Development of a Caco-2 Cell Line Carrying the Human Intestine-Type CES Expression Profile as a Promising Tool for Ester-Containing Drug Permeability Studies

Yuma Ishizaki, Tomomi Furihata, Yusuke Oyama, Kayoko Ohura, Teruko Imai, Masakiyo Hosokawa, Hidetaka Akita, and Kan Chiba

Carboxylesterase 2 (CES2), which is a member of the serine hydrolase superfamily, is primarily expressed in the human small intestine, where it plays an important role in the metabolism of ester-containing drugs. Therefore, to facilitate continued progress in ester-containing drug development, it is crucial to evaluate how CES2-mediated hydrolysis influences its intestinal permeability characteristics. Human colon carcinoma Caco-2 cells have long been widely used in drug permeability studies as an enterocyte model. However, they are not suitable for ester-containing drug permeability studies due to the fact that Caco-2 cells express CES1 (which is not expressed in human enterocytes) but do not express CES2. To resolve this problem, we created a new Caco-2 cell line carrying the human small intestine-type CES expression profile. We began by introducing short-hairpin RNA for CES1 mRNA knockdown into Caco-2 cells to generate CES1-deficient Caco-2 cells (Caco-2CES1KD cells). Then, we developed Caco-2CES1KD cells that stably express CES2 (Caco-2CES1KD cells) and their control Mock/Caco-2CES1KD cells. The results of a series of functional expression experiments confirmed that CES2-specific activity, along with CES2 mRNA and protein expression, were clearly detected in our CES2/Caco-2CES1KD cells. Furthermore, we also confirmed that CES2/Caco-2CES1KD cells retained their tight junction formation property as well as their drug efflux transporter functions. Collectively, based on our results clearly showing that CES2/Caco-2CES1KD cells carry the human intestinal-type CES expression profile, while concomitantly retaining their barrier properties, it can be expected that this cell line will provide a promising in vitro model for ester-containing drug permeability studies.

Key words carboxylesterase; Caco-2 cell; prodrug; drug permeability; small intestine

Carboxylesterases (CESs), which belong to the serine hydrolase superfamily, play an important role in metabolizing diverse types of ester compounds, including drugs. Among the CES members identified to date, CES1 and CES2 are primarily involved in drug metabolism in mammals. As such, they show a broad range of substrate specificities with some overlaps, but there is at least one clear difference between the two. CES1 prefers to hydrolyze substrates with bulky acyl moieties (such as irinotecan and aspirin), whereas CES2 preferentially recognizes substrates with bulky alcohol moieties (such as irinotecan and aspirin). Therefore, CES1 and CES2 play separate roles in drug metabolism.

One of the important tissues for drug metabolism is the human small intestine, where CES species are differentially expressed. It has been reported that CES2 is predominantly expressed in the small intestine (duodenum, jejunum, and ileum) over the liver in humans, and that, in the small intestine, CES2 can be expressed in enterocytes. On the other hand, CES1 is primarily expressed in the liver, and hardly expressed in the small intestine. Therefore, to facilitate the development of ester-containing drugs, it is crucial to determine whether or not new chemical entities can be a substrate for CES2. In cases where compounds are not hydrolyzed by CES2, they hold an increasing chance of being easily absorbed and moved into systemic circulation. Temocapril is an example of one such drug. That is, it can be readily uptaken via the human small intestine, and then be converted to its active metabolite (temocaprilat) by CES1 in the liver.

On the other hand, in cases where the drugs are susceptible to hydrolysis by CES2 in the human small intestine, evaluating the absorption rate of the parental drug and its metabolite is much more complicated. It is extremely difficult to estimate how much of the parent drug is metabolized during its transport across the enterocytes. Additionally, hydrolytic metabolites often exhibit remarkably different physiochemical properties from those of the parent drug. For these reasons, the CES2-mediated hydrolysis of an ester-containing drug is likely to be a critical factor in determining its overall absorption profile in humans.

Caco-2 cells, which are derived from human colon carcinoma, have long been widely used as an in vitro human small intestine model in drug development studies where they are used for predicting drug absorption properties in humans. One of the reasons for this is that Caco-2 cells express several drug efflux/influx transporters, such as P-glycoprotein (P-gp), breast cancer resistance protein (BCRP), organic anion transporting polypeptide 2B1 (OATP2B1), and peptide transporter 1 (PEPT1). Furthermore, it is well known that, when cul-

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turred for a few weeks under the confluent conditions, Caco-2 cells undergo differentiation to acquire microvilli-like morphology and form tight junctions.12,13)

However, Caco-2 cells are not useful as models in human small intestine studies involving ester-containing drug permeability due to apparent differences in the CES expression profiles between Caco-2 cells and the human small intestine cells.14) More specifically, Caco-2 cells highly express CES1 (but not CES2) that is clear contrast to the fact that CES2 (but not CES1) is primarily expressed in the human small intestine. This makes it unlikely that the permeability profiles of ester-containing drugs obtained from Caco-2 cells would be able to precisely reflect those observed in the human small intestine.

To resolve this problem, a Caco-2 cell clone line lacking CES1 activity has been isolated from heterogeneous parental cell populations.15,16) While this approach has been reasonable to improve CES1 substrate drug absorption profiles, the lack of CES2 activity in the CES1-deficient Caco-2 cell clone line has rendered it unsuitable for use as a general human small intestine model cell line in ester-containing drug permeability studies.

Given the above background, in this study, we sought to develop a new Caco-2 cell line that carries a human small intestine-type CES expression profile suitable for use in ester-containing drug permeability studies.

MATERIALS AND METHODS

**Cell Culture** Human colon cancer Caco-2 cells (passages 35–70) were obtained from DS Pharma Biomedical (Osaka, Japan) and grown with Dulbecco’s modified Eagle’s medium (DMEM) (Wako Pure Chemical Industries, Ltd., Osaka, Japan) supplemented with heat-inactivated 10% fetal bovine serum (FBS), 100 U/mL penicillin and 100 µg/mL streptomycin in a humidified atmosphere of 5% CO2/air at 37°C. Human embryonic kidney 293FT cells (passages 6–10) (Thermo Fisher Scientific, Waltham, MA, U.S.A.) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Caco-2 cells (parental Caco-2, pCaco-2) were seeded on a 25 cm2 culture flask (1.0 × 104 cells). The next day, lentivirus (6.0 × 105 TU/mL) was infected into pCaco-2 cells with 8 µg/mL polybrene (Nacalai Tesque, Kyoto, Japan). After the selection using 10 µg/mL puromycin (Wako), the cells were homogenized using a sonicator in a sodium pentobarbital (Nembutal, Abbott Laboratories, North Chicago, IL, U.S.A.) solution.

**Development of CES1-Deficient Caco-2 Cells** Lentivirus carrying short-hairpin RNA (shRNA) against human CES1A1 genes (TRC# are shown below) and control lentivirus (SHC002V) were purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Caco-2 cells (parental Caco-2, pCaco-2) were seeded on a 25 cm2 culture flask (1.0 × 104 cells). The next day, lentivirus (6.0 × 105 TU/mL) was infected into pCaco-2 cells with 8 µg/mL polybrene (Nacalai Tesque, Kyoto, Japan). After the selection using 10 µg/mL puromycin (Wako), the cells were individualized by a colony formation. CES1 mRNA expression levels were analyzed by quantitative real-time PCR (qPCR), as described below. Since the first trial with single shRNA (TRC00000371770) did not produce a clone in which the CES1 mRNA level was sufficiently knocked-down, a second round of lentiviral transduction using additional shRNA (TRC0000046934) and colony isolation was performed. Eventually, a CES1-deficient Caco-2 cell clone line (hereafter referred to as Caco-2 CES1KD cells) was obtained. Similarly, pCaco-2 cells carrying empty lentivirus were generated and designated as Caco-2Mock cells.

**Development of Caco-2CES1KD Cells Stably Expressing CES2** The retroviral vector pDON-A12-Neo (TaKaRa Bio, Shiga, Japan) carrying human CES2 cDNA was developed using human CES2 expression plasmid.17) The CES2/pDON-A12-Neo or empty pDON-A12-Neo, along with the packaging plasmids (a retrovirus packaging kit amphi [TaKaRa Bio]) were transfected with 293FT cells using Lipofectamin 3000 Reagent (Thermo Fisher Scientific). Three days after transfection, the culture supernatant containing the retrovirus was collected and concentrated by a Retro-X™ Concentrator (TaKaRa Bio). The retrovirus collection was then stored at −80°C until used.

Caco-2CES1KD cells were infected with the CES2 expression retrovirus (or empty retrovirus) in the presence of 8 µg/mL polybrene. One week later, successfully infected cells were selected using 600 µg/mL G418. Then Caco-2CES1KD cells stably expressing CES2 were individualized using a colony formation method. CES2 mRNA expression levels were then analyzed by qPCR, as described below. Based on these qPCR results, the Caco-2CES1KD cell clone line showing the highest CES2 expression level among those examined (hereafter referred to as CES2/Caco-2CES1KD cells) was identified. Likewise, Caco-2CES1KD cells carrying the empty retrovirus were generated and designated as Mock/Caco-2CES1KD cells.

**Total RNA Extraction, cDNA Synthesis, and PCR** Total RNA was isolated from Caco-2 cells using a NucleoSpin RNA II mini spin kit (TaKaRa Bio), and PCR was performed to confirm that no genomic DNA contamination had occurred. Human small intestine RNA was purchased from Agilent Technologies (Santa Clara, CA, U.S.A.). cDNA synthesis from total RNA (1 µg) was performed by the High Capacity cDNA Reverse Transcription Kit (Thermo Fisher Scientific) with random primers.

qPCR was performed using the KAPA SYBR Fast qPCR Kit (KAPA Biosystems, Boston, MA, U.S.A.) and the Eco Real-Time PCR System (Illumina Inc., San Diego, CA, U.S.A.). The CES mRNA expression levels were normalized by the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA level. RT-PCR was also performed using the KAPA qPCR Mix DNA polymerase (KAPA Biosystems). All of the primer sets used are shown in Table 1.

**Preparation of the S9 Fractions from Caco-2 Cells** The S9 fractions were prepared from Caco-2 cells that had been cultured for 14d. After washing with phosphate-buffered saline (PBS), the cells were homogenized using a sonicator in a

<table>
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<th>Primer names</th>
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<tr>
<td>CES1 R</td>
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<td>P-gp F</td>
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<td>P-gp R</td>
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<td>BCRP F</td>
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<tr>
<td>GAPDH R</td>
<td>GCCCAATACGACAAATCC</td>
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(a) F and R indicate forward and reverse primers, respectively.
SET buffer (240 mM sucrose, 1 mM ethylenediaminetetraacetic acid (EDTA), 10 mM Tris–HCl, pH=7.4). The homogenates were centrifuged at 9000×g, 4°C for 30 min, and the resulting supernatant was designated as the S9 fraction. Protein concentrations were determined by using a DC Protein Assay Kit (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

Western Blotting CES1 and CES2 protein detection by Western blotting was performed using the method described previously. Briefly, the Caco-2 cell S9 fractions (20 µg each) were solubilized with Laemmli sample buffer, and then boiled at 95°C for 3 min. After 10% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), the proteins were transferred onto a nitrocellulose membrane, which was blocked with 3% bovine serum albumin (BSA) suspended with Tris-buffered saline containing 0.05% Tween-20 (TBS-T). The primary and secondary antibodies are shown in Table 2. The anti-human CES2 peptide anti-serum used is the custom-made product developed by Sigma-Aldrich using the CES2 peptide TFGDLRREEYIGDNGDPQT, and its selectivity was confirmed using pre-immune serum as a comparison. The immunocomplex was detected via an ImageQuant LAS-4000 biomolecular imager (GE Healthcare Life Sciences, Pittsburgh, PA, U.S.A.) using an ImmunoStar LD Western blotting detection reagent (Wako).

Native PAGE Coupled with Esterase Staining Native-PAGE electrophoresis coupled with esterase staining was performed as described previously. In brief, 50 µg of Caco-2 S9 fraction was loaded into each lane. After electrophoresis, esterase activities in the polyacrylamide gel were detected using α-naphthyl acetate as a substrate (Tokyo Kasei, Tokyo, Japan).

Hydrolase Activity Determinations Hydrolisis activities toward p-nitrophenyl acetate (PNPA), which is a substrate of both CES1 and CES2, and fluorescein diacetate (FDA), which is a preferential substrate for CES2, were determined using the methods described elsewhere. p-Nitrophenol (absorbance at 405 nm) and fluorescein (excitation 483 nm, emission 525 nm) released were detected by the Wallac 1420 ARVOx multi-plate reader (PerkinElmer, Inc., Waltham, MA, U.S.A.), and their concentrations were determined using each standard curve.

Transepithelial Electric Resistance (TEER) Measurement of Cell Monolayer Caco-2 cells were seeded at a density of 1.0×10⁵ cells/well onto a transwell insert membrane (polyethylene telephthalate, 0.4 µm high density pores, 0.3 cm², BD Falcon, Franklin Lakes, NJ, U.S.A.), and cultured for 21 d in a transwell system. Immunocytochemistry was performed to detect occludin essentially based on the method described previously. The primary antibodies were shown in Table 2. The immunocomplex was detected via fluorescent microscopy using a Zeiss 710 Confocal Microscope (Carl Zeiss AG, Oberkochen, Germany).

Permeability Assays The apical to the basolateral movement of d−(−)-mannitol in the Caco-2 cell monolayers was examined. [³H]Mannitol (0.5 µCi) (American Radiolabeled Chemicals, St. Louis, MO, U.S.A.), together with cold mannitol (final concentration of 5 µM), was added to the apical side of the Caco-2 cell monolayers, which was then incubated for 90 min at 37°C. The medium was collected from the basolateral side at 30, 60, and 90 min and the [³H]mannitol concentration was determined by liquid scintillation counter (LSC-6100, Aloka Co., Ltd., Tokyo, Japan).

The vectorial transport assays for Rhodamine-123 (R123, Wako) and [³H]digoxin (PerkinElmer, Inc.) (together with cold digoxin) were also performed using the method described previously. The final concentrations of rhodamine-123 and digoxin were 5 and 1 µM, respectively. We began incubation with the addition of the substrate to either the apical or basolateral side, 60 min after which the medium was collected from the opposite side. R123 was detected using the Wallac 1420 ARVOx, and its concentrations were calculated using a standard curve. [³H]Digoxin concentrations were analyzed by liquid scintillation counter.

CES Substrate Metabolism/Transport Assays The permeability of temocapril, along with temocaprilat formation (a metabolite), were analyzed using Caco-2 cell monolayers as described previously. Briefly, temocapril (LKT Labs, St. Paul, MN, U.S.A.) was added to the apical side of Caco-2 cell monolayers at the final concentration of 100 µM, and then incubated for 120 min at 37°C. The medium was collected from the AP side at zero, 60, and 120 min, or the BL side at zero, 20, 40, 60, 100 and 120 min, after which the concentrations of temocapril and temocaprilat were analyzed by HPLC.

The FDA metabolism/transport assays were performed using Caco-2 cell monolayers, and the amount of fluorescein (a FDA metabolite) transported into either the apical or basolateral compartment was determined. FDA (5 mM in 10% acetonitrile-containing sterile water) was prepared. This FDA solution was added into the apical compartment of the Caco-2 monolayers at 100-fold dilution to start the reaction (time=0). The medium of either side (5 and 50 µL from the AP and BL sides, respectively) was collected at 5, 10, 20, 30 min, and then the amount of fluorescein in each sample was determined by the Wallac 1420 ARVOx multi-plate reader.

In the above experiments, Caco-2 cell monolayers with TEER levels above 400 (Ω cm²) were used, and the volume

<table>
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RESULTS

Development of CES1-Deficient Caco-2 Cells (Caco-2CES1KD Cells) After introducing shRNA targeting CES1 mRNA into Caco-2 cells in order to eliminate their CES1 activity, the resulting cells were referred to as Caco-2CES1KD cells. Our qPCR results showed that the CES1 mRNA expression levels in Caco-2 CES1KD cells were lower than those of pCaco-2 and Caco-2 Mock cells (Fig. 1A), and the results of Western blotting and native-PAGE electrophoresis coupled with esterase staining analyses showed that, in contrast to pCaco-2 and Caco-2 Mock cells, CES1 protein expression could not be detected in Caco-2 CES1KD cells (Figs. 1B, C). To further confirm the CES1 knockdown profile, the PNPA hydrolysis activities of those Caco-2 cells were determined and compared. The results showed that the activity level of Caco-2 CES1KD cells was lower than those of pCaco-2 and Caco-2 Mock cells (324 ± 24 vs. 35–76 [nmol/mg protein/min]), and the activity was abolished in the presence of loperamide, which is a CES2 specific inhibitor (Fig. 2D). Furthermore, the FDA hydrolysis activity level, which is an indicator of the CES2 functionality, was higher in CES2/Caco-2 CES1KD cells than in other cells (47.2 ± 8.9 vs. 0.5–0.6 [nmol/mg protein/min]) (Fig. 2E).

These results clearly show that CES1 expression has been successfully knocked-down to a negligible level in Caco-2 CES1KD cells.

Development of Caco-2 CES1KD Cells Carrying CES2 Activity (CES2/Caco-2 CES1KD) Next, we introduced CES2 cDNA to Caco-2 CES1KD cells in order to develop cells that stably express CES2 (CES2/Caco-2 CES1KD). The qPCR results showed that high CES2 mRNA levels were detected in CES2/Caco-2 CES1KD cells, but not in pCaco-2, Caco-2 CES1KD, and Mock/Caco-2 CES1KD cells (Fig. 2A). Consistently, the results of Western blotting, as well as native-PAGE electrophoresis coupled with esterase staining analyses, show that CES2 protein expression was detected in CES2/Caco-2 CES1KD cells, but not in the other Caco-2 cells (Figs. 2B, C, respectively). Additionally, CES2/Caco-2 CES1KD cells showed higher levels of PNPA hydrolysis activity when compared with those observed in the other cells (324 ± 24 vs. 35–76 [nmol/mg protein/min]), and the activity was abolished in the presence of loperamide, which is a CES2 specific inhibitor (Fig. 2D). Furthermore, the FDA hydrolysis activity level, which is an indicator of the CES2 functionality, was higher in CES2/Caco-2 CES1KD cells than in other cells (47.2 ± 8.9 vs. 0.5–0.6 [nmol/mg protein/min]) (Fig. 2E).

Therefore, these results clearly demonstrate that the CES2

removed from the receiver side was always replenished with the fresh transport buffer.
are functionally expressed in CES2/Caco-2CES1KD cells. **

**Tight Junction Formation in CES2/Caco-2CES1KD Cells**

TEER values (400 [Ω·cm²]) have been widely used as a threshold line for judging the practically minimum tight junction function of Caco-2 cells. With this point in mind, the TEER values of pCaco-2, Mock/Caco-2CES1KD, and CES2/Caco-2CES1KD cells were examined over the course of their monolayer formation (Fig. 3A). TEER values were measured for 21 d, and the highest TEER values were recorded during day seven to eleven as 1012 ± 68, 1156 ± 58 and 1656 ± 240 in pCaco-2, Mock/Caco-2CES1KD, and CES2/Caco-2CES1KD cells, respectively.

Next, the apical-to-basolateral mannitol flux rate, which is a marker of paracellular permeability, was measured in each Caco-2 cell monolayer after they had reached their maximum TEER values (Fig. 3B). The mannitol $P_{\text{app}}$ ($\times 10^{-5}$ cm/min) values were 3.8 ± 0.5, 4.7 ± 0.9, 4.9 ± 1.6 in the pCaco-2, Mock/Caco-2CES1KD, and CES2/Caco-2CES1KD cells, respectively.

To further validate tight junction formation in these Caco-2 cells, their tight junction protein expression and cellular localization features were analyzed after their TEER values reached their maximum levels (Fig. 3C). The immunocytochemistry results show that occludin was detected at the cell borders in each cell monolayer.

Taken together, the above-described results indicate that CES2/Caco-2CES1KD cells could form tight junctions with functional properties that were comparable to those of pCaco-2 and Mock/Caco-2CES1KD cells.

**Functional Expression of Efflux Transporters in CES2/Caco-2CES1KD Cells**

To confirm whether CES2/Caco-2CES1KD cells retain the functional expression of P-gp and BCRP, we performed RT-PCR and vectorial transport assays using R123 (a P-gp and BCRP substrate) and digoxin (a P-gp substrate). The RT-PCR results showed that, as with pCaco-2 and Mock/Caco-2CES1KD cells, P-gp and BCRP mRNA expression were detected in CES2/Caco-2CES1KD cells (Fig. 4A). Consistently, the efflux ratios ($P_{\text{app,BL-AP}}/P_{\text{app,AP-BL}}$) of R123 and digoxin in the CES2/Caco-2CES1KD cells were 3.3 and 2.8, respectively.
which compares well to those obtained from pCaco-2 and Mock/Caco-2 CES1KD cells (Figs. 4B, C). Therefore, these results confirm that P-gp and BCRP were functionally expressed in CES2/Caco-2 CES1KD cells at levels that are similar to those of pCaco-2 cells.

**Temocapril and FDA Metabolism/Transport Assays Using Caco-2 Cell Monolayers** Finally, we sought to provide an example showing that CES2/Caco-2 CES1KD cells can be used for ester-containing drug permeability assays. At first, we selected temocapril as a model compound, because it is a well-established selective CES1 substrate and has been used in permeability experiments in previous studies. The results showed that temocapril appeared to be extensively hydrolyzed only in pCaco-2 cells, and temocaprilat (a metabolite of temocapril) was transported into both the AP and BL sides. Temocaprilat transported into the AP side could be detected only in the pCaco-2 cells (e.g., 14.0±8.0 nmol at 120 min), and the transported amount of temocaprilat at the BL side was much higher in the pCaco-2 cells than those in Mock/Caco-2 CES1KD cells and CES2/Caco-2 CES1KD cells (e.g., 9.5±2.2, 0.8±0.4, and 0.5±0.4 nmol at 120 min, respectively) (Fig. 5).

Then we also examined FDA metabolism/transport in the Caco-2 monolayers in order to obtain evidence that CES2 in Caco-2 cells plays a crucial role in its substrate metabolism/transport profiles in *in vitro* permeability assays. The results showed that fluorescein formed appeared to be preferentially transported into the AP side, and that the transported fluorescein amounts at the AP side were much higher in CES2/Caco-2 CES1KD cells than those of pCaco-2 cells and Mock/Caco-2 CES1KD cells (e.g., 60.1±10.0, 39.0±5.2, and 32.3±0.8 pmol at 20 min respectively) (Fig. 6).

Based on these data, it is clear that the differential CES isofrom expression profile in Caco-2 cells significantly affects ester-containing drug permeability.

**DISCUSSION**

Accurate predictions of drug absorption profiles in the human small intestine from pre-clinical results has long been a bottleneck in drug development, and such predictions are likely to be especially difficult for ester-containing drugs due to CES expression profiles difference between Caco-2 cells and human small intestine cells, which often results in differential metabolic profiles for the ester-containing drugs under evaluation. Since hydrolytic metabolites can exhibit significantly different physicochemical properties and different active efflux susceptibilities from those of the parental ester-containing drug, it is unlikely that the results of ester-
containing drug permeability assays using Caco-2 cells could reliably assist in absorption profile predictions involving the human small intestine.

However, the establishment of Caco-2 cells carrying the human small intestine-type CES expression profile (CES2/Caco-2CES1KD cells) has the potential to revise the longstanding experimental premise in regard to the applicability of Caco-2 cells in intestinal drug absorption studies. This is because...

Fig. 4. Functional Expression of Representative Intestinal Drug Efflux Transporters in pCaco-2, Mock/Caco-2CES1KD, and CES2/Caco-2CES1KD Cells
A, mRNA expression of two representative intestinal drug efflux transporters, P-gp and BCRP, in pCaco-2, Mock/Caco-2CES1KD, and CES2/Caco-2CES1KD cells were measured by RT-PCR. GAPDH mRNA was used as an internal control. The experiments were repeated three times, and the representative results are shown. In B and C, the vectorial transport assays of R123 and digoxin (which are representative substrates for P-gp and/or BCRP) were performed in pCaco-2, Mock/Caco-2CES1KD, and CES2/Caco-2CES1KD cells. The efflux ratio (ER) was calculated by dividing the P_{P-gp-M} (open bars) by the P_{P-gp-A} (solid bars). AP and BL indicate apical and basolateral compartments, respectively. The data are shown as the mean ± S.D. of three independent determinations, each performed in duplicate.

Fig. 5. Temocapril Metabolism/Transport Assays across pCaco-2, Mock/Caco-2CES1KD, and CES2/Caco-2CES1KD Cells
The apical (AP)-to-basolateral (BL) transported amount of temocapril across pCaco-2, Mock/Caco-2CES1KD, and CES2/Caco-2CES1KD cell monolayers are shown as solid circles. Similarly, the transported amount of temocapril generated within the cells to the AP side or BL side is shown as open circles and solid triangles, respectively. The experiments were performed with cells cultured for 14 d that had TEER values in excess of 400 (Ω cm²). In all figures, the data are shown as the mean ± S.D. of three independent determinations, each performed in duplicate. Note that the amounts of temocapril at the AP side of Mock/Caco-2CES1KD and CES2/Caco-2CES1KD cell monolayers were under the detection limit, and thus the values are tentatively set as 0.
our results of FDA metabolism/transport assays suggest that the clear expression of CES2 in CES2/Caco-2\(^{CES1KD}\) cells will make metabolic profiles of ester-containing drugs like what is expected in human enterocytes. In this context, the nearly complete loss of CES1 expression is noteworthy, because the residual CES1 activity would otherwise give a misleading result, as exemplified by our results showing that the functional CES1 expression in pCaco-2 cells influences temocapril permeability. Furthermore, it should be underscored that CES2/Caco-2\(^{CES1KD}\) cells apparently retain their tight junction formation properties and efflux transporter functions to a degree that is comparable to those observed in the parental Caco-2 cells. In addition to exclusive CES2 expression, these barrier functions are indispensable if the cells to be used in intestinal drug permeability analysis.

Therefore, our CES2/Caco-2\(^{CES1KD}\) cells can be expected to open up a new methodology for predicting intestinal absorption properties of ester-containing drugs in humans, and their significant potential in drug development applications warrants substantial attention and is worth pursuing. As yet, however, we have only tested FDA, of which pharmacokinetic parameters in humans cannot be available, and, therefore, we cannot state so categorically how our results can relate to the human in vivo situation. In addition, it remains to be characterized how CES2/Caco-2\(^{CES1KD}\) cells can be used for quantitative evaluation. Thus, considerable research efforts are obviously necessary. For such future studies, it should be mentioned that in contrast to the results of Fig. 2E, its appreciable hydrolysis has been observed even in pCaco-2 and Mock/Caco-2\(^{CES1KD}\) cells in FDA metabolism/transport assays. The causal factors remain unclear, and we need to a truly CES2-specific substrate to be included in studies aimed at further characterization of CES2/Caco-2\(^{CES1KD}\) cell properties.

Meanwhile, other research efforts have been made towards the establishment of human small intestine model cells for drug absorption studies. For example, development of human enterocytes derived from induced pluripotent stem (iPS) cells and their functional features have recently been reported.\(^\text{25}\) Human iPS-derived enterocytes have been shown to express CES2, but not CES1, while also expressing representative intestinal drug metabolizing enzymes (including CYP3A4) and transporters (including PEPT1 and BCRP).\(^\text{26,27}\) Such results are further suggestive of their promising potential as a useful human intestinal cell model.

However, the existence of other human enterocyte models (such as human iPS-derived enterocytes) does not necessarily reduce the value of our cells, because each cell model has its own advantages. As for CES2/Caco-2\(^{CES1KD}\) cells, it should be highlighted that Caco-2 cells have a long history of use as an in vitro human small intestine model for evaluating drug permeability in drug development studies,\(^\text{28}\) which means that significant amounts of background have already been accumulated on their uses. Therefore, choosing the right model, in the right place based on the model’s particular characteristics, is sure to provide to accelerate ester-containing drug developments.

Those points to one side, it is worth reiterating that we have also, in parallel, developed Caco-2 cells stably lacking detectable CES1 activity (Caco-2\(^{CES1KD}\) or Mock/Caco-2\(^{CES1KD}\) cells). Since these cells also show tight junction formation properties, as well as efflux transporter function levels that are comparable to CES2/Caco-2\(^{CES1KD}\) and parental Caco-2 cells, they can not only be used for comparison references, but may also be regarded as a model for use in determinations as to whether a parental drug could be a substrate of efflux transporters, or extent of its intrinsic cellular membrane permeability.\(^\text{29}\) These CES1 mRNA-knockdown cells have several advantageous features compared with other CES1-deficient Caco-2 cells.\(^\text{15,16}\) One is that Caco-2\(^{CES1KD}\) cells do not require pre-treatment with BNPP, which allows researchers to perform high-throughput assay more easily and to avoid unexpected interactions between BNPP and test compounds. Another is that, unlike Caco-2\(^{CES1KD}\) cells, CES1-deficient Caco-2 cell subclone harbors a risk for CES1 re-emergence under certain culture conditions that release its repression. Therefore, it can be said that the Caco-2 cell series developed in this study has the potential to provide researchers with new and convenient ways to determine multiple possible parameters involved in...
drug absorption via the human small intestine.

Finally, we will additionally describe potential limitations of our cells. CES2/Caco-2_CES1KD cells possess the essential factors governing ester-containing drug absorption profiles, we have not yet examined all of the other related factors. Considering the possibility that virus integration into the genome DNA might disrupt certain genetic regions, and that a Caco-2 subpopulation harboring a specific genetic background might survive the cell individualization process, the functional expression of each drug absorption-related gene (such as multidrug resistance-associated protein 2 and OATP2B1^{30,31}) will need to be investigated separately.

Furthermore, since CES2/Caco-2_CES1KD cells essentially possess the Caco-2 cell background, it should be borne in mind that several general limitations associated with those cells (such as lack of CYP3A4 expression^{22}) remain to be resolved. Therefore, efforts aimed at detailed characterization and functional improvements will be necessary in order to firmly establish their value as a useful model for use in in vitro human small intestine absorption studies.

In conclusion, herein we reported on the development of Caco-2 cells exhibiting the human small intestine-type CES activity (CES2/Caco-2_CES1KD cells). Based on their functional CES2 expression under CES1-null background, as well as their tight junction formation properties and efflux transporter levels, CES2/Caco-2_CES1KD cells can be expected to provide a useful in vitro model for pre-clinical ester-containing drug permeability studies. Therefore, further investigations aimed to thoroughly validate and characterize their applicability will be warranted.

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Conflict of Interest The authors declare no conflict of interest.

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