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Review Article

Factors influencing cell differentiation and expressions of cytochrome P450s and transporters in Caco-2 cells

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Abstract

Absorption, transportation, and first-pass metabolism of xenobiotics, drugs and nutrients taken orally occurs in the intestine, which is a major organ expressing cytochrome P450 enzymes (CYP) and transporters. Caco-2 is a human colon carcinoma cell line widely employed as a model for xenobiotic absorption, transportation, and metabolism in the intestinal tract as it can spontaneously differentiate to become small intestinal enterocytes. Culturing factors influence differentiation of Caco-2 as well as expression of CYPs and drug transporters. Culturing period, medium composition, type of filter membrane inserts, and the seeding density, account for the major factors affecting cell differentiation and the expression of CYP and transporter. Therefore, determining the optimal conditions for Caco-2 culturing is of strong concern depending on the aim and experimental design of the study.

Keywords: cytochrome P450, transporter, Caco-2, differentiation, intestinal enterocyte

1. Introduction

Oral consumption is the main route for drugs and nutrients entering the body (Angelis & Turco, 2011), by which the gastrointestinal tract, particularly the intestine, is the main site of absorption (Ferrec & Fardel, 2012). In addition, the intestine is the first line of xenobiotic metabolism and transportation via Phase I cytochrome P450s (CYP) and many transporters that facilitate xenobiotics entering the blood circulation system (Kaminsky & Zhang, 2003; Xie, Ding, & Zhang, 2016). CYP is a superfamily of monooxygenase enzymes responsible for drug metabolism (Lampen *et al.*, 1998; Xie *et al.*, 2016) and their activity depends on abundance and expression in the intestine (Xie *et al.*, 2016). In addition, there are influx- and efflux-transporters in the intestine affecting xenobiotic transportation (Varma *et al.*, 2010).

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Caco-2 is a human colon carcinoma cell line which is commonly used as a model for absorption, transportation, and metabolism in the intestinal epithelium (Natoli *et al.*, 2011). Though Caco-2 originated from human colorectal carcinoma cell, it can spontaneously differentiate to provide either small intestinal enterocyte or colonocytic characteristics (Borlak & Zwadlo, 2003; Maubon *et al.*, 2007). Caco-2 first appears as a monolayer of adherent, cylindrical enterocytes on a culturing flask. The mature enterocyte and microvilli differentiation occurs after prolonged culturing for 2 to 3 weeks (Kumar, Karnati, Reddy, & Chandramouli, 2010; Sun, Chow, Liu, Du, & Pang, 2008). Caco-2 expresses various transporters, e.g. ATP-binding cassette subfamily B member 1 (ABCB1) and drug metabolizing enzymes such as CYPs, which both are located in the enterocytes of the small intestine (Sun *et al.*, 2008). Several studies have revealed that the culturing conditions, e.g. the passage number, culturing medium, culturing period, seeding density, and the characteristic of filter membrane inserts affect the differentiation and the expression of CYPs and transporters in Caco-2 cells (Behrens & Kissel, 2003; Lea, 2015; Natoli *et al.*, 2011; Sambuy *et al.*, 2005).

2. Expression of CYPs in Caco-2

CYP-mediated oxidation is the main intestinal metabolism to increase the polarity of metabolite(s) for elimination (Zanger & Schwab, 2013). Thereby, the metabolism via CYPs is considered as a factor limiting bioavailability when a drug passes through intestinal cells before entering the circulation (Xie *et al.*, 2016; Zanger & Schwab, 2013).

CYP1, CYP2, and CYP3 are mainly expressed along the length of the human small intestine but they are less abundant in colon (Ferrec & Fardel, 2012; Zanger & Schwab, 2013). In the enterocytes of human small intestine, 9 CYP mRNAs, namely CYP1A1, CYP1B1, CYP2C8, CYP2C9, CYP2C19 CYP2D6, CYP2E1, CYP3A4, and CYP3A5 are expressed (Ferrec & Fardel, 2012; Xie *et al.*, 2016; Zanger & Schwab, 2013) but only CYP1A1, CYP2C9, CYP2C19, CYP2D6, CYP3A4, and CYP3A5 proteins have been detected (Xie *et al.*, 2016; Zanger & Schwab, 2013). CYP3A4 and CYP3A5 account for ~80% of the CYPs in the human intestine (Ferrec & Fardel, 2012; Xie *et al.*, 2016) and ~50% of drugs are metabolized by CYP3A4. Hence, CYP3A4 is considered as the key determining factor of lower bioavailability (Ferrec & Fardel, 2012; Zanger & Schwab, 2013). CYP2C9 is the second most abundant isoform in the intestine (~15%) (Paine *et al.*, 2006; Xie *et al.*, 2016), followed by CYP2C19 (~2%), CYP2J2 (~1.4%), CYP2D6 (~0.7%), and CYP1A1 and CYP2E1 (~0.9%) (Paine *et al.*, 2006; Vaessen *et al.*, 2017; Xie *et al.*, 2016). Correlated to the intestinal CYPs expression, hepatic CYP3A is the highest expression (40%), followed by CYP2C (25%), CYP2D6 (2%), and CYP1A1 (1%). By contrast, hepatic CYP1A2, CYP2E1, CYP2A6, and CYP2B6 were expressed for 18, 9, 6, and <1%, respectively (Paine *et al.*, 2006; Zanger & Schwab, 2013). Though CYP3A was the most abundant CYP in the small intestine, it accounted for ~1% in the liver (Paine *et al.*, 2006).

The mRNA level of CYPs in Caco-2 cells is approximately 5-fold lower than in human jejunum. CYP3A5 showed the highest expression in Caco-2 cells followed by CYP2J2 > CYP1A1 > CYP2C9 and CYP2C18 > CYP3A4 > CYP2D6 > CYP2E1 (Vaessen *et al.*, 2017). Generally, CYP1A1, CYP1B1, CYP2C8, CYP2C9, CYP2C18, CYP2C19, CYP2D6, CYP2E1, CYP2J2, CYP3A4, and CYP3A5 were expressed at very low levels in Caco-2 cells compared to human jejunum (Vaessen *et al.*, 2017; Xie *et al.*, 2016). Many CYP inducers (β-naphthoflavone, benzimadole derivatives, 3-methylcholanthrene, benz[*a*]anthracene, benzo[*a*]pyrene, benzo[*e*]pyrene, phenobarbital, Aroclor 1254, and vitamin D derivatives) have been applied to induce expression of CYPs in Caco-2 cells (Borlak & Zwadlo, 2003; Ferrec & Fardel, 2012; Sun *et al.*, 2008; Vaessen *et al.*, 2017) (Table 1).

CYP1A1 mRNA has been detected at different levels in human hepatic total RNA (5 μg), human duodenal enterocytes (15 μg), and Caco-2 cells (20-40 μg) (Lampen *et al.*, 1998; Sun *et al.*, 2008). CYP1A1 was inducible in Caco-2 cells by β-naphthoflavone, benzimadole derivatives, 3-methylcholanthrene, benz[*a*]anthracene, benzo[*a*]pyrene, benzo[*e*]pyrene, and phenobarbital (Borlak & Zwadlo, 2003; Sambuy *et al.*, 2005). CYP1A1 is the main enzyme in the procarcinogen activating process, thereby the Caco-2 cell line is a suitable model for intestinal biotransformation by carcinogens such as 7,12-dimethylbenz[*a*]anthracene (Lampen *et al.*, 1998). Aroclor 1254 elevated expression of CYP1A1, CYP2C, CYP2E1, and CYP3A5 mRNA by 14, 10-, 2.4-, and 2.5-fold, respectively, while it decreased expression of CYP2D6 in Caco-2 cells (Borlak & Zwadlo, 2003) (Table 1). Several studies have revealed that CYP3A4, the most abundant CYP in human intestine, is absent or expressed only at low levels in Caco-2 cells. CYP3A4 expression in Caco-2 cells was 871 and 150 times lower than *ex vivo* human jejunum and duodenum, respectively (Ferrec & Fardel, 2012;

Table 1. Expression of CYPs in human intestine and Caco-2 cell by typical CYP inducers

	Level of Expression		Inducers	References
	Human intestine	Caco-2 cell		
CYP1A1	+++	++	β-naphthoflavone, benzimadole derivatives, 3-methylcholanthrene, benz[*a*]anthracene, benzo[*a*]pyrene, benzo[*e*]pyrene, DMBA, and phenobarbital	Vaessen *et al.*, 2017; Borlak & Zwadlo, 2003; Sambuy *et al.*, 2005
CYP1B1	+++	–	N/A	Zanger & Schwab, 2013; Xie *et al.*, 2016
CYP2C8	+++	–	N/A	Zanger & Schwab, 2013; Xie *et al.*, 2016
CYP2C9	+++	++	N/A	Vaessen *et al.*, 2017
CYP2C18/19	+++	++	N/A	Vaessen *et al.*, 2017
CYP2D6	+++	++	N/A	Borlak & Zwadlo, 2003; Vaessen *et al.*, 2017
CYP2E1	+++	+/-	Aroclor 1254	Borlak & Zwadlo, 2003; Vaessen *et al.*, 2017
CYP2J2	+++	++	N/A	Vaessen *et al.*, 2017
CYP3A4	++++	+	Aroclor 1254, 1α,25-dihydroxyvitamin D3 or vitamin D	Lampen *et al.*, 1998; Sun *et al.*, 2008; Vaessen *et al.*, 2017; Ferrec & Fardel, 2012
CYP3A5	+++	++	Aroclor 1254	Borlak & Zwadlo, 2003; Vaessen *et al.*, 2017

Note. +++ and ++++, Expression is higher than 5 and 100 folds compared to the expression in Caco-2 cell, respectively; + and ++, Expression is lower than 100 and 5 folds compared to the expression in human intestine (jejunum), respectively; +/-, Very low expression; –, Absence; N/A, No data available; CYP, Cytochrome P450; Caco-2, Human colon carcinoma cell line.

Lampen *et al.*, 1998; Sun *et al.*, 2008; Vaessen *et al.*, 2017). Incubation of Caco-2 with 1α,25-dihydroxyvitamin D3 or vitamin D increased expression of CYP3A4 (Ferrec & Fardel, 2012; Fisher *et al.*, 1999) (Table 1).

There are two Caco-2 cell lines designed for studies of CYP3A xenobiotic metabolism and biotransformation, namely CYP3A4-tranfected Caco-2 cell and Caco-2/TC7 clone (Sambuy *et al.*, 2005; Sun *et al.*, 2008). The CYP3A4-transfected Caco-2 cell line shows higher CYP3A4 expression than wild type, but expression declines with passage number because the half-life of the vector is three to four weeks (Sambuy *et al.*, 2005). The Caco-2/TC7 cell line is a Caco-2 clone arising from repeated subculturing of the Caco-2 cell line (198 times) that expresses more CYP3A4 and CYP3A5 mRNA than the parent (Ferrec & Fardel, 2012; Pereira, Costa, Sarmento, & Araújo, 2016). Moreover, the Caco-2/TC7 clone demonstrates similar morphology to parent Caco-2 cells, having microvilli on the apical surface and connecting with neighboring cells via tight-junctions (Pereira *et al.*, 2016). Hence, to study CYP3A metabolic activity, transportation, and biotransformation, the Caco-2/TC7 clone is preferable to the Caco-2/ATCC (American Culture Tissue Collection) cell line. Finally, the Caco-2/ATCC cell line can be induced to express CYP3A4/CYP3A5 at levels four times higher than the Caco-2/TC7 clone with repeated passage in the presence of vitamin D (Ferrec & Fardel, 2012; Sambuy *et al.*, 2005).

3. Expression of Transporters in Caco-2

Membrane transporters play an important role as a regulator of xenobiotic transportation into cells and organelles (Estudante, Morais, Soveral, & Benet, 2013). Transporters are divided into two super-families based on transportation direction; uptake transporters called solute carrier (SLC) transporters and efflux transporters called ATP-binding cassette (ABC) transporters (Estudante *et al.*, 2013; Murakami & Takano, 2008). Intestinal uptake and efflux transporters both influence pharmacodynamics and pharmacokinetics of drugs and xenobiotics (Estudante *et al.*, 2013).

ABC and SLC transporters are expressed on both apical and basolateral membranes of human intestinal enterocytes (Estudante *et al.*, 2013; Varma *et al.*, 2010). The ABC family transporters play a role as the primary active transporter requiring adenosine triphosphate (ATP) to facilitate xenobiotic moving out of the enterocyte (Murakami & Takano, 2008; Varma *et al.*, 2010). The ABC transporters expressed in human intestine include ABCB1, ABC subfamily G member 2 (ABCG2), and six members of ABC subfamily C (ABCC1-6) (Estudante *et al.*, 2013; Murakami & Takano, 2008; Varma *et al.*, 2010). ABCB1, ABCG2, and ABCC2 are expressed on the apical membrane whereas ABCC1, ABCC3, ABCC4, and ABCC5 are located on the basolateral membrane of enterocytes (Estudante *et al.*, 2013; Varma *et al.*, 2010). The SLC family transporters are responsible for intestinal absorption using Na^+/K^+-ATPase and Na^+/H^+-ATPase carriers (Murakami & Takano, 2008; Varma *et al.*, 2010). SLC transporters include SLC organic anion transporter family member 1A2 (SLCO1A2), SLCO2B1, SLC family 5 member 6 (SLC5A6), SLC7A5, SLC7A8, SLC15A1, SLC16A1, SLC22A3, SLC22A4, SLC22A5, and SLC36A1 located on the apical membrane of enterocytes (Estudante *et al.*, 2013; Müller *et al.*, 2005; Murakami & Takano, 2008; Varma *et al.*, 2010) and SLC22A1 and SLC22A2 found on the basolateral membrane of enterocytes (Estudante *et al.*, 2013) (Figure 1). SLC15A1, ABCG2, ABCC2, and ABCB1 are highly expressed in the human intestine, while SLC22A1 and SLC22A6 are the predominant isoforms in liver and kidney (Murakami & Takano, 2008).

Several studies have examined the expression of human intestinal transporters in jejunum and colon cell models (Seithel, Karlsson, Hilgendorf, Bjorquist, & Ungella, 2006; Taipalensuu *et al.*, 2001). A previous study reported that ABCG2 and ABCC2 were the most expressed transporters in jejunum, followed by ABCB1, ABCC3, ABCC6, and ABCC1, with the lowest ABCC4 (Taipalensuu *et al.*, 2001). Another study reported that SLC15A1 was the most expressed transporter in jejunum while ABCC2 and ABCB1 were the second most abundant, followed by ABCG2 > ABCC3 > SLC22A5 > SLC16A1 > SLCO2B1 > SLC22A7 > SLC22A1 and SLC22A3, respectively. The transporter expression in the colon showed that ABCC3 was the most abundant transporter, followed by ABCB1 > SLC16A1 and SLC22A5 > ABCG2 and SLC15A1 > SLCO2B1 and SLC22A3, with the lowest ABCC2 and SLC22A1 (Seithel *et al.*, 2006). ABCB1, ABCC2, ABCC3, ABCC4, ABCC5, ABCC6, SLCO1A2, SLCO2B1, SLC16A1, and SLC22A1 were expressed in Caco-2 cells at levels approximately 2-fold higher than in human jejunum, while SLC15A1 expression was lower in Caco-2 cells than in human jejunum (Maubon *et al.*, 2007; Taipalensuu *et al.*, 2001). Interestingly, ABCG2 was expressed 100-fold higher in human jejunum than in Caco-2 cells (Taipalensuu *et al.*, 2001). Furthermore, SLC22A1, SLC22A2, and SLC22A3 were all expressed in Caco-2 cells but SLC22A1 was only detected at a very low level compared to small intestine (Müller *et al.*, 2005; Seithel *et al.*, 2006). The most abundant transporters in Caco-2 cells were ABCC2, followed by SLCO2B1 > SLC15A1 > ABCB1 > SLC16A1, ABCC3, ABCG2 and SLC22A5 > SLC22A3 > SLC22A1. The lowest expressed transporter in Caco-2 cells was SLC22A7 (Seithel *et al.*, 2006) (Table 2). In Caco-2 cells, ABCC2, SLCO2B1, SLC15A1, ABCB1, SLC16A1, ABCG2, and SLC22A5 were expressed on the apical membrane of enterocytes while ABCC3, ABCC4, and ABCC6 were expressed on the basolateral membrane (Sun *et al.*, 2008). SLC22A1, SLC22A2, and SLC22A3 were located on the plasma membrane and within the cytoplasm of Caco-2 cells (Müller *et al.*, 2005) (Figure 1).

There are several typical inducers employed in Caco-2 models to establish drug-interactions resulting from induction of the ABC and SLC transporter families and they are listed in Table 2. ABCB1 is the main efflux transporter involved in drug-interaction events due to its wide range of substrates (Shirasaka *et al.*, 2006). The therapeutic agents rifampicin and venlafaxine are well-known inducers of ABCB1 (Ehret, Levin, Narasimhan, & Rathinavelu, 2007; Shirasaka *et al.*, 2006) along with aroclor1254, all-trans retinoic acid, and vinblastine (Borlak & Zwadlo, 2003; Li, Sai, Kato, Tamai, & Tsuji, 2003; Shirasaka *et al.*, 2006). Aroclor1254 also increased mRNA level of ABCC2-5 in Caco-2 cells (Borlak & Zwadlo, 2003) and quercetin up-regulated expression of ABCC3 (Li *et al.*, 2003). Ligands of the aryl hydrocarbon receptor and the peroxisome proliferator-activated receptor gamma such as β-naphthoflavone, rosiglitazone, and tert-butylhydroquinone increased

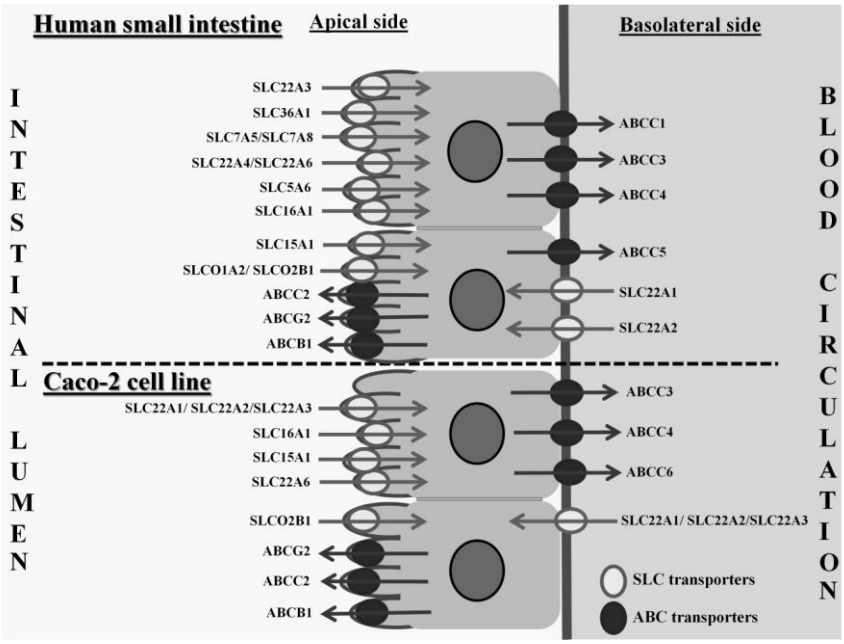

Figure 1. Expression of transporters in human small intestine and Caco-2 cell

Table 2. Expression of influx and efflux transporters in human intestine and Caco-2 cell by typical transporter inducers

	Level of Expression		Inducers	References
	Human intestine	Caco-2 cell		
ABCB1	++	+++	all-trans retinoic acid, aroclor1254, rifampicin, venlafaxine, and vinblastine	Borlak & Zwadlo, 2003; Ehret *et al.*, 2007; Maubon *et al.*, 2007; Shirasaka *et al.*, 2006; Taipalensuu *et al.*, 2001
ABCC1	+/-	–	venlafaxine	Ehret *et al.*, 2007; Taipalensuu *et al.*, 2001
ABCC2-6	++	+++	Aroclor1254 (ABCC2-5), quercetin (ABCC3), and venlafaxine	Borlak & Zwadlo, 2003; Ehret *et al.*, 2007; Maubon *et al.*, 2007; Seithel *et al.*, 2006; Taipalensuu *et al.*, 2001
ABCG2	++++	+	β-naphthoflavone, rosiglitazone, and tert-butylhydroquinone	Seithel *et al.*, 2006; Taipalensuu *et al.*, 2001; Wright *et al.*, 2011
SLCO1A2	++	+++	vitamin D_3	Eloranta *et al.*, 2012; Maubon *et al.*, 2007
SLCO2B1	++	+++	N/A	Maubon *et al.*, 2007; Seithel *et al.*, 2006
SLC15A1	+++	++	quercetin and short-chain fatty acid (butyrate)	Dalmasso *et al.*, 2008; Li *et al.*, 2003; Maubon *et al.*, 2007; Taipalensuu *et al.*, 2001
SLC16A1	++	+++	short-chain fatty acid (butyrate)	Borthakur *et al.*, 2008; Maubon *et al.*, 2007; Seithel *et al.*, 2006
SLC22A1	+/-	+/-	N/A	Müller *et al.*, 2005; Seithel *et al.*, 2006
SLC22A2	+/-	+/-	N/A	Müller *et al.*, 2005; Seithel *et al.*, 2006
SLC22A3	+/-	+/-	N/A	Müller *et al.*, 2005; Seithel *et al.*, 2006
SLC22A5	+/-	+/-	N/A	Seithel *et al.*, 2006
SLC22A7	+/-	+/-	N/A	Seithel *et al.*, 2006

Note. ++++, Expression is higher than 100 folds compared to the expression in Caco-2 cell; +++, Expression is higher than 2-folds compared to the expression in either human intestine (jejunum) or Caco-2 cell; ++, Expression is lower than 2-folds compared to the expression in either human intestine (jejunum) or Caco-2 cells; +, Expression is lower than 100-folds compared to the expression in human intestine (jejunum); +/-, Very low expression; –, Absence; N/A, No data available; Caco-2, Human colon carcinoma cell line

expression of ABCG2 in Caco-2 cells (Wright, Haslam, Coleman, & Simmons, 2011). For influx transporters, vitamin D_3 induced SLCO1A2 expression in Caco-2 cells (Eloranta, Hiller, Jüttner, & Kullak-Ublick, 2012), while a short-chain fatty acid arising from carbohydrate and fiber fermentation (butyrate) induced SLC15A1 and SLC16A1 (Borthakur *et al.*, 2008, Dalmasso *et al.*, 2008) and quercetin induced SLC15A1 in Caco-2 cells (Li *et al.*, 2003) (Table 2).

The expression of transporters has been shown to vary among Caco-2 cell lines and clones. Wild-type Caco-2/ATCC exhibited higher cyclosporine A-induced expression of ABCB1 at passage no. 70 to 110 than Caco-2/TC7 at passage no. 40 to 80 (Sambuy *et al.*, 2005). One study compared expression of SLC15A1 and SLC36A1 in three Caco-2 clones, namely Caco-2/ATCC, Caco-2/HD (German Cancer Research Center in Heidelberg), and Caco-2/MR (University Hospital in Marburg). Levels of SLC15A1 and SLC36A1 mRNA were around 2 times higher in Caco-2/HD than Caco-2/ATCC, while these two transporters were not detected in Caco-2/MR (Behrens, Kamm, Dantzig & Kissel, 2004).

4. Factors Influencing Cell Differentiation and Expression of Cyps and Transporters

Caco-2 standard culture conditions are shown in Table 3. Incubation is at 37 °C in 5% CO_2 under humidified atmosphere. Caco-2 medium consists of Dulbecco's modified Eagle medium (DMEM) supplemented with 20% fetal bovine serum (FBS), 1% non-essential amino acids (NEAA), 2 to 4 mM glutamine, and antibiotics, e.g. gentamicin (25 mg/ml) or penicillin (100 U/ml) with streptomycin (100 μg/ml) (Lea, 2015). The medium can be renewed 2-3 times per week (Angelis & Turco, 2011; Lea, 2015). Caco-2 cells require 4 to 7 days to reach 80% confluence and 14 to 21 days to differentiate (Lea, 2015). The cells can be subcultured at 50 to 80% confluence by rinsing with phosphate buffered saline (PBS) before trypsinization with trypsin (0.25%) containing ethylenediaminetetraacetic acid (EDTA, 0.2%) (Kumar *et al.*, 2010; Lea, 2015). A density of 100,000 to 400,000 cells/cm^2 is recommended for seeding (Ferrec & Fardel, 2012; Lea, 2015).

Table 3. Standard culturing condition for Caco-2 cell

Parameters	Conditions
Incubation condition	37 °C with 5% CO_2 in humidified atmosphere
Type of medium	Dulbecco's modified Eagle medium
Concentration of	
Fetal bovine serum	20%
Non-essential amino acids	1%
Glutamine	2 to 4 mM
Antibiotics	Gentamycin (25 mg/ml) or Penicillin (100 U/ml) with streptomycin (100 μg/ml)
Cell density for sub culturing	8×10^4 - 1×10^5 cell/cm^2 (80% confluence)
Cell density for seeding	1×10^5 - 4×10^5 cell/cm^2
Trypsinization	0.25% Trypsin with 0.2% EDTA
Period of medium change	2 to 3 times per week
Period of differentiation	14 to 21 days after confluence

4.1 Number of passages

Passage number of Caco-2 cells affected the expression of CYPs and transporters. SLC15A1 expression in Caco-2 cells was detectable for early passages (no. 33 to 43) and decreased with an increase in the passage numbers (Sambuy *et al.*, 2005). In contrast, no difference in expression of ABCB1, ABCC2, ABCC3, ABCG2, SLC22A5, SLC15A1, SLCO2B1, SLC22A7, SLC22A1, SLC22A3, SLC22A6, and SLC16A1 was seen in Caco-2 cells from passage no. 29 to 43 (Seithel *et al.*, 2006). Likewise, the activity of carrier-mediated transporters of cephalexin, cephradine, phenylalanine, L-proline, and taurocholic acid was lower among late passage Caco-2 cells (passage no. 93 to 108) than early passage cells (passage no. 28 to 36) (Sambuy *et al.*, 2005). In contrast, expression of vitamin D3-induced CYP3A4 was greater in late passage Caco-2/ATCC cells (passage no. 92 to 105) than in early passage cells (passage no. 20 to 30) of either Caco-2/ATCC or Caco-2/TC7 (Sambuy *et al.*, 2005). Constitutive expression levels of CYP3A4 were very low or absent in Caco-2 cells and showed high variation. For example, one study collected Caco-2 cells from Japanese laboratories at passage numbers ranging from 36 to 88 and determined CYP3A expression. Three of five Caco-2 cell lines showed no CYP3A expression while the other two cell lines expressed with high variation (Nakamura *et al.*, 2002).

4.2 Culturing period

Culturing period can also affect cell differentiation and expression of CYPs and transporters in Caco-2 (Sambuy *et al.*, 2005). Villin is an actin binding protein expressed in microvilli that can be used to determine maturity of microvilli. Villin content was increased 3-fold after prolonged culturing up to 28 days (Nakamura *et al.*, 2003). Culturing period was also shown to influence Caco-2 cells differentiation, in which heterogeneous differentiation occurred at day 0 to 20 while homogeneous differentiation occurred later, after day 30 following the cells reaching confluence (Sambuy *et al.*, 2005). In addition, mRNA levels of SLC15A1, ABCB1, ABCC2, SLCO2B1, and ABCG2 transporters in Caco-2 cells increased over five weeks culturing in a transwell assay (Seithel *et al.*, 2006). Correspondingly, SLC15A1 showed the highest expression in Caco-2 cells after culturing for more than 25 days (Sambuy *et al.*, 2005). Conversely, ABCB1 transporter expression in Caco-2 cells decreased by 85% from day 6 after seeding to day 14 then remained at this level to day 28 (Nakamura *et al.*, 2003). A few reports have suggested culturing time can affect expression of CYPs in Caco-2 cells; CYP3A mRNA level was increased approximately 20-fold after culturing for 28 days (Nakamura *et al.*, 2003; Sambuy *et al.*, 2005).

4.3 Seeding density

Seeding density is an important factor affecting Caco-2 cell differentiation and several studies have examined using different seeding densities (3,500 to 500,000 cells/cm^2) of Caco-2 cells. Differences in seeding density did not affect paracellular permeation of substances but it did affect monolayer formation and expression of transporters, e.g. SLC36A1 and ABCB1 (Behrens & Kissel, 2003; Sambuy *et al.*, 2005). Seeding Caco-2 cells at a density of 60,000 cell/cm^2 was optimal for cell differentiation. Higher seeding cell density (120,000 cells/cm^2) created a multilayer formation of Caco-2 while lower seeding cell density (10,000 cells/cm^2) resulted in a very thin monolayer with high expression of tight junctions between cells (Behrens & Kissel, 2003). However, the multilayer formation could be reduced by washing cells with PBS after seeding the cells (Sambuy *et al.*, 2005).

Expression of SLC15A1 mRNA in Caco-2 cells was not modified by different seeding density, while SLC36A1 and ABCB1 expression was reduced at the both lower and higher seeding densities (1×10^4 and 1.2×10^5 cells/cm^2 compared 6×10^4 cell/cm^2) (Behrens & Kissel, 2003).

4.4 Medium composition

Culture medium and supplements including FBS, glucose, glutamine, antibiotics, and other additional substances affected both cell proliferation and expression of CYPs and transporters in Caco-2 cells (Sambuy *et al.*, 2005). Glutamine is one factor affecting the cell growing process and formation of tight junctions between cells as it is an essential amino acid with a role as a precursor for amino acid and nucleic acid synthesis and a regulator of intestinal tract homeostasis (Sambuy *et al.*, 2005). Culturing Caco-2 cells without glutamine resulted in the lowest tight junction formation (Li, Lewis, Samuelson, Liboni, & Neu, 2004). Culturing four clones of Caco-2 including Caco-2/ATCC, Caco-2/TC7, Caco-2/15, and Caco-2/AQ in serum free conditions showed increases in permeation through the paracellular pathway due to less tight junction formation (Sambuy *et al.*, 2005). Culturing Caco-2/ATCC cells in FBS- or glutamine-free medium for 72 h did not affect expression of SLC15A1, ABCB1, ABCC2, and ABCC3 transporters. In contrast, culturing Caco-2/ATCC cells in glutamine-free medium supplemented with L-Lysine (20 mM) or ascorbic acid (10 μM) for 72 hrs induced CYP3A4 expression (Li *et al.*, 2003; Sambuy *et al.*, 2005). In addition, serum- and vitamin D3-free medium decreased enzymatic activity, especially CYP3A4, by around 70% compared to standard Caco-2 culturing conditions (Sambuy *et al.*, 2005). Correspondingly, a decrease in metabolism of midazolam, a specific CYP3A4 substrate, was observed in Caco-2 cells incubated in a medium lacking FBS and vitamin D3 for 24 h (Fisher *et al.*, 1999). Moreover, Caco-2 cells cultured with vitamin D3 at concentrations ranging from 0.25 to 0.50 μM for 2 weeks showed increased expression of CYP3A4 by approximately 40- to 80-fold (Sambuy *et al.*, 2005). Glucose depletion affected expression of transporters in Caco-2 cells; glucose-depleted medium significantly elevated the expression of ABCB1, ABCC2, and β-actin (Li *et al.*, 2003). Addition of other substances such as dipeptides (L-alanyl-L-glutamine or L-glycyl-L-glutamine), peptone to the culture medium increased expression of SLC15A1 and SLC36A1 mRNAs in Caco-2 cells (Sambuy *et al.*, 2005).

4.5 Filter membrane insert

Caco-2 cells are often cultured on a permeable filter membrane insert in a transwell to create a model for drug absorption, transportation, and metabolism (Angelis & Turco, 2011; Lea, 2015). There are two compartments in an insert culturing system, which include apical and basolateral compartments that represent intestinal lumen and serous site, respectively (Ferrec & Fardel, 2012; Lea, 2015). This condition mimics the physiological status of intestinal lumen, resulting in improvement of the morphological and functional differentiation of Caco-2 cells (Ferrec & Fardel, 2012). Caco-2 cells require 14 to 28 days in the insert culturing system to proliferate and differentiate into mature enterocytes with microvilli (Kurma *et al.*, 2010; Lea, 2015) (Figure 2).

The filter membrane insert can be made from several materials, e.g. nitrocellulose membrane, aluminium oxide, polycarbonate (PC), polyester (PE), polyethylene terephthalate (PET), and other inert materials with low specific protein binding (Kurma *et al.*, 2010; Sambuy *et al.*, 2005). To observe cell morphology and differentiation, a transparent filter membrane insert, e.g. aluminium oxide, PE, or PET, is recommended (Behrens & Kissel, 2003). The type of filter membrane insert does not affect mRNA expression of SLC15A1 and ABCB1 efflux transporters in Caco-2 cells. In contrast, Caco-2 cells grown on a PET filter membrane insert exhibited higher paracellular transportation activity (Behrens & Kissel, 2003; Sambuy *et al.*, 2005). PET and PE filter membrane inserts promoted Caco-2 cell growth as a tight monolayer via an increase in tight junctions compared to PC filter inserts. Caco-2 culturing on PET and PE created monolayer morphology, which were shown to be less thick and have higher actin in the cell under Fluoresceinyl-aminomethyldithiolano-phalloidin (FITC-phalloidin) staining. Moreover, culturing on a PET filter membrane insert resulted in Caco-2 differentiated microvilli appearing as a flower-like cluster on the apical surface due to the fusion of microvilli. In contrast, Caco-2 cells grown on PC and PE filters exhibited carpet-like microvilli, which resemble normal intestine microvilli (Behrens & Kissel, 2003). Differences in the insert-pore size affected differentiation and growth rate of Caco-2 cells (Kurma *et al.*, 2010; Lea, 2015). Inserts with 0.4 μm-pore diameter are widely used to study drug permeation while a larger one is employed in co-cultures of Caco-2 and other cell lines (Kurma *et al.*, 2010; Lea, 2015). However, a large pore size results in undesirable characteristics of intestinal models due to Caco-2 cells growing on both apical and

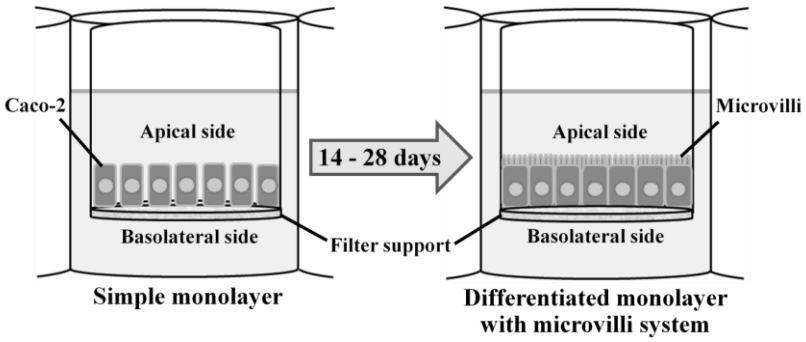

Figure 2. Inserted plate-Caco-2 cell culturing system

basolateral surfaces of the filter membrane insert (Kurma *et al.*, 2010). Protein coating, e.g. type I collagen or mitragel, on the filter membrane insert prevented Caco-2 cells from becoming loosened from the filter (Ferrec & Fardel, 2012; Lea, 2015). Growing Caco-2 cells on rat collagen-coated filters increased expression of ABCB1, SLC36A1, and SLC15A1 mRNAs (Behrens & Kissel, 2003). Hence, the properties of a filter membrane insert, including the base material, transparency, pore size, and protein coatings, should be considered for their effect on Caco-2 cell morphology and transporter expression in insert plate culturing systems.

5. Conclusions

The human intestine expresses many CYPs and influx/efflux transporters and it is the expression levels of these CYPs and transporters that are considered the main factor affecting xenobiotic bioavailability. Intestinal metabolism is mainly performed by CYPs, especially the CYP1, CYP2, and CYP3 subfamilies. CYP3A4 and CYP3A5 are the principal isoforms responsible for lowering xenobiotic bioavailability due to their abundance in the intestine. Unfortunately, CYP3A4 is expressed in Caco-2 cells at a level five times lower than other CYP isoforms. In addition, expression levels of influx/efflux transporters differ between intestine and Caco-2 cells, which might be associated with the origin and clones of the Caco-2 cell line. Insert plate Caco-2 culturing system is a popular system because this model mimics the physiological conditions of the intestinal lumen and blood circulation system and Caco-2 cells can spontaneously differentiate and develop microvilli in this system. Several culturing factors, including culturing period, seeding density, medium composition, and types of filter membrane insert, influence the Caco-2 morphology as well as the expression of CYPs and transporters. Culturing period and medium composition are the most important factors affecting expression of CYPs and transporters, while Caco-2 cell morphology is mainly affected by culturing period, seeding density, and types of filter membrane insert. Therefore, determining the origin of the Caco-2 cell-line and judicious control of culturing factors are essential aspects of any study on drug metabolism and transportation in Caco-2 cells.

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