Inhibition of mitogen-activated protein kinase kinase, DNA methyltransferase, and transforming growth factor- β promotes differentiation of human induced pluripotent stem cells into enterocytes

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Running Title:

Enhanced intestinal differentiation of human iPS cells

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Abstract

We previously reported that small-molecule compounds were effective in generating pharmacokinetically functional enterocytes from human induced pluripotent stem (iPS) cells. In this study, to determine whether the compounds promote the differentiation of human iPS cells into enterocytes, we investigated the effects of a combination of mitogen-activated protein kinase kinase (MEK), DNA methyltransferase (DNMT), and transforming growth factor (TGF)- β inhibitors on intestinal differentiation.

Human iPS cells cultured on feeder cells were differentiated into endodermal cells by activin A. These endodermal-like cells were then differentiated into intestinal stem cells by fibroblast growth factor 2. Finally, the cells were differentiated into enterocyte cells by epidermal growth factor and small-molecule compounds. After differentiation, mRNA expression levels and drug-metabolizing enzyme activities were measured.

The mRNA expression levels of the enterocyte marker sucrase–isomaltase and the major drug-metabolizing enzyme cytochrome P450 (CYP) 3A4 were increased by a combination of MEK, DNMT, and TGF- β inhibitors. The mRNA expression of CYP3A4 was markedly induced by 1 α ,25-dihydroxyvitamin D₃. Metabolic activities of CYP1A1/2, CYP2B6, CYP2C9, CYP2C19, CYP3A4/5, UDP-glucuronosyltransferase, and sulfotransferase were also observed in the differentiated cells. In conclusion, MEK, DNMT, and TGF- β inhibitors can be used to promote the differentiation of human iPS cells into pharmacokinetically functional enterocytes.

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Introduction

The small intestine is an important organ in the pharmacokinetics of orally administrated drugs, owing to the presence of drug transporters and drug-metabolizing enzymes [1-4]. Thus, Caco-2 cells, a human colon carcinoma cell line, are widely used in predicting the absorption of orally administered drugs. However, these cells have different characteristics than those of enterocytes because the expression pattern of drug transporters is different and the level of cytochrome P450 (CYP) 3A4, which is a major drug-metabolizing enzyme in the small intestine, is very low [5]. Thus, it is difficult to estimate drug absorption and metabolism appropriately in the small intestine using Caco-2 cells. For precisely predicting intestinal pharmacokinetics, it is desirable to use primary culture of small intestinal epithelial cells of humans. However, it is difficult to obtain these cells, in contrast to human primary hepatocytes, because primary small intestinal epithelial cells rapidly die when cultured *ex vivo* [6]. Thus, there is no appropriate model for intestinal pharmacokinetic prediction.

Human induced pluripotent stem (iPS) cells [7], have the potential to form almost all types of cells and are expected to be a useful tool in regenerative medicine and drug discovery research. Human iPS cells reportedly differentiate into many types of tissue cells, including cardiomyocytes, neuron cells, and hepatocytes [8-11]. However, there are few reports of intestinal differentiation of human iPS cells. Spence et al. reported the generation of three-dimensional gut-like organoids from human iPS cells and showed that the organoids had the morphological characteristics of intestinal-tract tissues [12]. Ogaki et al. showed that all four intestinal differentiated cell types, i.e., absorptive enterocytes and three types of secretory cells (such as goblet cells, enteroendocrine cells, and Paneth cells) were efficiently differentiated from human embryonic stem/iPS cells

[13]. However, the pharmacokinetic functions of organoids and intestinal cells were little investigated in these studies. We have established a method for differentiating human iPS cells into enterocytes [14, 15]. The enterocytes differentiated using three small-molecule compounds had pharmacokinetic functions such as peptide uptake through peptide transporters, drug-metabolizing activities, and CYP3A4 induction by $1\alpha,25$ -dihydroxyvitamin D_3 . We showed that these small-molecule compounds effectively promoted intestinal differentiation and increased pharmacokinetic functions [15]. In the present study, to determine whether these compounds are effective for intestinal differentiation, we investigated the effects of the combination of the following small-molecule compounds that have similar actions on the differentiation of human iPS cells into enterocytes: mitogen-activated protein kinase kinase (MEK), DNA methyltransferase (DNMT), and transforming growth factor (TGF)- β inhibitors. Although each of them has varieties, all the combinations using these three resulted in similar differentiations.

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). GW788388 hydrate, PD184352, PD-184161, SL327, 5-azacytidine, RG108, zebularine, RepSox, LY-364947, and dimethyl sulfoxide were purchased from Sigma-Aldrich Co. (St. Louis, MO). PD0325901, U0126, D4476, PD98059, 5-aza-2'-deoxycytidine, A-83-01, 1 α ,25-dihydroxyvitamin D₃, rifampicin, **SB431542**, and Y-27632 were purchased from Wako Pure Chemical Industries (Osaka, Japan). MEK inhibitor II was purchased from Merck Millipore (Billerica, MA). The RNeasy Mini Kit was purchased from QIAGEN (Valencia, CA). The PrimeScript RT Reagent Kit and TaKaRa SYBR Premix EX Taq II were purchased from Takara Bio Inc. (Otsu, 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 (Tokyo, Japan). 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% CO₂. 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 Cells

The human iPS cells were used for differentiation studies between the passages 30 and 50. When the cells reached a confluence of approximately up to 70%, 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 U/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 U/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 for 96 h. The cells were then treated for 1 h with the selective Rho-associated kinase inhibitor Y-27632 at 10 µM. The cells were then passaged on Matrigel-coated 24-well plates and cultured in DMEM/F12 containing 2% 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, and three small-molecule compounds (each of MEK, DNMT, and TGF-β inhibitors) for 19 days. Working solutions of small-molecule compounds were prepared in dimethyl sulfoxide or sterile water. Y-27632 was added at 10 µM during the initial 24 h after passage. The medium was changed at every 3 days. In the CYP3A4 induction study, the medium was replaced with 10 nM 1α,25-dihydroxyvitamin D₃ or 40 µM rifampicin for the final 48 h.

RNA Extraction and Reverse Transcription Reaction

1 Total RNA was isolated from differentiated iPS cells using the RNeasy Mini Kit.
2 First-strand cDNA was prepared from 500 ng of total RNA. The reverse transcription
3 reaction was performed using the PrimeScript RT Reagent Kit according to the
4 manufacturer's instructions.

6 **Real-Time Polymerase Chain Reaction (PCR) Analysis**

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

15 **Drug-Metabolizing Enzyme Activity Assay**

16 Measurement of drug-metabolizing enzyme activities was performed by a method
17 similar to that described in our previous reports [9, 15]. Differentiated cells were
18 incubated with medium containing probe substrates of CYPs,
19 UDP-glucuronosyltransferase (UGT), and sulfotransferase (SULT) [CYP1A,
20 phenacetin; CYP2B6, bupropion; CYP2C9, diclofenac; CYP2C19, *S*-mephenytoin;
21 CYP2D6, bufuralol; CYP3A4/5, midazolam; and UGT and SULT, 7-hydroxycoumarin]
22 for 24 h at 37°C. The reactions were then stopped by adding ice-cold acetonitrile
23 containing stable isotope-labeled internal standards for quantification. Metabolites (such
24 as acetaminophen, hydroxybupropion, 4'-hydroxydiclofenac, 4'-hydroxymephenytoin,

1 1'-hydroxybufuralol, 1'-hydroxymidazolam, 7-hydroxycoumarin glucuronide, and
2 7-hydroxycoumarin sulfate) were measured by ultra-performance liquid
3 chromatography–tandem mass spectrometry (UPLC–MS/MS).

4 To correct for drug-metabolizing enzyme activities, total protein of the
5 differentiated cells was measured using a Pierce™ BCA Protein Assay Kit (Thermo
6 Fisher Scientific Inc., Waltham, MA) according to the manufacturer's instructions.

7 **Statistical Analysis**

9 Multiple comparisons were performed using the analysis of variance followed by
10 Dunnett's test. Statistical analysis was performed on the PASW Statistics 18 system
11 software (IBM, USA).

Results

Effects of MEK, DNMT, and TGF- β Inhibitors on Intestinal Differentiation

To investigate whether the primary actions of PD98059, 5-aza-2'-deoxycytidine, and A-83-01 are effective for the differentiation of human iPS cells into enterocytes, we used a combination of MEK, DNMT, and TGF- β inhibitors during the process of differentiation from intestinal stem cells to enterocytes. These small-molecule compounds were listed in Table 2. The mRNA expression levels of leucine-rich repeat containing G-protein-coupled receptor 5 (LGR5), which is an intestinal stem cell marker [16], were lower in all small-molecule compound treatment groups than those in the control group (Fig. 1). In contrast, mRNA expression levels of sucrase-isomaltase, which is a specific enterocyte marker [17], in small-molecule compound treatment groups, except for RepSox, were equal to or higher than those in the control group. The mRNA expression levels of solute carrier family 15 member 1/peptide transporter 1 (SLC15A1/PEPT1), which is an intestinal peptide transporter [18], and CYP3A4 were increased in most small-molecule compound treatment groups than those in the control group. In particular, LGR5 and SLC15A1/PEPT1 mRNA expression levels were considerably lower and higher, respectively.

Effects of MEK Inhibitor II, Zebularine, and GW788388 on Intestinal Differentiation

Considering the abovementioned results and cytotoxicity with these compounds, we selected MEK inhibitor II, zebularine, and GW788388, which were promising compounds for the intestinal differentiation of human iPS cells, from among MEK, DNMT, and TGF- β inhibitors, respectively.

1 Compared with that of the control group, LGR5 mRNA expression level was
2 decreased up to 0.3-fold by MEK inhibitor II, zebularine, and GW788388 treatment
3 (Fig. 2). The sucrase–isomaltase mRNA expression level was increased up to 2.9-fold
4 by treatment with these small-molecule compounds; LGR5 and sucrase–isomaltase
5 mRNA expression levels were considerably lower and higher than the nontreatment
6 group, respectively. These results showed a tendency similar to that of the group
7 differentiated using PD98059, 5-aza-2'-deoxycytidine, and A-83-01.

8 9 **Drug-Metabolizing Enzyme Activities**

10 Drug-metabolizing enzyme activities in the cells differentiated using PD98059,
11 5-aza-2'-deoxycytidine, and A-83-01 were significantly increased compared with those
12 in the cells of the control group (Fig. 3). **CYP1A2 and CYP2B6 activities of the MEK**
13 **inhibitor II, GW788388, and zebularine treatment groups** were significantly higher than
14 those of the control group, whereas CYP2D6, UGT, and SULT activities were
15 unchanged. CYP2C9 and CYP2C19 activities were detected **in the both** small-molecule
16 compound **treatment groups** during intestinal differentiation, but not in the control
17 group.

18 19 **Induction of mRNA Expression and Activity of CYP3A4**

20 CYP3A4 is a major drug-metabolizing enzyme in the small intestine. Its
21 expression was induced by adding 1 α ,25-dihydroxyvitamin D₃ [19]. We evaluated the
22 mRNA expression, activity, and inducibility of CYP3A4. CYP3A4 mRNA expression
23 levels were increased up to **6.0- and 4.1-fold** by treatment with PD98059,
24 5-aza-2'-deoxycytidine, and A-83-01 and with MEK inhibitor II, zebularine, and

1 GW788388, respectively, compared with those in the control group (Fig. 4). By addition
2 of $1\alpha,25$ -dihydroxyvitamin D₃, mRNA was markedly induced up to 31.9- and 6.4-fold
3 in the treatment groups of PD98059, 5-aza-2'-deoxycytidine, and A-83-01 and of MEK
4 inhibitor II, zebularine, and GW788388, respectively, whereas it was not induced by
5 rifampicin. This is probably because mRNA expression level of PXR was low in all
6 groups (data not shown). CYP3A4/5 activity was also increased in the presence of these
7 compounds. Moreover, the activity was induced approximately up to 3-fold by adding
8 $1\alpha,25$ -dihydroxyvitamin D₃ in the presence of the compounds but not by that of
9 rifampicin. In particular, CYP3A4/5 activity levels were significantly higher than those
10 of the control and vehicle groups.

Discussion

The present study showed that inhibition of MEK, DNMT, and TGF- β is effective for the differentiation of human iPS cells into enterocytes. We previously reported that the three small-molecule compounds PD98059, 5-aza-2'-deoxycytidine, and A-83-01, promoted the intestinal differentiation of human iPS cells [15]. However, it was unclear whether the primary actions of these compounds contributed to the enhanced intestinal differentiation. In this study, we selected the small-molecule compounds MEK, TGF- β , and DNMT inhibitor having actions similar to PD98059, A-83-01, and 5-aza-2'-deoxycytidine and investigated whether MEK, TGF- β , and DNMT inhibitor acted to promote the differentiation of human iPS cells into enterocytes. The differentiated cells expressed the marker genes of intestinal stem cells and enterocytes and exhibited drug-metabolizing enzyme activities. These results indicated that human iPS cells were differentiated into functional enterocyte-like cells. From the findings of our study, it is observed that the LGR5 mRNA expression was lower in the MEK, DNMT, and TGF- β inhibitor treatment groups than that in the control group, whereas the expression levels of sucrase-isomaltase, SLC15A1/PEPT1, and CYP3A4 mRNAs were increased in the group of small-molecule compound treatment compared with those of the control group. Thus, we believe that the intestinal differentiation was enhanced by treatment with a combination of MEK, DNMT, and TGF- β inhibitors despite the degrees of effectiveness being different among compounds.

The small intestine has a variety of drug-metabolizing enzymes and drug transporters. These pharmacokinetics-associated proteins have great influence on the efficacy or side-effects of drugs. We analyzed drug-metabolizing enzyme activities for the evaluation of major intestinal function. Drug-metabolizing enzyme activities of

CYP1A1/2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, CYP3A4/5, UGT, and SULT were detected in the differentiated cells (Figs. 3 and 4). In particular, the activities of CYP2C9 and CYP2C19 were detected by treatment with small-molecule compounds, although these activities were not detected in the control group. Furthermore, the induction of mRNA expression and activity of CYP3A4 by 1 α ,25-dihydroxyvitamin D₃ were clearly observed in the differentiated cells using small-molecule compounds (Fig. 4). 1 α ,25-Dihydroxyvitamin D₃ is a ligand of the vitamin D receptor (VDR) and induces CYP3A4 expression through VDR in the intestinal cell lines [19]. Thus, we thought that the inducibility of CYP3A4 was a characteristic property of enterocytes. Our results showed that the differentiated cells not only expressed intestinal marker genes but also had intestinal pharmacokinetic functions. The combination of MEK, DNMT, and TGF- β inhibitors was effective for the differentiation of human iPS cells into pharmacokinetically functional enterocytes. However, the mRNA expression level and CYP3A4 activity were not induced by rifampicin because PXR expression was low. Thus, it is possible that P-glycoprotein, such as CYP3A4, is not induced by PXR ligands. Therefore, intestinal differentiation should be improved in the future.

We used MEK, DNMT, and TGF- β inhibitors for intestinal differentiation in this study. We now consider why these compounds promoted the intestinal differentiation of human iPS cells using the scheme in Fig. 5. First, mitogen-activated protein (MAP) kinase intracellular signaling pathways are involved in the regulation of various cellular functions. The Raf/MEK/extracellular signal-regulated kinase (ERK) pathway, which is one of the mitogen-activated protein kinase intracellular signaling pathways, plays major roles in the cellular growth regulation, differentiation, and proliferation [20, 21]. Inactivation of the ERK pathway is required for the induction of intestinal epithelial cell

terminal differentiation. The constitutive activation of the MEK/ERK cascade inhibits differentiation into enterocytes, in part through caudal-type homeobox (Cdx) 2/3 phosphorylation, resulting in the inhibition of its transcriptional activity [21]. Together, these reports suggested that MEK inhibitors enhance the induction of intestinal epithelial cell terminal differentiation by inhibiting the MEK/ERK signaling pathway. Second, epigenetic modifications stably influence gene expression without changing the underlying DNA sequences. The mechanisms include DNA methylation, histone tail modifications, chromatin remodeling, and noncoding RNA expression [22-24]. DNA methylation plays critical roles in many biological processes, including gene silencing, genomic imprinting, and X chromosome inactivation. In addition, DNA methylation contributes to genomic stability by suppressing transposable and repetitive elements [24]. Maintenance of DNA methylation by DNMT1 is required for the repression of adult intestinal stem cell genes to allow appropriate timing of differentiation. In intestinal differentiation, DNA methylation played an active role in the regulation of gene expression by controlling the accessibility of enhancers during intestinal epithelial differentiation [25]. Thus, DNMT inhibitors may be effective for intestinal differentiation by regulating the gene expression involved in transcription. Third, the mesenchyma is important for the maintenance and differentiation of enterocytes [26]. As a developmental process, cell differentiation depends on the epithelial–mesenchymal interaction [27]. The epithelial–mesenchymal interaction is regulated by BMP-2 expressed in enterocytes and BMP-4 expressed in mesenchyma [28].

The epithelial-to-mesenchymal transition (EMT) is a feature of embryogenesis that is vital for morphogenesis during embryonic development [29]. The process of EMT is triggered by molecular interplay among extra-cellular signals such as collagen,

1 TGF- β , FGF, and EGF [30]. TGF- β , a growth factor, can initiate and maintain EMT in
2 various biological systems and pathophysiological contexts by activating major
3 signaling pathways and transcriptional regulators integrated in extensive signaling
4 networks [31]. Type I receptors, referred to as ALK, lie at the center of the signaling
5 cascade and transduce TGF- β signals to the intracellular regulators of transcription
6 known as Smad proteins. A TGF- β type I receptor, ALK5, and its effector Smad
7 proteins mediate the EMT [32]. Thus, we thought that TGF- β inhibitors inhibit EMT by
8 inhibiting the Smad2/3 signaling pathway. Taking these results together, we suggest that
9 MEK, DNMT, and TGF- β inhibitors synergistically promote the intestinal
10 differentiation of human iPS cells by regulating the abovementioned signaling
11 pathways.

12 In conclusion, we have demonstrated a method for promoting differentiation
13 and/or maturation of enterocyte-like cells using small-molecule compounds. The
14 differentiated cells expressed intestinal marker and pharmacokinetic-related genes. The
15 cells also showed pharmacokinetic functions such as drug-metabolizing enzyme
16 activities and CYP3A4 induction by 1 α ,25-dihydroxyvitamin D₃. In addition, we found
17 that a combination of MEK, DNMT, and TGF- β inhibitors promoted the differentiation
18 of human iPS cells into pharmacokinetically functional enterocytes. Thus, these
19 compounds may be useful for generating enterocyte-like cells for use in drug
20 development studies.

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Authorship Contributions

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Participated in research design: Kodama, Iwao, Kondo, Nakamura, Matsunaga.

Conducted experiments: Kodama, Iwao, Kabeya, Horikawa, Niwa, Kondo.

Performed data analysis: Kodama, Iwao, Horikawa.

Wrote or contributed to the writing of the manuscript: Kodama, Iwao, Horikawa, Niwa,

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Footnotes

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Figure Legends

Fig. 1. Effects of small-molecule compounds on intestinal differentiation of human iPS 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 and of small-molecule compounds for 19 days. We selected two compounds from among PD98059, 5-aza-2'-deoxycytidine, and A-83-01 and one compound from another inhibitor group. After 26 days of differentiation, total RNA was extracted and mRNAs were analyzed by SYBR Green real-time PCR. The mRNA expression levels were normalized relative to those of GAPDH. Gene expression levels are represented relative to the level in the control group, which is set as 1. The group in the absence of small-molecule compounds (shown as an open column) was used as control (Ctrl.). The closed columns show our previously reported combination. Data are presented as the mean $\pm$ S.D. ($n = 2$ or 3). There are columns of $n = 2$ group because sufficient CYP3A4 mRNA was not obtained (A-83-01, D4476, RepSox, PD184352, PD-184161, SL327, and 5-aza-2'-deoxycytidine). Levels of statistical significance compared with the nontreatment group: * $P < 0.05$; ** $P < 0.01$.

Fig. 2. Relative mRNA expression levels of LGR5, sucrase-isomaltase, and SLC15A1/PEPT1 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 the medium containing FGF2 for 4 days and then in the presence of EGF and small-molecule compounds for 19 days. Small-molecule compounds were added with combinations of PD98059, 5-aza-2'-deoxycytidine, and A-83-01 or of MEK

inhibitor II, zebularine, and GW788388. After 26 days of differentiation, total RNA was extracted and mRNAs were analyzed by SYBR Green real-time PCR. The mRNA expression levels were normalized relative to that of GAPDH. Gene expression levels are represented relative to the level in the control group, which is set as 1. The group in the absence of small-molecule compounds (shown as an open column) was used as control (Ctrl). The closed columns show combinations previously reported. Data are presented as means $\pm$ S.D. ($n = 3$). Levels of statistical significance compared with the nontreatment group: $*P < 0.05$; $**P < 0.01$.

Fig. 3. Activities of CYP1A1/2, CYP2B6, CYP2C9, CYP2C19, CYP2D6, UGT, and SULT 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 the medium containing FGF2 for 4 days and then in the presence of EGF and small-molecule compounds for 19 days. Small-molecule compounds were added as combinations of PD98059, A-83-01, and 5-aza-2'-deoxycytidine or of MEK inhibitor II, GW788388, and zebularine.

After 26 days of differentiation, 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 group in the absence of small-molecule compounds (shown as an open column) was used as control (Ctrl). The closed columns show combinations previously reported. Data are presented as means $\pm$ S.D. ($n = 4$). N.D., not detected. Levels of statistical significance compared with the nontreatment group: $*P < 0.05$; $**P < 0.01$.

Fig. 4. Induction of CYP3A4 mRNA expression level and CYP3A4/5 activity in differentiated enterocyte-like cells by 1 α ,25-dihydroxyvitamin D₃ and rifampicin

Human iPS cells were cultured in the presence of activin A for 3 days. The cells were further cultured in the medium containing FGF2 for 4 days and then in the presence of EGF and small-molecule compounds for 19 days. Small-molecule compounds were added with the combination of PD98059, 5-aza-2'-deoxycytidine, and A-83-01 or of MEK inhibitor II, zebularine, and GW788388. 1 α ,25-dihydroxyvitamin D₃ (VD3) and rifampicin (RIF) were supplied for the last 48 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. The mRNA expression levels were normalized relative to that of GAPDH. For a drug-metabolizing enzyme activity analysis, the differentiated cells were incubated with the medium containing midazolam, a CYP3A4/5 probe substrate, for 24 h. The supernatant was recovered and metabolites were analyzed by UPLC–MS/MS. The group in the absence of small-molecule compounds (shown as an open column) was used as control (Ctrl). The closed columns show combinations previously reported. Induction of CYP3A4 is represented relative to the level in the nontreatment (vehicle), which is set as 1. Data are presented as means $\pm$ SD [$n = 3$ (A), $n = 4$ (B)]. $^{**}P < 0.01$. Levels of statistical significance compared with those of the nontreatment groups of VD3 and RIF: $^{\dagger\dagger}P < 0.01$.

Fig. 5. A scheme of the approach for systematic differentiation of human iPS cells into enterocytes

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

Gene names	Sense (5'→3')	Antisense (5'→3')	Product length (bp)
LGR5	TGCTCTTCACCAACTGCATC	CTCAGGCTCACCAGATCCTC	193
Sucrase–isomaltase	GGTAAGGAGAAACCGGGAAG	GCACGTCGACCTATGGAAAT	195
CYP3A4	CTGTGTGTTTCCAAGAGAAGTTAC	TGCATCAATTCCTCCTGCAG	298
SLC15A1/PEPT1	CACCTCCTTGAAGAAGATGGCA	GGGAAGACTGGAAGAGTTTTATCG	105
GAPDH	GAGTCAACGGATTTGGTCGT	GACAAGCTTCCCGTTCTCAG	185

(Revised manuscript, P. 30, Table 1)

Table 2. List of MEK, DNMT, and TGF- β inhibitors used in this study

Groups	Compounds	Final concentrations
MEK Inhibitor	PD184352	1 μ M
	PD98059	20 μ M
	PD0325901	1 μ M
	PD-184161	10 μ M
	SL327	10 μ M
	U0126	10 μ M
	MEK Inhibitor II	10 μ M
DNMT Inhibitor	5-Aza-2'-deoxycytidine	5 μ M
	5-Azacytidine	10 μ M
	RG108	10 μ M
	Zebularine	10 μ M
TGF- β Inhibitor	A-83-01	0.5 μ M
	SB431542	10 μ M
	D4476	10 μ M
	RepSox	10 μ M
	LY-364947	10 μ M
	GW788388 Hydrate	20 μ M

(Revised manuscript, P. 31, Table 2)

(Revised manuscript, Fig. 1)

(Revised manuscript, Fig. 2)

(Revised manuscript, Fig. 3)

(Revised manuscript, Fig. 4)

Figure 5