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cAMP signaling promotes the differentiation of human induced pluripotent stem cells into intestinal epithelial cells

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Running Title: cAMP promotes intestinal differentiation of iPS cells

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Nonstandard abbreviation

ABC, ATP-binding cassette; BCRP, breast cancer resistance protein; cAMP, cyclic adenosine monophosphate; CDX2, caudal type homeobox 2; CES, carboxylesterase; CYP, cytochrome P450; DAPI, 4',6-diamidino-2-phenylindole; DMEM, Dulbecco's Modified Eagle's medium; DNMT, DNA methyltransferase; DPP4, dipeptidyl peptidase-4; EGF, epidermal growth factor; ER, efflux ratio; FABP2, fatty acid-binding protein 2; FBS, fetal bovine serum; FGF, fibroblast growth factor; HBSS, Hanks' balanced salt solution; IBMX, 3-isobutyl-1-methylxanthine; iPS, induced pluripotent stem; ISX, intestine-specific homeobox; MAPK, mitogen-activated protein kinase; MDR1, multidrug resistance 1; NEAA, minimum essential medium nonessential amino acid solution; OATP, organic anion transporting polypeptide; P_{app}, apparent permeability coefficient; PBS, phosphate-buffered saline; PEPT1, peptide transporter 1; P-gp, P-glycoprotein; PXR, pregnane X receptor; RT-PCR, reverse transcription-polymerase chain reaction; SGLT1, sodium-glucose cotransporter 1; SLC, solute carrier; SULT, sulfotransferase; TEER, transepithelial electrical resistance; TGF, transforming growth factor; UPLC–MS/MS, ultraperformance liquid chromatography–tandem mass spectrometry; UGT, UDP-glucuronosyltransferase

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Abstract

To develop a novel *in vitro* system for predicting intestinal drug absorption using human induced pluripotent stem (iPS) cell-derived intestinal epithelial cells, the cells need to have sufficient drug-metabolizing enzyme and drug transporter activities. We found that cyclic adenosine monophosphate (cAMP) signaling plays an important role in the differentiation of human iPS cells into intestinal epithelial cells. In this study, we aimed to demonstrate the effects of signaling activation in the intestinal differentiation of human iPS cells and the pharmacokinetic characteristics of human iPS cell-derived intestinal epithelial cells. Human iPS cells were differentiated into intestinal stem cells using activin A and fibroblast growth factor 2. Subsequently, the intestinal stem cells were maturated into intestinal epithelial cells by treatment with 8-bromo-cyclic adenosine monophosphate (8-Br-cAMP) and 3-isobutyl-1-methylxanthine (IBMX), which activate cAMP signaling. The expression levels of intestinal markers and pharmacokinetics-related genes in the differentiated cells were markedly increased by using 8-Br-cAMP and IBMX. In the cells differentiated with the compound, cytochrome P450 (CYP) 3A4 inducibility via pregnane X receptor and vitamin D receptor was observed. The metabolic activities of CYP2C9, CYP2C19, CYP2D6, CYP3A4/5, and UDP-glucuronosyltransferase, which are expressed in the human small intestine, were also markedly increased. Furthermore, uptake of glycylsarcosine via peptide transporter 1 was markedly increased. The cells differentiated with the compounds also had drug transporter activities via organic anion

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transporters and P-glycoprotein. This study is the first to report that the activation of cAMP signaling promotes differentiation of human iPS cell-derived intestinal epithelial cells.

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Introduction

In drug disposition, the small intestine is involved not only in the absorption of orally administered drugs but also in their metabolism by cytochrome P450 (CYP) 3A (Paine et al., 2006; Yoshida et al., 2013; Xie et al., 2016). Therefore, the small intestine as well as the liver is a key organ related to the first-pass effect of drugs. It is very important to accurately predict intestinal drug absorption and metabolism in nonclinical studies. Human colon carcinoma cell line Caco-2 is commonly used to predict drug membrane permeability in the small intestine (Hidalgo et al., 1989; Yee, 1997). However, Caco-2 cells differ from normal human small intestine cells in the expression patterns of drug-metabolizing enzymes and drug transporters (Lennernäs et al.,1996; Schmiedlin-Ren et al., 1997; Nakamura et al., 2002; Sun et al., 2002). Using primary human intestinal epithelial cells for the evaluation of drug membrane permeability would be desirable, but they have poor viability and other characteristics, such as short life span, that limit their application (Aldhous et al., 2001; Chougule et al., 2012; Takenaka et al., 2014). Therefore, currently, there is no pharmacokinetic system that can simultaneously predict the intestinal metabolism and drug absorption.

In order to accurately predict the pharmacokinetics of the human small intestine, development of human induced pluripotent stem (iPS) cell-derived intestinal epithelial cells has been expected with functions similar to normal human intestinal epithelial cells. However, there are very few reports of differentiation of intestinal cells from human iPS cells (Spence et al., 2011; Ogaki et al., 2013; Iwao

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et al., 2014; Iwao et al., 2015; Ogaki et al., 2015; Ozawa et al., 2015). In addition, the pharmacokinetic characteristics of human iPS-derived intestinal cells have been insufficiently investigated. Spence et al. have reported the production of intestinal organoids, with intestinal morphological features, from human pluripotent stem cells using the Matrigel embedding method (Spence et al., 2011). However, quantitative evaluation of the intestinal absorption process using three-dimensional intestinal organoids is difficult. Therefore, intestinal epithelial cells with monolayers are suitable for the quantitative evaluation of pharmacokinetics.

Generally, small molecular compounds are useful as inducers of the differentiation of human iPS cells so that they can be stably synthesized in large quantities and their effect are stable. However, to the best of our knowledge, there are few studies on small molecular compounds that can promote the intestinal differentiation of iPS cells. Therefore, the kind of effect useful for differentiation is not well known. We have previously reported that three different inhibitors, transforming growth factor-β (TGF-β), DNA methyltransferase (DNMT), and mitogen-activated protein kinase (MAPK) inhibitors, promote the differentiation of human iPS cell-derived intestinal epithelial cells (Iwao et al., 2015; Kodama et al., 2016a). The intestinal epithelial cells differentiated using these inhibitors exhibit activities of drug-metabolizing enzymes, such as CYPs, UDP-glucuronosyltransferase (UGT), sulfotransferase (SULT), and carboxylesterase (CES), and drug transporters, such as solute carrier family (SLC) 15 member 1/peptide transporter 1 (SLC15A1/PEPT1) and ATP-binding cassette (ABC)

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subfamily G member 2/breast cancer resistance protein (ABCG2/BCRP) (Iwao et al., 2015; Kodama et al., 2016b; Kabeya et al., 2017). In addition, we demonstrated that fraction absorbed of drugs could be predicted from the apparent permeability coefficient (P_{app}) in human iPS cell-derived intestinal epithelial cells (Kodama et al., 2016b). However, sufficient transporter activity of P-glycoprotein (P-gp) was not observed and that of CYP3A4, a major drug-metabolizing enzyme in the human small intestine, was also low in these cells. In order to use human iPS cell-derived intestinal epithelial cells for pharmacokinetic studies, it is necessary to improve some of the pharmacokinetic functions. Therefore, we have conducted screening of compounds effective for the intestinal differentiation of human iPS cells.

Recently, we found that 8-bromo-cyclic adenosine monophosphate (8-Br-cAMP) and 3-isobutyl-1-methylxanthine (IBMX), which are small molecular compounds that activate cyclic AMP (cAMP) signaling, promote the differentiation of human iPS cell-derived intestinal epithelial cells. In this study, we demonstrated that cAMP signaling plays an important role in the differentiation of human iPS cells into intestinal epithelial cells. Furthermore, we analyzed the pharmacokinetic functions of the differentiated intestinal epithelial cells.

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Materials and Methods

Materials. Human iPS cell line Windy was provided by Dr. A. Umezawa of the National Center for Child Health and Development (Tokyo, Japan), and another cell line, 610B1, was purchased from RIKEN BRC (Tsukuba, Japan). Fibroblast growth factor 2 (FGF2), activin A, and epidermal growth factor (EGF) were purchased from PeproTech (Rocky Hill, NJ). BD Matrigel Matrix Growth Factor Reduced was purchased from BD Biosciences (Bedford, MA). PD98059, 5-aza-2′-deoxycytidine, A-83-01, ibuprofen, and sulfobromophthalein were purchased from Wako Pure Chemical Industries (Osaka, Japan). IBMX and PSC833 were purchased from Sigma-Aldrich (St. Louis, MO). Rhodamine 123 was purchased from Dojindo Laboratories (Kumamoto, Japan). 8-Br-cAMP was purchased from Enzo Life Sciences (New York, NY). Anti-villin and anti-PEPT1 antibodies were purchased from Santa Cruz Biotechnology (Dallas, TX). Anti-occludin antibody was purchased from Invitrogen Life Technologies/Thermo Fisher Scientific (Carlsbad, CA). Anti-P-glycoprotein antibody was purchased from Abcam (Cambridge, UK). Human adult small intestine total RNA (5 donors) was purchased from BioChain Institute. (Newark, CA). All other reagents were of the highest quality available.

Human iPS Cell Culture. Human iPS cell lines were cultured on a feeder layer using a 1:1 mixture of Dulbecco's Modified Eagle's medium (DMEM) and Ham's nutrient mixture F-12 (DMEM/F-12) supplemented with 20% KnockOut Serum Replacement, 2 mM L-glutamine, 1% minimum essential

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medium nonessential amino acid solution (NEAA), 0.1 mM 2-mercaptoethanol, and 5 ng/mL FGF2. Mitomycin C-treated mouse embryonic fibroblasts were used as feeder cells.

Differentiation of Human iPS Cells into Intestinal Epithelial Cells. Differentiation of the intestinal epithelial cells was performed according to the protocol reported previously (Iwao et al., 2015). Briefly, for definitive endoderm differentiation, human iPS cells were incubated with activin A for 3 days. For the induction of intestinal stem cells, the cells were subsequently incubated with FGF2 for 4 days. Then, the intestinal stem cells were cultured in the intestinal differentiation medium DMEM/F12 containing 2% fetal bovine serum (FBS), 2% B-27 supplement, 1% N2 supplement, 1% NEAA, 2 mM L-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, and 20 ng/mL EGF for 7 days. Subsequently, these cells were cultured for 12 days in the intestinal differentiation medium supplemented with PD98059, 5-aza-2′-deoxycitidine, and A-83-01. In addition, 1 mM 8-Br-cAMP and 500 μM IBMX were also added to the intestinal differentiation medium.

Caco-2 Cell Culture. Caco-2 cells (RCB0988, RIKEN BRC) were maintained in DMEM supplemented with 10% FBS, 1% NEAA, 100 U/mL penicillin, and 100 μg/mL streptomycin for 14 days.

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RNA Isolation and Reverse Transcription-Polymerase Chain Reaction (RT-PCR). Total RNA isolation and cDNA synthesis of human iPS cell-derived intestinal epithelial cells and Caco-2 cells were performed according to the previously reported protocol (Kabeya et al., 2017). Relative mRNA expression levels were determined using a KAPA SYBR Fast qPCR Kit (Genetics, Tokyo, Japan) and the Applied Biosystems 7300 Real-time PCR System with 7300 System SDS software version 1.4 (Applied Biosystems, Foster City, CA). The primer pairs are listed in Table 1. Hypoxanthine phosphoribosyltransferase (HPRT) was used as a housekeeping gene for normalizing mRNA expression levels of target genes.

Determination of Drug-Metabolizing Enzyme Activity. Drug metabolism study was performed according to the previously reported protocol (Iwao et al., 2015). At terminal differentiation, the differentiated cells were incubated with intestinal differentiation medium containing substrate cocktail for 24 h at 37 °C. In the metabolic study of midazolam, the differentiated and Caco-2 cells were incubated in the presence or absence of 10 μM ketoconazole. To normalize drug-metabolizing enzyme activities, the total protein contents of the differentiated and Caco-2 cells were measured using a TaKaRa BCA Protein Assay Kit (Takara Bio, Shiga, Japan), according to the manufacturer's instructions.

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Immunofluorescence Staining. For staining of villin and occludin, the differentiated cells were fixed and permeabilized in methanol (−20°C) for 5 min at 4°C. For staining of PEPT1 and P-gp, the cells were fixed in a 4% (w/v) paraformaldehyde solution for 30 min and permeabilized in a 0.1% Triton X-100 solution for 5 min at room temperature. After washing thrice with phosphate-buffered saline (PBS) without calcium and magnesium, the cells were blocked in PBS containing 5% FBS for 20 min at room temperature. The cells were then incubated for 60 min at room temperature with the primary antibodies. The primary antibodies were diluted as follows: villin, 1:100; occludin, 1:100; PEPT1, 1:50; and P-gp, 1:20. The experiments were performed according to the previously reported method (Iwao et al., 2015).

Uptake Study. Glycylsarcosine was chosen as a substrate to measure the activity of PEPT1, and 3 mM ibuprofen was used as the specific inhibitor (Omkvist et al., 2010). Before the uptake study, the differentiated cells were preincubated for 20 min with a transport buffer [Hanks' balanced salt solution (HBSS) containing 10 mM 4-morpholineethanesulfonic acid, pH 6.0], with or without the inhibitor. For the uptake study of organic anion transporting polypeptide (OATP), 5 nM [^{3}H]-estrone-3-sulfate and 200 μM sulfobromophthalein were used as the substrate and specific inhibitor, respectively (Sai et al., 2006). HBSS containing 10 mM HEPES (pH 7.4) was used as a transport buffer. The uptake studies were performed according to the previously reported protocol (Iwao et al., 2015). To normalize

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transporter activities, the total protein contents of the differentiated cells were measured using a TaKaRa BCA Protein Assay Kit, according to the manufacturer's instructions.

Bidirectional Transport Assay. The differentiated cells were seeded on the cell culture inserts. The transepithelial electrical resistance (TEER) values were measured using Millicell ERS-2 (Millipore, Bedford, MA). Rhodamine 123 was chosen as a substrate to measure the activity of P-gp, and 2 μM PSC833 was used as the specific inhibitor (Lee et al., 1994; Jouan et al., 2016). The cells were preincubated for 20 min with a transport buffer (HBSS containing 10 mM HEPES, pH 7.4) in the presence or absence of the inhibitor. After preincubation, the transport buffer containing 100 μM rhodamine 123 was added to the apical or basal chambers, and the cells were incubated at 37°C for 120 min in the presence or absence of the inhibitor. The samples were collected from the receiver chambers. The concentration of rhodamine 123 was quantified using SYNERGY HTX (BioTek, Winooski, VT). The excitation and emission wavelengths of rhodamine 123 were 485 and 535 nm, respectively.

Analysis of Apparent Membrane Permeability. The P_{app} value and efflux ratio (ER) of rhodamine 123 in bidirectional transport assay were calculated according to the analytical previously reported method (Kodama et al., 2016).

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$$P_{\mathrm{app}} = \frac{\mathrm{d}Q}{\mathrm{d}t} \times \frac{1}{A \times C_0}$$

where $\mathrm{d}Q/\mathrm{d}t$ is the amount of the compound permeated per unit of time, A is the membrane surface area and C_0 is the initial donor concentration of the compound. The ER of rhodamine 123 was calculated according following equation:

$$\mathrm{ER} = \frac{P_{\mathrm{app,BtoA}}}{P_{\mathrm{app,AtoB}}}$$

Where $P_{\mathrm{app,AtoB}}$ and $P_{\mathrm{app,BtoA}}$ are P_{app} values in apical-to-basal and basal-to-apical transport.

Statistical Analysis. The level of statistical significance was assessed using Student's *t*-test for comparisons between two groups or analysis of variance followed by Dunnett's test for multiple comparisons.

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Results

Effect of cAMP Signaling on Differentiation from Human iPS Cells into Intestinal Epithelial Cells. Human iPS cell-derived intestinal stem cells were treated with 1 mM 8-Br-cAMP or 500 μM IBMX at day 8–13 or day 13–26 during the intestinal differentiation. After terminal differentiation, the expression levels of intestinal specific markers, such as villin 1 (Boller et al., 1988; Landry et al., 1994), fatty acid-binding protein 2 (FABP2) (Gajda et al., 2015), dipeptidyl peptidase-4 (DPP4) (Darmoul et al., 1994), and pharmacokinetics-related genes such as CYP3A4 (Paine et al., 2006; Xie et al., 2016), SLC15A1/PEPT1, and ABCB1/multidrug resistance 1 (MDR1) (Hilgendorf et al., 2007; Yoshida et al., 2013), were markedly increased in the cells treated with 8-Br-cAMP and IBMX compared with cells differentiated without these compounds (control group) (Fig. 1). Based on these results, 8-Br-cAMP and IBMX were added on days 8–14 and 14–26, respectively. In the cells differentiated with these two compounds, the expression levels of villin 1, FABP2, intestine-specific homeobox (ISX) (Choi et al., 2006), caudal type homeobox 2 (CDX2) (Suh et al., 1996; Coskun et al., 2011), DPP4, pregnane X receptor (PXR), CYP2C9, CYP2C19, CYP3A4, CES2A1, SLC15A1/PEPT1, SLC5A1/sodium-glucose cotransporter 1 (SGLT1) (Wright et al., 2004), ABCB1/MDR1, and ABCG2/BCRP were markedly increased compared with the cells differentiated without the compounds (Fig. 2). The expression of the proteins villin, PEPT1, and P-gp was also increased (Fig. 3).

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CYP3A4 Inducibility and Drug-metabolizing Enzyme Activities. Before differentiation, 1α,25-dihydroxyvitamin D_3 or rifampicin, inducers of CYP3A4, was administered for 48 h. In CYP3A4, the mRNA expression level and CYP3A4/5 metabolic activity were significantly induced by these ligands in cells differentiated with 8-Br-cAMP and IBMX. CYP3A4/5 activity was increased by 1.8- or 1.3-fold by treatment with 1α,25-dihydroxyvitamin D_3 or rifampicin, respectively, compared with the vehicle group. The activity was strongly inhibited by ketoconazole, a CYP3A4 specific inhibitor. On the other hand, the mRNA expression level and metabolic activity were significantly increased only by 1α,25-dihydroxyvitamin D_3 in Caco-2 cells. CYP3A4/5 activity in Caco-2 cells was increased by 1.8-fold by treatment with 1α,25-dihydroxyvitamin D_3 (Fig. 4). In cells differentiated with 8-Br-cAMP and IBMX, the metabolic activities of CYP2C9, CYP2C19, CYP2D6, CYP3A4/5, and UGT were markedly increased by 78-, 48-, 1.6-, 3.7-, and 1.9-fold, respectively, compared with cells differentiated without the compounds (Table 2).

Uptake Transport Activities via PEPT1 and OATP. The activity of PEPT1 was measured using glycylsarcosine, a PEPT1 substrate. The activity of PEPT1 in the cells differentiated with 8-Br-cAMP and IBMX was 8.1 pmol/h/mg protein, and it was markedly increased 2.3-fold compared with the nontreatment group. The activities were dramatically suppressed by treatment with ibuprofen, a PEPT1 inhibitor, and at 4°C (Fig. 5A). The rate of uptake of estrone-3-sulfate, an OATP substrate, was 0.28

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pmol/h/mg protein. The activity was markedly inhibited by sulfobromophthalein, an OATP inhibitor (Fig. 5B).

Efflux Transport Activity via P-gp. On day 7 after differentiation, the cells were seeded on cell culture inserts and treated with 8-Br-cAMP and IBMX. The TEER values of the cells were increased in a time-dependent manner and finally reached 200–250 $\Omega \cdot cm^2$ (Fig. 6A). Occludin, a tight junction marker (Groschwitz et al., 2009), was expressed in the cells and localized along the plasma membrane (Fig. 6B). In the efflux transport study via P-gp using rhodamine 123 as the substrate, the apical-to-basal P_{app} values were 0.54 ± 0.31 and 1.47 ± 0.22 ($\times 10^{-6}$ cm/s) in the absence and presence of PSC833, a specific P-gp inhibitor, respectively. On the other hand, the basal-to-apical P_{app} values were 1.87 ± 0.34 and 1.66 ± 0.14 ($\times 10^{-6}$ cm/s) in the absence and presence of PSC833, respectively. The ER value, calculated from the apical-to-basal and basal-to-apical P_{app} values, was decreased from 3.5 to 1.1 by PSC833 (Fig. 6C)..

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Discussion

It has been reported that cAMP signaling promotes the differentiation of pluripotent stem cells into hepatocytes, pancreatic β cells, and vascular endothelial cells (Ogawa et al., 2013; Sakano et al., 2014; Ikuno et al., 2017). However, there is no such report on the differentiation of intestinal epithelial cells. The present study initially revealed that the activation of cAMP signaling contributes to the differentiation and maturation of human iPS cell-derived intestinal epithelial cells. The activation of cAMP signaling in intestinal cell lines has been reported to increase the expression of CDX2, which is a transcription factor involved in the differentiation into the intestine (Suh et al., 1996; Chen et al., 2005; Coskun et al., 2011). Indeed, the expression levels of intestinal markers, including CDX2 and pharmacokinetics-related genes, in human iPS cell-derived intestinal epithelial cells were markedly increased by using 8-Br-cAMP and IBMX during differentiation in our study (Fig. 2). According to immunostaining, the expression of intestinal markers villin, PEPT1, and P-gp was also observed in cells differentiated by a combination of 8-Br-cAMP and IBMX (Fig. 3). Furthermore, when another human iPS cell line, 610B1, was differentiated into intestinal epithelial cells by using 8-Br-cAMP and IBMX, the expression levels of intestinal markers and pharmacokinetics-related genes as well as those of the human iPS cell line Windy were increased (Supplemental Figure 1). These results suggested that the activation of cAMP signaling is useful for the promotion of differentiation into intestinal epithelial cells against multiple human iPS cell lines.

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The induction of CYP3A4 via nuclear receptors, such as PXR, vitamin D receptor, and glucocorticoid receptor, in human iPS cell-derived intestinal epithelial cells has been reported (Negoro et al., 2016). In our study, the CYP3A4 mRNA expression level and CYP3A4/5 activity were increased by 1α,25-dihydroxyvitamin D_3 and rifampicin in cells differentiated by a combination of 8-Br-cAMP and IBMX (Fig. 4). On the other hand, in Caco-2 cells, a marked increase was observed with treatment with 1α,25-dihydroxyvitamin D_3, but not with rifampicin. CYP3A4/5 activity in human iPS cell-derived intestinal epithelial cells was much higher than that in Caco-2 cells (Fig. 4). These results indicate that human iPS cell-derived intestinal epithelial cells would be a more useful model than Caco-2 cells for studies of CYP3A4 activity and induction mediated by nuclear receptors, including PXR. The human small intestine expresses multiple CYPs and metabolizes various clinical drugs (Paine et al., 2006; Xie et al., 2016). The metabolic activities of CYP2C9, CYP2C19, CYP2D6, CYP3A4/5, and UGT, which are expressed in the human small intestine, were markedly increased by using 8-Br-cAMP and IBMX (Table 2). These results suggested that the activation of cAMP signaling contributes to the enhancement of metabolic functions of human iPS cell-derived intestinal epithelial cells. On the other hand, the drug metabolic activities of CYP1A and CYP2B6, which are expressed in nonintestinal tissues, were markedly decreased (Table 2). From these results, the activation of cAMP signaling in human iPS cell-derived intestinal cells was believed to more specifically promote differentiation into intestinal epithelial cells, but not other tissue cells. However, the drug-metabolizing enzyme activities

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in the differentiated cells are still not high enough. To use human iPS cell-derived intestinal epithelial cells for pharmacokinetic studies, it is necessary to use better cell lines suitable for intestinal differentiation and to improve the protocol.

The peptide transporter SLC15A1/PEPT1 and the organic anion transporter SLCO2B1/OATP2B1 are expressed in intestinal epithelial cells and are involved in uptake transport from the lumen (Hilgendorf et al., 2007; 2010; Yoshida et al., 2013). Cells differentiated by using 8-Br-cAMP and IBMX have uptake transporter activities via these transporters (Fig. 5). Furthermore, the activity of PEPT1 was markedly increased by the two compounds (Fig. 5A). We have previously reported the method of differentiation into human iPS cell-derived intestinal epithelial cells with TGF-β, DNMT, and MAPK inhibitors and the characteristics of intestinal transport (Iwao et al., 2015; Kodama et al., 2016a; Kodama et al., 2016b). However, the cells still did not have sufficient P-gp activity. In the present study, by using 8-Br-cAMP and IBMX in addition to the three inhibitors, TEER values were increased and the human iPS cell-derived intestinal epithelial cells formed more robust tight junctions (Fig. 6A and B). The cells also had P-gp activity (Fig. 6C). Moreover, the activities of PEPT1 and OATP in Caco-2 cells were 3.7 and 0.07 pmol/h/mg protein, respectively (data not shown). In the efflux transport study via P-gp, the apical-to-basal P_{app} values were 0.49 ± 0.05 and 0.56 ± 0.01 ($\times 10^{-6}$ cm/s) in the absence and presence of PSC833, respectively. On the other hand, the basal-to-apical P_{app} values were 2.73 ± 0.26 and 0.63 ± 0.07 ($\times 10^{-6}$ cm/s) in the absence and presence of PSC833,

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respectively. The ER value, calculated from the apical-to-basal and basal-to-apical P_{app} values, was decreased from 5.7 to 1.1 by PSC833 (data not shown). Therefore, human iPS cell-derived intestinal cells have almost equivalent functions of uptake and efflux transporters to those of Caco-2 cells. From these results, human iPS cell-derived intestinal epithelial cells can serve as a useful tool for the evaluation of PEPT1-, OATP-, P-gp-, and BCRP-mediated transport in the intestinal absorption process.

The activation of cAMP signaling during differentiation of human intestinal epithelial cells from human iPS cells increases the expression levels of intestinal markers and pharmacokinetics-related genes and improves the activities of drug-metabolizing enzymes and drug transporters. Therefore, human iPS cell-derived intestinal epithelial cells differentiated using this novel method can be more useful for predicting the pharmacokinetics in the human small intestine than those differentiated by previously reported methods. This study is the first to prove that activation of cAMP signaling is effective for differentiation into human iPS cell-derived intestinal epithelial cells.

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Author Contributions

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Conducted experiments: Kabeya, Qiu, Hibino, Nagasaki, and Kodama.

Performed data analysis: Kabeya, Qiu, Hibino, Nagasaki, and Kodama.

Wrote or contributed to the writing of the manuscript: Kabeya, Iwao, and Matsunaga.

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Footnotes

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Figure Legends

Fig. 1. Effect of 8-Br-cAMP and IBMX on differentiation of human iPS cells into intestinal epithelial cells.

Human iPS cells were differentiated into intestinal stem cells by treatment with activin A and FGF2. The intestinal stem cells were then cultured in the intestinal differentiation medium, as described in Materials and Methods section. In addition, 1 mM 8-Br-cAMP and 500 μM IBMX were added on days 8–13 or 13–26 during differentiation. At terminal differentiation, total RNA was extracted and the gene expression levels of intestinal markers and pharmacokinetics-related genes were measured by real-time RT-PCR. HPRT expression levels were used to normalize target gene expression levels. Gene expression levels in the control group were defined as 1. All data are presented as mean ± standard deviation ($n = 3$). Levels of statistical significance compared with the control group: $*P < 0.05$; $**P < 0.01$.

Fig. 2. Relative gene expression levels of intestinal markers and pharmacokinetics-related genes in differentiated intestinal epithelial cells.

During the differentiation, human iPS cell-derived intestinal stem cells were differentiated in the presence or absence of 8-Br-cAMP and IBMX in combination. 1 mM 8-Br-cAMP and 500 μM IBMX were added on days 8–14 and 14–26, respectively. At terminal differentiation, total RNA was extracted

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and the gene expression levels of intestinal markers and pharmacokinetics-related genes were measured by real-time RT-PCR. HPRT expression levels were used to normalize target gene expression levels. Gene expression levels in adult small intestine were defined as 100. All data are presented as means ± standard deviation (n = 3). Level of statistical significance compared with the group without compounds: $^{**}P < 0.01$.

Fig. 3. Immunofluorescence staining of villin (A), PEPT1 (B), and P-gp (C) in the differentiated intestinal epithelial cells by 8-Br-cAMP and IBMX in combination.

After differentiation, the differentiated cells were stained with villin (green), PEPT1 (green), P-gp (green), and DAPI (blue). Scale bar = 100 μm.

Fig. 4. Induction of CYP3A4 mRNA expression levels (A) and CYP3A4/5 activity (B) in differentiated intestinal epithelial cells by 8-Br-cAMP and IBMX in combination.

Differentiated cells and Caco-2 cells were treated with 10 nM 1α,25-dihydroxyvitamin D_3 (VD3) or 40 μM rifampicin for 48 h at terminal differentiation. For analysis of CYP3A4 mRNA expression, total RNA was extracted and the gene expression levels were measured by real-time RT-PCR after differentiation. HPRT expression levels were used to normalize target gene expression levels. For analysis of CYP3A4/5 activity at terminal differentiation, the differentiated cells were incubated with

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intestinal differentiation medium containing 5 μM midazolam for 24 h at 37 °C in the presence or absence of 10 μM ketoconazole. The supernatants were collected, and the metabolites were measured by UPLC–MS/MS. Gene expression levels in adult small intestine were defined as 100. All data are presented as mean ± standard deviation (n = 3–5). Levels of statistical significance: *P < 0.05; **P < 0.01 compared with the vehicle group in iPS cell-derived intestinal epithelial cells. ††P < 0.01 compared with the vehicle group in Caco-2 cells.

Fig. 5. Uptake transporter activities of PEPT1 (A) and OATP (B) in differentiated intestinal epithelial cells.

At terminal differentiation, the cells were incubated with the transport buffer containing glycylsarcosine or estrone-3-sulfate at 37°C in the presence or absence of the inhibitors, or at 4°C for 60 min. The uptake rates of these substrates in the differentiated cells with or without 8-Br-cAMP and IBMX in combination were measured. All data are presented as mean ± standard deviation (n = 3). Levels of statistical significance: **P < 0.01 compared with the vehicle group without compounds and ††P < 0.01 compared with the vehicle group with compounds.

Fig. 6. TEER values, protein expression, and bidirectional permeability of rhodamine 123 in differentiated intestinal epithelial cells by 8-Br-cAMP and IBMX in combination.

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(A) The TEER values of the cells seeded on culture inserts were measured during intestinal differentiation. (B) The differentiated cells were stained with villin (red), occludin (green), and DAPI (blue) at terminal differentiation. Scale bar = 50 μm. (C) The cells were incubated with the transport buffer containing rhodamine 123 at 37°C for 120 min in the presence or absence of the inhibitor.

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Table 1. Sequences of primers for real-time PCR analysis.

Gene name	Sence 5' → 3'	Antisence 5' → 3'
ABCB1/MDR1	CCCATCATTGCAATAGCAGG	TGTTCAAACTTCTGCTCCTGA
ABCG2/BCRP	AGATGGGTTTCCAAGCGTTCAT	CCAGTCCCAGTACGACTGTGACA
CDX2	ACCTGTGCGAGTGGATGC	TCCTTTGCTCTTGCGGTTCT
CES2A1	CTGGGGAGTCTTGTCCATGT	GGAAGGGAAGGTCATGTTGA
CYP2C9	GACATGAACAACCCTCAGGACTTT	TGCTTGTCGTCTCTGTCCCA
CYP2C19	GAACACCAAGAATCGATGGACA	TCAGCAGGAGAAGGAGAGCATA
CYP3A4	CTGTGTGTTTCCAAGAGAAGTTAC	TGCATCATCAATTTCCTCCTGCAG
DPP4	CAAATTGAAGCAGCCAGACA	GGAGTTGGGAGACCCATGTA
FABP2	TTGGAAGGTAGACCGGAGTG	AGGTCCCCCTGAGTTCAGTT
HPRT	CTTTGCTTTCCTTGGTCAGG	TCAAGGGCATATCCTACAACA
ISX	CAGGAAGGAAGGAAGAGCAA	TGGGTAGTGGGTAAAGTGGAA
PXR	AGGATGGCAGTGTCTGGAAC	AGGGAGATCTGGTCCTCGAT
SLC5A1/SGLT1	CAACATCGCCTATCCAACCT	TAAACAACCTTCCGGCAATC
SLC15A1/PEPT1	CACCTCCTTGAAGAAGATGGCA	GGGAAGACTGGAAGAGTTTTATCG
Villin 1	AGCCAGATCACTGCTGAGGT	TGGACAGGTGTTCCTCCTTC

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Table 2. Drug-metabolizing enzyme activities of CYP1A, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP3A4/5, UGT, and SULT in the differentiated intestinal epithelial cells.

Enzymes	Substrate	Metabolite	Metabolic activities (pmol/24h/mg protein)		Ratio (+/-)
			Compounds (-)	Compounds (+)	
CYP1A	Phenacetin	Acetaminophen	81.5 ± 8.4	45.8 ± 7.6**	0.56
CYP2B6	Bupropion	Hydroxybupropion	6.8 ± 0.9	4.0 ± 0.8**	0.59
CYP2C9	Diclofenac	4'-Hydroxydiclofenac	0.2 ± 0.2	15.5 ± 3.4**	77.5
CYP2C19	(s)-Mephenytoin	4'-Hydroxymephenytoin	0.6 ± 0.1	28.6 ± 4.4**	47.7
CYP2D6	Bufuralol	1'-Hydroxybufuralol	0.5 ± 0.1	0.8 ± 0.1**	1.60
CYP3A4/5	Midazolam	1'-Hydroxymidazolam	23.7 ± 2.6	88.2 ± 7.9**	3.72
UGT	7-hydroxycoumarin	7-Hydroxycoumarin glucuronide	2499 ± 220	4696 ± 539**	1.88
SULT		7-Hydroxycoumarin sulfate	9949 ± 3061	5744 ± 909*	0.58

At terminal differentiation, the differentiated cells were incubated with intestinal differentiation medium containing 40 μM phenacetin, 50 μM bupropion, 5 μM diclofenac, 47.5 μM (*S*)-mephenytoin, 5 μM bufuralol, 5 μM midazolam, and 10 μM 7-hydroxycoumarin for 24 h at 37°C. The supernatants were collected and the metabolites in the differentiated cells with or without 8-Br-cAMP and IBMX in combination were measured by UPLC–MS/MS. All data are presented as mean ± standard deviation (n = 4). Levels of statistical significance compared with the group without

compounds: *$P < 0.05$; **$P < 0.01$.

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Figure 1

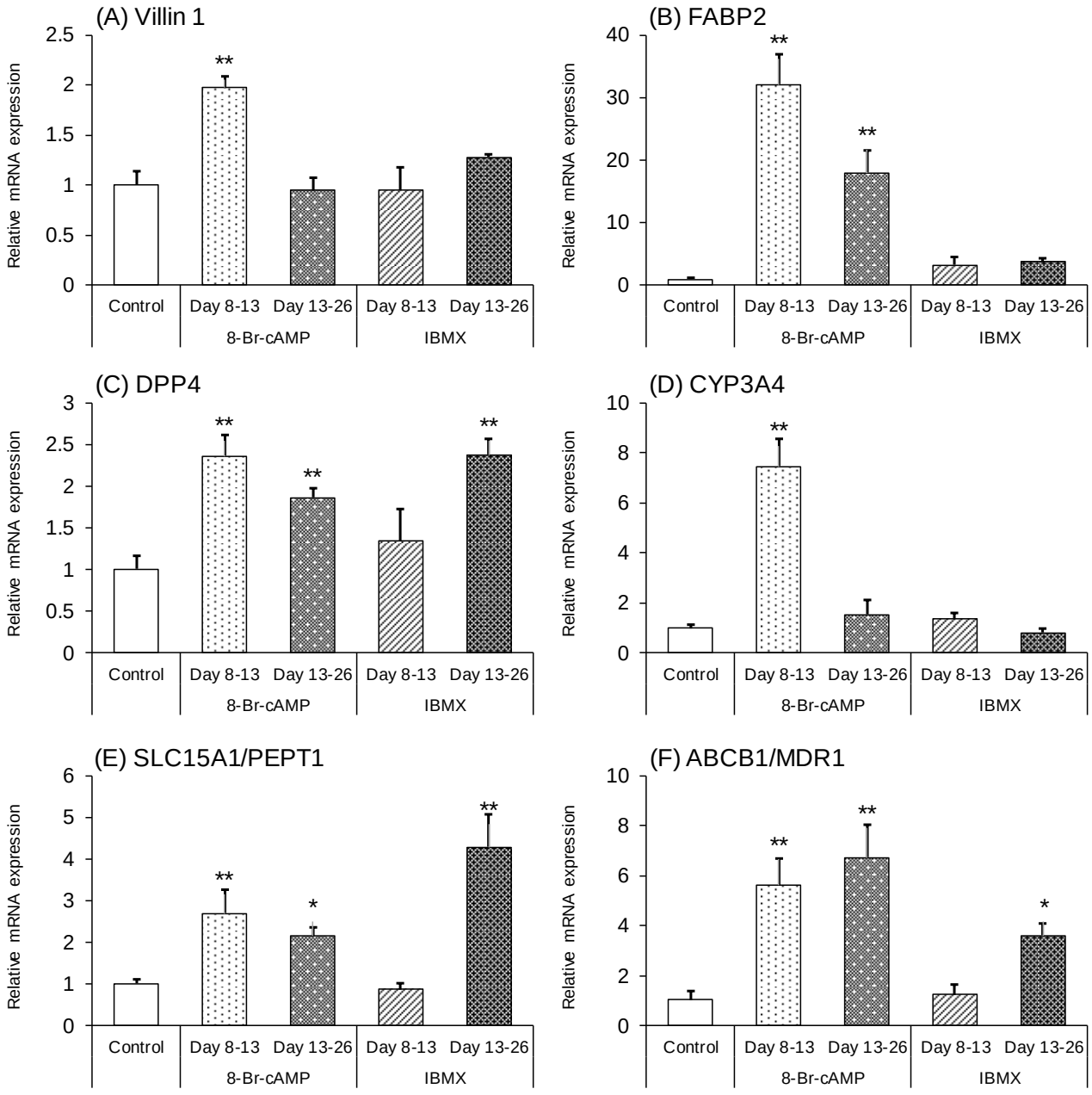

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Figure 2

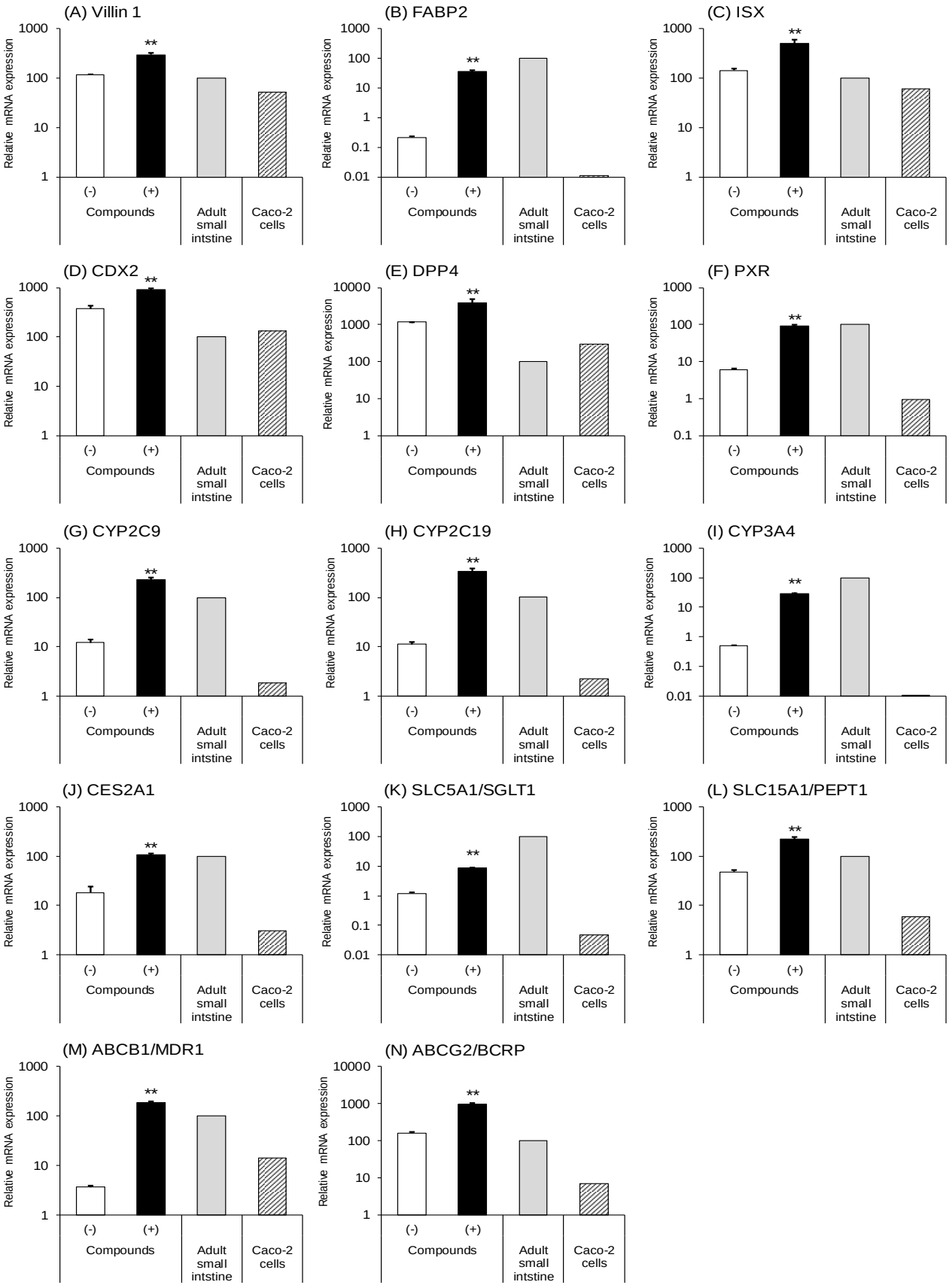

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Figure 3

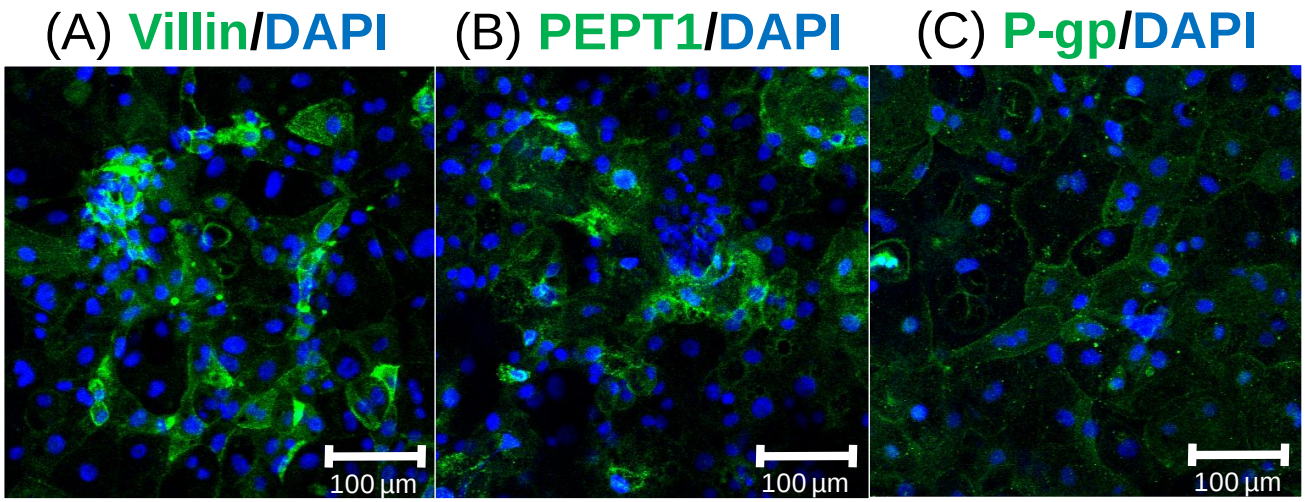

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Figure 4

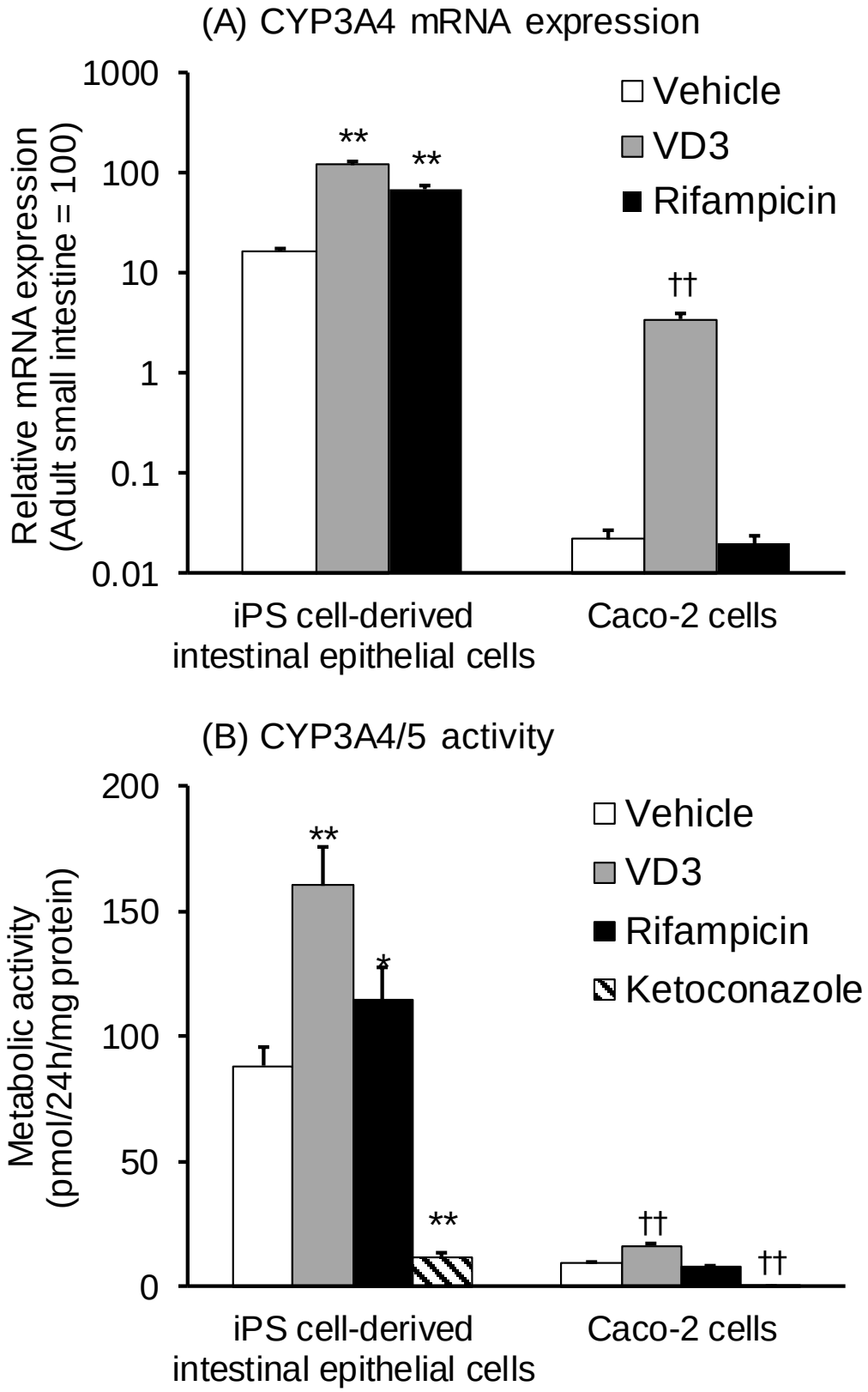

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Figure 5

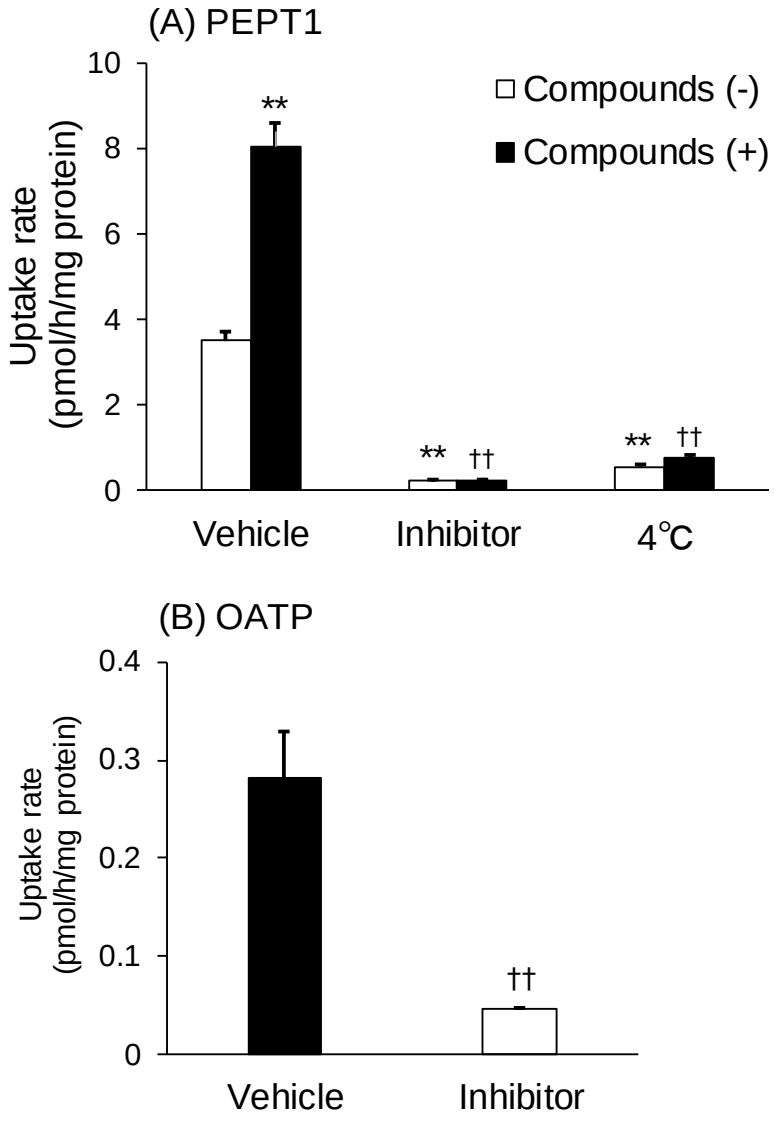

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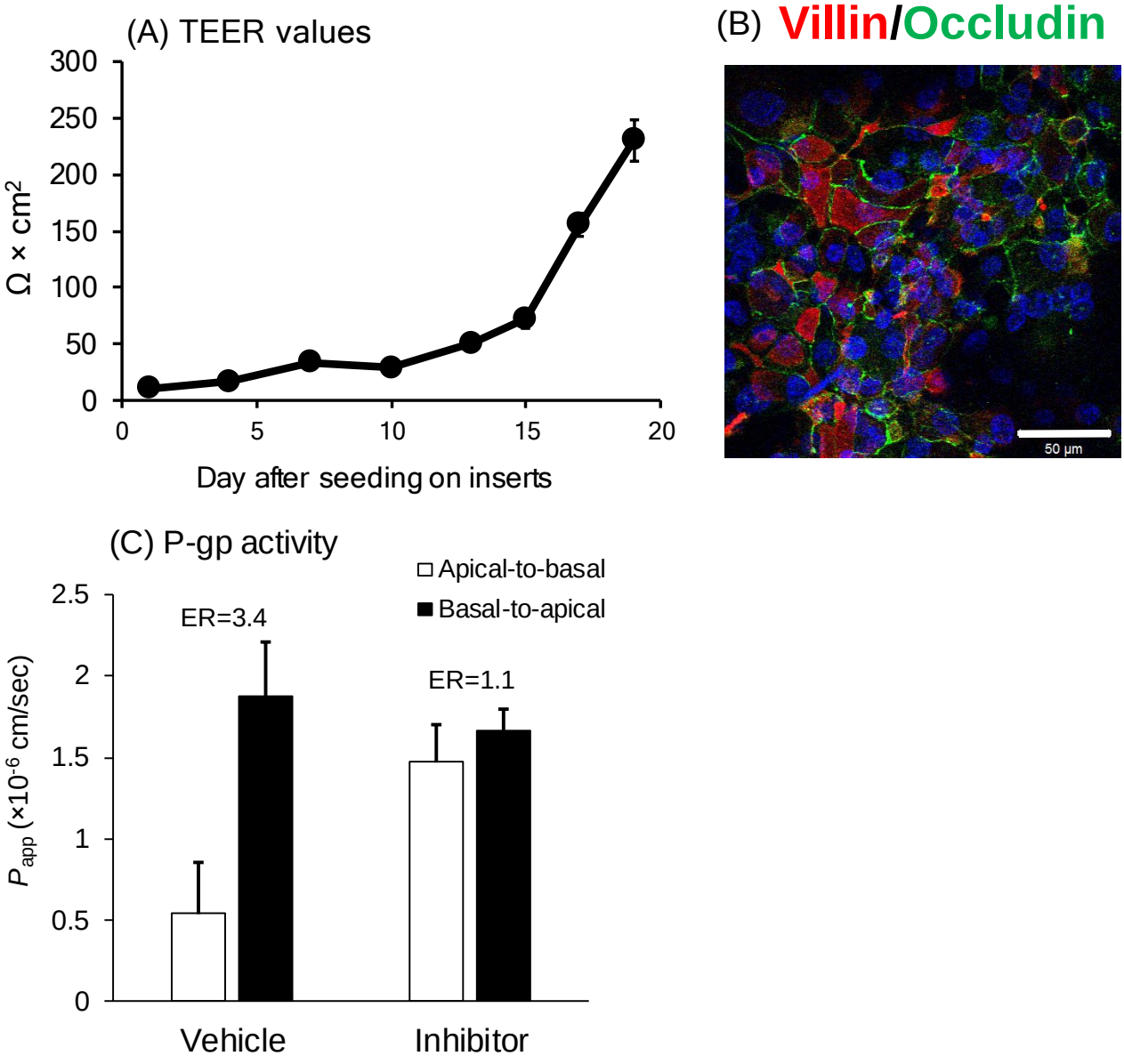
Figure 6



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