Human Small Intestinal Epithelial Cells Differentiated from Adult Intestinal Stem Cells as a Novel System for Predicting Oral Drug Absorption in Humans

Toru Takenaka, Naomoto Harada, Jiro Kuze, Masato Chiba, Takahiro Iwao, and Tamihide Matsunaga

Discovery Drug Metabolism and Pharmacokinetics, Pharmacokinetics Research Laboratories (T.T., J.K., M.C.), and Evaluation Research Laboratory (N.H.), Tsukuba Research Center, Taiho Pharmaceutical Co. Ltd., Tsukuba, Ibaraki, Japan; and Department of Clinical Pharmacy, Graduate School of Pharmaceutical Sciences, Nagoya City University, Nagoya, Japan (T.I., T.M.).

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ABSTRACT

Adult intestinal stem cells (ISCs) possess both a long-term proliferation ability and differentiation capability into enterocytes. As a novel in vitro system for the evaluation of drug absorption, we characterized a human small intestinal epithelial cell (HIEC) monolayer that differentiated from adult ISCs. Continuous proliferation/differentiation from ISCs consistently conferred the capability of maturation of enterocytes to HIECs over 25 passages. The morphologically matured HIEC monolayer consisted of polarized columnar epithelia with dense microvilli, tight junctions, and desmosomes 8 days after seeding onto culture inserts. Transepithelial electrical resistance across the monolayer was 9-fold lower in HIECs (98.9 \( \Omega \times \text{cm}^2 \)) than in Caco-2 cells (800 \( \Omega \times \text{cm}^2 \)), which indicated that the looseness of the tight junctions in the HIEC monolayer was similar to that in the human small intestine (approximately 40 \( \Omega \times \text{cm}^2 \)). No significant differences were observed in the overall gene expression patterns of the major drug-metabolizing enzymes and transporters between the HIEC and Caco-2 cell monolayers. Furthermore, the functions of P-glycoprotein and breast cancer resistance protein in the HIEC monolayer were confirmed by the vectorial transport of marker substrates and their disappearance in the presence of specific inhibitors. The apparent drug permeability values of paracellularly transported compounds (fluorescein isothiocyanate-dextran 4000, atenolol, and terbutaline) and nucleoside transporter substrates (didanosine, ribavirin, and doxifluoridine) in the HIEC monolayer were markedly higher than those of Caco-2 cells, whereas transcellularly transported drugs (pindolol and midazolam) were equally well permeated. In conclusion, the HIEC monolayer can serve as a novel and superior alternative to the conventional Caco-2 cell monolayer for predicting oral absorption in humans.

Introduction

Oral absorption often needs to be predicted in humans to assess the feasibility of drug development so that development candidates can be selected and prioritized at the discovery stage. Many techniques have served as tools for the prediction of intestinal absorption and metabolism in humans. For example, Lennernäs et al. (1997) demonstrated that the human jejunal perfusion method could predict human oral absorption for both passively and carrier-mediated transported drugs. The Ussing-chamber model has also been used as a system that can not only accurately predict absorption, but can also transepithelial metabolism (Rozehnal et al., 2012; Sjöberg et al., 2013). However, neither technique is frequently used as a routine assay at the discovery stage of new drug candidates (Irvine et al., 1999; Tavelin et al., 2003b; Skolnik et al., 2010). The Caco-2 cell line is one of the most widely used cell systems. Although the Caco-2 cell line retains very similar morphologic properties to human enterocytes, its tight junctions are markedly tighter than those in the small intestine, which may reduce the permeability of drugs with significant paracellular absorption (Lennernäs et al., 1996; Saïto et al., 2004), thereby resulting in the underestimation of intestinal absorption for those compounds (Tavelin et al., 2003b; Saïto et al., 2004; Matsson et al., 2005). The utilization of human primary normal enterocytes was expected to be a promising solution for the aforementioned discrepancy between in vivo and in vitro models. Although several methods have been used to isolate primary enterocytes from the human small intestine (Perreault and Beaulieu, 1998; Alldous et al., 2001; Grossmann et al.,

Cell-based assay methods such as Caco-2 cells (human colon adenocarcinoma cells), MDCK cells (Madin–Darby canine kidney cells), and 2/4/A1 cells (conditionally immortalized rat intestinal cells) have also been used to evaluate membrane permeability and the transport of drug candidates (Irvine et al., 1999; Tavelin et al., 2003b; Skolnik et al., 2010). The Caco-2 cell line is one of the most widely used cell systems. Although the Caco-2 cell line retains very similar morphologic properties to human enterocytes, its tight junctions are markedly tighter than those in the small intestine, which may reduce the permeability of drugs with significant paracellular absorption (Lennernäs et al., 1996; Saïto et al., 2004), thereby resulting in the underestimation of intestinal absorption for those compounds (Tavelin et al., 2003b; Saïto et al., 2004; Matsson et al., 2005). The utilization of human primary normal enterocytes was expected to be a promising solution for the aforementioned discrepancy between in vivo and in vitro models. Although several methods have been used to isolate primary enterocytes from the human small intestine (Perreault and Beaulieu, 1998; Alldous et al., 2001; Grossmann et al.,
Adult stem cells are found in many organs, including the intestine, after development, and possess a long-term proliferation ability and differentiation capability into several distinct cell types. Therefore, adult stem cells are responsible for adult tissue homeostasis and the regeneration of damaged tissue. The small intestinal epithelium is rapidly self-renewed via cell proliferation and migration along the crypt-villus axis. This systematic process consists of the proliferation of adult intestinal stem cells (ISCs) at the bottom of the crypt, migration into the villi with differentiation/maturation, and eventual apoptosis at the tips of the villi (Yeung et al., 2011). Suzuki et al. (2010) recently reported that adult ISCs expressing leucine-rich repeat-containing G protein–coupled receptor 5 (LGR5), a marker of ISCs, were contained in commercially available primary normal human small intestinal epithelial cells (HIECs). The authors demonstrated that ISCs were maintained in culture with continuous proliferation and spontaneous differentiation into four distinct epithelial cell lineages including not only absorptive enterocytes, but also secretory lineages such as enteroendocrine cells, goblet cells, and Paneth cells. Suzuki et al. (2010) also found that these differentiated cells formed polarized monolayers with dome-like structures. However, to the best of our knowledge, the utility of this differentiated HIEC for evaluating the permeability of drug candidates to predict absorption in humans has not yet been assessed.

In this study, we characterized the HIEC monolayer as a novel tool for the evaluation of oral drug absorption. The HIEC had the ability to form a monolayer with a tight and matured in vivo–like morphology. HIECs also displayed the long-term proliferating ability of ISCs and continuous differentiation potency into enterocytes from ISCs. The looseness of tight junctions in the HIEC monolayer, which had a transepithelial electrical resistance (TEER) value of 98.9 Ω cm², was similar to that of the human small intestine (TEER = approximately 40 Ω cm²) (Sjoberg et al., 2013), whereas that of Caco-2 cells was not (TEER = 900 Ω cm²). The HIEC monolayer also distinctly expressed concentrative nucleoside transporter (CNT) 3, which was absent in Caco-2 cells. As a result, the permeability values of paracellularly transported and nucleoside transporter substrates were markedly higher in the HIEC monolayer than in Caco-2 cells, whereas transcellularly transported drugs were equally well permeated. This study demonstrated that the HIEC monolayer could serve as a novel and superior alternative to the conventional Caco-2 cell monolayer for predicting oral absorption in humans; its accuracy could serve as a novel and superior alternative to the conventional Caco-2 cell monolayer for predicting oral absorption in humans.

**Materials and Methods**

HIECs (ACBRIS159) and Caco-2 cells (HTB-37) were obtained from Cell Systems (Kirkland, WA) and the American Type Culture Collection (Rockville, VA), respectively. Dulbecco’s modified Eagle’s medium (DMEM), DMEM mixed 1:1 with Ham’s F-12 (DMEM/F12), 0.25% trypsin-EDTA, Hanks’ balanced salt solution, nonessential amino acids, penicillin-streptomycin, and GlutaMAX were obtained from Life Technologies (Carlsbad, CA). Bovine pituitary extract was obtained from Kohjin Bio (Saitama, Japan). Fetal bovine serum (FBS) was purchased from Life Technologies (Carlsbad, CA). Bovine pituitary extract was obtained from Kohjin Bio (Saitama, Japan). Fetal bovine serum (FBS) was purchased from APET Journals on October 14, 2017 dmd.aspetjournals.org Downloaded from

**TABLE 1**

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences (5'–3')</th>
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<tbody>
<tr>
<td>CDX2</td>
<td>Forward ACGCATTGCGAGTGGATGCT</td>
</tr>
<tr>
<td>IFABP</td>
<td>Forward ACAATCTAGCGAGCCGAACT</td>
</tr>
<tr>
<td>ASBT</td>
<td>Forward TGGCCCTAAAAAGGAAA</td>
</tr>
<tr>
<td>MCT1</td>
<td>Forward CGCGCGATATAACGATTTAT</td>
</tr>
<tr>
<td>MRP3</td>
<td>Forward GTGCCGAGAATGGAGCTTGT</td>
</tr>
</tbody>
</table>

ASBT, apical sodium-dependent bile acid transporter; MCT1, monocarboxylate transporter 1.
were normalized to the Ct value of glyceraldehyde-3-phosphate dehydrogenase (ΔCt). Relative mRNA expression was determined using the 2−ΔΔCt method. We selected Ct values > 35 as the cut-off for the absence of expression.

The mRNA levels of other transporters, including organic cation transporter 1, organic anion-transporting peptide 2B1, P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), and MRPI and MRP2, and the drug-metabolizing cytochrome P450 (P450) enzymes CYP2C9, CYP2C19, CYP2D6, CYP3A4, and CYP3A5, and UDP-glucuronosyltransferases (UGTs) UGT1A1, UGT1A3, UGT1A4, and UGT2B7 were analyzed using the Quantigene Plex 2.0 Assay Kit (Affymetrix, Santa Clara, CA) according to the manufacturer’s instructions. HIECs and Caco-2 cells cultured on membrane inserts for 8 and 20 days, respectively, were lysed with the Quantigene Sample Processing Kit (Affymetrix). Samples were analyzed using a Bio-Plex 200 suspension array system (Bio-Rad, Hercules, CA). The expression level of hypoxanthine phosphoribosyl-transferase was used to normalize mRNA expression data from the target genes.

Transmission Electron Microscopy of HIECs. HIECs seeded on culture inserts as described above were fixed with 2% paraformaldehyde and 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) and were then cooled to 4°C. Thereafter, they were fixed with 2% glutaraldehyde in 0.1 M phosphate buffer (pH 7.4) at 4°C overnight. After three washes with 0.1 M phosphate buffer, cells were postfixed with 2% osmium tetroxide in 0.1 M phosphate buffer at 4°C for 90 minutes, and dehydrated through a graded ethanol series (50–100%). The samples were embedded in resin, and ultrathin sections were stained with 2% uranyl acetate and lead stain solution. The specimens were examined using a JEM-1200 EX transmission electron microscope (JEOL, Tokyo, Japan).

Transport Assay. Prior to the addition of substrates, growth medium was removed and monolayers were rinsed twice with transport medium (TM) (Hanks’ balanced salt solution with 4.2 mM NaHCO3 and 20 mM glucose) adjusted to pH 7.4 by 10 mM HEPES. Monolayers were preincubated in TM (pH 7.4) for 30 minutes at 37°C in 95% humidity. Transport assays were carried out with apical and basal buffers consisting of TM adjusted to pH 6.5 by 10 mM MES and TM (pH 7.4) with 4.5% (w/v) bovine serum albumin, respectively. The following buffer volumes were used for the apical and basal chambers: 0.4 ml and 1.2 ml, respectively, for HIECs; and 0.5 ml and 1.5 ml, respectively, for Caco-2 cells. Donor concentrations for the different test compounds were set at 50 µM, except for didanosine (100 µM), ribavirin (100 µM), doxifluridine (100 µM), and FD-4 (500 µg/ml) due to low quantification sensitivity. Monolayers were incubated at 120 minutes at 37°C in 95% humidity with 80 rpm reciprocal shaking. Basal compartments were sampled at 30, 60, and 120 minutes. Except for topotecan, samples were added to twice the volume of methanol/acetonitrile (2:1, v/v) and centrifuged for 15 minutes at 10,000g before being analyzed with a liquid chromatography–mass spectrometry system. Regarding topotecan, twice the volume of methanol/7% perchloric acid (1:1, v/v) was added to samples instead of methanol/acetonitrile (2:1, v/v) to quantify the total of its lactone form instead of methanol/acetonitrile (2:1, v/v) to quantify the total of its lactone form. The concentrations of test compounds were the same as those used for the transport assays in any of the experiments.

The efflux ratio (ER) was determined from the ratio of

\[ \frac{Q_{app}}{Q_{app}^{secretory}} \]

where \( Q_{app} \) and \( Q_{app}^{secretory} \) represent the total amount of the test compound transported to the acceptor chamber per unit time, the surface area of the transport membrane (0.33 and 1 cm² for HIECs and Caco-2 cells, respectively), and the initial compound concentration in the donor chamber, respectively.

The efflux ratio (ER) was determined from the ratio of \( Q_{app} \) to \( Q_{app}^{secretory} \) to that in absorptive (apical-to-basal) directions. The \( Q_{app}^{secretory} \) for the PAMPA assay was calculated using eq. 2:

\[ Q_{app}^{secretory} = - \left( 1 - C_A \times \frac{V_A + V_B}{C_D \times V_A + C_A \times V_B} \right) + \left( A \times \frac{1}{V_B} + \frac{1}{V_A} \times t \right) \]

where \( C_D \) and \( C_A \) represent the final concentrations in the donor and acceptor wells, respectively; \( V_A \) and \( V_B \) represent the volumes of the donor and acceptor wells, respectively; and \( A \) and \( t \) represent the membrane surface area (0.3 cm²) and incubation time, respectively.

The relationship between the \( Q_{app} \) values and known fraction absorbed in humans (Fa) data were described using eq. 3 (Amidon et al., 1988):

\[ Fa = 100 \times \left( 1 - \exp(-a \times Q_{app}) \right) \]

where \( a \) is the scaling factor. The best fitting curves were calculated by nonlinear regression using XLfit software (IDBS, Guildford, UK).

Results

Morphology of HIECs. HIECs survived and proliferated under our culture condition as previously demonstrated (Suzuki et al., 2010), and formed domes, which were similar to those in Caco-2 cells, after confluence on culture dishes (Fig. 1A). As monolayers were formed in HIECs and Caco-2 cells, TEER values increased and reached plateaus at

Sample Analysis. An analysis of samples was performed using the liquid chromatography–tandem mass spectrometry system, which consisted of a Waters Quattro Micro Mass Spectrometer and Waters Alliance 2795 HT (Waters, Milford, MA), except for FD-4. The ionization source was an electrospray. The multiple-reaction monitoring mode was used to monitor ions as follows: digoxin (779.1 > 649.2), mitoxantrone (445.3 > 88.0), vinblastine (811.3 > 224.1), topotecan (4220.0 > 171.0), didanosin (2351.7 > 137.0), atenolol (267.2 > 145.0), terbutaline (226.2 > 152.0), ribavirin (245.0 > 112.9), doxifluridine (245.0 > 171.0), pindolol (249.2 > 116.0), midazolam (320.6 > 222.7), 1'-hydroxymidazolam (341.9 > 323.9), and 4-hydroxymidazolam (342.0 > 324.9). The Cosmосil 5C18 AR-II column (50 mm, 4.6 mm i.d.; Nacalai Tesque, Kyoto, Japan) was used for the chromatographic separation of analytes for digoxin, mitoxantrone, vinblastine, topotecan, atenolol, terbutaline, pindolol, midazolam, 1'-hydroxymidazolam, and 4-hydroxymidazolam. The Capcellpak C18 AQ column (50 mm, 4.6 mm i.d.; Shiseido, Tokyo, Japan) was used for the chromatographic separation of analytes for didanosine, ribavirin, and doxifluridine. The following gradient condition was used for the elution of digoxin, mitoxantrone, vinblastine, topotecan, atenolol, terbutaline, pindolol, midazolam, 1'-hydroxymidazolam, and 4-hydroxymidazolam. The flow rate was 0.2 ml/min and the injection volume was 5 µl. Column temperatures were maintained at 40°C. All data processing was performed with Waters QuanLynx software (Waters, Woburn, MA).

Calculations. The apparent drug permeability (Q D P) was calculated for cellular transport in the HIEC and Caco-2 cell monolayers according to eq. 1 by using the linear part of the time versus transported amount:

\[ P_{app} = \frac{dQ}{dt} \times \frac{1}{A \times C_0} \]

where \( dQ/dt \), \( A \), and \( C_0 \) represent the volume of the test compound transported to the acceptor chamber per unit time, the surface area of the transport membrane (0.33 and 1 cm² for HIECs and Caco-2 cells, respectively), and the initial compound concentration in the donor chamber, respectively.

The efflux ratio (ER) was determined from the ratio of \( Q_{app} \) to \( Q_{app}^{secretory} \) to that in absorptive (apical-to-basal) directions. The \( Q_{app}^{secretory} \) for the PAMPA assay was calculated using eq. 2:
was examined by measuring TEER values (Fig. 2D). The TEER values remained on a plateau at least until day 11 in the HIEC monolayer, and the value in the HIEC monolayer was more than 9-fold lower than that in the differentiated Caco-2 cell monolayer.

In agreement with these results, transmission electron microscopy confirmed the morphologic maturation of the HIEC monolayers after 8 days of growth on culture inserts (Fig. 1, C and D). HIECs grew as monolayers of polarized columnar epithelia (Fig. 1C) with straight dense microvilli, tight junctions, and desmosomes being observed under a higher magnification (Fig. 1D). These results indicated that HIECs differentiated into enterocytes with morphologically mature characteristics under our culture condition.

**Long-Term Proliferation Ability, Maintained Population of ISCs, and Differentiation Potency into Enterocytes.** Under our culture conditions, HIECs constantly proliferated over 25 passages without reaching senescence (Fig. 2A), which implied that ISCs continuously existed and underwent cell division, leading to a long-term proliferating ability and formation capability of HIEC monolayers. To confirm this hypothesis, we examined the mRNA expression levels of CDX2 and LGR5, a hindgut marker and an ISC marker (Barker and Clevers, 2010; Iwao et al., 2014), respectively, at various passages. The mRNA expression levels in both HIECs and Caco-2 cells were 6-fold and 17-fold higher in the HIEC monolayer than in Caco-2 cells, respectively, whereas the mRNA expression levels of CYP2C9, CYP2C19, UGT2B7, and apical sodium-dependent bile acid transporter in Caco-2 cells were 7.0% of those in HIECs. CYP3A4, UGT1A1, UGT1A4, and CNT1 mRNA expression levels were 6-fold and 17-fold higher in the HIEC monolayer than in Caco-2 cells, respectively, whereas the mRNA expression levels of CYP2C9, CYP2C19, UGT2B7, and apical sodium-dependent bile acid transporter in Caco-2 cells were >17-fold higher than those in HIECs. CYP3A4, UGT1A1, UGT1A4, and CNT1 mRNA expression levels in both HIECs and Caco-2 cells were < 7.0% of those in the human small intestine. Among the mRNAs for transporters tested, CNT2 was not detected (Ct > 35) in either monolayers in this study.

**Bidirectional Transport across HIEC and Caco-2 Cell Monolayers.** Bidirectional (apical-to-basal and basal-to-apical) permeability coefficients for digoxin, a P-gp marker substrate (Pauli-Magnus et al., 2000), and mitoxantrone, a BCRP marker substrate (Matsson et al., 2000), were determined in both HIEC and Caco-2 cell monolayers to examine the functions of efflux transport mediated by these transporters. The values of ER, calculated from the ratio of $P_{app}$ in secretory (basal-to-apical) to that in absorptive (apical-to-basal) directions, of digoxin in HIEC and Caco-2 cells were 2.2 and 3.1, respectively (Fig. 4A). In addition, verapamil, a P-gp inhibitor (Pauli-Magnus et al., 2000), reduced the ER value to almost 1 in both monolayers. Regarding BCRP-mediated transport, mitoxantrone was transported with markedly larger ER values in HIEC (ER = 38.2) than in Caco-2 cells (ER = 4.0) (Fig. 4B). The ER values of mitoxantrone in both cells were reduced by 98.9 ± 17.5 Ω × cm² on day 5 and at 900 ± 23 Ω × cm² on day 20 after seeding of HIECs and Caco-2 cells, respectively (Fig. 1B). The TEER values remained on a plateau at least until day 11 in the HIEC monolayer, and the value in the HIEC monolayer was more than 9-fold lower than that in the differentiated Caco-2 cell monolayer.

**mRNA Analysis of Drug-Metabolizing Enzymes and Transporters.** The gene expression profiles of drug-metabolizing enzymes and transporters in HIECs and Caco-2 cells are summarized in Fig. 3. The mRNA expression levels of CYP3A5, UGT1A3, UGT1A6, organic cation transporter 1, organic anion-transporting peptide 2B1, monocarboxylate transporter 1, ENT1, ENT2, ENT3, P-gp, BCRP, MRP1, MRP2, and MRP3 in HIECs were within 10-fold of those in the human small intestine, whereas those of other P450/UGT isoforms and transporters in HIECs were markedly lower (<10%) than those in the human small intestine. UGT1A6 and CNT3 mRNA expression levels were 6-fold and 17-fold higher in the HIEC monolayer than in Caco-2 cells, respectively, whereas the mRNA expression levels of CYP2C9, CYP2C19, UGT2B7, and apical sodium-dependent bile acid transporter in Caco-2 cells were >17-fold higher than those in HIECs. CYP3A4, UGT1A1, UGT1A4, and CNT1 mRNA expression levels in both HIECs and Caco-2 cells were < 7.0% of those in the human small intestine. Among the mRNAs for transporters tested, CNT2 was not detected (Ct > 35) in either monolayers in this study.
Ko143, a specific BCRP inhibitor (Matsson et al., 2009). These results indicated that these efflux transporters were functionally active in the HIEC monolayer, similar to that in Caco-2 cells.

Permeability of 10 Test Compounds across HIEC and Caco-2 Cell Monolayers and PAMPA. The \( P_{\text{app}} \) values for 10 test compounds across the HIEC and Caco-2 cell monolayers and in PAMPA are listed in Table 2. The compounds tested included those with diverse characteristics for intestinal absorption, such as the \( F_a \), major route for absorption (paracellular versus transcellular), transporters involved in net absorption (P-gp, BCRP, and nucleoside transporters), and physicochemical properties (molecular weight, partition coefficient, and polar surface area). The \( P_{\text{app}} \) values of 10 test compounds in HIECs, Caco-2 cells, and PAMPA ranged from 0.56 to 3.0 \( \times \) 10\(^{-6}\) cm/s, 0.035 to 1.7 \( \times \) 10\(^{-6}\) cm/s, and 0.0046 to 4.0 \( \times \) 10\(^{-6}\) cm/s, respectively. Transcellularly absorbed drugs (pindolol and midazolam) showed similarly high \( P_{\text{app}} \) values in all three models: 2.2 to 7.9 \( \times \) 10\(^{-6}\) cm/s for pindolol and 3.0 to 1.7 \( \times \) 10\(^{-6}\) cm/s for midazolam. The formation of the primary metabolites of midazolam (1\(^{\beta}\)-hydroxymidazolam and 4-hydroxymidazolam) was less than the lower limit of quantification (3 nM) in the apical and basal buffers after a 2-hour incubation in both HIECs and Caco-2 cells, which was consistent with the low mRNA expression of CYP3A4 (Fig. 3). In agreement with the looseness of the tight junction in the HIEC monolayer being lower, as demonstrated by the lower TEER value than that of the Caco-2 monolayer (Fig. 1B), the \( P_{\text{app}} \) values for paracellularly absorbed compounds (FD-4, atenolol, and terbutaline) in the HIEC monolayer were markedly higher than those in both the Caco-2 monolayer and PAMPA. Possibly due to the higher expression levels of nucleoside transporters (CNT3, ENT1, ENT2, and ENT3) in HIECs than in Caco-2 cells (Fig. 3), didanosine, ribavirin, and doxifluridine, all substrates of nucleoside transporters, permeated more than 5-fold, 45-fold, and 24-fold faster, respectively, across the HIEC monolayer than the Caco-2 monolayer and by more.

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Fig. 2. Long-term proliferation ability, maintained population of ISCs, and differentiation potency into enterocytes. (A) Effect of the passage number on the relative cell proliferation activity. Data were represented as relative value to that at passage number 8. Data represent the mean ± S.D. (\( n = 6 \)). (B and C) Effects of the passage number and culture time on the relative expression levels of CDX2, LGR5, IFABP, and SI. Quantitative real-time polymerase chain reaction analyses were performed with total RNA derived from HIECs at subconfluence and on day 8 after seeding onto culture inserts. The expression levels of each gene were normalized to those of glyceraldehyde-3-phosphate dehydrogenase, and are represented as the ratios of expression levels at subconfluence or on day 8 at various passage numbers to those at subconfluence at passage number 8. Data represent the mean ± S.D. (\( n = 3 \)), except for the data at subconfluence. (D) Effects of the passage number on TEER values in HIEC monolayers. TEER measurements were conducted in culture medium on day 8 after seeding. Data represent the mean ± S.D. (\( n = 3 \)).

Fig. 3. Relative mRNA expression levels of drug-metabolizing enzymes (A) and transporters (B) in HIECs and Caco-2 cells. Expression levels were normalized to those of glyceraldehyde-3-phosphate dehydrogenase or hypoxanthine phosphoribosyltransferase, and are represented as relative values (%) to the human small intestine. Data represent the mean ± S.D. (\( n = 3 \)). ASBT, apical sodium-dependent bile acid transporter; MCT1, monocarboxylate transporter 1; OATP2B1, organic anion-transporting peptide 2B1; OCT1, organic cation transporter 1.

Permeability of 10 Test Compounds across HIEC and Caco-2 Cell Monolayers and PAMPA. The \( P_{\text{app}} \) values for 10 test compounds across the HIEC and Caco-2 cell monolayers and in PAMPA are listed in Table 2. The compounds tested included those with diverse characteristics for intestinal absorption, such as the \( F_a \), major route for absorption (paracellular versus transcellular), transporters involved in net absorption (P-gp, BCRP, and nucleoside transporters), and physicochemical properties (molecular weight, partition coefficient, and polar surface area). The \( P_{\text{app}} \) values of 10 test compounds in HIECs, Caco-2 cells, and PAMPA ranged from 0.56 to 3.0 \( \times \) 10\(^{-6}\) cm/s, 0.035 to 1.7 \( \times \) 10\(^{-6}\) cm/s, and 0.0046 to 4.0 \( \times \) 10\(^{-6}\) cm/s, respectively. Transcellularly absorbed drugs (pindolol and midazolam) showed similarly high \( P_{\text{app}} \) values in all three models: 2.2 to 7.9 \( \times \) 10\(^{-6}\) cm/s for pindolol and 3.0 to 1.7 \( \times \) 10\(^{-6}\) cm/s for midazolam. The formation of the primary metabolites of midazolam (1\(^{\beta}\)-hydroxymidazolam and 4-hydroxymidazolam) was less than the lower limit of quantification (3 nM) in the apical and basal buffers after a 2-hour incubation in both HIECs and Caco-2 cells, which was consistent with the low mRNA expression of CYP3A4 (Fig. 3). In agreement with the looseness of the tight junction in the HIEC monolayer being lower, as demonstrated by the lower TEER value than that of the Caco-2 monolayer (Fig. 1B), the \( P_{\text{app}} \) values for paracellularly absorbed compounds (FD-4, atenolol, and terbutaline) in the HIEC monolayer were markedly higher than those in both the Caco-2 monolayer and PAMPA. Possibly due to the higher expression levels of nucleoside transporters (CNT3, ENT1, ENT2, and ENT3) in HIECs than in Caco-2 cells (Fig. 3), didanosine, ribavirin, and doxifluridine, all substrates of nucleoside transporters, permeated more than 5-fold, 45-fold, and 24-fold faster, respectively, across the HIEC monolayer than the Caco-2 monolayer and by more.
than 6-fold, 137-fold, and 34-fold faster, respectively, than PAMPA. PAMPA had the highest permeability for vinblastine (a P-gp substrate) and topotecan (a BCRP substrate) among assay systems tested, which was consistent with the lack of both P-gp and BCRP in PAMPA. The sigmoidal relationship between the $P_{\text{app}}$ values of 10 test compounds and the $F_a$ values were observed in HIECs and Caco-2 cells, but not in PAMPA (Fig. 5). The correlation coefficient in HIECs (0.779) was higher than that in Caco-2 cells (0.373).

### Discussion

In this study, we showed that the HIEC monolayer, which differentiated from ISCs, displayed mature morphologic features consisting of polarized columnar epithelia with dense microvilli, tight junctions, and desmosomes. The looseness of the tight junctions in the HIEC monolayer was similar to those of the human small intestine (Tavelin et al., 1999, 2003a). These HIEC-like characteristics led to the similarly high permeability values of paracellularly absorbed compounds (FD-4, atenolol, and terbutaline) were higher in HIECs than in Caco-2 cells (Table 2). These results indicated that the HIEC monolayer had a leakier paracellular route than Caco-2 cells. Furthermore, the permeability for these compounds of PAMPA, which completely lacks a paracellular pathway, was even lower. This result was consistent with the lower TEER value in the HIEC monolayer (98.9 $\Omega \times \text{cm}^2$) than in Caco-2 cells (900 $\Omega \times \text{cm}^2$) (Fig. 1). TEER values in the human duodenum, jejunum, and ileum have been reported to be 45, 34, and 37 $\Omega \times \text{cm}^2$, respectively (Sjöberg et al., 2013), and indicate that the TEER value of HIECs was closer to the human small intestine than that of Caco-2 cells. Previous studies reported that the oral absorption of compounds that permeate via the paracellular route was poorly predicted in the Caco-2 monolayer, and this was attributed to its excessively tight junctions (Tavelin et al., 2003b; Saitoh et al., 2004; Mattson et al., 2005). The monolayer of 2/4/A1 cells, conditionally immortalized rat intestinal cells, has been reported to possess a radius of tight junction pores and TEER value that are similar to those of the human small intestine (Tavelin et al., 1999, 2003a). These HIEC-like characteristics led to the similarly high $P_{\text{app}}$ values of paracellularly absorbed drugs between the 2/4/A1 cell monolayer and human jejunum (Tavelin et al., 1999, 2003b). However, in contrast with HIECs, the activities of efflux transporters, such as P-gp, BCRP, and MRP, are completely absent in 2/4/A1 cells (Tavelin et al., 2003a). Therefore, the utility of the HIEC monolayer as a superior alternative to the 2/4/A1 cell monolayer remains to be confirmed by side-by-side comparisons with diverse compounds including paracellularly absorbed compounds and the substrates of these efflux transporters.

The permeability of the HIEC monolayer to didanosine, ribavirin, and doxifluuridine, substrates of multiple nucleoside transporters (CNT2, CNT3, and BCRP, and abundant mRNA expression of CNT3 made HIECs a valuable tool for studies on the intestinal absorption of these substrates.

Paracellular absorption refers to permeation across cell monolayers through pores in the tight junction, and the oral absorption of hydrophilic molecules generally relies on the paracellular pathway. In this study, the permeability values of paracellularly absorbed compounds (FD-4, atenolol, and terbutaline) were higher in HIECs than in Caco-2 cells (Table 2). Therefore, the utility of the HIEC monolayer as a superior alternative to the 2/4/A1 cell monolayer remains to be confirmed by side-by-side comparisons with diverse compounds including paracellularly absorbed compounds and the substrates of these efflux transporters.

### Table 2

$P_{\text{app}}$ values represent the mean ± S.D. ($n = 3$).

<table>
<thead>
<tr>
<th>Compound</th>
<th>$F_a$ (%)</th>
<th>$P_{\text{app}}$ (HIEC)</th>
<th>$P_{\text{app}}$ (Caco-2 Cells)</th>
<th>$P_{\text{app}}$ (PAMPA)</th>
<th>Major Route for Absorption or Transport</th>
<th>Molecular Weight</th>
<th>LogP</th>
<th>PSA</th>
<th>$V_{c}%$</th>
</tr>
</thead>
<tbody>
<tr>
<td>FD-4</td>
<td>0</td>
<td>0.56 ± 0.01</td>
<td>0.035 ± 0.009</td>
<td>0.0046 ± 0.0017</td>
<td>Paracellular</td>
<td>4000</td>
<td>—</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>25</td>
<td>2.6 ± 0.1</td>
<td>0.57 ± 0.02</td>
<td>4.1 ± 0.8</td>
<td>Transcellular/P-gp</td>
<td>811.0</td>
<td>3.7</td>
<td>154.1</td>
<td></td>
</tr>
<tr>
<td>Topotecan</td>
<td>30</td>
<td>0.78 ± 0.14</td>
<td>0.35 ± 0.06</td>
<td>1.5 ± 0.1</td>
<td>Transcellular/BCRP</td>
<td>421.4</td>
<td>0.8</td>
<td>103.2</td>
<td></td>
</tr>
<tr>
<td>Didanosine</td>
<td>42</td>
<td>0.62 ± 0.12</td>
<td>0.11 ± 0.02</td>
<td>0.11 ± 0.01</td>
<td>Nucleoside transporters</td>
<td>236.2</td>
<td>—1.2</td>
<td>88.7</td>
<td></td>
</tr>
<tr>
<td>Atenolol</td>
<td>50</td>
<td>0.68 ± 0.09</td>
<td>0.34 ± 0.05</td>
<td>0.022 ± 0.005</td>
<td>Paracellular</td>
<td>266.3</td>
<td>0.2</td>
<td>84.6</td>
<td></td>
</tr>
<tr>
<td>Terbutaline</td>
<td>62</td>
<td>0.70 ± 0.05</td>
<td>0.069 ± 0.006</td>
<td>0.014 ± 0.001</td>
<td>Paracellular</td>
<td>225.3</td>
<td>0.9</td>
<td>72.7</td>
<td></td>
</tr>
<tr>
<td>Ribavirin</td>
<td>85</td>
<td>4.1 ± 0.3</td>
<td>0.092 ± 0.003</td>
<td>0.030 ± 0.002</td>
<td>Nucleoside transporters</td>
<td>244.2</td>
<td>—1.9</td>
<td>143.7</td>
<td></td>
</tr>
<tr>
<td>Doxifluuridine</td>
<td>90</td>
<td>4.7 ± 0.6</td>
<td>0.20 ± 0.03</td>
<td>0.14 ± 0.03</td>
<td>Nucleoside transporters</td>
<td>246.2</td>
<td>—1.4</td>
<td>99.1</td>
<td></td>
</tr>
<tr>
<td>Pindolol</td>
<td>90</td>
<td>7.9 ± 0.7</td>
<td>0.36 ± 0.3</td>
<td>2.2 ± 0.1</td>
<td>Transcellular</td>
<td>248.3</td>
<td>1.8</td>
<td>57.3</td>
<td></td>
</tr>
<tr>
<td>Midazolam</td>
<td>100</td>
<td>30 ± 3</td>
<td>170 ± 7</td>
<td>40 ± 4</td>
<td>Transcellular</td>
<td>325.8</td>
<td>3.1</td>
<td>30.2</td>
<td></td>
</tr>
</tbody>
</table>

LogP, partition coefficient; PSA, polar surface area.

*Footnotes:*  
1. $F_a$ values were obtained from published data (Yamashita et al., 2000; Sugano et al., 2002; Tavelin et al., 2003b; Dixit and Perelson, 2006; Skolnik et al., 2010; Varma et al., 2010; Lin et al., 2011; Sjöberg et al., 2013).
2. Data obtained from www.drugbank.ca.
4. Data obtained from a reference (Rodgers and Rowland, 2006).
The overall lower expression profiles of drug-metabolizing enzymes in Caco-2 cells than in the human small intestine (Fig. 3) have been well documented in previous studies (Schmiedlin-Ren et al., 1997; Sun et al., 2002; Bourgine et al., 2012). In this study, the expression profile of mRNA for the P450/UGT isoforms in HIECs was similar to that in Caco-2 cells, except for CYP2C9, CYP2C19, UGT1A6, and UGT2B7. Although the causes of these exceptions are not known, the differences of origins (normal versus tumoral tissue) between HIECs and Caco-2 cells (Bourgine et al., 2012) and medium additives, such as insulin (Martínez-Jiménez et al., 2006) and dexamethasone (Jemmiz et al., 2002), may contribute these observations. Among the mRNAs whose expression was examined, the mRNA expression (Fig. 3) and function (data not shown) of CYP3A4 were negligible in both HIECs and Caco-2 cells. The reason for the lack of CYP3A4 in HIECs may be partly explained by interindividual variations in the expression and activity of CYP3A4. The expression and function levels of CYP3A4 are known to have a large interindividual variation (Pain et al., 1997), which could explain the lack of CYP3A4 expression and activity in the HIECs used in this study, which was isolated from the ileum of a single donor (19-year-old Caucasian female). Alternatively, our culture condition may not have been sufficient for full differentiation to express CYP3A4 and other enzymes; therefore, further optimization could be required.

Transmission electron microscopy analysis showed that differentiated HIECs formed polarized columnar monolayers with mature morphologic features including microvilli, tight junctions, and desmosomes, which were similar to those observed in vivo and in the Caco-2 monolayer (Hidalgo et al., 1989). However, the features observed in this study were different from previous morphologic findings (Suzuki et al., 2010) in which flat-shaped cells were noted in differentiated HIECs. Although the cause for this discrepancy was not clear, the fibrillar collagen coating used in this study could accelerate maturation of the HIEC monolayer because this coating is known to accelerate the development of the Caco-2 monolayer (Swiderski and Mannuzzu, 1997).

Continuously maintained ISCs conferred the long-term proliferation ability and differentiation capacity of enterocytes to HIECs over 25 passages (Fig. 2). The mRNA of LGR5, an ISC marker, was detected at similar levels during the 8-day cultivation period, whereas the mRNA of IFABP and SI, both enterocyte markers, increased by >10-fold. These results were consistent with the hypothesis that the ISC undergoes asymmetric division to one intestinal stem cell and one progenitor cell, and consequently, the population of ISCs is maintained to constantly generate progenitor cells that can proliferate and differentiate into mature enterocytes over a long period of time (Barker and Clevers, 2010).

The formation of intestinal organoids consisting of enterocytes, goblet cells, enteroendocrine cells, and Paneth cells from human induced pluripotent stem (iPS) cells via ISC was previously demonstrated (Spence et al., 2011); however, the functional characteristics of drug-metabolizing enzymes and transporters were not explored. Iwao et al. (2014) recently demonstrated that enterocyte-like cells that differentiated from human iPS cells via ISCs expressed the mRNAs of peptide transporter 1, also known as soluble carrier family 15 member 1, and CYP3A4. These differentiated cells from iPS cells are expected to have similar gene expression levels for drugs and other xenobiotics as solute carrier family 15 and CYP3A4. These differentiated cells from iPS cells are expected to have similar gene expression levels for drug-metabolizing enzymes and transporters to the human small intestine. In addition, differentiated iPS cells retain the genetic background of the donors; thus, they may facilitate studies on interindividual differences in drug absorption and metabolism. Therefore, advances in the development of in vitro assay systems with HIECs and enteroocyte-like cells differentiated from human iPS cells will mutually improve the prediction and elucidation for intestinal function, transport, and metabolism involved in the absorption process in humans.

In conclusion, we established conditions for the long-term culture of adult ISCs with differentiation capability into enterocytes. To the best of

CNT3, ENT2, and ENT3 for didanosine; CNT2, CNT3, ENT1, and ENT2 for ribavirin; CNT1, CNT3, ENT1, and ENT2 for doxifluridine (Mangravite et al., 2003; Yamamoto et al., 2007; Zhang et al., 2007; Young et al., 2013) was considerably higher than that of Caco-2 cells and PAMPA (Table 2). Na⁺-dependent CNTs and Na⁺-independent ENTs are expressed in the human small intestine (Meier et al., 2007). Nucleosides and their analog drugs generally have hydrophilic properties and diffuse slowly across the cell membrane; therefore, nucleoside transporters expressed in the small intestine largely contribute to their oral absorption (Endres et al., 2009; Okayama et al., 2012; Ishida et al., 2013). Previous studies reported that Caco-2 cells did not express CNT1, CNT2, or CNT3 (Ward and Tse, 1999; Bourgine et al., 2012), which was consistent with the very low expression of CNTs observed in our study (Fig. 3B). In contrast with the Caco-2 monolayer, the significantly high expression of CNT3 mRNA (16% of human small intestine) in HIECs may have conferred an absorption capability for didanosine, ribavirin, and doxifluridine to the HIEC monolayer (Table 2). Ribavirin and doxifluridine, which are good substrates for CNT3 (Hu et al., 2006), had higher P_app values than didanosine, a relatively poor substrate for CNT3 (Hu et al., 2006), in the HIEC monolayer, and the rank order of their P_app values was in agreement with that of F_a values.

Directional transport by P-gp and BCRP was confirmed in the HIEC monolayer by the efflux transport of the corresponding selective substrates (digoxin and mitoxantrone, respectively) and decreases in vectorial transport in the presence of specific inhibitors (verapamil and Ko143, respectively) (Fig. 4). Therefore, the HIEC monolayer can serve as a useful in vitro tool for the evaluation of P-gp- and BCRP–mediated transport during the absorption process. The ratio of P_app for the basolateral-to- apical direction to that for the apical-to-basal direction (ER) of mitoxantrone was approximately 10-fold higher in HIECs (38.2) than in Caco-2 cells (4.0) despite the lower mRNA expression of BCRP in HIECs than in Caco-2 cells (Fig. 3B). Ohtsuki et al. (2012) reported that the protein levels of several transporters including BCRP in the plasma membrane did not correlate with the respective mRNA levels in the human liver, and suggested that a post-transcriptional process and/or intracellular trafficking may play a key role in regulating the functional protein levels of transporters.
our knowledge, this is the first study to describe the application of a HIEC monolayer to the permeability assay for diverse compounds that are known to be absorbed paracellularly or transcellularly, and/or transported by P-gp, BCRP, and nucleoside transporters. The HIEC monolayer had similarly loose tight junctions to the human small intestine and the distinct mRNA expression of CNT3, which was absent in Caco-2 cells. This study demonstrated that the HIEC monolayer can serve as a novel and superior alternative to the conventional Caco-2 monolayer for predicting oral absorption in humans.

**Authorship Contributions**

**Participated in research design:** Takenaka, Harada, Kuze, Chiba, Iwao, Matsunaga.

**Conducted experiments:** Takenaka, Harada.

**Performed data analysis:** Takenaka, Harada.

**Wrote or contributed to the writing of the manuscript:** Takenaka, Harada, Kuze, Chiba, Iwao, Matsunaga.

**References**


