Establishment of a novel culture method for maintaining intestinal stem cells derived from human induced pluripotent stem cells

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INTRODUCTION

Intestinal enterocytes are subject to constant turnover, undergoing continuous and rapid regeneration by intestinal stem cells (ISCs), which have the ability to self-renew and generate undifferentiated transit-amplifying (TA) cells in the process. TA cells then differentiate into various cell types, including absorptive enterocytes, goblet cells, enteroendocrine cells, Paneth cells, M cells and tuft cells (Noah et al., 2011), localization of which differ according to cell type. Although ISC are located at the bottom of the crypt, most differentiated cells migrate up the crypt villus in the small intestine. In contrast, Paneth cells, which represent an essential component of the ISC niche, are also located at the bottom of the crypt (Meran et al., 2017). The small intestine plays a major role in the absorption of orally administered drugs. This absorption is influenced by a number of factors, including the presence of drug transporters and drug-metabolizing enzymes. However, few appropriate methods exist to investigate intestinal pharmacokinetics. Induced pluripotent stem (iPS) cells can form various types of cells and represent a potentially useful tool for drug discovery. We previously reported that differentiated enterocytes from human iPS cells are useful for pharmacokinetic studies; however, the process is time and resource intensive. Here, we established a new two-dimensional culture method for maintaining human iPS-cell-derived intestinal stem cells (ISCs) with differentiation potency and evaluated their ability to differentiate into enterocytes exhibiting appropriate pharmacokinetic function. The culture method used several factors to activate signalling pathways required for maintaining stemness, followed by differentiation into enterocytes. Functional evaluation was carried out to verify epithelial-marker expression and inducibility and activity of metabolic enzymes and transporters. Our results confirmed the establishment of an ISC culture method for maintaining stemness and verified that the differentiated enterocytes from the maintained ISCs demonstrated proper pharmacokinetic function. Thus, our findings describe a time- and cost-effective approach that can be used as a general evaluation tool for evaluating intestinal pharmacokinetics.

KEY WORDS: Human iPS cells, Intestinal stem cells, Pharmacokinetics
As a solution to these issues, maintaining and culturing ISCs has been considered. However, it is difficult to simply cultivate ISCs alone, as they lose cellular stemness and proliferation potential with repeated passages and normally maintain stemness by utilizing a special niche environment localized near the crypt bottom. It was reported that use of three-dimensional (3D) cultures extended the period during which intestinal cells can be cultured (Jung et al., 2011; Sato et al., 2011, 2009). Moreover, the organoids in 3D cultures display a villus-like structure similar to intestinal tissue and contain several cells that are consistent with the crypt niche of the intestines (Sawant-Basak et al., 2018; Onozato et al., 2018; Tamminen et al., 2015; Foulke-Abel et al., 2014; Jung et al., 2011; Spence et al., 2011; Sato et al., 2011, 2009). Although stem cell characteristics can reportedly be maintained by mimicking the environment and structure of the living intestine, the exchange and passage of medium in 3D cultures are complicated. Additionally, because organoids are usually cultured in a Matrigel containing extracellular matrix, cellular passage and recovery are complicated, and their shape and size are varied. Furthermore, the use of Matrigel is unsuitable for large-scale cultures because of its gel form. The quantitative evaluation of intestinal absorption using 3D intestinal organoids is not very feasible because of the difficulty in accessing apical and basal compartments. Recently, Capeling et al. reported that organoids can be passaged and cultured using alternative methods to Matrigel, and some researchers have shown that organoids can be dissociated and seeded onto Transwell inserts (Capeling et al., 2019; Van der Hee et al., 2018; Múnera et al., 2017; Fernando et al., 2017). In addition, accessible organ-on-a-chip to both compartments has also been reported. However, the number of such reports is still low, and the function of these cells has not been sufficiently evaluated. These findings suggest that intestinal enterocytes with monolayers and two-dimensional (2D) culture are more suitable for quantitative pharmacokinetic and pharmacological evaluation.

In this study, in order to resolve these issues, we attempted to establish a new 2D culture method for maintaining human iPS-cell-derived ISCs capable of differentiation into enterocytes by using factors that enhance intestinal stemness and lineage. Additionally, we evaluated whether the resulting enterocytes demonstrated appropriate pharmacokinetic functions.

RESULTS
Schematic outline of the differentiation of human iPS cells into enterocytes
The process of human iPS cell differentiation into enterocytes is presented in Fig. 1A. Cells on day 7 after initiation of differentiation...
were established as ISCs. At this stage, we repeatedly passaged and cultured the cells on iMatrix-511-coated dishes using a medium containing several factors. ISCs from P0 to P7 were differentiated into enterocytes by changing to the differentiation medium. The morphologies of human iPS cells, ISCs (P0 and P3), and enterocytes (P0 and P3) are shown in Fig. 1B. No marked morphological difference was observed between P0 and P3 ISCs and enterocytes.

**Effects of foetal bovine serum (FBS) and KnockOut serum replacement (KSR) on mRNA levels of intestinal markers in ISCs derived from human iPS cells**

Although FBS is widely used in cell-culture media, ISCs cannot be cultured in the presence of FBS, because it commonly contains growth factors and other proteins that can initiate their differentiation. KSR is a commercially available serum-free supplement often used to culture embryonic stem cells and in other serum-free cultures. Therefore, we evaluated the ability to substitute KSR for FBS in ISC cultures without affecting intestinal stemness and lineage (Fig. 2A,B).

We found that mRNA levels of the leucine-rich repeat-containing G protein-coupled receptor 5 (LGR5), an ISC marker, decreased along with increased FBS concentration. However, we observed a smaller decrease in LGR5 levels in the presence of KSR. Additionally, we observed no obvious differences in the mRNA levels of caudal type homeobox 2 (CDX2), an intestinal lineage marker, between FBS and KSR cultures. From these results, KSR appears to have less influence on cellular stemness and intestinal lineage when applied at a concentration of 10% to ISC cultures.

**Effects of CHIR99021 and valproic acid (VPA) on LGR5 and CDX2 mRNA levels in ISC**

Many studies have reported that both WNT and Notch signalling are necessary for the maintenance of cellular stemness. Thus, we evaluated the influence of CHIR99021 and VPA on intestinal stemness and lineage in ISCs derived from human iPS cells. CHIR99021 inhibits GSK-3β-mediated β-catenin degradation and activates WNT signalling. VPA is a histone-deacetylase inhibitor that activates Notch signalling (Greenblatt et al., 2007), although the details of the underlying mechanism remain unclear. We found that CHIR99021 increased LGR5 and CDX2 mRNA levels in a concentration-dependent manner (Fig. 2C,D). VPA slightly increased LGR5 mRNA expression in a concentration-dependent manner, but had little effect on CDX2 mRNA expression at all concentrations. Quantification of changes in mRNA levels in the presence of combined 10 µM CHIR99021 and 1 mM VPA treatment revealed that LGR5 and CDX2 levels were 1.3- and 1.5-fold higher, respectively, than in the presence of CHIR99021 alone.

**Effects of CHIR99021 and VPA on ISC growth**

We observed that the ISC number was approximately two-fold higher than that of the control in the presence of up to 10 µM CHIR99021 (Fig. 2E), whereas the cell number at 30 µM CHIR99021 was comparable to that of the control. Furthermore, the ISC number decreased with increasing concentrations of CHIR99021 and VPA and at concentrations >60 µM CHIR99021 or 3 mM VPA relative to the control. Combination treatment with 10 µM CHIR99021 and 1 mM VPA did not affect cell number relative to the results in the presence of 1 mM VPA alone. Although VPA had little effect on intestinal stemness and lineage, given that these results and previous reports support the importance of Notch signalling in cell stemness (Greenblatt et al., 2007), we conducted subsequent studies in the presence of 10 µM CHIR99021 and 1 mM VPA.

**Alterations in LGR5, Ki67, and CDX2 mRNA and protein levels according to ISC passage**

To investigate whether repeated passage of ISCs maintained intestinal stemness and lineage, we evaluated mRNA and protein levels according to ISC passage.

![Figure 2](image-url) Fig. 2. Effect of FBS, KSR, CHIR99021 and VPA on CDX2 and LGR5 mRNA levels and cell growth in ISCs. (A,B) Effect of FBS and KSR on CDX2 and LGR5 mRNA levels in ISCs derived from human iPS cells. LGR5 and CDX2 mRNA levels relative to levels in the human adult small intestine (set at 100). (C,D) Effect of CHIR99021 and VPA on CDX2 and LGR5 mRNA levels and cell growth in ISCs derived from human iPS cells. CDX2 and LGR5 mRNA levels are presented relative to levels in the human adult small intestine (set at 100). Cell number was counted using an automated cell counter. C0V0, concentrations of CHIR99021 and VPA (0); C10V1, concentrations of CHIR99021 and VPA (10 µM and 1 mM, respectively). Data represent the mean±s.d. (n=3); **P<0.01 compared with the C0V0.
levels of LGR5, Ki67, and CDX2 (Fig. 3A,B). Ki67 is a stem cell and proliferation marker. Although the mRNA expression levels of LGR5 and Ki67 tended to decrease with passage, the expression levels were higher than or similar to those in human adult small intestine. In contrast, levels of CDX2 were higher in P1 to P7 relative to P0 and human adult small intestine. The fractional changes in LGR5, Ki67 and CDX2 expression levels in P1 to P7 relative to P0 are shown in Fig. S3. Immunofluorescence analysis detected LGR5 and Ki67 in P0 ISC whereas CDX2 was not detected, which is consistent with the low CDX2 mRNA levels observed at this passage. In contrast, LGR5, Ki67 and CDX2 were detected in P3 ISCs. Almost all cells were LGR5-, Ki67- and CDX2-positive in P3. These results show that intestinal stemness and lineage were maintained at the mRNA and protein levels.

**Alterations in mRNA and protein levels of enterocyte-differentiation markers according to ISC passage**

To investigate whether the repeatedly-passaged ISCs maintained the potency to differentiate into functional enterocytes, we evaluated mRNA and protein levels of several enterocyte markers (Fig. 4A,B). The fractional changes in differentiation markers in P1–P7 enterocytes relative to P0 enterocytes (set at 100) are shown in Fig. S4. The mRNA level of BCRP was higher in P0–P7 enterocytes than in human adult intestine, and that of P-gp was almost maintained from P0–P7 enterocytes. Additionally, the levels of several markers – including villin, sucrase-isomaltase, and mucin 2 (MUC2) – in P3 enterocytes were similar to those in P0 enterocytes and showed a decreasing trend with repeated passage, as did other markers such as CYP2C9, CYP2C19, CYP3A4, UDP glucuronyltransferase 1A1 (UGT1A1), peptide transporter 1 (PEPT1), organic anion transporting polypeptide 2B1 (OATP2B1), and sodium glucose transport protein 1 (SGLT1). To evaluate differentiation potency, we then performed an immunofluorescence analysis of protein levels of several differentiation markers, including BCRP, villin, occludin and E-cadherin. All four of these markers were detected in P0 and P3 enterocytes, with microvilli observed on the surface of each enterocyte by scanning electron microscopy (SEM). Tight junctions were also observed in P0 enterocytes.

**Induction of CYP3A4 and P-gp expression by rifampicin and 1α,25-dihydroxyvitamin D₃ in enterocytes derived from human iPS cells**

We investigated whether CYP3A4 and P-gp expression was induced in P0 and P3 enterocytes (Fig. 5A,B). To assess the induction of CYP3A4 and P-gp expression, we used 30 μM rifampicin and 30 nM 1α,25-dihydroxyvitamin D₃, ligands of the pregnane X receptor and vitamin D receptor, respectively. In P0 and P3 enterocytes, CYP3A4 mRNA levels ranged from 1.2- to 2.5-fold and 4.1- to 41.5-fold higher following rifampicin and 1α,25-dihydroxyvitamin D₃ administration, respectively. Additionally, P-gp mRNA levels increased from 1.3- to 2.9-fold following rifampicin administration; however, levels showed only minimal increases following 1α,25-dihydroxyvitamin D₃ administration.

**Formation of 1'-hydroxy midazolam in enterocytes**

To evaluate CYP3A4 activity, enterocytes were incubated with 150 μM midazolam for 6 h and the formation of 1'-hydroxy midazolam, a specific metabolite of CYP3A4, was then measured. Metabolic activity was detectable in enterocytes derived from P0 to P3 ISCs, with levels of 1'-hydroxy midazolam formation comparable in P0 to P3 enterocytes (Fig. 5C).
Permeability of Rhodamine 123 and Hoechst 33342 across enterocyte monolayers

P-gp and BCRP play an important role in limiting the intestinal absorption and tissue distribution of various drugs, and both transporters are highly expressed in the apical membrane of small intestinal enterocytes. Therefore, we conducted bidirectional-transport assays in order to examine enterocyte layers on the cell-culture inserts (Fig. 5D–G).

In the P-gp assays, the apparent permeability coefficient ($P_{\text{app}}$) values of Rhodamine 123, a P-gp substrate, in the basal-to-apical direction were higher than those in the apical-to-basal direction in P0 and P3 enterocytes (Fig. 5D,E). The efflux ratio (ER), which is calculated from the directional ratio of $P_{\text{app}}$, was 14.6 and 7.2 for P0 and P3, respectively. Following the addition of verapamil, a P-gp inhibitor, the basal-to-apical $P_{\text{app}}$ values decreased, whereas the apical-to-basal $P_{\text{app}}$ values increased along with a reduction in ER values to 0.7 and 1.0 in P0 and P3 cells, respectively.

In BCRP assays, the $P_{\text{app}}$ values of the BCRP substrate Hoechst 33342 in the basal-to-apical direction were higher than those for the apical-to-basal direction in P0 and P3 enterocytes, with directional ERs of 11.6 and 9.8, respectively (Fig. 5F,G). Following addition of Ko143, a BCRP inhibitor, basal-to-apical $P_{\text{app}}$ values decreased, whereas apical-to-basal $P_{\text{app}}$ values increased along with reductions in ER to 1.5 and 1.4 in P0 and P3 cells, respectively. These results indicated that enterocytes derived from P0 and P3 membranes displayed P-gp and BCRP activity.
123 or Hoechst 33342 was derived from human iPS cells. The permeability of Rhodamine 123 and Hoechst 33342 across enterocyte monolayers. The efflux ratio (ER) for Rhodamine concentration (Fig. 2A), whereas KSR did not affect these levels observed decreased LGR5 mRNA levels with increased FBS differentiation (Ma and Liu, 2011). In the present study, we reported that FBS promotes murine corneal epithelial cell differentiation from iPS cells into hepatocyte-like cells compared with results observed in human iPS cells (Kabeya et al., 2018; Kodama et al., 2018; Zhang et al., 2018; Ozawa et al., 2015; Ogaki et al., 2015), important for maintaining intestinal stem cell stemness (Demitrack and Samuelson, 2016; Horvay et al., 2013), and VPA represents an activator of Notch signalling (Greenblatt et al., 2008, 2007). Notch signalling is also important for maintaining intestinal stem cell stemness (Fevr et al., 2007). In the present study, CHIR99021 was shown to dramatically increase LGR5 and CDX2 levels (Fig. 2C,D). Notch signalling is also important for maintaining intestinal stem cell stemness (Fevr et al., 2007). In the present study, CHIR99021 was shown to dramatically increase LGR5 and CDX2 levels (Fig. 2C,D), an effect was observed when VPA was administered in combination with CHIR99021, resulting in maintenance of LGR5, Ki67 and CDX2 levels up to P7 (Fig. 3A). Moreover, we verified protein levels of LGR5, Ki67, and CDX2 in P3 ISC (Fig. 3B).

We then evaluated whether repeatedly passaged ISCs have the potential to differentiate into functional enterocytes. We verified that the mRNA levels of several differentiation markers were maintained after several passages (Fig. 4A), and that their protein levels were still observed in P3 enterocytes (Fig. 4B). We also confirmed the presence of microvilli, which are characteristic structures of the intestine and tight junctions in P0 enterocytes (although they were not observed in P3 enterocytes). These results indicate that repeatedly-passaged ISCs maintain the potential to differentiate into enterocytes.

Pharmacokinetic evaluation was performed using enterocytes derived from non-passaged and repeatedly-passaged ISCs. We observed similar CYP3A4 activity in P0 to P3 enterocytes and confirmed the induced expression of CYP3A4 by 1α,25-dihydroxyvitamin D3 and rifampicin and of P-gp by rifampicin in P0 and P3 enterocytes (Fig. 5A–C). Furthermore, P-gp and BCRP protein levels of LGR5, Ki67, and CDX2 in P3 ISC (Fig. 3B).

DISCUSSION
The majority of drugs are absorbed in the small intestine, because the presence of villi and microvilli significantly increases the absorptive area. The intestine regulates the extent of orally administered drug absorption due to first-pass metabolism (Kharasch et al., 2003; Gibaldi et al., 1971). A number of metabolic enzymes, including CYP3A4 and transporters, such as P-gp and BCRP, are abundantly expressed in intestinal enterocytes, where they are heavily involved in drug metabolism and absorption. As a result, evaluation of intestinal pharmacokinetics is very important. Several studies have been undertaken with a focus on differentiating human iPS cells into intestinal cells (Negoro et al., 2018; Zhang et al., 2018; Ozawa et al., 2015; Ogaki et al., 2015), and the uses of intestinal cells have received increasing attention. We previously reported the usefulness of intestinal enterocytes derived from human iPS cells (Kabeya et al., 2018; Kodama et al., 2016; Iwao et al., 2015, 2014). Because enterocytes derived from human iPS cells express important metabolic enzymes and transporters, they serve as useful tools for evaluating pharmacokinetics in the intestinal tract. However, due to the time-and resource-intensive nature of experiments to differentiate human iPS cells into enterocytes, these issues must be addressed in order to increase the availability of these cells. In the present study, we established a new culture method for maintaining ISCs at the stage of differentiation from iPS cells and then evaluated whether the maintained ISCs retained the potential to differentiate into functional enterocytes.

We first examined the effect of FBS on ISC stemness. FBS is an essential supplement in cell culture and contains several hormone factors necessary for cell growth and proliferation. However, we previously reported that FBS promotes differentiation of human iPS cells into hepatocyte-like cells compared with results observed in the presence of KSR (Kondo et al., 2014). Notably, Ma et al. reported that FBS promotes murine corneal epithelial cell differentiation (Ma and Liu, 2011). In the present study, we observed decreased LGR5 mRNA levels with increased FBS concentration (Fig. 2A), whereas KSR did not affect these levels relative to FBS. Our findings suggest that KSR is suitable for culturing ISCs.

Maintenance of ISCs is difficult because the cells are located at the base of intestinal crypts and surrounded by a complex environment (i.e. the niche). Therefore, we used several factors (Table 1) involved in the niche to enhance or maintain ISC stemness. Among these factors, the most important was CHIR99021, an inhibitor of GSK-3β that reportedly improves stem cell properties by activating WNT signalling, which is critical for the maintenance of ISC stemness (Fevr et al., 2007). In the present study, CHIR99021 was shown to dramatically increase LGR5 and CDX2 levels (Fig. 2C,D). Notch signalling is also important for maintaining intestinal stem cell stemness (Demitrack and Samuelson, 2016; Horvay et al., 2013), and VPA represents an activator of Notch signalling (Greenblatt et al., 2008, 2007).

Although VPA did not clearly affect LGR5 or CDX2 levels (Fig. 2C,D), an effect was observed when VPA was administered in combination with CHIR99021, resulting in maintenance of LGR5, Ki67 and CDX2 levels up to P7 (Fig. 3A). Moreover, we verified protein levels of LGR5, Ki67, and CDX2 in P3 ISC (Fig. 3B).

We then evaluated whether repeatedly passaged ISCs have the potential to differentiate into functional enterocytes. We verified that the mRNA levels of several differentiation markers were maintained after several passages (Fig. 4A), and that their protein levels were still observed in P3 enterocytes (Fig. 4B). We also confirmed the presence of microvilli, which are characteristic structures of the intestine and tight junctions in P0 enterocytes (although they were not observed in P3 enterocytes). These results indicate that repeatedly-passaged ISCs maintain the potential to differentiate into enterocytes.
Table 1. Summary of compounds used in intestinal stem cell culture

| Compound          | Function                  | Concentration | References                  |
|-------------------|---------------------------|---------------|-----------------------------|
| A83-01            | ALK4,5,7 inhibitor        | 1.5 μM        | Sato et al., 2011           |
| SB202190          | p38 inhibitor             | 10 μM         | Sato et al., 2011           |
| Wnt3a             | Wnt agonist               | 100 ng/ml     | Krausova and Korinek, 2014  |
| CHIR99021         | GSK-3β inhibitor          | 10 μM         | Naujok et al., 2014         |
| Nicotinamide      | PARP-1 inhibitor          | 10 mM         | Sato et al., 2011           |
| N-acetylcysteine  | ROS scavenger, inhibition of apoptosis | 1 nm | Sato et al., 2011 |
| Valproic acid     | HDAC inhibitor            | 1 mM          | Greenblatt et al., 2007     |
| R-spondin 1       | Wnt agonist               | 100 ng/ml     | Krausova and Korinek, 2014  |
| Noggin            | BMP inhibitor             | 100 ng/ml     | Haramis et al., 2004        |
| EGF               | Growth factor             | 100 ng/ml     | Suzuki et al., 2010         |
| FGF2              | Growth factor             | 30 ng/ml      | Danopoulos et al., 2017, Lotz et al., 2013 |
| N-2 Supplement (100X) | Serum-free supplement      | 1X            | Sato et al., 2011           |
| B-27 Supplement (50X) | Serum-free supplement      | 1X            | Sato et al., 2011           |

(FIG. 5D–G). Several studies have reported that metabolic enzymes and transporters, such as CYP3A4 and P-gp, in the small intestine work in coordination to reduce the intracellular concentration of drugs and, therefore, drug–drug interactions (DDIs) based on this interplay (Li et al., 2017; Zhang and Benet, 2001; Benet et al., 1999). Because few allow the simultaneous evaluation of metabolic enzymes and transporters, iPS-cell-derived enterocytes represent a promising model for evaluating the pharmacokinetics of the human small intestine and might better reflect enterocyte function than other model cells like Caco-2, HT-29, and LS-180 (Yamaura et al., 2016; Angelis and Turco, 2011; Walter et al., 1996). However, the activity of the CYP3A4 enzyme in enterocytes derived from human iPSCs was low compared to that in human primary enterocytes (Li et al., 2018). Although several studies have already reported methods for differentiating human iPSC cells into enterocytes, these cells also reportedly express low levels of CYP3A4 enzyme compared with primary enterocytes. Therefore, further intestinal enterocyte maturation studies are required. Nonetheless, iPS-cell-derived enterocytes are considered useful as an in vitro model for the evaluation of intestinal pharmacokinetics and drug screening tool, because they are of non-carcinogenic origin and express more or equivalent intestinal markers, transporters and enzymes than Caco-2 cells. In addition, they show metabolic enzyme and transport activity and inducibility, and morphological feature such as microvilli. Induction of CYP3A by rifampicin has been reported in human intestinal enterocytes, while Caco-2 cells show no inducibility (Alba et al., 2005; Glaeser et al., 2005; Kolars et al., 1992). Recently, several reports have suggested that intestinal cells can be maintained and cultured for a long period of time (Moorefield et al., 2018; Zhang et al., 2018; Wang et al., 2015). However, to the best of our knowledge, there are few reports evaluating intestinal pharmacokinetics in detail, including enzyme metabolic activity, inducibility and transport activity by using enterocytes derived from maintained ISCs. In the present study, CYP3A4-specific metabolic activity, transporter activity and inducibility of CYP3A4 and P-gp expression were observed in P0 and P3 enterocytes, with no notable differences between P0 and P3, except for a reduction in CYP3A4 inducibility. These results thus suggest that enterocytes, at least up to P3, are functional and suitable for pharmacokinetic evaluation. Further, they support the use of this method to reduce differentiation time and cost, as well as to supply sufficient quantities of enterocytes. For enterocytes after P3, further improvement of the maintenance of stem cells is necessary because the mRNA expression levels of differentiation markers were decreased relative to P0. In addition, the maintained ISCs after P3 showed cell detachment during the differentiation period, making it difficult to perform intestinal pharmacokinetics study. Furthermore, although we evaluated only CYP3A4 activity in the present study, other drug metabolizing enzymes such as carboxylesterase (CES), UDP-glucuronosyltransferase (UGT), sulfotransferase (SULT), N-acetyl transferases (NAT), flavin-containing monoxygenase (FMO) and monoamine oxidase (MAO) are also expressed in the small intestine and are involved in the intestinal metabolizing pathway. In our previous study, we reported that the patterns of CES expression and activity in the enterocytes were similar to those in the small intestine, and also reported activity of other CYP isoforms, UGT, and SULT (Kabeya et al., 2018, 2017). However, in addition to these enzymes, we also need to investigate expression and activity of enzymes such as NAT, FMO and MAO. In summary, we established a method for culturing ISCs while maintaining cellular stemness. Our results showed that the differentiated enterocytes from the non-maintained and maintained ISCs demonstrated appropriate pharmacokinetic function. This culture method can potentially reduce the time and cost associated with generating large quantities of iPS-cell-derived enterocytes.

MATERIALS AND METHODS

Materials

Materials were purchased from the following sources: fibroblast growth factor (FGF) 2 and activin A (PeproTech, Rocky Hill, NJ, USA); iMatrix-511 (Nippi, Tokyo, Japan); CHIR99021 and Y-27632 (Focus Biomolecules, Plymouth Meeting, PA, USA); KSR, DMEM, DMEM/F-12, Advanced DMEM/F-12, N2 supplement and B27 serum-free supplement (Thermo Fisher Scientific, Waltham, MA, USA); FBS (Nichirei Biosciences, Tokyo, Japan); R-spondin 1, Noggin and epidermal growth factor (EGF) (GenScript, Piscataway, NJ, USA); N-acetylcysteine (Sigma-Aldrich, St Louis, MO, USA); 8-BrcAMP (Enzo Life Sciences, New York, NY, USA); PD98059 and A-83-01 (AdooQ BIOSCIENCE, Irvine, CA, USA); 5-aza-2′- deoxycytidine (Chem-impex International, Wood Dale, IL, USA); L-glutamine, minimum essential medium nonessential amino acid solution (NEAA), penicillin-streptomycin solution, valproic acid and nicotinamide (Wako Pure Chemical Industries, Osaka, Japan); and total RNA from human small intestine samples (5 donors; BioChain Institute, Newark, CA, USA). All other reagents were of the highest quality available, including SB 202190 (ChemSciene, Monmouth Junction, NJ, USA) and WNT3a (ATGen Co. Ltd., Gyeonggi-do, South Korea).

Human iPS cell culture

Human iPS cells (Windy #51) were kindly provided by Dr A. Umezawa of the National Institute for Child Health and Development (Tokyo, Japan). Human iPS cells were maintained on a feeder layer of mitomycin C-treated mouse embryonic fibroblasts with DMEM/F-12 supplemented with 20% KSR, 2 mM L-glutamine, 1% NEAA, 0.1 mM 2-mercaptoethanol and 5 ng/ml FGF2. The medium was changed daily. Since mouse embryonic fibroblasts cannot survive for a long period due to mitomycin C treatment,
they were considered to be dead in the process of repeated passage of ISCs or differentiation period into enterocytes.

**Differentiation of human iPS cells into ISCs**

Human iPS cells were differentiated into endoderm by the addition of 100 ng/ml activin A for 3 days and then differentiated into ISCs by 250 ng/ml FGF2 for 4 days. We established the cells as ISCs on day 7 after the initiation of differentiation. The schematic outline of the protocol for the differentiation of human iPS cells into ISCs is shown in Fig. S1.

**Evaluation of the effect of FBS and KSR on intestinal stemness and lineage and ISC lineage**

After differentiation of the human iPS cells into ISCs, we passaged the ISCs on iMatrix-511-coated 24-well plates and cultured the cells with different concentrations of FBS or KSR for 3 days. After completion of the culture, the cells were collected, and LGR5 and CDX2 mRNA levels were measured.

**Evaluation of the effect of CHIR99021 and VPA on intestinal stemness and ISC growth and lineage**

After the differentiation of human iPS cells into ISCs, we passaged the ISCs on iMatrix-511-coated 24-well plates and cultured the cells with different concentrations of CHIR99021 or VPA for 3 days. After completion of the culture, the cells were collected, and LGR5 and CDX2 mRNA levels were measured, and the number of cells was counted. Controls were treated with dimethyl sulfoxide.

**Culture of ISCs derived from human iPS cells**

Human iPS cells were induced into ISCs, and the cells were passaged on iMatrix-511-coated dishes in culture medium (Advanced DMEM/F-12 supplemented with several factors shown in Table 1). The effect of these supplements is shown in Table 1. For cell passage, Y-27632 (10 μM) was added in the first 24 h, and the medium was subsequently changed every 2–3 days.

**Differentiation of ISCs into enterocytes**

ISCs (P0–P7) were differentiated into enterocytes based on the methods described in our previous report. In brief, the maintained ISCs were passaged on iMatrix-511-coated 24-well plates or cell-culture inserts and cultured with medium containing EGF and 8-Br-cAMP. After 7 days of differentiation, PD98059, 5-aza-2′,25-dihydroxyvitamin D3 or 30 μM rifampicin, known inducers of CYP3A4 and P-gp induction assays

| Gene name | Forward primer sequence (5′→3′) | Reverse primer sequence (5′→3′) |
|-----------|-------------------------------|-------------------------------|
| LGR5      | AGAAGGCCACGCTATGACGAC         | CAGCGTTACAGACACACTGTCCT       |
| CDX2      | AGCCAGATCCTGCTAAGGGCT         | TGGACAGGTGTTTCCCTCCTTC        |
| Ki67      | GATCCTGTTGTTGCTATGACGAC       | ACCCGCTTCTTTTATGAGT           |
| Villin    | GATCCTGTTGTTGCTATGACGAC       | TATGCTTGTGTTGCTATGACGAC       |
| P-gp      | GTGACATGACCTGCTATGACGAC       | GTGCTTGTGTTGCTATGACGAC        |
| BCRP      | GTGACATGACCTGCTATGACGAC       | GTGCTTGTGTTGCTATGACGAC        |
| CYP3A4    | GTGACATGACCTGCTATGACGAC       | GTGCTTGTGTTGCTATGACGAC        |
| UGT1A     | GTGACATGACCTGCTATGACGAC       | GTGCTTGTGTTGCTATGACGAC        |
| MUC2      | GTGACATGACCTGCTATGACGAC       | GTGCTTGTGTTGCTATGACGAC        |
| OATP2B1   | GTGACATGACCTGCTATGACGAC       | GTGCTTGTGTTGCTATGACGAC        |
| Sucrase-isomaltase | GTGACATGACCTGCTATGACGAC   | GTGCTTGTGTTGCTATGACGAC        |
| PEPT1     | GTGACATGACCTGCTATGACGAC       | GTGCTTGTGTTGCTATGACGAC        |
| CYP2C9    | GTGACATGACCTGCTATGACGAC       | GTGCTTGTGTTGCTATGACGAC        |
| CYP2C19   | GTGACATGACCTGCTATGACGAC       | GTGCTTGTGTTGCTATGACGAC        |
| SGLT1     | GTGACATGACCTGCTATGACGAC       | GTGCTTGTGTTGCTATGACGAC        |
| HPRT1     | GTGACATGACCTGCTATGACGAC       | GTGCTTGTGTTGCTATGACGAC        |

**Immunofluorescence staining**

IPSCs (P0 and P3) and enterocytes differentiated from ISCs (P0 and P3) were washed with phosphate-buffered saline (PBS) and fixed and permeabilized with cold methanol for 5 min at 4°C. After washing with PBS, the cells were blocked in PBS containing 0.5% FBS for 20 min at room temperature. After the blocking step, the cells were incubated with primary antibody at room temperature for 60 min and subsequently incubated overnight at 4°C. The cells were then washed with PBS and incubated with secondary antibody for 60 min at room temperature. The antibodies used and their dilutions are shown in Table 3. After washing with PBS, the cells were incubated with 1 μg/ml of 4’,6-diamidino-2-phenylindole for 5 min at room temperature, followed by washing with PBS. The cells were analysed using an ECLIPSE Ti-S microscope (Nikon, Tokyo, Japan).

**Transmission electron microscopy (TEM)**

Enterocytes differentiated from ISCs (P0 and P3) were fixed with 2.5% glutaraldehyde overnight at 4°C and post-fixed with 1% osmium tetroxide for 2 h at 4°C. Subsequently, the samples were dehydrated with ethanol and embedded in resin. Then, embedded samples were cut into 0.1-μm sections, which were stained with uranyl acetate and observed using a Hitachi H7600 transmission electron microscope (JEOL, Tokyo, Japan).

**Scanning electron microscopy (SEM)**

Enterocytes differentiated from maintained ISCs (P0 and P3) were fixed and dehydrated as described for TEM preparations. Subsequently, the samples were dehydrated with ethanol and critical point drying was performed using a CPD 300 (Leica, Wetzlar, Germany). Osmium coating was performed with a plasma coater (OPC80A, Filgen, Nagoya, Japan). Images of the fractured surface of the freeze-dried samples were obtained using an S-4800 scanning electron microscope (Hitachi, Tokyo, Japan).

**CYP3A4 and P-gp induction assays**

Enterocytes differentiated from ISCs (P0 and P3) were treated with 30 nM 1α,25-dihydroxyvitamin D3 or 30 μM rifampicin, known inducers of CYP3A4 and P-gp expression, respectively, for 48 h. Controls were treated with dimethyl sulfoxide. Inducibility was evaluated by quantifying the mRNA levels of CYP3A4 and P-gp.
**Determination of CYP3A4 activity**

Enterocytes differentiated from iSCs (P0 and P3) were incubated with Hanks’ balanced salt solution (HBSS) containing 150 µM midazolam for 6 h at 37°C. After incubation, 200 µl of the reaction medium was collected and added to 200 µl of ice-cold acetonitrile containing 3.6 µM chlorpropamide as an internal standard. Enterocytes were lysed with 150 µl of 1 M NaOH, with an equal amount of 1 M HCl added for neutralization. The protein content was then measured with a Pierce BCA protein assay kit (Thermo Fisher Scientific). The formation of 1’-hydroxylated metabolites was measured using ultra-performance liquid chromatography–tandem mass spectrometry (UPLC–MS/MS) system (ACQUITY UPLC® System and Micromass® Quattro Premier® XE, Waters, Milford, MA, USA) equipped with an XBridge BEH C18 column (2.1×50 mm, 3.5 µm; Waters). Samples were centrifuged at 20,600 g for 5 min at 4°C. Ten microliters of supernatant were injected into the UPLC-MS/MS system. The mobile phase comprised 10 mM ammonium formate (A) and acetonitrile (B), and the sample and column temperatures were 4°C and 54°C, respectively. The following gradient program was used: 0–0.5 min: 5% B, 0.5–2.0 min: 5–95% B (linear), 2.1–3.5 min: 95% B, 3.6–5.4 min: 95–5% B (linear). The flow rate was set at 0.25 ml/min (except for 2.1–3.5 min; 0.55 ml/min). Multiple-reaction monitoring in positive ion electrospray was used for the detection of 1’-hydroxy midazolam derivatives (m/z 342.03→168.40). All data were analysed with Mass Lynx 4.1 software (Waters).

**Transport study of Rhodamine 123**

iSCs (P0 and P3) were seeded on the inserts of Corning Transwells (Corning, NY, USA) and cultured for 19 days. After pre-incubation with HBSS (pH 7.4) for 20 min, Rhodamine 123 solution was added to the apical side to a final concentration of 1 µM and then drawn out of the basal side at 30-min intervals for up to 120 min at 37°C in the presence or absence of 30 µM of the P-gp inhibitor verapamil. An equal volume of HBSS was added to the basal compartment at time zero and the donor-compartment concentration at time zero and time was calculated. The ER was defined as $P_{app}$ (basal-to-apical)/$P_{app}$ (apical-to-basal).

**Transport study of Hoechst 33342**

We used the same methods as those described for Rhodamine 123 transport to analyse the transport of Hoechst 33342. Hoechst 33342 was used at a final concentration of 20 µM. Ko143 was used as BCRP inhibitor at a final concentration of 1 µM. Fluorescence was measured at 352 nm excitation and 461 nm emission, and $P_{app}$ was calculated.

**Statistical analysis**

Statistical significance was assessed with Student’s t-tests for analysis of variance, followed by Duncan’s new multiple range test. Data are represented as the mean±s.d. from three biological replicates. Significant differences are indicated by $P$-values listed in the figure legends. A $P$-value <0.05 was considered to be statistically significant.

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**Competing interests**

The authors declare no competing or financial interests.

**Author contributions**

Conceptualization: S.K., T.H., T.I., T.M.; Methodology: S.K., T.H., T.I., T.M.; Validation: S.K., S.M., T.I., T.M.; Formal analysis: S.K., S.M.; Investigation: S.K., S.M., T.I., T.M.; Data curation: S.K.; Writing - original draft: S.K.; Writing - review & editing: S.K., T.H., T.I., T.M.; Supervision: T.I., T.M.; Funding acquisition: T.I., T.M.

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**Supplementary information**

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**References**

Alba, T., Susa, M., Fukumori, S. and Hashimoto, Y. (2005). The effects of culture conditions on CYP3A4 and MDR1 mRNA induction by 1α,25-dihydroxyvitamin D(3) in human intestinal cell lines, Caco-2 and LS180. *Drug Metab. Pharmacokinet.* 20, 268-274. doi:10.1013/dmpk.20.268

Angelis, I. D. and Turco, L. (2011). Caco-2 cells as a model for intestinal absorption. *Curr. Protoc. Toxicol.* 47, 20.1.1-20.1.15. doi:10.1002/0471140856.ch20_1.6.

Benet, L. Z., Izumi, T., Zhang, Y., Silverman, J. A. and Wacher, V. J. (1999). Intestinal MDR transport proteins and P-450 enzymes as barriers to oral drug delivery. *J. Control. Release* 62, 25-31. doi:10.1016/S0168-3656(99)00034-6

Capeling, M. M., Czerwinski, M., Huang, S., Tsai, Y. H., Wu, A., Nagy, M. S., Julian, B., Sundaram, N., Song, Y., Han, W. M. et al. (2019). Nonadhesive alginate hydrogels support growth of pluripotent stem cell-derived intestinal organoids. *Stem Cell Reports.* 12, 381-394. doi:10.1016/j.stemcr.2018.12.001

DanaPoulos, S., Schlieve, C., Grikseit, T. C. and Al Alam, D. (2017). Fibroblast growth factors in the gastrointestinal tract: twists and turns. *Dev. Dyn.* 246, 344-352. doi:10.1002/dvdy.24491

Demitrack, E. S. and Samuelson, L. C. (2016). Notch regulation of gastrointestinal stem cells. *J. Physiol.* 594, 4791-4803. doi:10.1113/jphysiol.2016.137443

Fernando, E. H., Dicay, M., Stahl, M., Gordon, M. H., Vegso, A., Baggio, C., Alston, L., Lopes, F., Baker, K., Hirotta, S. et al. (2017). A simple, cost-effective method for generating murine colonic 3D enteroids and 2D monolayers for studies of primary epithelial cell function. *Am. J. Physiol. Gastrointest. Liver Physiol.* 313, 467-475. doi:10.1152/ajpgi.00152.2017

Fevr, T., Robine, S., Louvard, D. and Huelsken, J. (2007). Wnt/beta-catenin is essential for intestinal homeostasis and maintenance of Intestinal Stem Cells. *Dev. Biol.* 307, 7551-7559. doi:10.1102/MCB.01344-07

Foulke-Abel, J., In, J., Kovbasnjuk, O., Zachos, N. C., Ettayebi, K., Blutt, S. E., Hyser, J. M., Zeng, X. L., Crawford, S. E., Broughman, J. R. et al. (2014). Human enteroids as an ex vivo model of host-pathogen interactions in the...
gastrointestinal tract. Exp. Biol. Med. 239, 1124-1134. doi:10.1177/1535370214529398

Gibaldi, M., Boyes, R. N. and Feldman, S. (1971). Influence of first-pass effect on availability of drugs. J. Pharm. Sci. 60, 1338-1340. doi:10.1002/js.2620600909

Glazer, N. J., Friend, S. H., Eichelbaum, M. and Fromm, M. F. (2005). Influence of rifampicin on the expression and function of human intestinal cytochrome P450 enzymes. Br. J. Clin. Pharmacol. 59, 199-206. doi:10.1111/j.1365-2125.2004.02265.x

Greenblatt, D. Y., Vaccaro, A. M., Jaskula-Sztul, R., Ling, N., Haymart, M., Kurosu, T. I., Nobl, J., and Chao, H. (2007). Valproic acid activates notch-1 signaling and regulates the neuroendocrine phenotype in carcinoid cancer cells. Oncologist 12, 942-951. doi:10.1634/oncologist.2-942

Greenblatt, D. Y., Cayo, M. A., Adler, J. T., Ling, N., Haymart, M. R., Kummimaliyaan, M. and Chen, H. (2008). Valproic acid activates Notch1 signaling and regulates expression of morphological and carcinoid cancer cell. Ann. Surg. 247, 1036-1040. doi:10.1097/SLA.0b013e3181758bde

Grossmann, J., Mohr, S., Lapentina, E. G., Fiocchi, C. and Levine, A. D. (2016). Real-time quantitative polymerase chain reaction for MDR1, MRP1, MRP2, and CYP3A mRNA levels in Caco-2 cell lines, human duodenal enterocytes, normal colorectal tissues, and colorectal adenocarcinomas. Drug Metab. Dispos. 44, 3-6. doi:10.1124/dmd.049064.

Haramis, A. P., Begthel, H., van den Born, M., van Es, J., Jonkheer, S., Offerhaus, G. J. and Clevers, H. (2004). De novo crypt formation and juvenile polyposis on BMP inhibition in mouse intestine. Science 303, 1684-1686. doi:10.1126/science.1093589.

Harwood, M. D., Achour, B., Neuhoff, S., Russell, M. R., Carlson, G., Warhurst, G. and Amin, R. H. (2016). In vitro and in vivo extrapolation scaling factors for intestinal P-glycoprotein and breast cancer resistance protein: Part I: a cross- laboratory comparison of transporter protein abundances and relative expression factors in human intestine and Caco-2 cells. Drug Metab. Dispos. 44, 297-307. doi:10.1124/dmd.115.067637.

Horvay, K. and Abud, H. E. (2013). Regulation of ISC's by Wnt and Notch Signalling. Adv. Exp. Med. Biol. 786, 175-186.

Iwao, T., Toyota, M., Miyagawa, Y., Okita, H., Kiyokawa, N., Akutsu, H., Umezawa, A., Nagata, K. and Matsunaga, T. (2014). Characterization of human induced pluripotent stem cells into functional enterocyte-like cells using a simple method. Drug Metab. Pharmacokinet. 29, 44-51. doi:10.2133/dmpk.DMPK-13-RG-005

Iwao, T., Kodama, N., Kondo, Y., Kabeya, T., Nakamura, K., Horikawa, T., Niwa, T., Kurose, K. and Matsunaga, T. (2010). Generation of human intestinal epithelium with phenotypic functions from induced pluripotent stem cells using small-molecule compounds. Drug Metab. Dispos. 40, 603-610. doi:10.1124/dmd.109.036804.

Jung, P., Sato, T., Merlos-Suárez, A., Barriga, F. M., Iglésias, M., Rosselli, D., Auer, M., Höchli, M., Blasco, M. A., Sancho, E. et al. (2011). Isolation and in vitro expansion of human colonic stem cells. Nat. Med. 17, 1225-1227. doi:10.1038/nm.2298.

Kabeya, T., Matsunaga, M., Iwao, T., Hosokawa, M. and Matsunaga, T. (2017). Functional analysis of carboxylesterase in human induced pluripotent stem cell-derived enterocytes. Biochem. Biophys. Res. Commun. 486, 143-148. doi:10.1016/j.bbrc.2016.03.014.

Kabeya, T., Qiu, S., Hibino, M., Nagasaki, M., Kodama, N., Iwao, T. and Matsunaga, T. (2018). Cyclic AMP signalling promotes the differentiation of human induced pluripotent stem cells into intestinal epithelial cells. Drug Metab. Dispos. 46, 414-419. doi:10.1124/dmd.117.092123.

Kharasch, E. D., Hoffer, C., Whittington, D. and Sheffels, P. (2017). Stained levels of GF2 maintain undiffrated stem cell cultures with biweekly feeding. PLoS ONE 8, e56629. doi:10.1371/journal.pone.0056629.

Krausova, M. and Korinek, V. (2017). Intestinal stem cell niche: the extracellular matrix and cellular components. Stem Cells Int. 2017, 970385. doi:10.1155/2017/970385.

Lotz, S., Goderie, S., Tokas, N., Hirsch, S. E., Ahmad, F., Comeo, B., Le, S., Banerjee, A., Kane, R. S., Stern, J. H. et al. (2013). Sustained levels of GF2 maintain undifferentiated stem cell cultures with biweekly feeding. PLoS ONE 8, e56629. doi:10.1371/journal.pone.0056629.

Li, M., de Graaf, I. A., van de Steeg, E., de Jager, M. H. and Groothuis, G. M. (2017). The consequence of regional gradients of P-gp and CYP3A4 for drug-drug interactions by P-gp inhibitors and the P-gp/CYP3A4 interplay in the human intestine ex vivo. Toxicol. in Vitro. 40, 26-33. doi:10.1016/j.tiv.2016.12.002.

Li, A. P., Alam, N., Amaral, K., Ho, M. D., Loretz, C., Mitchell, W. and Yang, Q. (2018). Cryopreserved human intestinal mucosal epithelium: a novel in vitro experimental system for the evaluation of enteric drug metabolism, cytochrome P450 induction, and enterotoxigenicity. Drug Metab. Dispos. 46, 1562-1571. doi:10.1124/dmd.118.082875.

Lotz, S., Goderie, S., Tokas, N., Hirsch, S. E., Ahmad, F., Comeo, B., Le, S., Banerjee, A., Kane, R. S., Stern, J. H. et al. (2013). Sustained levels of GF2 maintain undiffrated stem cell cultures with biweekly feeding. PLoS ONE 8, e56629. doi:10.1371/journal.pone.0056629.
human microphysiology systems: intestine, liver, kidney proximal tubule, blood-brain barrier and skeletal muscle. Sci. Rep. 7, 42296. doi:10.1038/srep42296

Walter, E., Janich, S., Roessler, B. J., Hilfinger, J. M. and Amidon, G. L. (1996). HT29-MTX/Caco-2 cocultures as an in vitro model for the intestinal epithelium: in vitro-in vivo correlation with permeability data from rats and humans. J. Pharm. Sci. 85, 1070-1076. doi:10.1021/js960110x

Wang, X., Yamamoto, Y., Wilson, L. H., Zhang, T., Howitt, B. E., Farrow, M. A., Kern, F., Ning, G., Hong, Y., Khor, C. C. et al. (2015). Cloning and variation of ground state intestinal stem cells. Nature 11, 173-178. doi:10.1038/nature14484

Yamaura, Y., Chapron, B. D., Wang, Z., Himmelfarb, J. and Thummel, K. E. (2016). Functional comparison of human colonic carcinoma cell lines and primary small intestinal epithelial cells for investigations of intestinal drug permeability and first-pass metabolism. Drug Metab. Dispos. 44, 329-335. doi:10.1124/dmd.115.068429

Zanger, U. M. and Schwab, M. (2013). Cytochrome P450 enzymes in drug metabolism: regulation of gene expression, enzyme activities, and impact of genetic variation. Pharmacol. Ther. 138, 103-141. doi:10.1016/j.pharmthera.2012.12.007

Zhang, Y. and Benet, L. Z. (2001). The gut as a barrier to drug absorption: combined role of cytochrome P450 3A and P-glycoprotein. Clin. Pharmacokinet. 40, 159-168. doi:10.2165/00003088-200140030-00002

Zhang, R. R., Koido, M., Tadokoro, T., Ouchi, R., Matsuno, T., Ueno, Y., Sekine, K., Takebe, T. and Taniguchi, H. (2018). Human iPSC-derived posterior gut progenitors are expandable and capable of forming gut and liver organoids. Stem Cell Reports. 10, 780-793. doi:10.1016/j.stemcr.2018.01.006