

Vinblastine treatment decreases the undifferentiated cell contamination of human iPSC-derived intestinal epithelial-like cells

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Human induced pluripotent stem cell-derived intestinal epithelial cells (hiPSC-IECs) are expected to be utilized in regenerative medicine. To perform a safe transplantation without the risk of tumor formation, residual undifferentiated hiPSCs must be removed from hiPSC-IECs. In this study, we examined whether vinblastine (a multiple drug resistance 1 [MDR1] substrate) could remove residual undifferentiated hiPSCs in hiPSC-IECs and attempted to generate hiPSC-IECs applicable to transplantation medicine. We found that the expression levels of pluripotent markers were largely decreased and those of intestinal markers were increased by vinblastine treatment. The treatment of undifferentiated hiPSCs with vinblastine significantly decreased their viability. These results suggested that undifferentiated hiPSCs can be eliminated from hiPSC-IECs by vinblastine treatment. We hypothesized that MDR1-negative cells (such as undifferentiated hiPSCs) die upon vinblastine treatment because they are unable to excrete vinblastine. As expected, the cell viability of MDR1-knockout hiPSC-IECs was significantly decreased by vinblastine treatment. Furthermore, teratomas were formed by subcutaneous transplantation of hiPSC-IECs mixed with undifferentiated hiPSCs into mice, but they were not observed when the transplanted cells were pre-treated with vinblastine. Vinblastine-treated hiPSC-IECs would be an effective cell source for safe regenerative medicine.

INTRODUCTION

Intestinal epithelial cells (IECs), which are generated from human pluripotent stem cells, including human induced pluripotent stem cells (hiPSCs), are expected to be used in drug discovery and regenerative medicine. Because it is difficult to obtain and culture primary human IECs, many researchers have tried to establish a differentiation method for generating IECs from hiPSCs.¹⁻⁷ Although the application of hiPSC-derived intestinal epithelial cells (hiPSC-IECs) to pharmacokinetic studies has been attempted, these cells have not been sufficiently applied to regenerative medicine.

The need for intestinal regenerative medicine has been increasing in recent years. In inflammatory bowel diseases, such as ulcerative colitis and Crohn's disease, it is known that chronic inflammation by unknown cause develops. It is difficult to cure these diseases due to the residual refractory ulcer site. The number of patients with these diseases is estimated to be about 5 million worldwide,⁸ and thus the development of new intestinal regenerative medicine technologies is imperative. Although cell therapy using biopsy-derived intestinal organoids has been developed,⁹⁻¹² ethical and immunity issues would be a hurdle to widespread use of this approach.

In previous studies, we developed a method for generating functional IECs from hiPSCs.¹³⁻¹⁷ Since we developed these cells for pharmaceutical use, their application to regenerative medicine has not been fully verified. Therefore, in order to meet the recent needs for intestinal regenerative medicine, we decided to investigate the usefulness of hiPSC-IECs in regenerative medicine. As an initial part of this investigation, we needed to address the risk of teratoma formation that arises from the transplantation of any hiPSC derivatives. In order to avoid teratoma formation, it is essential to remove residual undifferentiated cells in hiPSC-IECs.

Here, we focused on multiple drug resistance 1 (MDR1) (P-glycoprotein [P-gp]) to eliminate residual undifferentiated cells in hiPSC-IECs. MDR1 is a drug excretion transporter that is highly expressed in IECs¹⁸ but is hardly expressed in undifferentiated hiPSCs.¹⁹ Many drugs, including anticancer drugs, have been reported as substrates for MDR1.²⁰⁻²³ Because MDR1 is selectively expressed in

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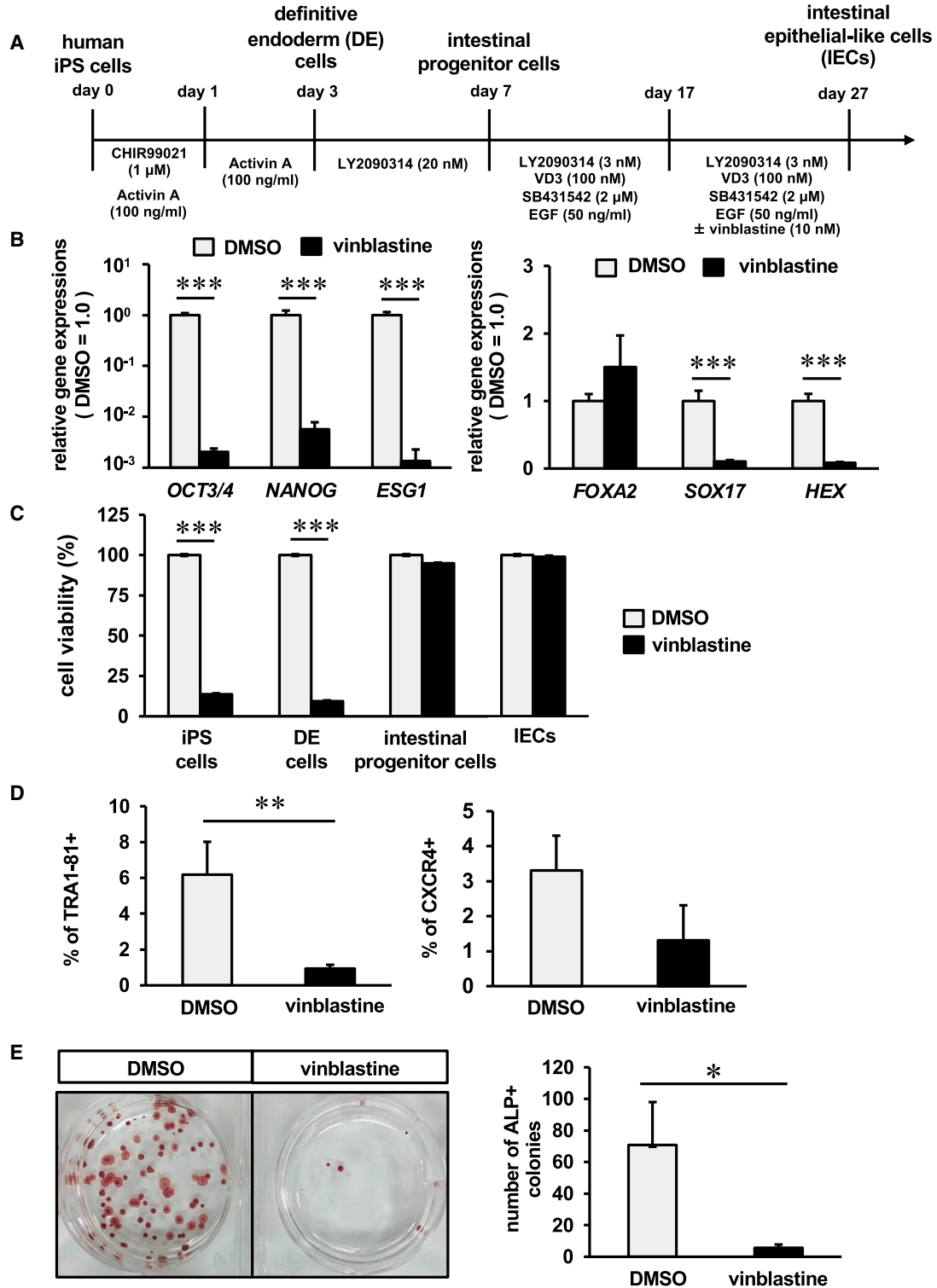


Figure 1. Removal of residual undifferentiated hiPSCs by vinblastine treatment

(A) The schematic overview shows the protocol of intestinal differentiation of hiPSCs. Details are described in materials and methods section. (B) The hiPSC-IECs (YOW) were treated with 10 nM vinblastine during the intestinal maturation step (from day 17 to 27). The gene expression levels of pluripotent markers (*NANOG*, *OCT3/4*, and *ESG1*) and

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IECs, it is expected that undifferentiated hiPSCs could be eliminated by using a cytotoxic MDR1 substrate.

In this study, we used vinblastine as a candidate compound and examined whether it could eliminate residual undifferentiated cells in hiPSC-IECs. Moreover, we examined whether hiPSC-IECs could be safely transplanted after treatment with vinblastine.

RESULTS

Vinblastine eliminates undifferentiated cells from hiPSC-IECs

Vinblastine, a substrate for MDR1, has been reported to have cytotoxicity.²⁴ To examine the effect of vinblastine on the functions of hiPSC-IECs, hiPSCs were differentiated into IECs (Figure 1A). Gene expression analysis was performed on hiPSC-IECs treated with vinblastine during the intestinal maturation step (from day 17 to 27) (Figure 1B). Vinblastine treatment significantly reduced the gene expression levels of undifferentiated markers (*octamer-binding transcription factor 3/4* [OCT3/4], *Nanog homeobox* [NANOG], and *embryonal stem cell-specific gene 1* [ESG1]) and definitive endoderm (DE) markers (*SRY-box transcription factor 17* [SOX17] and *hematopoietically expressed homeobox* [HEX]). To investigate the effect of vinblastine treatment on undifferentiated cells, hiPSCs, DE cells, intestinal progenitor cells, or IECs, the cell viability was measured 3 days after vinblastine treatment. Vinblastine treatment did not alter the cell viability of intestinal progenitor cells and IECs. On the other hand, vinblastine treatment significantly reduced the cell viability of undifferentiated hiPSCs and DE cells (Figure 1C). These results suggest that vinblastine treatment could remove residual undifferentiated cells and DE cells in hiPSC-IECs. The percentage of cells expressing undifferentiated cell marker, tumor-related antigen-1-81 (TRA1-81), and DE cell marker, C-X-C motif chemokine receptor 4 (CXCR4), in vinblastine-treated hiPSC-IECs was measured using flow cytometry. The percentage of TRA1-81-positive cells decreased as the differentiation of hiPSC-IECs progressed (Figure S1). Vinblastine treatment reduced the percentage of TRA1-81-positive cells remaining in hiPSC-IECs from 6.1% to 0.9%. Furthermore, vinblastine treatment reduced the percentage of CXCR4-positive cells from 3.3% to 1.3% (Figure 1D). A colony formation test was performed to evaluate the contamination rate of functional undifferentiated cells in hiPSC-IECs. The hiPSC-IECs treated with or without vinblastine were cultured under conditions suitable for undifferentiated hiPSCs, and the number of alkaline phosphatase (ALP)-positive colonies was counted. The number of ALP-positive colonies was significantly reduced by vinblastine treatment (Figure 1E). These results indi-

cated that the residual undifferentiated hiPSCs in hiPSC-IECs were eliminated by vinblastine treatment.

Vinblastine-induced cytotoxicity is dependent on MDR1 expression

Vinblastine is a substrate for MDR1 and exhibits cytotoxicity in a concentration-dependent manner (Figure S2).²⁴ Therefore, if vinblastine is excreted extracellularly by MDR1, cytotoxicity due to vinblastine would be avoided. We investigated whether the elimination of undifferentiated hiPSCs by vinblastine was due to the absence of MDR1 expression. First, the gene expression level of MDR1 during the intestinal differentiation process was examined. MDR1 was hardly expressed in undifferentiated cells and DE cells, while it increased during the intestinal progenitor and intestinal epithelial cell differentiation steps (Figure 2A). We have previously succeeded in establishing MDR1-knockout (KO) hiPSCs.²⁵ The gene expression levels of the intestinal epithelial cell markers (VIL1, CYP3A4, and breast cancer resistance protein [BCRP]) were not changed by MDR1-KO (Figure S3). To examine whether elimination of undifferentiated and DE cells by vinblastine was indeed due to MDR1, hiPSC-IECs were differentiated from wild-type (WT) or MDR1-KO hiPSCs in the presence of vinblastine (from day 17 to 27). Vinblastine treatment did not change the cell viability of WT hiPSC-derived IECs but markedly reduced that of MDR1-KO hiPSC-derived IECs (Figure 2B). The cell viability of undifferentiated WT hiPSCs treated with vinblastine was rescued by adenovirus (Ad) vector-mediated MDR1 transductions (Figure 2C). These results suggest that the elimination of undifferentiated hiPSCs by vinblastine was mediated through MDR1.

Vinblastine treatment enhances the functions of hiPSC-IECs

Next, we investigated whether vinblastine treatment could improve the functions of hiPSC-IECs. We found that vinblastine treatment increased *villin1* (VIL1), *sucrase-isomaltase* (SI), and *fatty acid binding protein 2* (FABP2) gene expression levels in a concentration-dependent manner. To examine the effect of vinblastine on drug metabolism and drug transport, we also examined the gene expression levels of *cytochrome P450 family 3 subfamily A member 4* (CYP3A4), *MDR1*, *BCRP*, and *peptide transporter 1* (PEPT1), and found that their expression levels increased depending on the concentration of vinblastine (Figure 3A). We examined the protein expressions by western blotting and found that CYP3A4 was the most highly expressed in hiPSC-IECs treated with 10 nM vinblastine (Figure 3B). CYP3A4 activity was the highest in hiPSC-IECs treated with 10 nM vinblastine (Figure 3C). Similar results were obtained in two other

definitive endoderm (DE) markers (*FOXA2*, *SOX17*, and *HEX*) were examined by real-time RT-PCR. The gene expression levels in the DMSO-treated cells were taken as 1.0. The results are represented as means \pm SD ($n \geq 3$, technical replicate). (C) The undifferentiated hiPSCs, DE cells, intestinal progenitor cells, and hiPSC-IECs were treated with 10 nM vinblastine for 3 days, and the cell viability was measured. The cell viability of DMSO-treated cells was taken as 100%. The results are represented as means \pm SD ($n \geq 3$, technical replicate). (D) The percentage of TRA1-81-positive cells and CXCR4-positive cells in DMSO- or vinblastine (10 nM)-treated hiPSC-IECs was examined by flow cytometry. The results are represented as means \pm SD ($n = 3$, technical replicate). (E) To estimate the number of residual undifferentiated cells in DMSO- or vinblastine (10 nM)-treated hiPSC-IECs, a colony formation assay was performed. DMSO- or vinblastine (10 nM)-treated hiPSC-IECs were cultured with AK02N medium on iMatrix-511 for 7 days. The cells were stained by alkaline phosphatase (ALP), and the number of ALP-positive colonies was counted. The results are represented as means \pm SD ($n = 3$, technical replicate). Statistical analyses were performed using the unpaired two-tailed Student's t test (* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$).

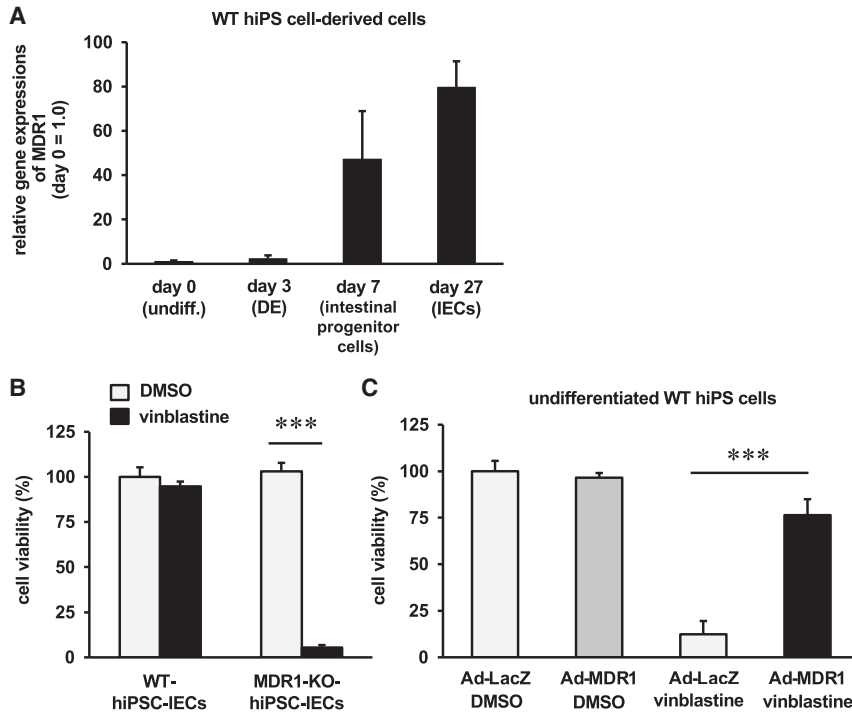


Figure 2. Elimination of MDR1-negative cells by vinblastine treatment

(A) The hiPSCs (YOW) were differentiated into IECs. The gene expression levels of *MDR1* in undifferentiated hiPSCs (day 0), DE cells (day 3), intestinal progenitor cells (day 7), and hiPSC-IECs (day 28) were examined by real-time RT-PCR. The results are represented as means \pm SD ($n \geq 3$, technical replicate). (B) After 10 nM vinblastine treatment, the cell viability of wild-type (WT) hiPSC-IECs and MDR1-knockout hiPSC-IECs was examined. The cell viability of DMSO-treated WT hiPSC-IECs was taken as 100%. (C) The hiPSCs were transduced with 1,000 VP/mL of Ad-LacZ or Ad-MDR1 for 90 min. At 48 h post transduction, hiPSC-IECs were treated with DMSO or vinblastine (10 nM) for 10 days, and the cell viability was examined. The cell viability of the Ad-LacZ-transduced hiPSCs that were treated with DMSO was taken as 100%. The data of (B) and (C) are represented as means \pm SD ($n = 3$, technical replicate). Statistical analyses were performed using the unpaired two-tailed Student's *t* test (** $p < 0.001$).

hiPSC lines, Tic and FCL (Figures S4A, S4B, and S5). These results suggest that vinblastine treatment improved the functions of hiPSC-IECs.

Importantly, vinblastine treatment did not reduce the percentage of ALP (membrane enzyme expressed in villi) and VIL1-positive cells and the expression of VIL1 and E-cadherin in hiPSC-IECs (Figures 3D, 3E, and S6). In order to evaluate the barrier function of hiPSC-IEC monolayers, we measured transepithelial electrical resistance (TEER) and calculated the membrane permeability coefficient of fluorescein isothiocyanate-dextran (FD4), which is a substrate for intercellular transport. The TEERs in DMSO- or vinblastine-treated hiPSC-IECs were both about $200\Omega \times \text{cm}^2$, and the membrane permeation coefficient of FD4 of these cells was less than $1.5 \text{ cm/s} \times 10^{-6}$ (Figures 3F and 3G). These results showed that vinblastine treatment can improve the functions of hiPSC-IECs without affecting the formation of tight junctions of hiPSC-IECs.

Vinblastine treatment can avoid the risk of teratoma formation

Finally, to determine whether vinblastine-treated hiPSC-IECs can avoid the risk of teratoma formation *in vivo*, we performed transplantation experiments using immunodeficient mice. Undifferentiated hiPSCs and hiPSC-IECs were mixed at a ratio of 1:1 and cultured in a medium containing vinblastine for 48 h, and then these cells were subcutaneously transplanted into immunodeficient mice. This method is often used in teratoma formation studies of differentiated cells.^{26,27} Teratoma formation was assessed at 8 weeks after the transplantation. Teratomas were observed in mice transplanted

with DMSO-treated hiPSC-IECs but not in mice transplanted with vinblastine-treated hiPSC-IECs (Figures 4A and 4B). Hematoxylin and eosin (H&E) staining images of teratomas also showed the presence of mesoderm, endoderm, and ectoderm tissues (Figure 4C). These results indicate that the risk of teratoma formation can be reduced by removing residual undifferentiated hiPSCs using vinblastine.

DISCUSSION

We succeeded in eliminating residual undifferentiated hiPSCs and improving the functions of hiPSC-IECs by vinblastine treatment. Vinblastine treatment eliminated not only undifferentiated cells but also DE cells (Figure 1). Undifferentiated cells form teratomas, while endodermal cells differentiate into lung epithelium and other cells.²⁸ Undifferentiated cells and endodermal cells have low expression of MDR1 and cannot excrete vinblastine, resulting in cell death and being eliminated by vinblastine treatment. Because it is very important to eliminate both undifferentiated cells and endodermal cells to prevent the generation of unintended tissues, the vinblastine-treated hiPSC-IECs should be suitable for transplantation medicine.

Vinblastine is one of the typical plant alkaloids extracted from *Catharanthus roseus* and is known to exhibit anti-cancer effects by inhibiting the formation of microtubules involved in cell division.^{29,30} Since vinblastine is a substrate for MDR1, we conducted various evaluations focusing on MDR1 in the present study. It is known that several other transporters, including BCRP and multidrug resistance associated protein 1 (MRP1), are expressed in IECs.³¹ Since BCRP and MRP1 are known to be expressed in undifferentiated iPSCs,¹⁹ it is thought that drugs that serve as substrates for BCRP and MRP1 cannot be used to eliminate residual undifferentiated hiPSCs

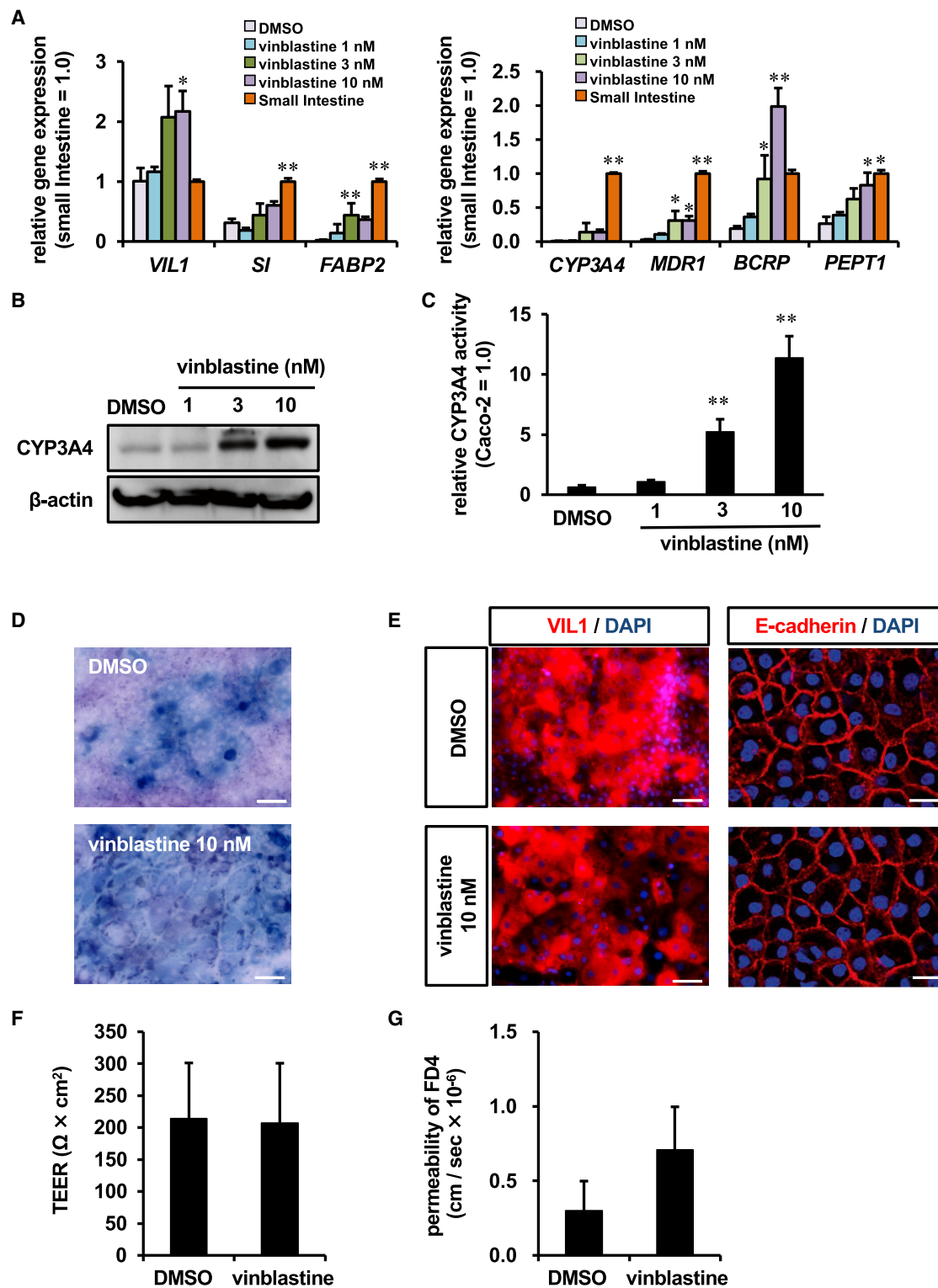


Figure 3. Enhancement of intestinal functions of hiPSC-IECs by vinblastine treatment

The hiPSCs (YOW) were differentiated into IECs. During the intestinal maturation step (from day 18 to 28), the hiPSC-IECs were treated with 10 nM vinblastine. (A) The gene expression levels of intestinal markers (*VIL1*, *SI*, and *FABP2*) and drug-metabolizing enzyme and transporters (*CYP3A4*, *MDR1*, *BCRP*, and *PEPT1*) in hiPSC-IECs were

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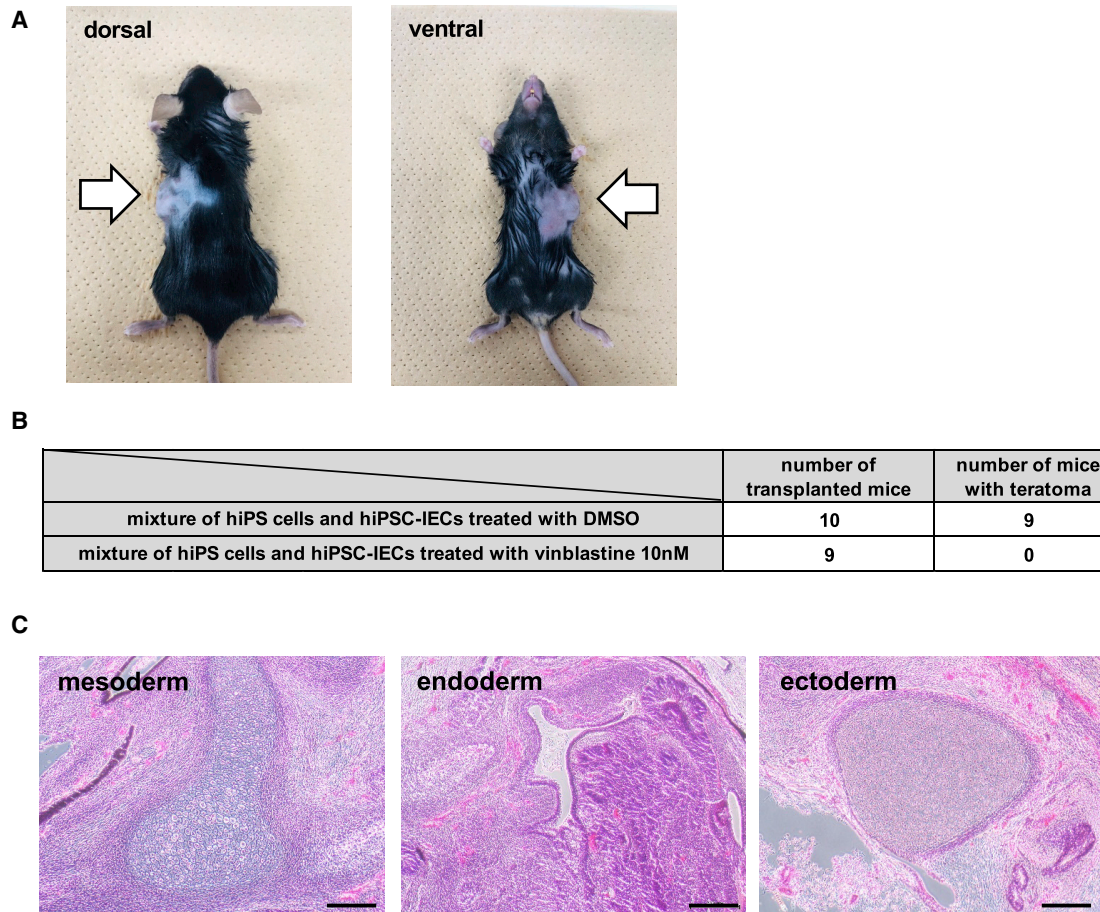


Figure 4. Safety assessment of hiPSC-IECs

(A and B) Dorsal and ventral view of the same mouse transplanted with DMSO-treated hiPSC-IECs (YOW). To perform the teratoma assay, a mixture of undifferentiated hiPSCs (5×10^6 cells/mouse) and hiPSC-IECs (5×10^6 cells/mouse) was subcutaneously transplanted after the DMSO or vinblastine (10 nM) treatment. At 8 weeks from the transplantation, we determined the presence or absence of teratomas. (C) H&E staining of teratoma sections. Scale bars, 200 μ m.

and improve the function of hiPSC-IECs, even if they have the same effect as vinblastine. In other words, it is important to be at least a substrate for MDR1 to achieve similar effects with compounds other than vinblastine.

There are several methods and molecules that were used to eliminate residual undifferentiated cells, such as the cell lines to express the herpes simplex virus thymidine kinase (HSV-tk) gene that enables selection with ganciclovir,³² use of survivin to induce apoptosis in undif-

ferentiated cells and teratomas,³³ immunodepletion with antibodies against pluripotency surface markers (SSEA-5, CD9, CD90, CD50, and CD200) to remove teratoma-formation potential,³⁴ increased copy number of tumor suppressors p53 or Ink4a/ARF to downregulate tumorigenicity of iPSCs,³⁵ and use of anti-podocalyxin-like protein-1 antibody to induce cell death of undifferentiated cells.³⁶ As compared with these methods, the method presented in this study might have an advantage, because vinblastine is a clinically used compound and has a clear mechanism to eliminate residual

examined by real-time RT-PCR. The gene expression levels in the human small intestine were taken as 1.0. The results are represented as means \pm SD ($n = 3$, technical replicate). (B) The protein expression levels of CYP3A4 in DMSO- or vinblastine (1, 3, 10 nM)-treated hiPSC-IECs were examined by western blotting. (C) The CYP3A4 activities in DMSO- or vinblastine (1, 3, 10 nM)-treated hiPSC-IECs were examined. (D) The ALP staining was performed in DMSO- or vinblastine (10 nM)-treated hiPSC-IECs. (E) The VIL1 and E-cadherin expression levels were examined by immunostaining analysis. Nuclei were counterstained with DAPI. Scale bars, 50 μ m. (F) Transepithelial electrical resistance (TEER) values of the DMSO- or vinblastine (10 nM)-treated hiPSC-IEC monolayers were measured by Millicell-ERS2. The results are represented as means \pm SD ($n \geq 10$, technical replicate). (G) Apical-to-basolateral permeability of FD4 (FITC-dextran 4 kDa) across the DMSO- or vinblastine (10 nM)-treated hiPSC-IEC monolayers was measured. The results are represented as means \pm SD ($n = 3$, technical replicate). Statistical significance was evaluated by one-way ANOVA followed by Dunnett's post hoc test (* $p < 0.05$, ** $p < 0.01$, compared with control).

undifferentiated cells. Like the intestinal tract, the cells in the blood-brain barrier (BBB), liver, and kidney express MDR1.^{37–42} Even in these organ cells, it would be possible to eliminate residual undifferentiated hiPSCs by vinblastine treatment. Indeed, we confirmed that vinblastine treatment decreased the gene expression level of undifferentiated markers in hiPSC-derived hepatocyte-like cells (Figure S7).

In the present study, we generated hiPSC-IECs containing almost no undifferentiated cells by vinblastine treatment. In order to apply the hiPSC-IECs to regenerative medicine in the future, we would like to confirm the therapeutic effects on damaged intestinal tissue by transplanting these cells into intestinal inflammation model mice.

MATERIALS AND METHODS

Human iPSCs

YOW-iPSCs and FCL-iPSCs generated in our previous report were used in this study.⁴³ The human iPSC line, Tic, was provided from the JCRB Cell Bank (Tic, JCRB: JCRB1331). MDR1-KO hiPSC line was recently established.²⁵ hiPSCs were cultured with StemFit AK02N medium (Ajinomoto) containing 0.1 $\mu\text{g}/\text{cm}^2$ iMatrix-511 (Nippi).

In vitro intestinal differentiation

Before the initiation of intestinal epithelial cell differentiation, hiPSCs were dissociated into single cells by using TrypLE Select Enzyme and plated onto growth factor-reduced BD Matrigel basement membrane matrix (BD Biosciences). These cells were cultured with StemFit AK02N medium until they reached approximately 80% confluence.

The differentiation protocol for the induction of DE cells and intestinal progenitor cells was described previously.¹⁵ Briefly, hiPSCs were cultured in RPMI 1640 medium (Sigma-Aldrich) containing 1 μM CHIR99021 (FUJIFILM Wako), 100 ng/mL activin A (R&D Systems), 1 \times GlutaMAX (Thermo Fisher Scientific), penicillin-streptomycin (P/S; Nacalai Tesque), and 1 \times B27 supplement minus vitamin A (Thermo Fisher Scientific) for 1 day, and then in RPMI 1640 medium (Sigma-Aldrich) containing 100 ng/mL activin A (R&D Systems), 1 \times GlutaMAX (Thermo Fisher Scientific), P/S, and 1 \times B27 supplement minus vitamin A (Thermo Fisher Scientific) for 2 days. For the induction of intestinal progenitor cells, the DE cells were cultured for 4 days in an intestinal differentiation medium, DMEM high-glucose medium (FUJIFILM Wako) containing 10% KO serum replacement (KSR; Thermo Fisher Scientific), 1% non-essential amino acid solution (NEAA; Thermo Fisher Scientific), P/S and 1 \times GlutaMAX supplemented with 20 nM LY2090314 (MedChem Express). For the induction of intestinal epithelial-like cells, the intestinal progenitor cells were cultured for 20 days in intestinal differentiation medium, DMEM high-glucose medium (FUJIFILM Wako) containing 10% KO serum replacement (KSR; Thermo Fisher Scientific), 1% NEAA (Thermo Fisher Scientific), P/S, and 1 \times GlutaMAX supplemented with 2 μM SB431542 (FUJIFILM Wako), 3 nM LY2090314 (MedChem Express), 1 α ,25-dihydroxyvitamin D3 (Calcitriol, Cayman Chemical), and 50 ng/mL epidermal growth factor (EGF; R&D Systems).

Real-time RT-PCR

Total RNA was isolated from hiPSCs and their derivatives using ISOGENE (Nippon Gene). cDNA was synthesized using 500 ng of total RNA with a Superscript VILO cDNA synthesis kit (Thermo Fisher Scientific). Total RNA of Human Adult Normal Tissue: Small Intestine was purchased from BioChain Institute. Real-time RT-PCR was performed with SYBR Green PCR Master Mix (Thermo Fisher Scientific) using a StepOnePlus real-time PCR system (Thermo Fisher Scientific). The relative quantitation of target mRNA levels was performed by using the $2^{-\Delta\Delta\text{CT}}$ method. The values were normalized by those of the housekeeping gene, *glyceraldehyde 3-phosphate dehydrogenase (GAPDH)*. The PCR primer sequences used in this study are described in Table S2.

Immunocytochemistry

To perform the immunocytochemistry, the hiPSCs and their derivatives were fixed with 4% paraformaldehyde (FUJIFILM Wako) in PBS for 10 min. After blocking the cells with PBS containing 2% bovine serum albumin (BSA) and 0.2% Triton X-100 for 20 min, the cells were incubated with primary antibodies at 4°C overnight and, finally, with secondary antibodies at room temperature for 1 h. All antibodies used in this report are described in Table S1.

Western blotting

The cells were homogenized with RIPA Lysis and Extraction buffer (Thermo Fisher Scientific) containing a protease inhibitor mixture (Sigma Aldrich). After being frozen and thawed, the homogenates were centrifuged at 15,000 $\times g$ at 4°C for 10 min, and the supernatants were collected. The lysates were subjected to SDS-PAGE on 7.5% polyacrylamide gel and then transferred onto polyvinylidene fluoride membranes (Millipore). After the reaction was blocked with 1% skim milk in Tris-buffered saline (TBS) containing 0.1% Tween 20 at room temperature for 1 h, the membranes were incubated with primary antibodies at 4°C overnight, followed by reaction with secondary antibodies at room temperature for 1 h. The band was visualized by Chemi-Lumi One Super (Nacalai Tesque), and the signals were read using an LAS-4000 imaging system (FUJIFILM). All antibodies used in this report are described in Table S1.

CYP3A4 activity

To measure the CYP3A4 activity, lytic assay was performed by using a P450-Glo CYP3A4 Assay with Luciferin-IPA (Promega) according to the manufacturer's instructions. The luminescence was read by a Lumat LB 9507 (Berthold Technologies). The CYP3A4 activity was normalized with the protein content per well, which was evaluated with Pierce BCA Protein Assay Kit (Thermo Fisher Scientific).

Teratoma formation

hiPSC-IECs (5×10^6 cells) and undifferentiated hiPSCs (5×10^6 cells) were subcutaneously injected into *Rag2/Il2rg* double KO mice (Taconic Biosciences). Eight weeks after the injection, teratomas were collected and processed for H&E staining. All animal experimental procedures used in this study were performed in accordance

with the institutional guidelines for animal experiments at Osaka University (approval number #28-4).

Flow cytometry

Single-cell suspensions of hiPSC-derived cells were fixed with 4% paraformaldehyde at 4°C for 10 min and then incubated with primary antibodies, followed by secondary antibodies. MACSQuant Analyzer (Miltenyi Biotec) and FlowJo software (<https://www.flowjo.com/>) were used for analysis. All antibodies used in this report are described in Table S1.

TEER measurements

TEER values were measured by Millicell-ERS2 (Merck Millipore). hiPSC-IECs were cultured on BD Falcon cell culture inserts (6 well plate, 0.4 µm pore size, 2.0×10^6 pores/cm², BD Biosciences) from day 0 of differentiation. The raw data were converted to $\Omega \times \text{cm}^2$ based on the culture insert area (4.2 cm²).

FD4 permeability tests

hiPSC-IECs, which were cultured on the cell culture inserts, were rinsed with HBSS (Hank's balanced salt solution). We added the 1.5 mL of HBSS containing 1.0 mg/mL FD4 (average mol wt 3,000–5,000, Sigma-Aldrich) to the apical chamber, and added 2.6 mL of HBSS to the basolateral chamber. After 90 min of incubation at 37°C, the solution was collected from the basolateral side. The FD4 fluorescent signal was measured with a fluorescence plate reader (TriStar LB 941, Berthold Technologies) using 490 nm excitation and 520 nm emission filters. FD4 concentrations were calculated using the standard curve generated by serial dilution of FD4.

Cell viability

To examine the cell viability, we performed WST (Water soluble Tetrazolium salts) -8 assay by using Cell Counting Kit-8 (Dojindo Laboratories) according to the manufacturer's instructions. The cell viability was calculated as a percentage of that in the cells treated with vehicle (DMSO) only.

Detection of residual undifferentiated cells

DMSO- or vinblastine-treated hiPSC-IECs were cultured on iMatrix-511 with AK02N medium for 10 days. The ALP-positive colonies were counted. ALP staining was performed using an ALP detection kit (Chemicon) according to the manufacturer's instructions. The efficiency of colony formation of undifferentiated hiPSCs was also measured.

Caco-2 cell culture and differentiation

The human colorectal adenocarcinoma cell line, Caco-2 (HTB-37), was obtained from the American Type Culture Collection. Caco-2 cells were cultured with DMEM high-glucose medium containing 10% FBS, 1 × NEAA, P/S, and 1 × GlutaMAX. Caco-2 cells were cultured for 21 days after they reached confluence for the differentiation. The passage number of Caco-2 cells was between 20 and 40.

Ad vectors

Ad vectors were constructed by an improved *in vitro* ligation method.^{44,45} The human MDR1 gene (NCBI: NM_000927.4) was amplified by PCR. The human MDR1 genes were inserted into pHMEF5,⁴⁶ which contains the elongation factor 1 alpha (EF1α) promoter, resulting in pHMEF5-MDR1. The pHMEF5-MDR1 was digested with *I-CeuI/PI-SceI* and ligated into *I-CeuI/PI-SceI*-digested pAdHM41-K7,⁴⁷ resulting in pAd-MDR1. The LacZ-expressing Ad vectors (Ad-LacZ) were constructed previously.⁴⁸ All of the Ad vectors contain a stretch of lysine residue (K7) peptides in the C-terminal region of the fiber knob for more efficient transduction of human pluripotent stem cells and their derivatives, in which the transfection efficiency was almost 100%, and were purified as described previously.⁴⁹ The vector particle (VP) titer was determined by using a spectrophotometric method.⁵⁰

Data and statistical analysis

Data are presented as means ± SD. Statistical analysis was performed using Student's unpaired t test. A value of p < 0.05 was considered statistically significant. All calculations were carried out using Easy R (EZR) software.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at <https://doi.org/10.1016/j.omtm.2021.01.005>.

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AUTHOR CONTRIBUTIONS

M.I., R.N., and H.M. designed the experiments. M.I., R.N., K.K., and T.Y. performed the experiments. M.I., R.N., K.K., and K.T. analyzed the data. M.I. and H.M. wrote the manuscript.

DECLARATION OF INTERESTS

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REFERENCES

- Kauffman, A.L., Gyurdieva, A.V., Mabus, J.R., Ferguson, C., Yan, Z., and Hornby, P.J. (2013). Alternative functional *in vitro* models of human intestinal epithelia. *Front. Pharmacol.* 4, 79.
- Ogaki, S., Morooka, M., Otera, K., and Kume, S. (2015). A cost-effective system for differentiation of intestinal epithelium from human induced pluripotent stem cells. *Sci. Rep.* 5, 17297.
- Spence, J.R., Mayhew, C.N., Rankin, S.A., Kuhar, M.F., Vallance, J.E., Tolle, K., Hoskins, E.E., Kalinichenko, V.V., Wells, S.I., Zorn, A.M., et al. (2011). Directed

- differentiation of human pluripotent stem cells into intestinal tissue in vitro. *Nature* 470, 105–109.
4. Iwao, T., Kodama, N., Kondo, Y., Kabeya, T., Nakamura, K., Horikawa, T., Niwa, T., Kurose, K., and Matsunaga, T. (2015). Generation of enterocyte-like cells with pharmacokinetic functions from human induced pluripotent stem cells using small-molecule compounds. *Drug Metab. Dispos.* 43, 603–610.
 5. 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.
 6. Kondo, S., Mizuno, S., Hashita, T., Iwao, T., and Matsunaga, T. (2020). Establishment of a novel culture method for maintaining intestinal stem cells derived from human induced pluripotent stem cells. *Biol. Open* 9, 1–11.
 7. Yoshida, S., Miwa, H., Kawachi, T., Kume, S., and Takahashi, K. (2020). Generation of intestinal organoids derived from human pluripotent stem cells for drug testing. *Sci. Rep.* 10, 5989.
 8. Ng, S.C., Shi, H.Y., Hamidi, N., Underwood, F.E., Tang, W., Benchimol, E.I., Panaccione, R., Ghosh, S., Wu, J.C.Y., Chan, F.K.L., et al. (2018). Worldwide incidence and prevalence of inflammatory bowel disease in the 21st century: a systematic review of population-based studies. *Lancet* 390, 2769–2778.
 9. Fordham, R.P., Yui, S., Hannan, N.R.F., Soendergaard, C., Madgwick, A., Schweiger, P.J., Nielsen, O.H., Vallier, L., Pedersen, R.A., Nakamura, T., et al. (2013). Transplantation of expanded fetal intestinal progenitors contributes to colon regeneration after injury. *Cell Stem Cell* 13, 734–744.
 10. Fukuda, M., Mizutani, T., Mochizuki, W., Matsumoto, T., Nozaki, K., Sakamaki, Y., Ichinose, S., Okada, Y., Tanaka, T., Watanabe, M., and Nakamura, T. (2014). Small intestinal stem cell identity is maintained with functional Paneth cells in heterotopically grafted epithelium onto the colon. *Genes Dev.* 28, 1752–1757.
 11. Nakamura, T., and Sato, T. (2017). Advancing Intestinal Organoid Technology Toward Regenerative Medicine. *Cell. Mol. Gastroenterol. Hepatol.* 5, 51–60.
 12. Sugimoto, S., Ohta, Y., Fujii, M., Matano, M., Shimokawa, M., Nanki, K., Date, S., Nishikori, S., Nakazato, Y., Nakamura, T., et al. (2018). Reconstruction of the Human Colon Epithelium In Vivo. *Cell Stem Cell* 22, 171–176.e5.
 13. Ozawa, T., Takayama, K., Okamoto, R., Negoro, R., Sakurai, F., Tachibana, M., Kawabata, K., and Mizuguchi, H. (2015). Generation of enterocyte-like cells from human induced pluripotent stem cells for drug absorption and metabolism studies in human small intestine. *Sci. Rep.* 5, 16479.
 14. Negoro, R., Takayama, K., Nagamoto, Y., Sakurai, F., Tachibana, M., and Mizuguchi, H. (2016). Modeling of drug-mediated CYP3A4 induction by using human iPSC-derived enterocyte-like cells. *Biochem. Biophys. Res. Commun.* 472, 631–636.
 15. Negoro, R., Takayama, K., Kawai, K., Harada, K., Sakurai, F., Hirata, K., and Mizuguchi, H. (2018). Efficient Generation of Small Intestinal Epithelial-like Cells from Human iPSCs for Drug Absorption and Metabolism Studies. *Stem Cell Reports* 11, 1539–1550.
 16. Takayama, K., Negoro, R., Yamashita, T., Kawai, K., Ichikawa, M., Mori, T., Nakatsu, N., Harada, K., Ito, S., Yamada, H., et al. (2019). Generation of Human iPSC-Derived Intestinal Epithelial Cell Monolayers by CDX2 Transduction. *Cell. Mol. Gastroenterol. Hepatol.* 8, 513–526.
 17. Kawai, K., Negoro, R., Ichikawa, M., Yamashita, T., Deguchi, S., Harada, K., Hirata, K., Takayama, K., and Mizuguchi, H. (2019). Establishment of SLC15A1/PEPT1-Knockout Human-Induced Pluripotent Stem Cell Line for Intestinal Drug Absorption Studies. *Mol. Ther. Methods Clin. Dev.* 17, 49–57.
 18. Bremer, S., Hoof, T., Wilke, M., Busche, R., Scholte, B., Riordan, J.R., Maass, G., and Tümmler, B. (1992). Quantitative expression patterns of multidrug-resistance P-glycoprotein (MDR1) and differentially spliced cystic-fibrosis transmembrane-conductance regulator mRNA transcripts in human epithelia. *Eur. J. Biochem.* 206, 137–149.
 19. Hirata, N., Nakagawa, M., Fujibayashi, Y., Yamauchi, K., Murata, A., Minami, I., Tomioka, M., Kondo, T., Kuo, T.F., Endo, H., et al. (2014). A chemical probe that labels human pluripotent stem cells. *Cell Rep.* 6, 1165–1174.
 20. Shirasaka, Y., Kawasaki, M., Sakane, T., Omatsu, H., Moriya, Y., Nakamura, T., Sakaeda, T., Okumura, K., Langguth, P., and Yamashita, S. (2006). Induction of human P-glycoprotein in Caco-2 cells: development of a highly sensitive assay system for P-glycoprotein-mediated drug transport. *Drug Metab. Pharmacokinet.* 21, 414–423.
 21. Pal, D., and Mitra, A.K. (2006). MDR- and CYP3A4-mediated drug-drug interactions. *J. Neuroimmune Pharmacol.* 1, 323–339.
 22. Anderle, P., Niederer, E., Rubas, W., Hilgendorf, C., Spahn-Langguth, H., Wunderli-Allenspach, H., Merkle, H.P., and Langguth, P. (1998). P-Glycoprotein (P-gp) mediated efflux in Caco-2 cell monolayers: the influence of culturing conditions and drug exposure on P-gp expression levels. *J. Pharm. Sci.* 87, 757–762.
 23. Manda, V.K., Avula, B., Dale, O.R., Ali, Z., Khan, I.A., Walker, L.A., and Khan, S.I. (2017). PXR mediated induction of CYP3A4, CYP1A2, and P-gp by *Mitragyna speciosa* and its alkaloids. *Phytother. Res.* 31, 1935–1945.
 24. Tsuruo, T., Iida, H., Tsukagoshi, S., and Sakurai, Y. (1981). Overcoming of Vincristine Resistance in p388 Leukemia in Vivo and in Vitro Through Enhanced Cytotoxicity of Vincristine and Vinblastine by Verapamil. *Cancer Res* 41, 1967–1972.
 25. Negoro, R., Kawai, K., Ichikawa, M., Deguchi, S., Takayama, K., and Mizuguchi, H. (2020). Establishment of MDR1-knockout human induced pluripotent stem cell line. *Drug Metab. Pharmacokinet.* 35, 288–296.
 26. Ben-David, U., Gan, Q.F., Golan-Lev, T., Arora, P., Yanuka, O., Oren, Y.S., Leikin-Frenkel, A., Graf, M., Garippa, R., Boehringer, M., et al. (2013). Selective elimination of human pluripotent stem cells by an oleate synthesis inhibitor discovered in a high-throughput screen. *Cell Stem Cell* 12, 167–179.
 27. Doi, D., Magotani, H., Kikuchi, T., Ikeda, M., Hiramatsu, S., Yoshida, K., Amano, N., Nomura, M., Umekage, M., Morizane, A., and Takahashi, J. (2020). Pre-clinical study of induced pluripotent stem cell-derived dopaminergic progenitor cells for Parkinson's disease. *Nat. Commun.* 11, 3369.
 28. Zorn, A.M., and Wells, J.M. (2009). Vertebrate endoderm development and organ formation. *Annu. Rev. Cell Dev. Biol.* 25, 221–251.
 29. Strahs, K.R., and Sato, H. (1973). Potentiation of vinblastine crystal formation in vivo by puromycin and colcemid. *Exp. Cell Res.* 80, 10–14.
 30. Amos, L.A., Jubb, J.S., Henderson, R., and Vigers, G. (1984). Arrangement of protofilaments in two forms of tubulin crystal induced by vinblastine. *J. Mol. Biol.* 178, 711–729.
 31. Kuo, T.F., Mao, D., Hirata, N., Khambu, B., Kimura, Y., Kawase, E., Shimogawa, H., Ojika, M., Nakatsuji, N., Ueda, K., and Uesugi, M. (2014). Selective elimination of human pluripotent stem cells by a marine natural product derivative. *J. Am. Chem. Soc.* 136, 9798–9801.
 32. Schuldiner, M., Itskovitz-Eldor, J., and Benvenisty, N. (2003). Selective ablation of human embryonic stem cells expressing a “suicide” gene. *Stem Cells* 21, 257–265.
 33. Blum, B., Bar-Nur, O., Golan-Lev, T., and Benvenisty, N. (2009). The anti-apoptotic gene survivin contributes to teratoma formation by human embryonic stem cells. *Nat. Biotechnol.* 27, 281–287.
 34. Tang, C., Lee, A.S., Volkmer, J.-P., Sahoo, D., Nag, D., Mosley, A.R., Inlay, M.A., Ardehali, R., Chavez, S.L., Pera, R.R., et al. (2011). An antibody against SSEA-5 glycan on human pluripotent stem cells enables removal of teratoma-forming cells. *Nat. Biotechnol.* 29, 829–834.
 35. Menendez, S., Camus, S., Herreria, A., Paramonov, I., Morera, L.B., Collado, M., Pekarik, V., Maceda, I., Edel, M., Consiglio, A., et al. (2012). Increased dosage of tumor suppressors limits the tumorigenicity of iPSC cells without affecting their pluripotency. *Aging Cell* 11, 41–50.
 36. Choo, A.B., Tan, H.L., Ang, S.N., Fong, W.J., Chin, A., Lo, J., Zheng, L., Hentze, H., Philp, R.J., Oh, S.K.W., and Yap, M. (2008). Selection against undifferentiated human embryonic stem cells by a cytotoxic antibody recognizing podocalyxin-like protein-1. *Stem Cells* 26, 1454–1463.
 37. Schinkel, A.H., Wagenaar, E., van Deemter, L., Mol, C.A.A.M., and Borst, P. (1995). Absence of the *mdr1a* P-Glycoprotein in mice affects tissue distribution and pharmacokinetics of dexamethasone, digoxin, and cyclosporin A. *J. Clin. Invest.* 96, 1698–1705.
 38. Schuetz, E.G., Beck, W.T., and Schuetz, J.D. (1996). Modulators and substrates of P-glycoprotein and cytochrome P4503A coordinately up-regulate these proteins in human colon carcinoma cells. *Mol. Pharmacol.* 49, 311–318.
 39. Schinkel, A.H., Wagenaar, E., van Deemter, L., Mol, C.A.A.M., and Borst, P. (1995). Absence of the *mdr1a* P-Glycoprotein in Mice Affects Tissue Distribution and

- Pharmacokinetics of Dexamethasone, Digoxin, and Cyclosporin A. *J. Clin. Invest.* 96, 1698–1705.
40. Hartley, D.P., Dai, X., He, Y.D., Carlini, E.J., Wang, B., Huskey, S.E.W., Ulrich, R.G., Rushmore, T.H., Evers, R., and Evans, D.C. (2004). Activators of the rat pregnane X receptor differentially modulate hepatic and intestinal gene expression. *Mol. Pharmacol.* 65, 1159–1171.
 41. Albermann, N., Schmitz-Winnenthal, F.H., Z'graggen, K., Volk, C., Hoffmann, M.M., Haefeli, W.E., and Weiss, J. (2005). Expression of the drug transporters MDR1/ABCB1, MRP1/ABCC1, MRP2/ABCC2, BCRP/ABCG2, and PXR in peripheral blood mononuclear cells and their relationship with the expression in intestine and liver. *Biochem. Pharmacol.* 70, 949–958.
 42. Miller, D.S., Bauer, B., and Hartz, A.M.S. (2009). Modulation of P-glycoprotein at the Blood-Brain Barrier: Opportunities to Improve Central Nervous System Pharmacotherapy. *Pharmacol. Rev.* 60, 196–209.
 43. Takayama, K., Morisaki, Y., Kuno, S., Nagamoto, Y., Harada, K., Furukawa, N., Ohtaka, M., Nishimura, K., Imagawa, K., Sakurai, F., et al. (2014). Prediction of inter-individual differences in hepatic functions and drug sensitivity by using human iPSC-derived hepatocytes. *Proc. Natl. Acad. Sci. USA* 111, 16772–16777.
 44. Mizuguchi, H., and Kay, M.A. (1998). Efficient construction of a recombinant adenovirus vector by an improved in vitro ligation method. *Hum. Gene Ther.* 9, 2577–2583.
 45. Mizuguchi, H., and Kay, M.A. (1999). A simple method for constructing E1- and E1/E4-deleted recombinant adenoviral vectors. *Hum. Gene Ther.* 10, 2013–2017.
 46. Kawabata, K., Sakurai, F., Yamaguchi, T., Hayakawa, T., and Mizuguchi, H. (2005). Efficient gene transfer into mouse embryonic stem cells with adenovirus vectors. *Mol. Ther.* 12, 547–554.
 47. Koizumi, N., Mizuguchi, H., Utoguchi, N., Watanabe, Y., and Hayakawa, T. (2003). Generation of fiber-modified adenovirus vectors containing heterologous peptides in both the HI loop and C terminus of the fiber knob. *J. Gene Med.* 5, 267–276.
 48. Tashiro, K., Kawabata, K., Sakurai, H., Kurachi, S., Sakurai, F., Yamanishi, K., et al. (2008). Efficient adenovirus vector-mediated PPAR gamma gene transfer into mouse embryoid bodies promotes adipocyte differentiation. *J. Gene Med.* 10, 598–507.
 49. Tashiro, K., Kawabata, K., Inamura, M., Takayama, K., Furukawa, N., Sakurai, F., Katayama, K., Hayakawa, T., Furue, M.K., and Mizuguchi, H. (2010). Adenovirus vector-mediated efficient transduction into human embryonic and induced pluripotent stem cells. *Cell. Reprogram.* 12, 501–507.
 50. Maizel, J.V., Jr., White, D.O., and Scharff, M.D. (1968). The polypeptides of adenovirus. I. Evidence for multiple protein components in the virion and a comparison of types 2, 7A, and 12. *Virology* 36, 115–125.