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

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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 \( \Omega \) cm\(^{-2} \)) than in Caco-2 cells (900 \( \Omega \) 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 \( \Omega \) 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 metabolize (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; Saitoh et al., 2004), thereby resulting in the underestimation of intestinal absorption for those compounds (Tavelin et al., 2003b; Saitoh et al., 2004; Mattsson 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; Aldhous et al., 2001; Grossmann et al., 2005).

Start of Notes

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; Saitoh et al., 2004), thereby resulting in the underestimation of intestinal absorption for those compounds (Tavelin et al., 2003b; Saitoh et al., 2004; Mattsson 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; Aldhous et al., 2001; Grossmann et al., 2005).
2003; Chougule et al., 2012), their applications remain limited, which has been attributed to their poor viability and short life span (Aldhous et al., 2001; Grossmann et al., 2003).

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 was more pronounced for paracellularly transported drugs and nucleoside transporter substrates.

Materials and Methods

HIECs (ACBR519) 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 BioChain Institute (Tokyo, Japan). All other chemicals and reagents were of analytical grade. Dulbecco’s phosphate-buffered saline, nonessential amino acids, penicillin-streptomycin, and GlutaMAX were added to the culture medium for Caco-2 cells. The culture medium for HIECs was supplemented with 1 ng/ml insulin, 20 ng/ml epidermal growth factor, 50 μM 2-mercaptoethanol, 50 U/ml penicillin, and 50 μg/ml streptomycin. Caco-2 cells were grown on culture flasks in DMEM with 4.5 g/l glucose, supplemented with 10% FBS, 1× nonessential amino acids, 1× GlutaMAX, 50 U/ml penicillin, and 50 μg/ml streptomycin. Both cell lines were cultured at 37°C in a humidified atmosphere of 5% CO₂ and 95% air. Before reaching confluence (every 4 to 5 days), HIECs and Caco-2 cells were treated with 0.25% trypsin-EDTA, and subcultured at a split ratio of 1:4, or seeded at 1 × 10⁶ cells/well onto 24-well collagen-coated inserts and at 6.3 × 10⁵ cells/well onto 12-well noncoated membrane inserts, respectively. HIECs were fed tridaily with culture medium, supplemented with 50 μg/ml bovine pituitary extract. The culture medium for Caco-2 cells was replaced once in the first week and every other day thereafter. HIECs and Caco-2 cells were grown for 8 to 9 days and 18–20 days, respectively, before assays were performed. TEER values were measured to check the integrity of the monolayer using Millipore-ERS (Millipore, Bedford, MA).

Proliferation Analysis. HIECs were seeded on type I collagen-coated 96-well plates at 5 × 10⁴ cells/well. Cell proliferation analysis was performed 48 hours after seeding by using a colorimetric bromodeoxyuridine (BrdU) cell proliferation assay kit (Millipore) according to the manufacturer’s instructions. A cell viability assay was also performed 48 hours after seeding by using Cell Counting Kit-8 (Dojindo, Kumamoto, Japan). BrdU incorporation into the DNA of proliferating cells as well as cell viability were measured with a microplate reader (SpectraMax Gemini; Molecular Devices, Sunnyvale, CA) at 450 nm with a reference wavelength of 650 nm. To compensate for variability of viable cell numbers, the absorbance in the BrdU incorporation assay was divided by the absorbance in the cell viability assay.

mRNA Quantification. Total RNA was extracted using the PureLink RNA Mini Kit (Life Technologies) from HIECs and Caco-2 cells on 8 and 20 days, respectively, after cell seeding onto membrane inserts. Total RNA from subconfluent HIECs was also extracted in the same manner. These total RNAs and human small intestinal total RNA (5-donor pooled; BioChain Institute, Newark, CA) were used to prepare cDNA using the PrimeScript RT reagent kit (Takara; Shiga, Japan). The quantitative real-time polymerase chain reaction amplification of cDNA corresponding to 25 ng of total RNA was performed in a reaction mixture (20 μl) containing 1× concentration of FAST SYBR Green Master Mix (Applied Biosystems, Foster City, CA) and 0.2 μM primer pairs (Table 1) for caudal type homeobox 2 (CDX2), intestinal fatty acid-binding protein (IFABP), apical sodium-dependent bile acid transporter, monocarboxylate transporter 1, and multidrug resistance protein (MRP) 3, or containing 1× concentration of the TaqMan fast universal PCR master mix (Applied Biosystems) and the following TaqMan Gene Expression Assay with individual assay IDs: LGR5 (Hs00173664_m1), sucrase-isomaltase (SI) (Hs00356112_m1), CNT1 (Hs00609440_m1), CNT2 (Hs00188407_m1), CNT3 (Hs00910439_m1), equilibrative nucleoside transporter (ENT) 1 (Hs01085704_g1), ENT2 (Hs00155426_m1), ENT3 (Hs00217911_m1), and glyceraldehyde-3-phosphate dehydrogenase (Hs02758991_g1) on a 7500 FAST Real-Time PCR system (Applied Biosystems). Thermal cycling conditions included 95°C for 30 seconds. Threshold cycle (Ct) values were determined with 7500 software (Applied Biosystems). Ct values for target genes

<table>
<thead>
<tr>
<th>Gene</th>
<th>Primer Sequences (5’-3’)</th>
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<tbody>
<tr>
<td>CDX2</td>
<td>ACGTGTGCGAGTGATGCTC</td>
</tr>
<tr>
<td></td>
<td>TCTTGGTCTGCTGCGTTTC</td>
</tr>
<tr>
<td>IFABP</td>
<td>ACAAATCTAGACGACGGACACT</td>
</tr>
<tr>
<td></td>
<td>TTGGCTTCTACTTCCTTACA</td>
</tr>
<tr>
<td>ASBT</td>
<td>TGGCCCCAAAAAGCACA</td>
</tr>
<tr>
<td></td>
<td>AACGGTGCGACCTGTCAT</td>
</tr>
<tr>
<td>MCT1</td>
<td>CGCCGCAATATAAAGCATATT</td>
</tr>
<tr>
<td></td>
<td>ATCCAACTTGGACCTCAA</td>
</tr>
<tr>
<td>MP3</td>
<td>GTCGGCAGAATGGCAGGTG</td>
</tr>
<tr>
<td></td>
<td>TACAACCTTGGGGATCATTT</td>
</tr>
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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 MRP1 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 Quantifine 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 phosphoribosyltransferase 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 for 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 its volume of methanol/acetonitrile (2:1, v/v) and centrifuged for 15 minutes at 10,000g before being analyzed with a liquid chromatography–tandem 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. The Cosmosil 5C18 AR-II (150 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 Capcell Pak 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, didanosine, terbutaline, ribavirin, doxifluridine, pindolol, midazolam, 1′-hydroxymidazolam, and 4-hydroxymidazolam: at 0: 1.5, 4, 4.1, and 8.5 minutes, the percentages of acetonitrile were 2, 95, 95, and 2, respectively, with 0.1% formic acid as the aqueous mobile phase. The gradient condition for atenolol was as follows: at 0, 1.5, 3, 3.1, and 8 minutes, the percentages of methanol were 5, 80, 80, 5, and 5, respectively, with 10 mM ammonium acetate as the aqueous mobile phase. 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).

Drug Transport across a Differentiated Enterocyte Monolayer

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 (771.9 > 649.2), mitoxantrone (445.3 > 88.0), vinblastine (811.3 > 224.1), topotecan (4220.5 > 171.0), didanosine (2531.7 > 137.0), atenolol (267.2 > 145.0), terbutaline (226.2 > 152.0), ribavirin (245.0 > 112.9), doxifluridine (245.0 > 171.0), pindolol (249.9 > 116.0), midazolam (326.0 > 222.7), 1′-hydroxymidazolam (341.9 > 323.9), and 4-hydroxymidazolam (342.0 > 324.9). The Cosmosil 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 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 (P_app) 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:

\[
P_{\text{app}} = \frac{dQ}{dt} \times \frac{1}{A \times C_0} \quad (1)
\]

where \(dQ/dt\), \(A\), and \(C_0\) 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 \(P_{\text{app}}\) in secretory (basal-to-apical) to that in absorptive (apical-to-basal) directions.

The \(P_{\text{app}}\) for the PAMPA assay was calculated using eq. 2:

\[
P_{\text{app}} = -\ln\left(1 - \frac{C_A}{C_D} \times \frac{V_{D} + V_{A}}{C_D \times V_{D} + C_A \times V_A} + \left[\frac{A \times \left(\frac{1}{V_D} + \frac{1}{V_A}\right) \times t}{V_D + V_A}\right]\right) \quad (2)
\]

where \(C_D\) and \(C_A\) represent the final concentrations in the donor and acceptor wells, respectively; \(V_D\) and \(V_A\) 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 \(P_{\text{app}}\) values and known fraction absorbed in humans (Fa) data were described using eq. 3 (Amidon et al., 1988):

\[
Fa = 100 \times \left(1 - \exp\left(-a \times P_{\text{app}}\right)\right) \quad (3)
\]

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 contact with culture dishes (Fig. 1A). As monolayers were formed in HIECs and Caco-2 cells, TEER values increased and reached plateaus at

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was examined by measuring TEER values (Fig. 2D). The TEER values
Moreover, the integrity of the HIEC monolayer during serial passage
that ISCs had retained the potential to be differentiated into enterocytes.
IFABP and SI on day 8 after seeding at any passage number indicated
2014) (Fig. 2C). The increase observed in the expression levels of
entiated enterocytes (Levy et al., 2009; Suzuki et al., 2010; Iwao et al.,
Clevers, 2010; Iwao et al., 2014), respectively, at various passages. The
CDX2 and LGR5, a hindgut marker and an ISC marker (Barker and
confirmed this hypothesis, we examined the mRNA expression levels of
proliferating ability and formation capability of HIEC monolayers. To
continuously existed and underwent cell division, leading to a long-term
characteristics under our culture condition.
HIECs differentiated into enterocytes with morphologically mature
under a higher magnification (Fig. 1D). These results indicated that
HIECs differentiates into enterocytes with morphologically mature
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 of CDX2 and LGR5 at subconfluence re-
mained almost constant up to 25 passages (Fig. 2B). In addition, there
was no consistent pattern of change in LGR5 levels between sub-
confluence and day 8 after seeding on culture inserts. These results
indicated that the markedly high number of ISCs was maintained over a
long period of time, even after the formation of monolayers. Diff-
ferentiation potency into enterocytes was also examined by measuring
the mRNA expression levels of IFABP and SI, markers for differ-
entiated enterocytes (Levy et al., 2009; Suzuki et al., 2010; Iwao et al.,
2014) (Fig. 2C). The increase observed in the expression levels of
IFABP and SI on day 8 after seeding at any passage number indicated
that ISCs had retained the potential to be differentiated into enterocytes.
Moreover, the integrity of the HIEC monolayer during serial passage
was examined by measuring TEER values (Fig. 2D). The TEER values
of HIEC monolayers on day 8 after seeding were almost constant over
the passage numbers tested, which suggested that HIECs formed mono-
layers with equal tightness over long-term continuous passages.

mRNA Analysis of Drug-Metabolizing Enzymes and Trans-
porters. 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, or-
ganic cation transporter 1, organic anion-transporting peptide 2B1,
monocarboxylate transporter 1, CNT3, 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 mono-
layer 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 expres-
sion 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 Mono-
layers. 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.,
2009), were determined in both HIECs 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
HIECs 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
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 30 cm/s, 0.035 to 170 cm/s, and 0.0046 to 40 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 cm/s for pindolol and 30 to 170 cm/s for midazolam. The formation of the primary metabolites of midazolam (1-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 $Fa$ 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 that in the human small intestine, whereas those of Caco-2 cells were not, and the HIEC monolayer also had a high $P_{\text{app}}$ for paracellularly absorbed compounds. Furthermore, the presence of the functions of P-gp 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). 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 doxifluridine, substrates of multiple nucleoside transporters (CNT2, CNT3), has 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.

**TABLE 2**

<table>
<thead>
<tr>
<th>Compound</th>
<th>$Fa^e$</th>
<th>$P_{\text{app}}$</th>
<th>Major Route for Absorption or Transporter</th>
<th>Molecular Weight</th>
<th>LogP$^f$</th>
<th>PSA$^g$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[cm$^2$]</td>
<td>[cm$^2$/s]</td>
<td>[cm$^2$/s]</td>
<td>[cm$^2$/s]</td>
<td>[cm$^2$/s]</td>
<td>[cm$^2$/s]</td>
</tr>
<tr>
<td>FD-4</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>3.7</td>
</tr>
<tr>
<td>Vinblastine</td>
<td>26 ± 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>0.8</td>
</tr>
<tr>
<td>Topotecan</td>
<td>30 ± 0.14</td>
<td>0.35 ± 0.06</td>
<td>1.5 ± 0.1</td>
<td>Transcellular/BCRP</td>
<td>421.4</td>
<td>0.2</td>
</tr>
<tr>
<td>Didanosine</td>
<td>42 ± 0.12</td>
<td>0.11 ± 0.02</td>
<td>0.11 ± 0.01</td>
<td>Nucleoside transporters</td>
<td>236.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Atenolol</td>
<td>50 ± 0.09</td>
<td>0.34 ± 0.05</td>
<td>0.022 ± 0.005</td>
<td>Paracellular</td>
<td>266.3</td>
<td>0.9</td>
</tr>
<tr>
<td>Terbutaline</td>
<td>60 ± 0.05</td>
<td>0.069 ± 0.006</td>
<td>0.014 ± 0.001</td>
<td>Paracellular</td>
<td>225.3</td>
<td>0.97</td>
</tr>
<tr>
<td>Ribavirin</td>
<td>85 ± 0.3</td>
<td>0.092 ± 0.003</td>
<td>0.030 ± 0.002</td>
<td>Nucleoside transporters</td>
<td>254.3</td>
<td>0.9</td>
</tr>
<tr>
<td>Doxifluridine</td>
<td>90 ± 0.6</td>
<td>0.20 ± 0.03</td>
<td>0.14 ± 0.03</td>
<td>Nucleoside transporters</td>
<td>254.3</td>
<td>0.9</td>
</tr>
<tr>
<td>Pindolol</td>
<td>90 ± 0.7</td>
<td>0.36 ± 0.3</td>
<td>0.21 ± 0.1</td>
<td>Transcellular</td>
<td>248.3</td>
<td>1.8</td>
</tr>
<tr>
<td>Midazolam</td>
<td>100 ± 3</td>
<td>0.17 ± 0.7</td>
<td>0.40 ± 0.4</td>
<td>Transcellular</td>
<td>325.8</td>
<td>3.1</td>
</tr>
</tbody>
</table>

LogP, partition coefficient; PSA, polar surface area.

$^e$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).

$^f$Data obtained from the interview form of Fortulon capsules.

$^g$Data 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).

$^h$Data obtained from www.drugbank.ca.


$^j$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; Bourvine 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 (Bourvine et al., 2012) and medium additives, such as insulin (Martínez-Jiménez et al., 2006) and dexamethasone (Fennitz et al., 2002), may contribute to 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 (Paine 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., 2013). Previous studies reported that Caco-2 cells did not express 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; Bourvine 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 \( P_{\text{app}} \) values than didanosine, a relatively poor substrate for CNT3 (Hu et al., 2006), in the HIEC monolayer, and the rank order of their \( P_{\text{app}} \) values was in agreement with that of Fa 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 \( P_{\text{app}} \) 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.

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

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