Title

Generation of Enterocyte-Like Cells with Pharmacokinetic Functions from Human Induced Pluripotent Stem Cells Using Small-Molecule Compounds

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Nonstandard abbreviations

ABC, ATP-binding cassette; ABCB1/MDR1, ATP-binding cassette, subfamily B, member 1/multi-drug resistance gene 1; ABCG2/BCRP, ATP-binding cassette, subfamily G, member 2/breast cancer resistance protein; BMP, bone morphogenetic protein; CDX2, caudal type homeobox 2; CYP, cytochrome P450; DAPI, 4′,6-diamidino-2-phenylindole; DMEM/F12, Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12; EGF, epidermal growth factor; EMT, epithelial-to-mesenchymal transition; EphB2, EHP receptor B2; FBS, fetal bovine serum; FGF, fibroblast growth factor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSK, glycogen synthase kinase; iPS cells, induced pluripotent stem cells; ISX, intestine specific homeobox; KSR, KnockOut Serum Replacement; LGR5, leucine-rich repeat-containing G-protein-coupled receptor 5; Matrigel, Matrigel matrix Growth Factor Reduced; MEFs, murine embryonic fibroblasts; MEK, mitogen-activated protein kinase kinase; NEAA, MEM nonessential amino acid solution; RPMI, Rosewell Park Memorial Institute; PBS, phosphate-buffered saline; PCR, polymerase chain reaction; PXR, pregnane X receptor; SLC: solute carrier; SLC15A1/PEPT1, solute carrier family 15 member 1/peptide transporter 1; SULT, sulfotransferase; TGF-β, transforming growth factor-β; UGT, UDP-glucuronosyltransferase; UPLC–MS/MS, ultra-performance liquid chromatography–tandem mass spectrometry; VDR, vitamin D receptor.

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Abstract

The small intestine plays an important role in all aspects of pharmacokinetics, but there is no system for the comprehensive evaluation of small-intestinal pharmacokinetics, including drug metabolism and absorption. In this study, we aimed to construct an intestinal pharmacokinetics evaluation system and to generate pharmacokinetically functional enterocytes from human induced pluripotent stem (iPS) cells. These cells were differentiated into intestinal stem cell-like cells using activin A and fibroblast growth factor 2. The resulting cells were differentiated into enterocytes in a medium containing epidermal growth factor and small-molecule compounds. The differentiated cells expressed intestinal marker genes and drug transporters. The expression of sucrase–isomaltase, an intestine-specific marker, was markedly increased by small-molecule compounds. The cells exhibited activities of drug-metabolizing enzymes expressed in enterocytes, including CYP1A1/2, CYP2C9, CYP2C19, CYP2D6, CYP3A4/5, UGT, and SULT. Fluorescence-labeled dipeptide uptake into the cells was observed and was inhibited by ibuprofen, an inhibitor of the intestinal oligopeptide transporter SLC15A1/PEPT1. CYP3A4 mRNA expression level was increased by these compounds and induced by the addition of 1α,25-dihydroxyvitamin D_3. CYP3A4/5 activity was also induced by 1α,25-dihydroxyvitamin D_3 in cells differentiated in the presence of the compounds. All these results show that we have generated enterocyte-like cells having pharmacokinetic functions and identified small-molecule compounds that are effective for promoting intestinal differentiation and the gain of pharmacokinetic functions. Our enterocyte-like cells would be useful material for developing a novel evaluation system to predict human intestinal pharmacokinetics.

Introduction

The small intestine is a tissue critically involved in the pharmacokinetics of orally administered drugs. One of the reasons is that various drug-metabolizing enzymes, such as cytochrome P450 (CYP) and UDP-glucuronosyltransferase (UGT), and drug transporters, such as ATP-binding cassette (ABC) and solute carrier (SLC) transporters, are expressed in the small intestine (Zhang et al., 1999; Paine et al., 2006; Hilgendorf et al., 2007; Yamanaka et al., 2007; Giacomini et al., 2010). Currently, human colon adenocarcinoma cell line Caco-2 cells are frequently used for the prediction of drug absorption in the small intestine, owing to the polarized morphological similarity of the monolayer to the small intestine (microvilli and tight junction formation) and the expression of many transporters. However, in Caco-2 cells, the drug transporter expression levels and carrier mediated drug permeabilities are different from those of the human duodenum, and the expression level of CYP3A4, a major drug-metabolizing enzyme in the small intestine, is very low (Nakamura et al., 2002; Sun et al., 2002; Borlak and Zwadlo, 2003). CYP3A4 mRNA expression was induced by 1α,25-dihydroxyvitamin D_3, but was not induced by rifampicin (Schmiedlin-Ren et al., 2001; Martin et al., 2008) because the pregnane X receptor (PXR) was not expressed in Caco-2 cells (Thummel et al., 2001). Recently, a human small intestinal epithelial cell (HIEC) monolayer has been reported to be useful as a novel *in vitro* system to predict oral absorption in humans (Takenaka et al., 2014). The HIEC monolayer expressed various drug transporters and could be used to evaluate the permeability of paracellularly absorbed compounds. However, further exploration of its pharmacokinetic functional characteristics is desirable, considering that accurate prediction of intestinal absorption in this cell line is difficult. There are some published

reports on the isolation and cultivation of human primary enterocytes (Perreault and Beaulieu, 1996; Grossmann et al., 2003; Chougule et al., 2012). However, these applications remain limited because there are some difficulties such as poor viability, short life span, limitation of passage number, and difficulty of obtaining human tissue samples. Thus, although the use of primary human enterocytes would be ideal, it is realistically impossible to obtain primary cells for an intestinal pharmacokinetics assay. Thus, there is no system suitable for the comprehensive evaluation of small intestinal pharmacokinetics including drug metabolism and absorption.

Human induced pluripotent stem (iPS) cells, which were generated by Takahashi et al. (Takahashi et al., 2007), are expected to be applicable not only in regenerative medicine but also in drug development such as pharmacokinetic and toxicokinetic studies. To date, many authors have reported the differentiation of human iPS cells into hepatocytes, which, like enterocytes, are important in drug pharmacokinetics (Si-Tayeb et al., 2010; Takayama et al., 2012; Ma et al., 2013). We have also established a method for the differentiation of human iPS cells into functional hepatocytes showing drug-metabolizing enzyme activities and inducibility (Kondo et al., 2014a). We found that histone deacetylase inhibitors promoted the hepatic differentiation of human iPS cells (Kondo et al., 2014b).

In contrast, there are few reports of differentiation from human iPS cells into enterocytes. To our knowledge, the only such reports are those of Spence et al. and Ogaki et al. (Spence et al., 2011; Ogaki et al., 2013). However, pharmacokinetically functional features, such as drug-metabolizing and -transporting activities, were not investigated in these studies. We have succeeded in generating enterocyte-like cells from human iPS cells (Iwao et al., 2014). The enterocyte-like cells expressed several

drug transporters and CYP3A4 and had peptide uptake functions. In the present study, we aimed to establish a prediction system for intestinal pharmacokinetics, and succeeded in the differentiation of human iPS cells into enterocytes with pharmacokinetic functions. For more effective differentiation, we modified our previously reported intestinal differentiation method using small-molecule compounds. We demonstrated that differentiated enterocyte-like cells had functions of drug-metabolizing enzyme activities, inducibility, and active peptide transport. We also identified small-molecule compounds that were effective for intestinal differentiation. Our results indicate that the human iPS cell-derived enterocytes would be useful as a novel evaluation system of intestinal metabolism and drug absorption.

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Materials and Methods

Materials. Fibroblast growth factor (FGF) 2, activin A, and epidermal growth factor (EGF) were purchased from PeproTech Inc. (Rocky Hill, NJ). BD Matrigel matrix Growth Factor Reduced (Matrigel) was purchased from BD Biosciences (Bedford, MA). KnockOut Serum Replacement (KSR) was purchased from Invitrogen Life Technologies Co. (Carlsbad, CA). Y-27632, dorsomorphin, PD98059, 5-aza-2′-deoxycitidine, A-83-01, and 1α,25-dihydroxyvitamin D_3 were purchased from Wako Pure Chemical Industries (Osaka, Japan). Glycogen synthase kinase (GSK)-3 Inhibitor XV was purchased from Merck Millipore (Billerica, MA). Human adult small intestine total RNA from a 66-year-old male donor was purchased from BioChain Institute Inc. (Newark, CA). Murine embryonic fibroblasts (MEFs) were obtained from Oriental Yeast Co. (Tokyo, Japan). All other reagents were of the highest quality available.

Human iPS Cell Culture. A human iPS cell line (Windy) was provided by Dr. Akihiro Umezawa of the National Center for Child Health and Development. Human iPS cells were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium and Ham's nutrient mixture F-12 (DMEM/F12) containing 20% KSR, 2 mM L-glutamine, 1% MEM nonessential amino acid solution (NEAA), 0.1 mM 2-mercaptoethanol, and 5 ng/ml FGF2 at 37°C in humidified air with 5% CO_2. The human iPS cells were cultured on a feeder layer of mitomycin C-treated MEFs, and the medium was changed every day.

Differentiation into Enterocyte-like Cells. The human iPS cells were used for

differentiation studies between passages 40 and 50. When the cells reached approximately 70% confluence, differentiation was initiated by replacing the medium with Roswell Park Memorial Institute (RPMI) 1640 medium containing 2 mM GlutaMAX, 0.5% fetal bovine serum (FBS), 100 ng/ml activin A, 100 units/ml penicillin, and 100 μg/ml streptomycin. After 48 h, the medium was replaced with RPMI 1640 medium containing 2 mM GlutaMAX, 2% FBS, 100 ng/ml activin A, 100 units/ml penicillin, and 100 μg/ml streptomycin, and the cells were cultured for 24 h. The culture medium was then replaced with DMEM/F12 containing 2% FBS, 2 mM GlutaMAX, and 250 ng/ml FGF2 and incubated for 96 h. The cells were then treated for 1 h with 10 μM Y-27632. The cells were then passaged on Matrigel-coated 24- or 96-well plates and cultured in basal medium (DMEM/F12 containing 2% FBS, 2% B-27 supplement, 1% N2 supplement, 1% NEAA, 2 mM L-glutamine, 100 units/ml penicillin, 100 μg/ml streptomycin, and 20 ng/ml EGF) for 19 days. Y-27632 was added at 10 μM during the initial 24 h of culture after passage. Small-molecule compounds were added in basal medium as follows: 125 nM GSK-3 Inhibitor XV and 1 μM dorsomorphin on days 8–14 and then 20 μM PD98059, 5 μM 5-aza-2′-deoxycytidine, and 0.5 μM A-83-01 on days 14–26, or 20 μM PD98059, 5 μM 5-aza-2′-deoxycytidine, and 0.5 μM A-83-01 on days 8–26 or days 14–26. The medium was changed every 3 days. In the induction study, the medium was replaced with 10 nM 1α,25-dihydroxyvitamin D_3 for the final 48 h.

RNA Extraction and Reverse Transcription Reaction. Total RNA was isolated from differentiated iPS cells using the RNeasy Mini Kit (QIAGEN, Valencia, CA). First-strand cDNA was prepared from 500 ng of total RNA. The reverse transcription

reaction was performed using the PrimeScript RT Reagent Kit (Takara Bio Inc., Otsu, Japan) according to the manufacturer's instructions.

Real-Time Polymerase Chain Reaction (PCR) Analysis. Relative mRNA expression levels were determined using SYBR Green real-time quantitative reverse transcription-PCR. Real-time PCR analysis was performed on the Applied Biosystems 7300 Real Time PCR System using 7300 System SDS software version 1.4 (Applied Biosystems, Carlsbad, CA). PCR was performed with the primer pairs listed in Table 1 using SYBR Premix EX Taq II (Takara Bio Inc.). mRNA expression levels were normalized relative to that of the housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

Determination of Drug-Metabolizing Enzyme Activity. A drug metabolism study was performed by a method similar to that described in our previous report (Kondo et al., 2014b). After 26 days of differentiation, the differentiated cells were incubated with basal medium containing 40 μM phenacetin, 50 μM bupropion, 5 μM diclofenac, 100 μM (*S*)-mephenytoin, 5 μM bufuralol, 5 μM midazolam, and 10 μM 7-hydroxycoumarin for 24 h at 37°C. After incubation, 36-μL aliquots of reaction medium were collected and the reactions were stopped by the addition of 24 μL ice-cold acetonitrile containing stable isotope-labeled internal standards for quantification. Metabolites were measured using ultra performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS). CYP1A1/2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP3A4/5, UGT, and sulfotransferase (SULT) activities were determined by the measurement of O-de-ethylation of phenacetin, hydroxylation of bupropion, 4′-hydroxylation of

diclofenac, 4′-hydroxylation of mephenytoin, 1′-hydroxylation of bufuralol, 1′-hydroxylation of midazolam, glucuronidation of 7-hydroxycoumarin, and sulfation of 7-hydroxycoumarin, respectively.

To correct for drug-metabolizing enzyme activities, the differentiated cells were lysed, and the total protein content was measured using a Pierce BCA protein assay kit (Thermo Fisher Scientific Inc., Waltham, MA) according to the manufacturer's instructions.

Immunofluorescence Staining. Differentiated cells were washed thrice with phosphate-buffered saline (PBS) without calcium and magnesium, fixed for 30 min at room temperature in 4% paraformaldehyde, and permeabilized in PBS containing 0.1% Triton X-100 for 5 min at room temperature. After washing thrice with PBS, the cells were blocked in PBS with 2% skim milk for 20 min at room temperature and incubated with anti-sucrase–isomaltase antibody (Sigma-Aldrich Co., St. Louis, MO) diluted at 1:200 for 60 min at room temperature. Rabbit serum was used as a negative control. The cells were washed thrice with PBS and incubated with a 1:500 dilution of Alexa Fluor 568-labeled secondary antibody for 60 min at room temperature. After washing thrice with PBS, the cells were incubated with 1 μg/ml 4′,6-diamidino-2-phenylindole (DAPI) for 5 min at room temperature and washed with PBS. The cells were mounted on a glass slide using a 9:1 mixture of glycerol and PBS and viewed using an LSM 510Meta confocal microscope (Carl Zeiss Inc., Oberkochen, Germany).

Uptake Study of β-Ala-Lys-AMCA. The differentiated cells were rinsed several times with PBS and incubated with DMEM/F12 containing 25 μM β-Ala-Lys-AMCA

for 4 h at 37°C with or without 10 mM ibuprofen, or 4°C. After incubation, the uptake of β-Ala-Lys-AMCA was stopped by washing with ice-cold PBS. The cells were fixed for 30 min at room temperature in 4% paraformaldehyde. The cells were mounted using a 9:1 mixture of glycerol and PBS and viewed using an LSM 510Meta confocal microscope.

Statistical analysis. Levels of statistical significance were assessed using the Student's *t*-test, and multiple comparisons were performed using analysis of variance (ANOVA) followed by Dunnett's test.

Results

Differentiation into Enterocyte-like Cells. To investigate the effects of GSK-3 inhibitor XV, dorsomorphin, PD98059, 5-aza-2′-deoxycytidine, and A-83-01 on the intestinal differentiation of human iPS cells, the expression levels of various genes were measured in the differentiated cells. mRNA expression levels of leucine-rich repeat-containing G-protein-coupled receptor 5 (LGR5) and EPH receptor B2 (EphB2), which are intestinal stem cell markers (Barker et al., 2007; Jung et al., 2011), in the groups treated with small-molecule compounds were comparable with those in the non-treatment group (Fig. 1). In contrast, mRNA expression level of sucrase–isomaltase, which is an intestine-specific marker (Boudreau et al., 2002; Gu et al., 2007), was markedly increased by 39–57-fold in the small-molecule compound treated groups (Fig. 2A). In the group treated with PD98059, 5-aza-2′-deoxycytidine, and A-83-01 from day 14 to 26, the mRNA expression level of sucrase–isomaltase was significantly increased. The expression levels of villin 1 and intestine specific homeobox (ISX), which are intestinal marker genes (Robine et al., 1985; Boller et al., 1988; Seino et al., 2008), were insignificantly changed (Fig. 2B, C). mRNA expression levels of solute carrier family 15 member 1/peptide transporter 1 (SLC15A1/PEPT1), ATP-binding cassette, subfamily G, member 2/breast cancer resistance protein (ABCG2/BCRP) and ATP-binding cassette, subfamily B, member 1/multi-drug resistance gene 1 (ABCB1/MDR1), which are drug transporters expressed in the small intestine (Hilgendorf et al., 2007; Giacomini et al., 2010), were increased by 1.3–2.3-, 2.4–3.2-, and 2.8–3.1-fold, respectively (Fig. 2D–F). In immunofluorescence staining, the protein expression of sucrase–isomaltase was also increased in comparison with the non-treatment group (Fig. 3). The morphology of differentiated enterocyte-like cells is

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shown in Figure 4. Undifferentiated human iPS cells had little cytoplasm and were small. However, differentiated enterocyte-like cells exhibited a cobbled shape similar to human intestinal epithelial cells (Chougule et al., 2012; Takenaka et al., 2014).

Drug-Metabolizing Enzyme Activities. Drug metabolism is one of the pharmacokinetically important functions of the small intestine. We performed a drug metabolism study to investigate whether the differentiated enterocyte-like cells had this function. Therefore, CYP1A1/2, 2C9, 2C19, 2D6, UGT, and SULT activities were observed in the differentiated enterocyte-like cells on treatment with small-molecule compounds (Fig. 5). In particular, CYP2C9 and SULT activities were increased by 1.1–3.2- and 4.9–7.8-fold, respectively. The differentiated cells showed no CYP2C19 activity in the non-treatment group, but this activity was detected on treatment with five small-molecule compounds or PD98059, 5-aza-2′-deoxycytidine, and A-83-01. In contrast, CYP2D6 activity was decreased by 0.3–0.5-fold. CYP1A and UGT activities were changed by 0.5–1.8- and 0.7–1.1-fold. CYP2B6 activity was undetected in all groups. The effects of these small-molecule compounds were different in other drug-metabolizing enzyme activities. We did not detect CYP2C19 activity in the groups that were treated with PD98059, 5-aza-2′-deoxycytidine, and A-83-01 for 18 days. Long-term treatment of these compounds may potentially affect the expression and transcription of CYPs, although the mechanism for this remains unclear.

Induction of CYP3A4 mRNA Expression and Activity. In the small intestine, CYP3A4 is a major drug-metabolizing enzyme, and its expression was induced by 1α,25-dihydroxyvitamin D_3 through the vitamin D receptor (VDR) (Thummel et al.,

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2001; Pavek et al., 2010). We evaluated CYP3A4 expression, activity, and inducibility. CYP3A4 mRNA was detected in the differentiated enterocyte-like cells. The expression level of CYP3A4 mRNA was increased 12–31-fold by treatment with small-molecule compounds, compared with that of the non-treatment group (Fig. 6A). In the group treated with PD98059, 5-aza-2′-deoxycytidine, and A-83-01 from day 14 to 26, the expression level of CYP3A4 mRNA was significantly increased. The addition of 1α,25-dihydroxyvitamin D_3 led to a 6.7–16-fold increase in expression in the presence and a 1.8-fold increase in the absence of the compounds. In the group treated with PD98059, 5-aza-2′-deoxycytidine, and A-83-01 from day 8 to 26, the induction of expression level was significant. Not only CYP3A4 mRNA expression but also CYP3A4/5 activity was significantly increased by 2.3–3.3-fold on treatment with the small-molecule compounds as compared with that of the non-treatment group (Fig. 6B). Moreover, after the addition of 1α,25-dihydroxyvitamin D_3, the activity was also significantly increased 1.6–2.6-fold in the presence but not absence of the compounds.

Uptake of β-Ala-Lys-AMCA. The oligopeptide transporter SLC15A1/PEPT1 is expressed in intestinal enterocytes and involved in peptide transport from the lumen (Giacomini et al., 2010). To investigate SLC15A1/PEPT1-mediated peptide transport, we performed an uptake study using β-Ala-Lys-AMCA, a fluorescence-labeled dipeptide that is a substrate of SLC15A1/PEPT1 (Groneberg et al., 2001). In the differentiated enterocyte-like cells treated with PD98059, 5-aza-2′-deoxycytidine, and A-83-01, the uptake of β-Ala-Lys-AMCA was observed and suppressed by ibuprofen, a noncompetitive inhibitor (Omkvist et al., 2010) (Fig. 7A and B, respectively). Uptake was also suppressed when the temperature was lowered from 37°C to 4°C (Fig. 7C).

Discussion

Many studies involving the differentiation of human iPS cells into various cells and tissues are performed worldwide, because the cells are expected to be applicable to regenerative medicine and drug development. However, there are few reports concerning the intestinal differentiation of human iPS cells. In the first report, Spence et al. reported the generation of intestinal tissues, which were called organoid, from human iPS cells by a three-dimensional culture method (Spence et al., 2011). Ogaki et al. then reported that human iPS and embryonic stem cells differentiated to various types of intestinal cells such as absorptive enterocytes, goblet cells, enteroendocrine cells and Paneth cells, on feeder cells in the presence of Wnt and Notch inhibitors (Ogaki et al., 2013). These reports had a great impact and attracted high interest. However, pharmacokinetics-related gene expression and pharmacokinetic functions were almost unexplored in these reports. Moreover, it is desirable to cultivate cells two-dimensionally, without mixture with other cells, for a precise evaluation of intestinal pharmacokinetics. We have also reported differentiation of human iPS cells into enterocyte-like cells with peptide uptake function, using a simple two-dimensional culture method without feeder cells (Iwao et al., 2014). In the present study, we performed the intestinal differentiation of human iPS cells by the addition of small-molecule compounds during the differentiation stage, and evaluated drug metabolism and inducibility. Differentiated enterocyte-like cells were morphologically similar to the intestinal epithelial cells previously reported by Chougule et al. (Chougule et al., 2012; Takenaka et al., 2014). Intestinal marker genes were expressed and expression levels of sucrase–isomaltase and CYP3A4 were markedly increased (Figs. 2 and 6A). The expression levels of several drug transporters were slightly increased.

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Sucrase–isomaltase is an index of intestinal differentiation that is expressed specifically in the brush border membrane in the small intestine. In immunofluorescence staining, sucrase–isomaltase positive cells were increased by the addition of small-molecule compounds (Fig. 3). The increase in sucrase–isomaltase-positive cells on the addition of small-molecule compounds suggested that these compounds promoted differentiation from intestinal stem cells into enterocytes. In contrast, intestinal stem cell marker gene expression was unchanged by treatment with small-molecule compounds (Fig. 1). We infer that these compounds had little influence on the differentiation of intestinal stem cells at early stages of intestinal differentiation.

Hepatic CYP3A4 is induced by rifampicin and regulated mainly by PXR (Drocourt et al., 2002). Intestinal CYP3A4 is induced by 1α,25-dihydroxyvitamin D_3 through VDR and is also induced by rifampicin (Kolars et al., 1992; Glaeser et al., 2005; van de Kerkhof et al., 2008). In our differentiated enterocytes, not only CYP3A4 mRNA expression but also CYP3A4/5 activity were induced by 1α,25-dihydroxyvitamin D_3 (Fig. 6). Not only CYP3A4 but also CYP1A, CYP2C, CYP2D, UGT, and SULT are expressed in the small intestine (Zhang et al., 1999; Paine et al., 2006; Teubner et al., 2007; Yamanaka et al., 2007; Riches et al., 2009). SLC15A1/PEPT1 as a typical uptake transporter is also expressed. It is also important that activities of these drug-metabolizing enzymes and peptide uptake through peptide transporter were detected (Figs. 5 and 7). We infer that the differentiated cells have intestinal-characteristic features, and the cells would be very useful for application in drug development studies.

Various drug transporters are expressed in Caco-2 cells, but expression levels of drug-metabolizing enzymes are very low (Bourgine et al., 2012). Thus, using Caco-2

cells we cannot predict drug–drug interactions, such as the induction or inhibition of drug-metabolizing enzymes and/or transporters by either parent drugs or metabolites, in a condition close to those *in vivo*. Moreover, the interaction between CYP3A4 and P-glycoprotein has also been reported (Siissalo and T. Heikkinen, 2013). Our enterocyte-like cells would thus be useful for evaluating such complex intestinal pharmacokinetics if the transporter functions of these cells were also elucidated in detail.

In this study, we identified small-molecule compounds effective in the intestinal differentiation of human iPS cells. We considered that these compounds promoted intestinal differentiation, considering mRNA expression of sucrase–isomaltase and CYP3A4 was markedly increased, whereas those of intestinal stem cell markers were decreased after the treatment with the compounds. CYP3A4 induction by 1α,25-dihydroxyvitamin D_3 was also observed. In particular, PD98059, 5-aza-2′-deoxycytidine, and A-83-01 were useful in enhancing the differentiation and gain of pharmacokinetic function. The constitutive activation of mitogen-activated protein/extracellular signal-regulated kinase kinase (MEK) led to decreased caudal type homeobox 2 (CDX2), a major transcription factor associated with intestinal development and differentiation, and resulted in the inhibition of intestinal differentiation (Lemieux et al., 2011). We accordingly speculated that PD98059, which is a MEK inhibitor, promoted intestinal differentiation through this pathway. DNA methylation and histone deacetylation epigenetically regulate gene expression. PXR promoter methylation was involved in the regulation of CYP3A4 and PXR expression in colon cancer cell lines (Habano et al., 2011). In that study, the expression of CYP3A4 and PXR were increased on treatment with 5-aza-2′-deoxycytidine, an inhibitor of DNA

methylation, in several cell lines. In another study, the expression of CYP3A genes was also increased in HepG2 cells, a human hepatoblastoma cell line (Dannenberg and Edenberg, 2006). We accordingly hypothesized that pharmacokinetics-associated genes would be upregulated by a DNA methyltransferase inhibitor in enterocyte-like cells. It has become apparent that transforming growth factor (TGF)-β production increases during the epithelial-to-mesenchymal transition (EMT). TGF-β was recently found to be one of the most important EMT inducers (Zavadil and Böttinger, 2005). In this intestinal differentiation study, A-83-01, a potent and selective TGF-β pathway inhibitor, inhibited EMT and might contribute to induce the differentiation of epithelial cells. GSK-3 inhibitor XV and dorsomorphin, a selective bone morphogenetic protein (BMP) inhibitor, is expected to be effective for inducing the proliferation of intestinal stem cells, considering Wnt signaling is activated and BMP signaling is suppressed in intestinal crypts (Scoville et al., 2008). Taking these results together, we suggest that these small-molecule compounds were effective at promoting the intestinal differentiation and gain of function. However, the detailed mechanism awaits further investigation.

In conclusion, we generated enterocyte-like cells from human iPS cells. The differentiated cells expressed intestinal marker genes and displayed pharmacokinetic functions such as drug-metabolizing activities, inducibility of CYP3A4, and active peptide transport. We also found that several small-molecule compounds were effective in inducing the differentiation of human iPS cells into enterocytes and gain of pharmacokinetic function. Enterocyte-like cells may be useful as an *in vitro* evaluation system for predicting intestinal pharmacokinetics.

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Footnotes

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Figure legends

Fig. 1. Relative mRNA expression levels of intestinal stem cell markers LGR5 (A) and EphB2 (B) in differentiated enterocyte-like cells

Human iPS cells were cultured in the presence of activin A for 3 days. The cells were further cultured in medium containing FGF2 for 4 days and then in the presence of EGF for 7 days. GSK-3 Inhibitor XV/dorsomorphin (GSKi/BMPi) or PD98059/5-aza-2′-deoxycytidine/A-83-01 (PD/5-aza-dC/A) was added on days 8–14. After 14 days of differentiation, total RNA was extracted and mRNAs were analyzed by SYBR Green real-time PCR. mRNA expression levels were normalized relative to that of GAPDH. The gene expression levels are represented relative to the level in the adult small intestine, which is set to 100. The adult small intestine (shown as a light gray column) was used as a positive control. Data are expressed as mean ± S.D. (n = 4), except for the adult small intestine. Levels of statistical significance compared with the non-treatment group; *$P < 0.05$, **$P < 0.01$.

Fig. 2. Relative mRNA expression levels of intestinal markers sucrase–isomaltase (A) villin 1 (B), ISX (C), SLC15A1/PEPT1 (D), ABCG2/BCRP (E), and ABCB1/MDR1 (F) in differentiated enterocyte-like cells

Human iPS cells were cultured in the presence of activin A for 3 days. The cells were further cultured in a medium containing FGF2 for 4 days and then in the presence of EGF for 19 days. GSK-3 Inhibitor XV/dorsomorphin (GSKi/BMPi) was added on days 8–14 and PD98059/5-aza-2′-deoxycytidine/A-83-01 (PD/5-aza-dC/A) was then added on days 14–26, or PD98059/5-aza-2′-deoxycytidine/A-83-01 (PD/5-aza-dC/A) were added on days 8–26 or 14–26. After 26 days of differentiation, total RNA was extracted

and mRNAs were analyzed by SYBR Green real-time PCR. mRNA expression levels were normalized relative to that of GAPDH. Gene expression levels are represented relative to the level in the adult small intestine, which was set to 100. The adult small intestine (shown as a light gray column) was used as a positive control. Data are expressed as mean ± S.D. (n = 3), except for the adult small intestine. Levels of statistical significance compared with the non-treatment group; *P < 0.05, **P < 0.01.

Fig. 3. Immunofluorescence analysis of sucrase–isomaltase in differentiated enterocyte-like cells

Human iPS cells were cultured in the presence of activin A for 3 days. The cells were further cultured in a medium containing FGF2 for 4 days and then in the presence of EGF for 19 days. PD98059/5-aza-2′-deoxycytidine/A-83-01 (PD/5-aza-dC/A) was added on days 14–26. After 26 days of differentiation, differentiated cells were stained with anti-sucrase–isomaltase antibody. Nuclei were counterstained with DAPI. DAPI-stained DNA (A, D, G, J); immunofluorescence staining of sucrase–isomaltase (B, E, H, K); overlay image of sucrase–isomaltase and DAPI (C, F, I, L). Scale bar, 500 μm (A–F), 100 μm (G–L).

Fig. 4. Morphology of human iPS cells and differentiated enterocyte-like cells

Human iPS cells were cultured in the presence of activin A for 3 days. The cells were further cultured in a medium containing FGF2 for 4 days and then in the presence of EGF for 19 days. PD98059/5-aza-2′-deoxycytidine/A-83-01 (PD/5-aza-dC/A) was added on days 14–26. Human iPS cells (A); differentiated enterocyte-like cells (B). Scale bar, 100 μm.

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Fig. 5. Drug-metabolizing enzyme activities of CYP1A1/2 (A), CYP2C9 (B), CYP2C19 (C), CYP2D6 (D), UGT (E), and SULT (F) in differentiated enterocyte-like cells

Human iPS cells were cultured in the presence of activin A for 3 days. The cells were further cultured in a medium containing FGF2 for 4 days and then in the presence of EGF for 19 days. GSK-3 Inhibitor XV/dorsomorphin (GSKi/BMPi) was added on days 8–14 and PD98059/5-aza-2′-deoxycytidine/A-83-01 (PD/5-aza-dC/A) was subsequently added on days 14–26, or PD98059/5-aza-2′-deoxycytidine/A-83-01 (PD/5-aza-dC/A) was added on days 8–26 or 14–26. After 26 days of differentiation, the differentiated cells were incubated with basal medium containing CYP, UGT, and SULT probe substrates for 24 h. Supernatant was recovered and metabolites were analyzed by UPLC–MS/MS. Data are expressed as mean ± S.D. (n = 3). N.D., not detected. Levels of statistical significance compared with the non-treatment group; *P < 0.05, **P < 0.01.

Fig. 6. Induction of CYP3A4 mRNA expression level (A) and CYP3A4/5 activity (B) in differentiated enterocyte-like cells by 1α,25-dihydroxyvitamin D_3

Human iPS cells were cultured in the presence of activin A for 3 days. The cells were further cultured in a medium containing FGF2 for 4 days and then in the presence of EGF for 19 days. GSK-3 Inhibitor XV/dorsomorphin (GSKi/BMPi) was added on days 8–14 and PD98059/5-aza-2′-deoxycytidine/A-83-01 (PD/5-aza-dC/A) was subsequently added on days 14–26, or PD98059/5-aza-2′-deoxycytidine/A-83-01 (PD/5-aza-dC/A) was added on days 8–26 or 14–26. 1α,25-dihydroxyvitamin D_3 was added for the last 48

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h. After 26 days of differentiation, in CYP3A4 mRNA expression analysis, total RNA was extracted and mRNAs were analyzed by SYBR Green real-time PCR. mRNA expression levels were normalized relative to that of GAPDH. In metabolic activity analysis, the differentiated cells were incubated with basal medium containing CYP, UGT, and SULT probe substrates for 24 h. The supernatant was recovered and metabolites were analyzed by UPLC–MS/MS. The induction of CYP3A4 is represented relative to the level in the cell differentiated in the absence of the small-molecule compounds (vehicle), which is defined as 1. Data are expressed as the mean ± S.D. (n = 3). The vehicle groups are represented by white and black bars and 1α,25-dihydroxyvitamin D_3 addition groups are represented by gray bars. Levels of statistical significance compared with the non-treatment group; $^{**}P < 0.01$; and compared with the vehicle group; $^{\dagger}P < 0.05$, $^{\dagger\dagger}P < 0.01$.

Fig. 7. The uptake of β-Ala-Lys-AMCA into differentiated enterocyte-like cells

Human iPS cells were cultured in the presence of activin A for 3 days. The cells were further cultured in a medium containing FGF2 for 4 days and then in the presence of EGF for 19 days. PD98059/5-aza-2′-deoxycytidine/A-83-01 (PD/5-aza-dC/A) was added on days 14–26. After 26 days of differentiation, the differentiated cells were incubated with 25 μM β-Ala-Lys-AMCA (blue) for 4 h at 37°C with or without 10 mM ibuprofen, or at 4°C. After uptake was stopped, the differentiated cells were fixed. Control (A); 10 mM ibuprofen (B); 4°C (C). Scale bar, 500 μm.

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

Gene name	Sense (5′→3′)	Antisense (5′→3′)	Product length (bp)
LGR5	TGCTCTTCACCAACTGCATC	CTCAGGCTCACCAGATCCTC	193
EphB2	AAAAGGGCTTGGGAGATTCAT	GTCCATCTGTCCCGTCCTC	215
Sucrase–isomaltase	GGTAAGGAGAAACCGGGAAG	GCACGTCGACCTATGGAAAT	195
Villin 1	AGCCAGATCACTGCTGAGGT	TGGACAGGTGTTCCTCCTTC	169
ISX	CAGGAAGGAAGGAAGAGCAA	TGGGTAGTGGGTAAAGTGGAA	96
SLC15A1/PEPT1	CACCTCCTTGAAGAAGATGGCA	GGGAAGACTGGAAGAGTTTTATCG	105
ABCG2/BCRP	AGATGGGTTTCCAAGCGTTCAT	CCAGTCCCAGTACGACTGTGACA	91
ABCB1/MDR1	CCCATCATTGCAATAGCAGG	TGTTCAAACTTCTGCTCCTGA	158
CYP3A4	CTGTGTGTTTCCAAGAGAAGTTAC	TGCATCAATTTCCTCCTGCAG	298
GAPDH	GAGTCAACGGATTTGGTCGT	GACAAGCTTCCCGTTCTCAG	185

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Figure 1

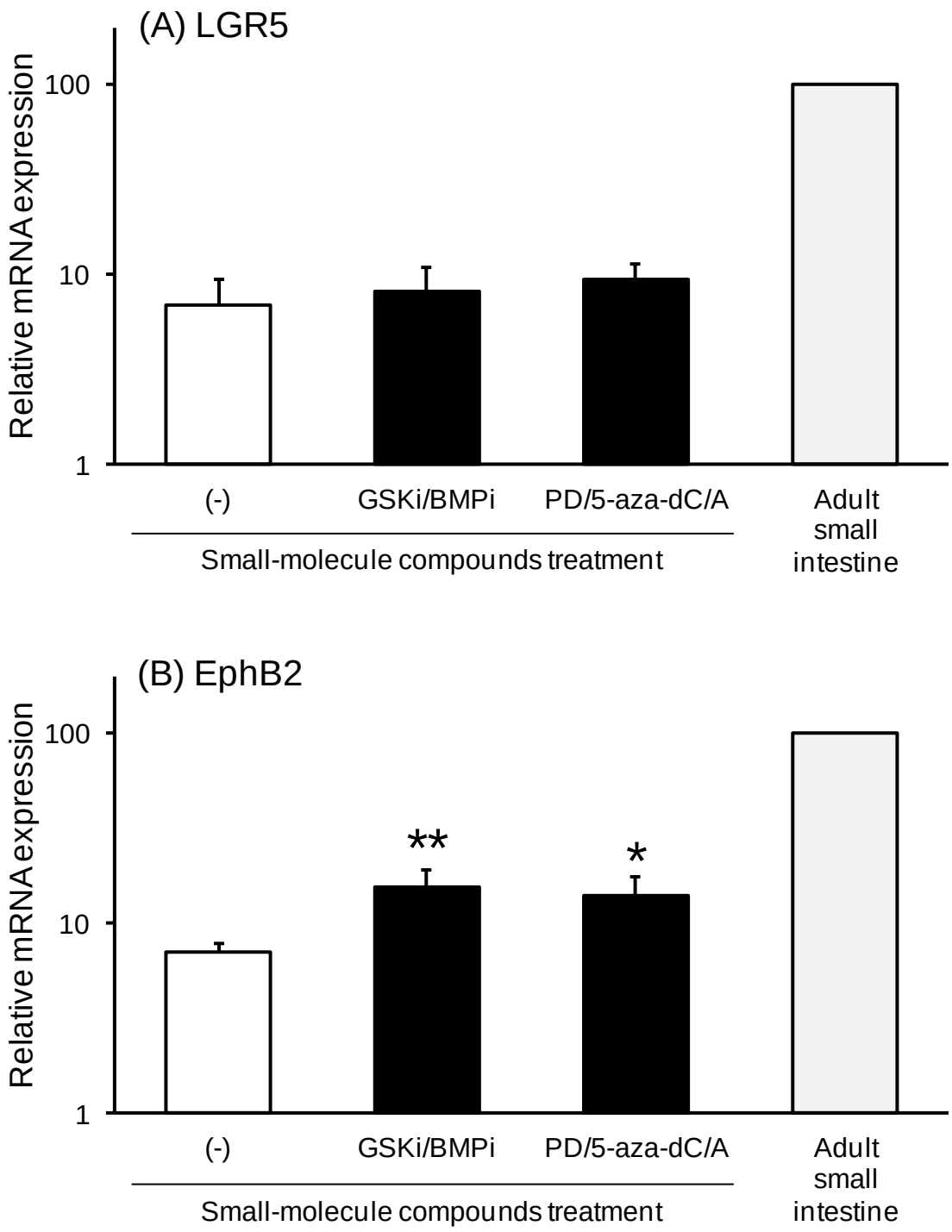

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Figure 2

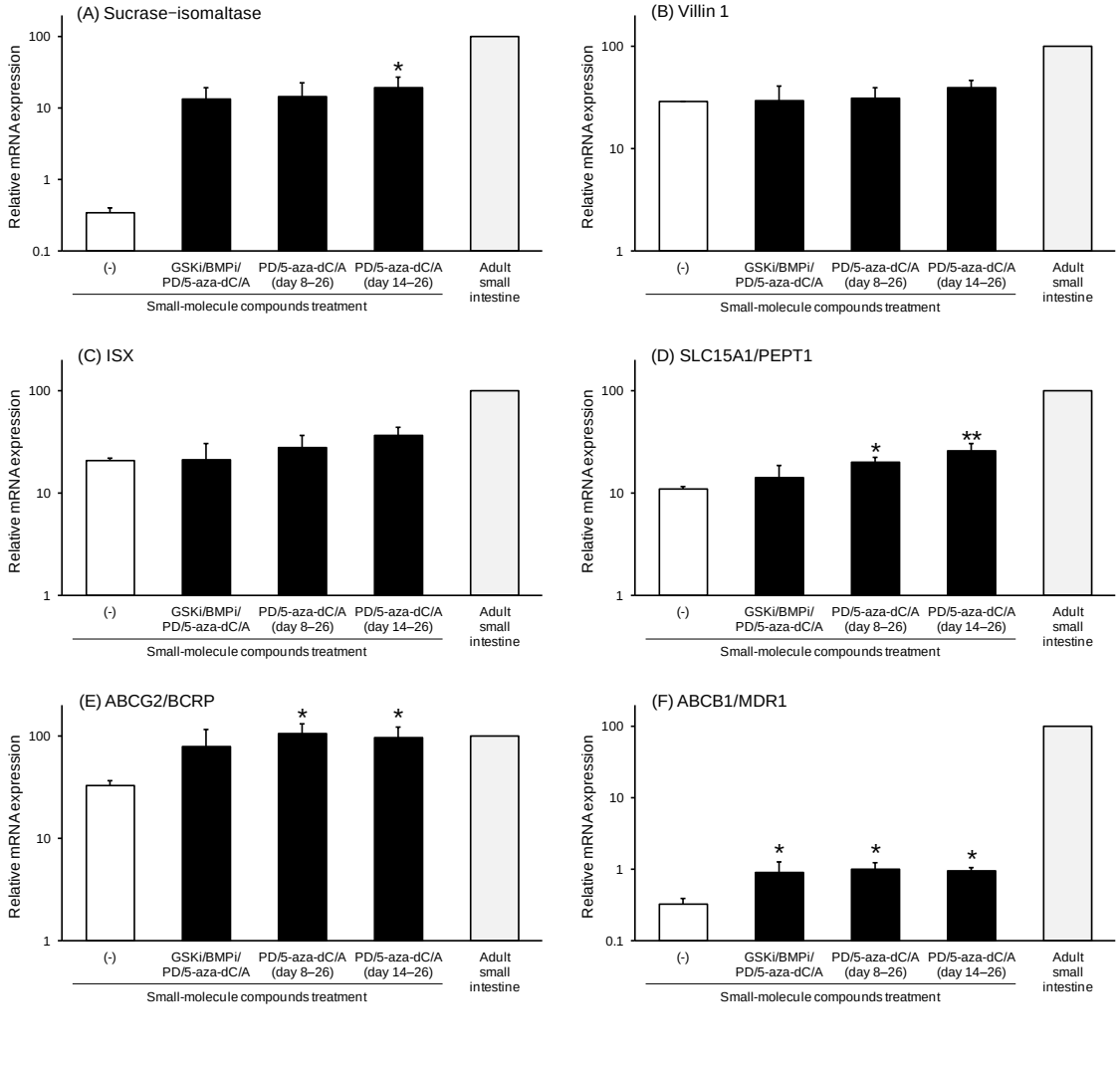

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Figure 3

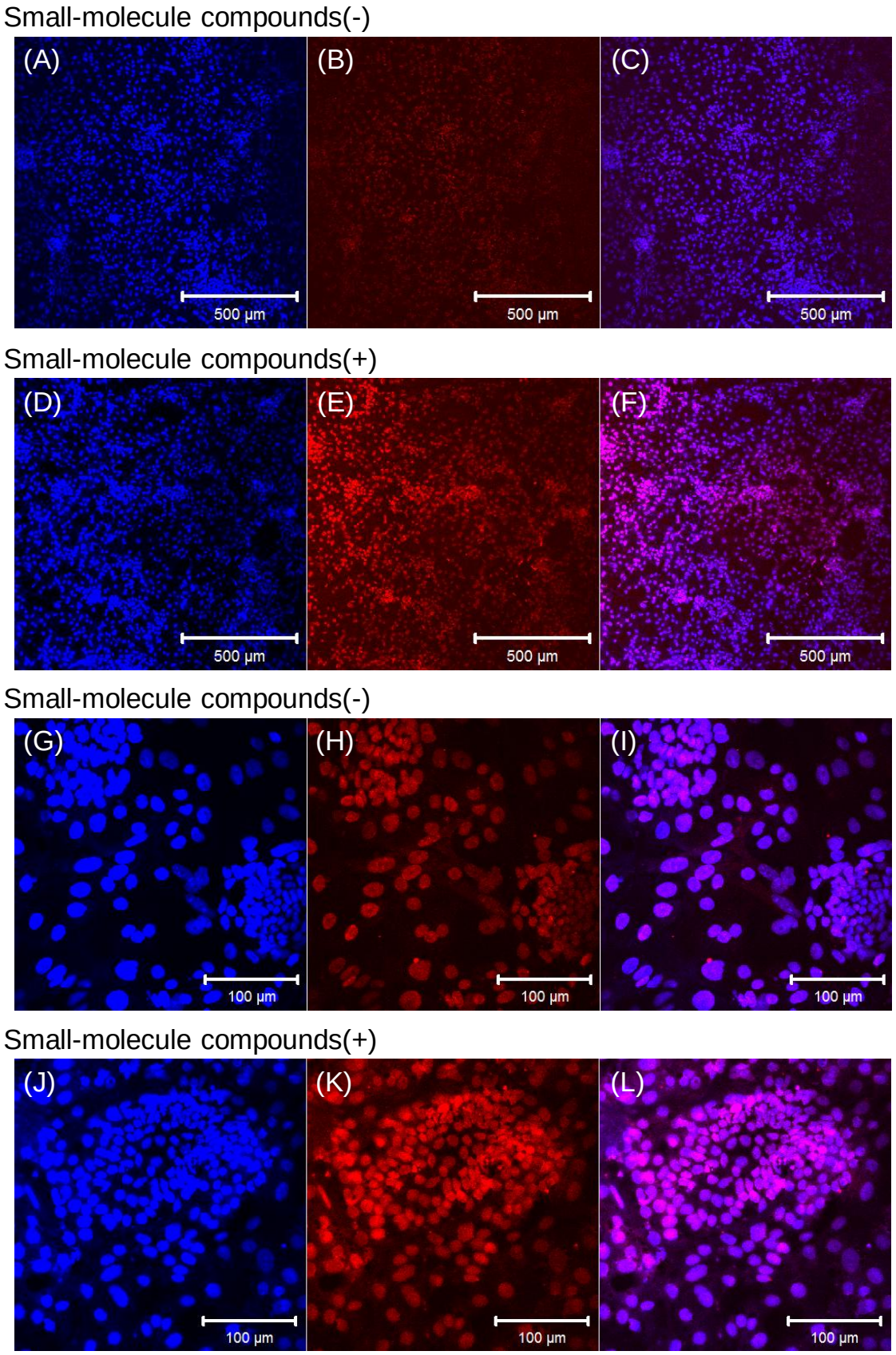

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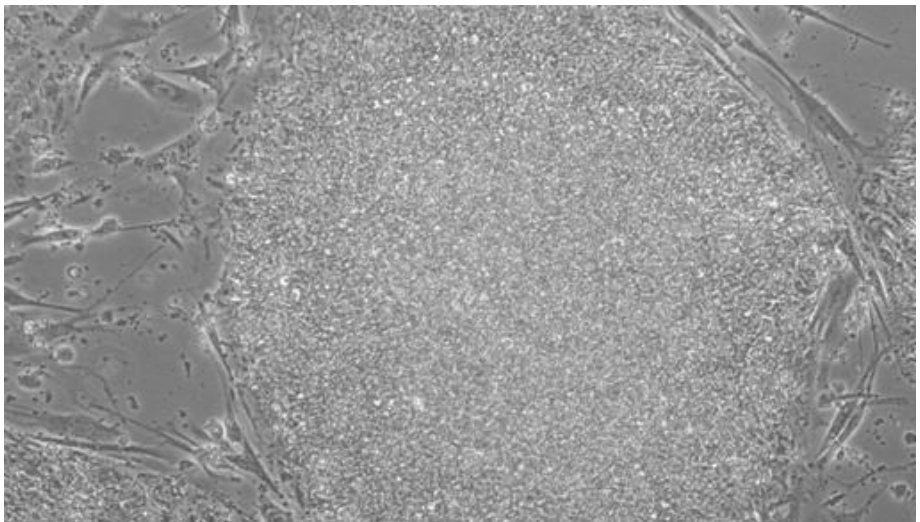
Figure 4

(A) Human iPS cells



(B) Differentiated enterocyte-like cells

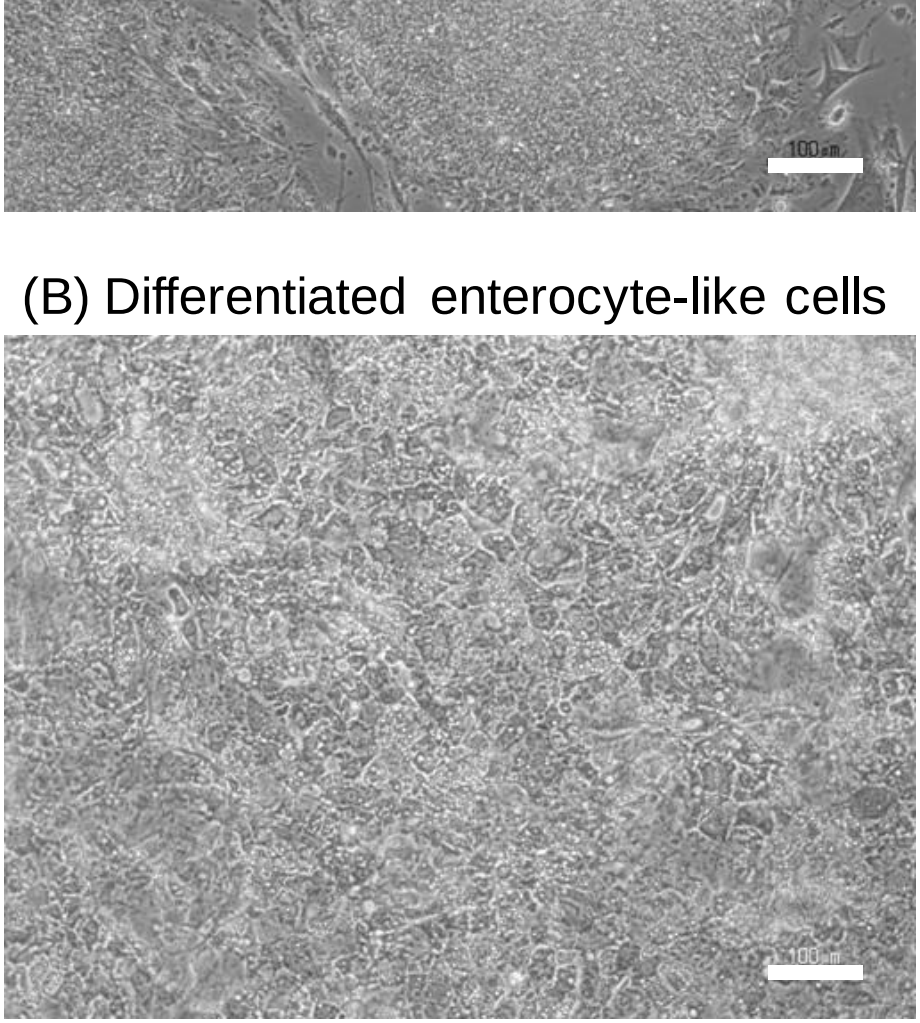


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Figure 5

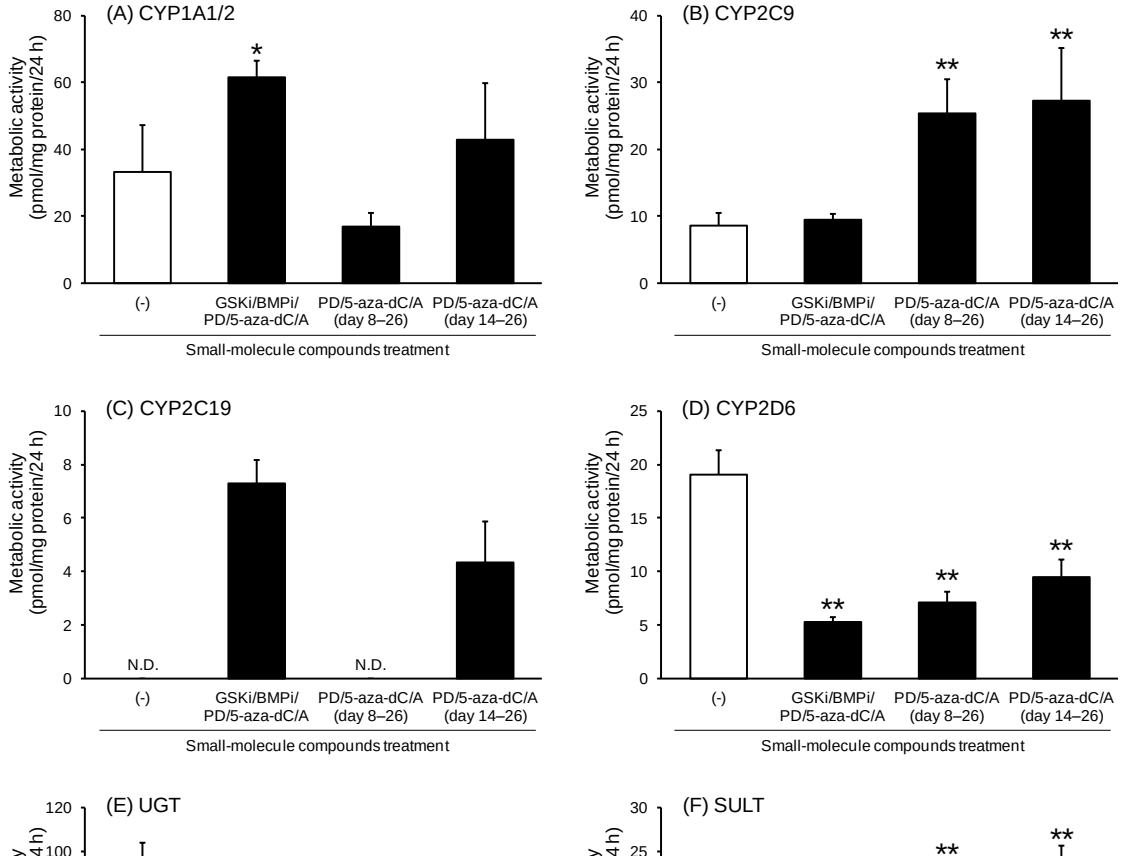

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Figure 6

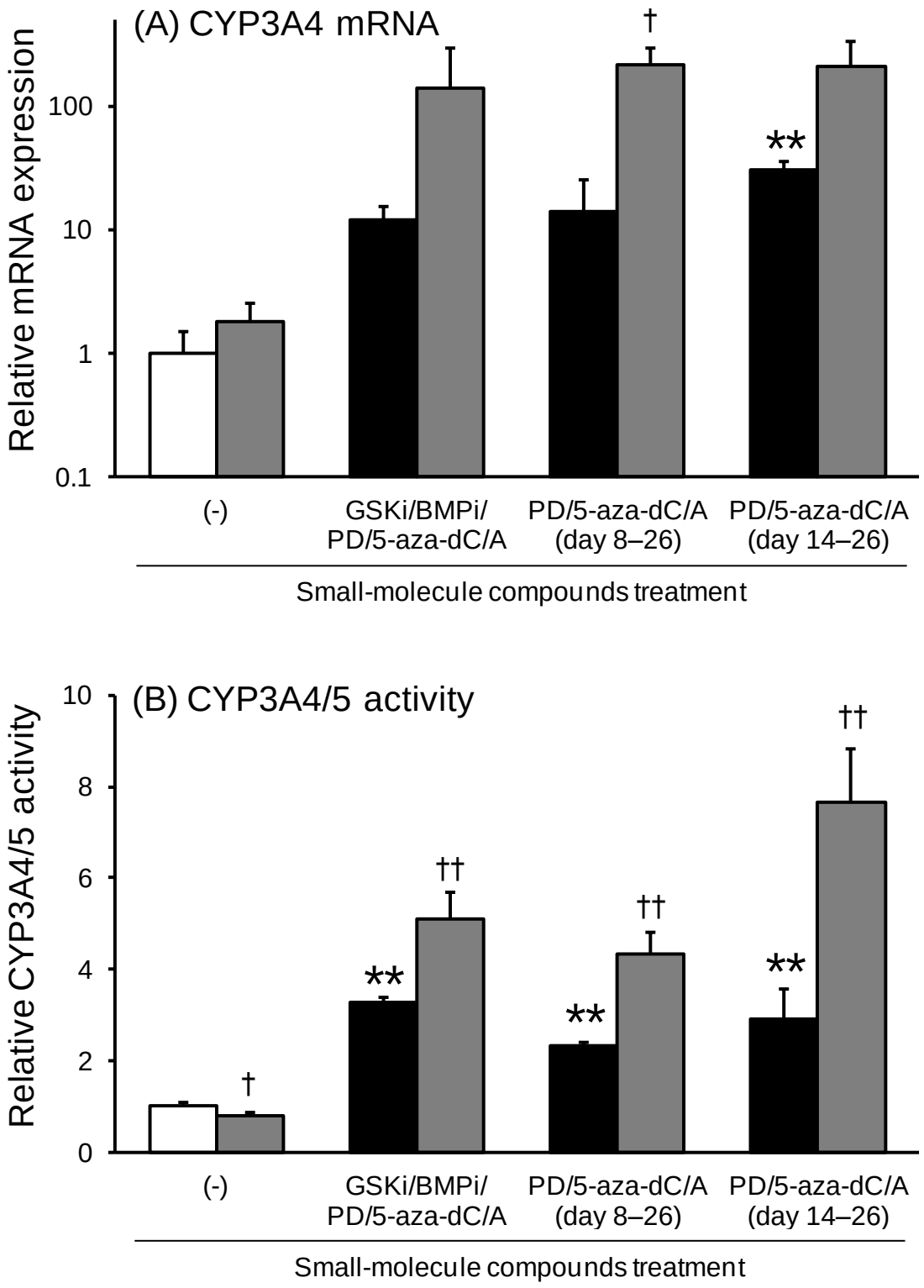

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Figure 7