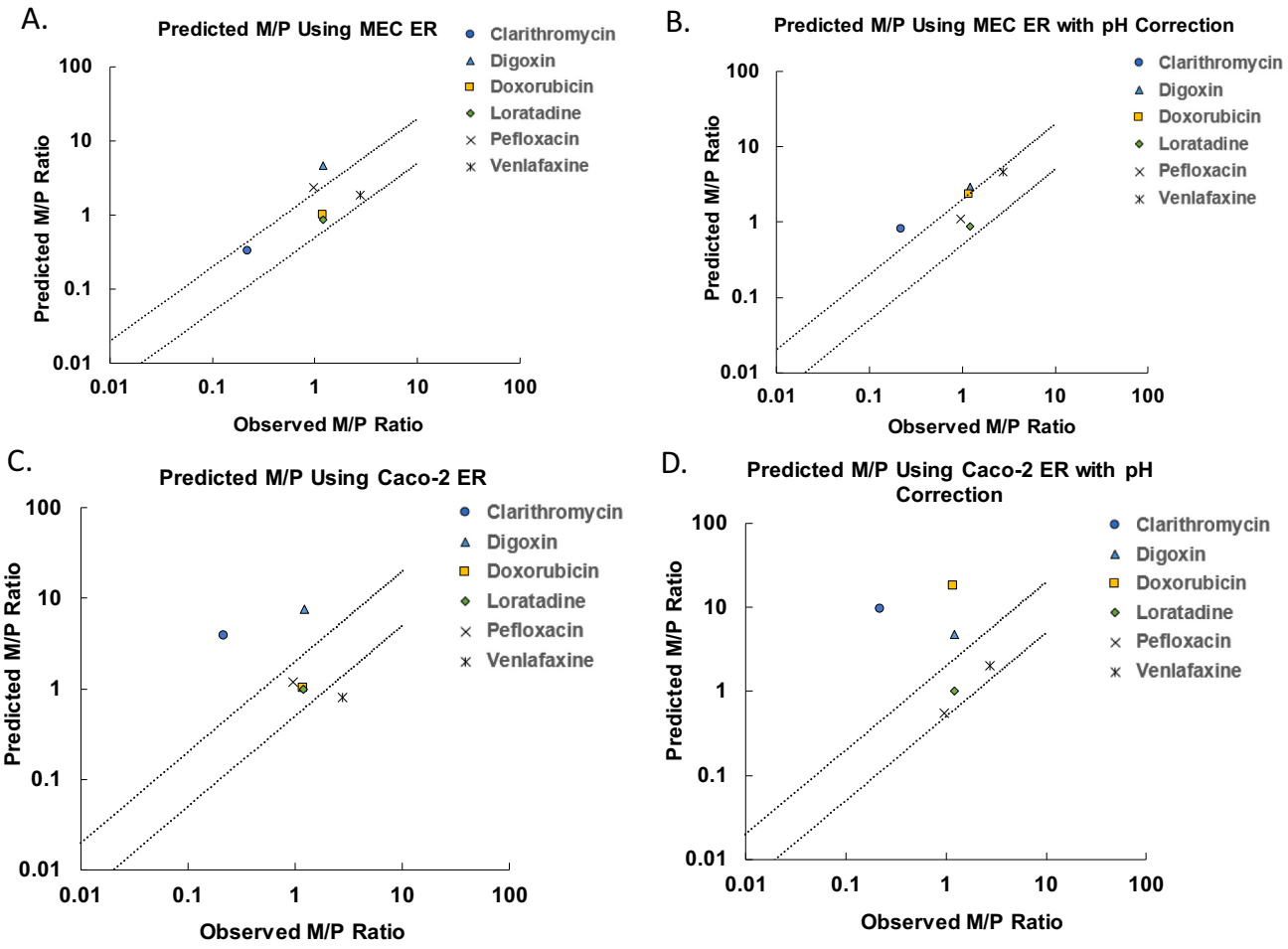

Fig. 6. Comparison of M/P ratio prediction using human MEC permeability data versus Caco-2 permeability data. The MCF10F and Caco-2 cell determined permeability values for clarithromycin, doxorubicin, digoxin, loratadine, pefloxacin, and venlafaxine were used to predict M/P ratios and compared with observed M/P ratios. A. Prediction of M/P ratios using MCF10F permeability results with extraction ratio (ER) approach. B. Prediction of M/P ratios using MCF10F permeability results with extraction ratio (ER) and correction for pH difference between plasma and milk approach. C. Prediction of M/P ratios using Caco-2 permeability results with extraction ratio (ER) approach. D. Prediction of M/P ratios using Caco-2 permeability results with extraction ratio (ER) and correction for pH difference between plasma and milk approach. Dash lines represent 2-fold deviations.

Glucocorticoids are involved in proliferation, morphogenic and lactogenic differentiation of MECs, which also induces TJ closure (Neville et al., 2002). Consistent with this, our results show that hydrocortisone promotes TJ formation as well (Fig. 1B). Insulin is present from pregnancy to lactogenesis I and II, but its absence doesn't seem to prevent lactogenesis II or decrease milk yield (Neville, McFadden et al. 2002). Insulin may have a role in mammary lipogenesis and lactose synthesis, but no direct evidence supports this. While main role of insulin in lactation is to regulate nutrient flux to the mammary gland (Neville et al., 1993; Griinari et al., 1997a, 1997b), its effect on TJ permeability has not been reported and our results show that insulin can promote TJ formation (Fig. 1B). EGF stimulates ductal growth and proliferation of mammary epithelial cells (Neville et al., 2002), and we show that EGF can promote TJ formation (Fig. 1B). Growth hormone (GH) is produced by the pituitary gland, and recently it is found that mammary epithelium itself can produce GH (Neville et al., 2002). GH is not needed for alveolar development and its effect on lactation is complex and controversial. 17-β-Estradiol (E2) is required for both ductal growth and lobuloalveolar development during pregnancy, and it is reported to stimulate casein synthesis and lactose synthetase activity. In our study, GH and 17-β-Estradiol (E2) didn't show a clear effect on TJ formation. Prolactin involves in MEC proliferation and differentiation, regulates the synthesis and secretion of milk, and probably plays a role in maintenance of TJ as well (Nguyen and Neville, 1998; Truchet and Honvo-Houeto, 2017). When prolactin was added to the medium in our study, the appearance of large lipid droplets suggest prolactin may contribute to mammary lipogenesis and lactose synthesis.

The human Caco-2 cell line was derived from a colon carcinoma and has been used extensively by academia and pharmaceutical industries for decades as an in vitro model to estimates drug permeability. Caco-2 cells are able to spontaneously differentiate into columnar cells with microvilli on the apical surface with high apical expression of P-gp (Crowe, 2021). Caco-2 cells require 3–4 weeks to differentiate, form tight junctions and express P-gp. The overexpression of P-gp during this time seemed to time dependent (Crowe, 2021). Consistent with this, our data also showed a dramatic increase of P-gp expression in 4-week Transwell-grown Caco-2 cells compared with Caco-2 cells grown on petri dish. Researchers have used various approaches to produce P-gp overexpression Caco-2 cells (Shirasaka et al., 2006; Lee et al., 2013; Lumen et al., 2013; Crowe, 2021). While these cells are useful for the purpose of identifying P-gp substrates, they resulted in large variability of permeability data due to the inherent difference of P-gp expression in Caco-2 cells and the modulation of P-gp expression by different conditions. In contrast, the MCF10F cells did not show much difference in P-gp expression under different culture conditions as shown in our study. Furthermore, in vitro and preclinical data revealed that P-gp expression was decreased in lactating mammary gland (Alcorn et al., 2002; Yagdiran et al., 2016). Therefore, the MCF10F cells is a better option

compared to Caco-2 cells in term of estimating blood to milk transfer of P-gp drug substrates. However, further studies to assess P-gp expression in MCF10F cells compared to that in human lactating mammary epithelial cells is needed to further validate this assay and support in vitro and in vivo extrapolation

.

5. Conclusion

In the present study, we developed a human MEC cell-based permeability assay for M/P predictions for small-molecule drugs. A number of factors were identified to promote MEC cells to form tight junctions when grown on the Transwell inserts. The assay showed a good prediction accuracy for a few P-gp substrates. We believe this assay will be a useful tool in predicting M/P ratios for small molecule drugs, especially P-gp substrates. The performance of this assay can be further validated with more drugs and its application to substrates of other transporters (i.e., BCRP, OATs and OCTs) is possible upon future evaluation.

Credit author statement

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Tao Zhang: Conceptualization; Data curation; Formal analsis; Funding acquisition; Investigation; Methodology; Project administration; Supervision; Writing - original; Writing - editing.
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Declaration of Competing Interest

All authors declare no conflicts of interest relevant to the content of this article.

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