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Supplemental information

Monolayer platform using human biopsy-derived duodenal organoids for pharmaceutical research

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Supplemental Materials and Methods

Maintenance and differentiation media

A 1:1 mixture of components A and B of Intesticult organoid growth medium (STEMCELL Technologies) was used as the maintenance medium. A 1:1 mixture of component A and DMEM/F-12 with 15 mM HEPES (36254, STEMCELL Technologies) was used as the differentiation medium. Human intestinal organoid differentiation was initiated by replacing the maintenance medium with the differentiation medium.

FACS analysis

Single-cell suspensions of the human iPS cell-derived cells were fixed with 4% PFA at 4°C for 10 min, and then incubated with the primary antibody, followed by the secondary antibody. Analysis was performed on a MACSQuant Analyzer (Miltenyi Biotec, Bergisch Gladbach, Germany) and FlowJo software (FlowJo LLC, http://www.flowjo.com/). All the antibodies are listed in **Table S2**.

Human iPS cell culture

The human iPS cell line (Tic, JCRB Number: JCRB1331) was maintained on a feeder layer of mitomycin C-treated mouse embryonic fibroblasts (Merck) with ReproStem medium (ReproCELL, Yokohama, Japan) supplemented with 10 ng/ mL fibroblast growth factor 2 (Katayama Chemical Industries, Osaka, Japan).

In vitro differentiation of human iPS cell-derived intestinal epitherial cells

The *in vitro* differentiation of human iPS cell-derived intestinal epitherial cells (iPS-IECs) was conducted according to previously reported method with some modifications.[1] Before the initiation of intestinal differentiation, iPS cells were dissociated into clumps by using dispase (4942078001; Roche Diagnostics, IN, USA) and plated onto the Matrigel–coated apical chamber of cell culture inserts. These cells were cultured in the mouse embryonic fibroblast–conditioned medium for 2–3 days. The differentiation protocol for the induction of definitive endoderm cells was described previously.[2,3] Briefly, for the definitive endoderm differentiation, human iPS cells were cultured for 4 days in RPMI1640

medium (R8758-500ML; Merck) containing 100 ng/mL Activin A (338-AC; R&D Systems, MN, USA), 1×GlutaMAX, penicillin-streptomycin, and 1×B27 Supplement Minus Vitamin A (12587001; Thermo Fisher Scientific). During the definitive endoderm differentiation, the mesendoderm cells (day 2) were transduced with 3000 vector particles (VPs)/cell of Ad-FOXA2, adenovirus vector expressing FOXA2 (forkhead box A2), for 1.5 hours to promote definitive endoderm differentiation. For the induction of intestinal progenitor cells, the definitive endoderm cells were cultured for 4 days in the intestinal differentiation medium (Dulbecco's modified Eagle medium, high glucose (043-30085; FUJIFILM Wako Pure Chemical) containing 1×MEM Non-Essential Amino Acids Solution, penicillin-streptomycin (2625384; Nacalai tesque), 1×GlutaMAX, 100 mM 2-mercaptoethanol (21985023; Thermo Fisher Scientific), and 10% KnockOut Serum Replacement (10828028; Thermo Fisher Scientific)) supplemented with 5 mM 6-Bromoindirubin-30-oxime (BIO) (361550; Calbiochem, CA, USA) and 10 mM N-[(3,5-difluorophenyl) acetyl]-L-alanyl-2-phenyl, 1-dimethylethyl ester-glycine (DAPT) (3219-v; Peptide Institute, Osaka, Japan). For the induction of intestinal epithelial cell monolayers, the intestinal progenitor cells were cultured for 11 days in intestinal differentiation medium supplemented with 1 mM BIO and 2.5 mM DAPT, and then cultured for 15 days in the Wnt-3A–conditioned intestinal differentiation medium supplemented with 0.1 mM BIO, 1 mM DAPT, 250 ng/mL epidermal growth factor, and 10 mM SB431542. During the intestinal differentiation, the intestinal progenitor cells (day 8) were transduced with 3000 VPs/cell of Ad-CDX2, adenovirus vector expressing CDX2 (caudal type homeobox 2), for 1.5 hours to promote intestinal differentiation.

CYP3A4 activity

Quantification of 6β-hydroxytestosterone in the solution was conducted according to the previously reported methods.[4] The solutions were mixed with 10-fold volume of quench solution (20% acetonitrile/distilled water) containing 0.835 μM D_3-6β-hydroxytestosterone as an internal standard. Mixed solutions were centrifuged for 1 min at 5,900 g. Then, the supernatant was analyzed by UPLC-MS/MS to measure the concentration of 6β-hydroxytestosterone according to a standard curve. UPLC analysis was performed using an ExionLC (AB Sciex), and MS/MS was performed on a 5500 QTRAP (AB Sciex).

The mass spectrometer was set to the MRM mode and was operated with the electrospray ionization source in negative ion mode. The MRM transition (*m*/*z* of precursor ion/*m*/*z* of product ion) for 6β-hydroxytestosterone was 305.2/269.1. For transition, the Ion spray voltage and collision energy were set at 5500 V, 23 eV. The dwell time for each MRM transition was set at 150 ms. LC separations were carried out at 40℃ with an X-terra MS C18 column (5 μm, 2.1×50 mm, Waters). The mobile phase was delivered at a flow rate of 0.5 mL/min using a gradient elution profile consisting of solvent A (5 mM ammonium formate containing 0.05% formic acid) and solvent B (95% acetonitrile, 4.95% methanol and 0.05% formic acid). The initial composition of the binary solvent was 0% solvent B. Solvent B was increased from 0% to 21% during from 0 to 0.4 min, 21% to 72.5% during from 0.4 to 3.0 min. The composition of solvent remained for 0.4 min at 72.5% solvent B. 5 μL of sample solution was injected into the column.

Supplemental Data Items

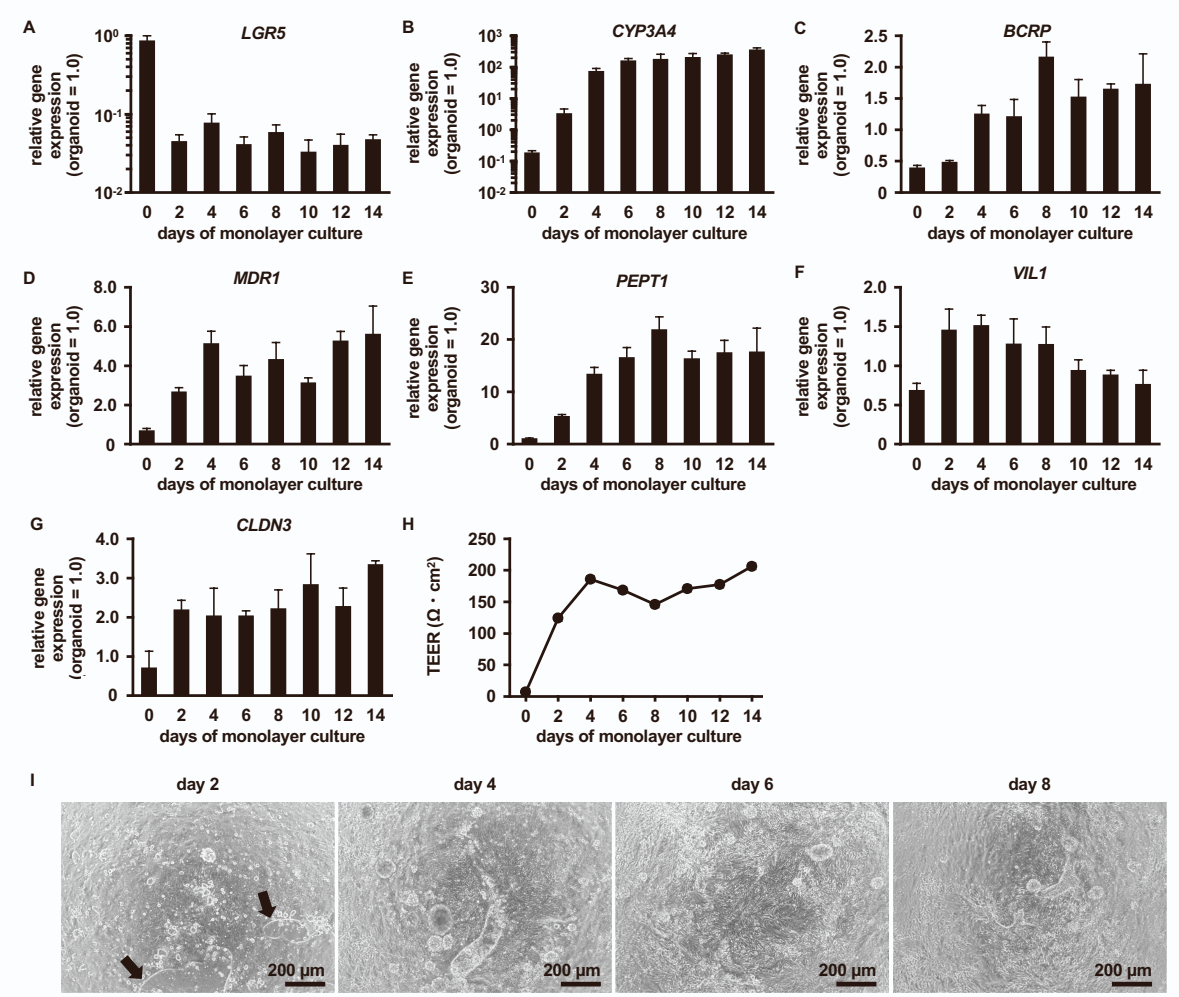

Figure S1. Determination of culture period of the human duodenal organoid-derived monolayer.

Changes over time in the gene expression levels of (A) a stem cell marker (*LGR5*), (B) a drug metabolizing enzyme (*CYP3A4*), (C-E) transporters (*BCRP*, *MDR1*, and *PEPT1*), (F) a brush border marker (*VIL1*), and (G) a tight junction marker (*CLDN3*) in the human duodenal organoid-derived monolayer were examined. (H) TEER values of the human duodenal organoid-derived monolayer were measured every other day after seeding. Data are expressed as means ± SD ($n = 3$). The human duodenal organoid-derived monolayer at 8 hours after seeding was shown as "day 0". The gene expression levels in human duodenal organoids in the maintenance culture were taken as 1.0. (I) Phase-contrast images of the

human duodenal organoid-derived monoloayer at day 2, 4, 6, and 8 of monolayerization. There were some gaps at day 2 (black allow), but the cells reached confluence after day 4.

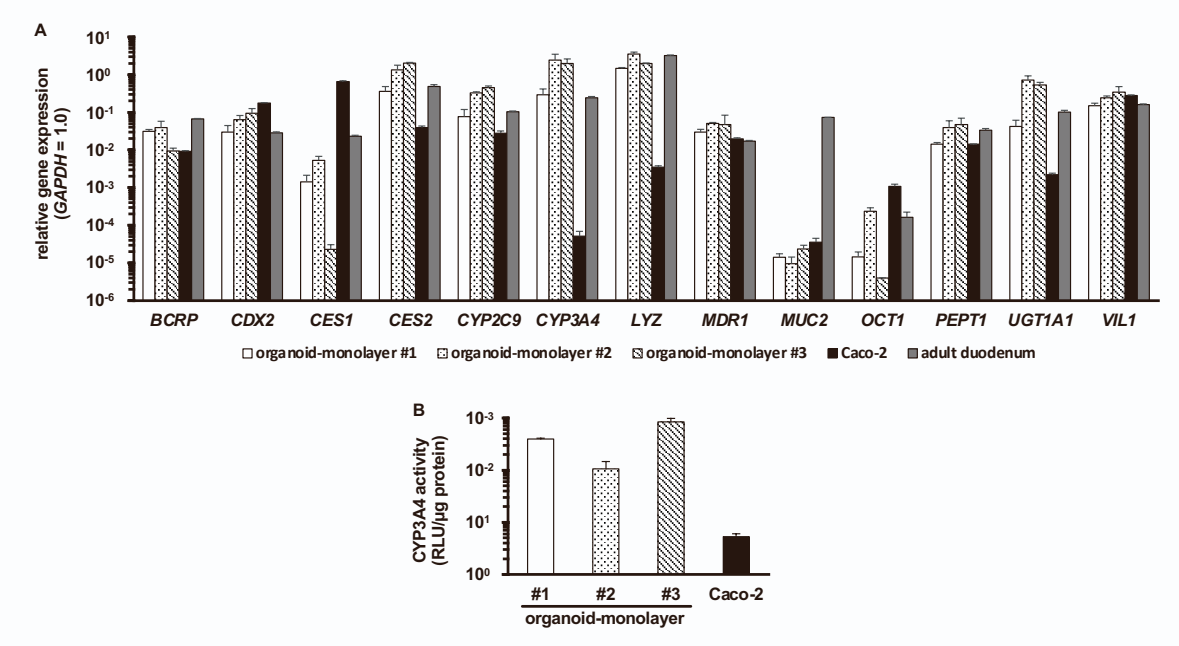

Figure S2. Analysis in duodenal organoid cultures from different donors.

(A) The gene expression levels in the human duodenal organoid-derived monolayer (organoid-monolayer) from three donors (#1, #2, and #3), Caco-2 cells (Caco-2), and adult duodenum (total RNA was purchased from the BioChain Institute) were measured. The organoid monolayer at 8 days after seeding is shown. (B) CYP3A4 activities in the human duodenal organoid-derived monolayer (organoid-monolayer) from three donors (#1, #2, and #3) and Caco-2 cells (Caco-2) are shown. CYP3A4 activity was examined by using a P450-Glo CYP3A4 assay kit. Data are expressed as means ± SD ($n = 3$).

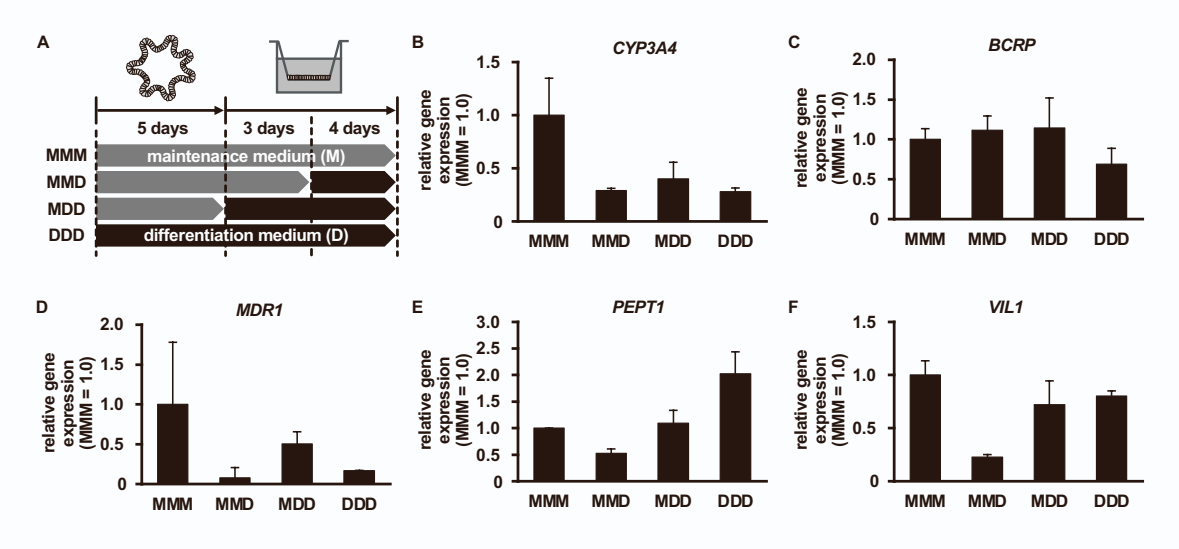

Figure S3. Analysis of the effect of medium composition on gene expression of pharmacokinetic factors.

(A) A schematic of the experiment is shown. The three letters in the group name correspond to 5 days at the end of the organoid culture, days 0–3 and days 4–7 after monolayerization, respectively, and indicate whether the maintenance (M) or differentiation (D) medium was used during the period. The gene expression levels of (B) a drug metabolizing enzyme (*CYP3A4*), (C-E) transporters (*BCRP*, *MDR1*, and *PEPT1*), and (F) a brush border marker (*VIL1*) in the human duodenal organoid-derived monolayer were examined at day 7 after seeding. Data are expressed as means ± SD ($n = 3$). The gene expression levels were expressed as a ratio to that in the human duodenal organoids in maintenance culture for 12 days (MMM).

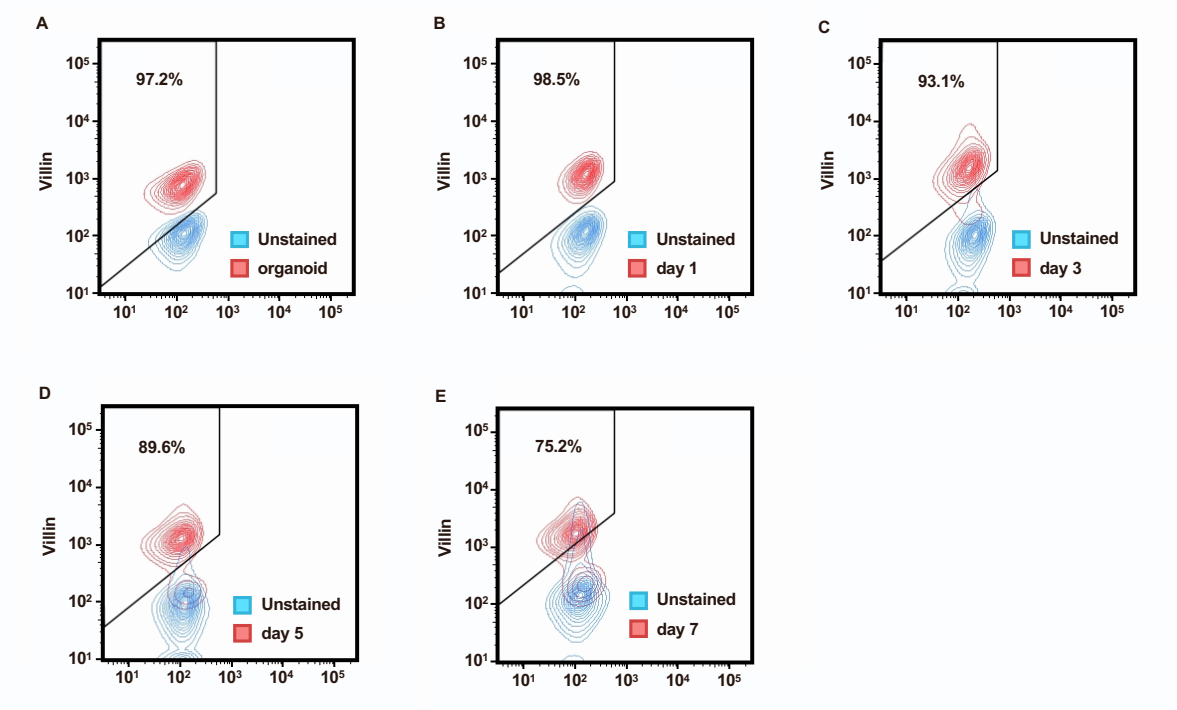

Figure S4. Characterization of the cell population in the human duodenal organoid-derived monolayer.

The percentages of villin-positive cells in the human duodenal organoids in maintenance culture (organoid) and the human duodenal organoid-derived monolayer at days 1, 3, 5, and 7 after seeding were measured by FACS analysis.

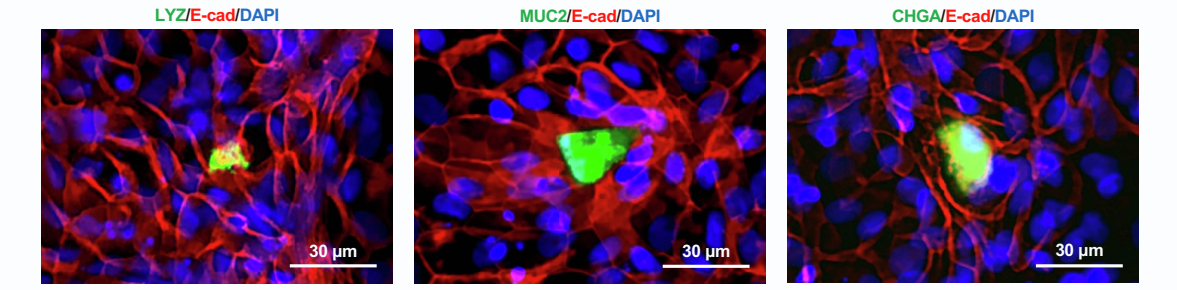

Figure S5. Detection of rare cell types in the human duodenal organoid-derived monolayer.

Immunostaining analysis of a Paneth cell marker (LYZ), a goblet cell marker (MUC2), an enteroendocrine cell marker (CHGA) and an epithelial cell marker (E-cad) was performed in the human duodenal organoid-derived monolayer. Nuclei were stained with DAPI (blue).

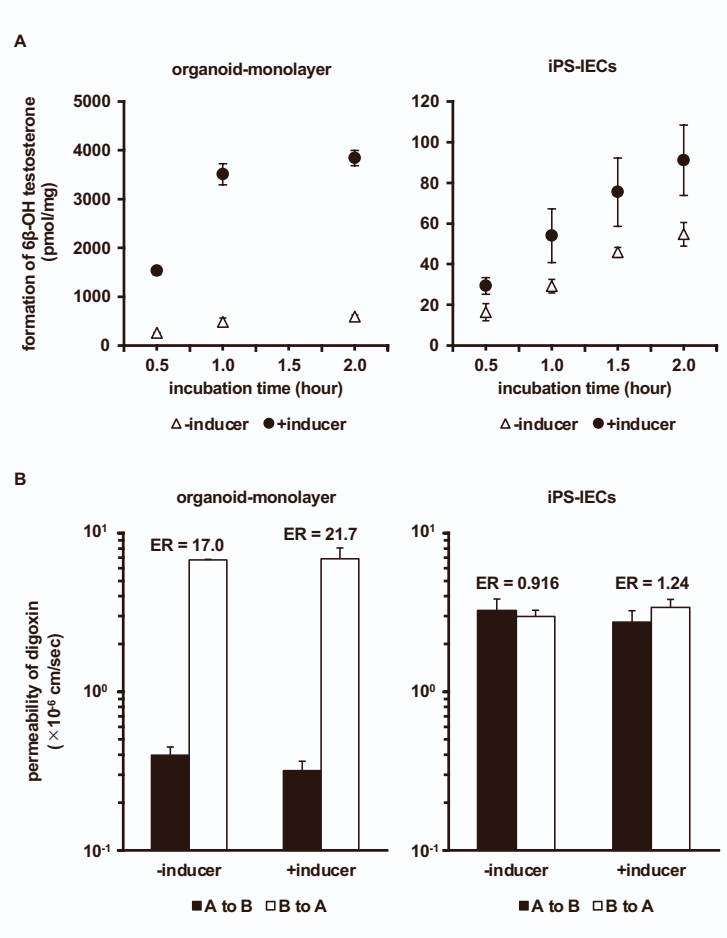

Figure S6. Comparison of the drug metabolizing activity and efflux transport activity of the human duodenal organoid-derived monolayer and human iPS cell-derived intestinal epithelial cells.

(A) CYP3A4 activities and (B) P-gp activities in the human duodenal organoid-derived monolayer (organoid-monolayer) and human iPS cell-derived intestinal epithelial cells (iPS-IECs) were evaluated by using testosterone and digoxin, respectively. The human duodenal organoid-derived monolayers at 3 days after seeding were subjected to assays in this figure. To induce CYP3A4 and P-gp, 20 mM rifampicin (RIF) and 100 nM 1a,25-dihydroxyvitamin D3 (VD3) were added to the culture for the last 48 hours of the culture period. Data are expressed as means ± SD ($n = 3$).

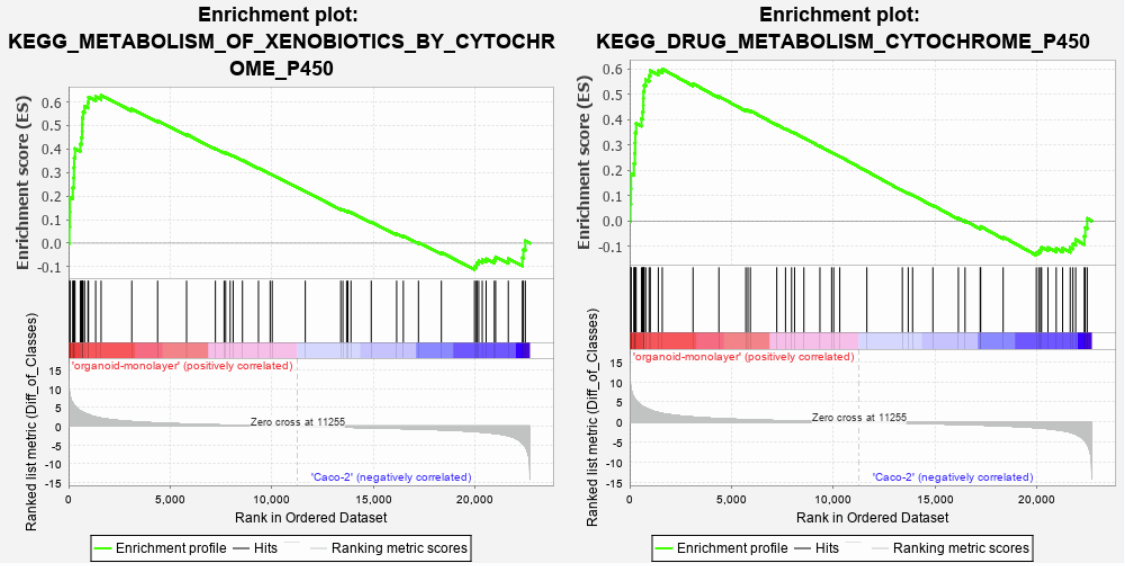

Figure S7. Comparison of the human duodenal organoid-derived monolayer with Caco-2 cells by comprehensive gene expression analysis.

The results of GSEA of the microarray data of the human duodenal organoid-derived monolayer (organoid-monolayer) were compared to those for Caco-2 cells (Caco-2). The analysis was run using curated gene signatures (see the **Materials and Methods** for details). The full results are listed in **Table S1**.

Table S1. Significantly enriched KEGG pathways detected by GSEA of microarray data of human duodenal organoid-derived monolayer and Caco-2 cells

Gene set name	NES[a]	P-value	Q-value
GRAFT_VERSUS_HOST_DISEASE	2.425	0.000	0.000
INTESTINAL_IMMUNE_NETWORK_FOR_IGA_PRODUCTION	2.356	0.000	0.000
TYPE_I_DIABETES_MELLITUS	2.324	0.000	0.000
ALLOGRAFT_REJECTION	2.293	0.000	0.000
ASTHMA	2.187	0.000	0.000
LEISHMANIA_INFECTION	2.109	0.000	0.000
VIRAL_MYOCARDITIS	2.108	0.000	0.000
AUTOIMMUNE_THYROID_DISEASE	2.093	0.000	0.000
LINOLEIC_ACID_METABOLISM	2.081	0.000	0.000
ANTIGEN_PROCESSING_AND_PRESENTATION	1.996	0.000	0.000
GLYCOLYSIS_GLUCONEOGENESIS	1.987	0.000	0.000
METABOLISM_OF_XENOBIOTICS_BY_CYTOCHROME_P450	1.957	0.000	0.001
NOD_LIKE_RECEPTOR_SIGNALING_PATHWAY	1.955	0.000	0.000
FRUCTOSE_AND_MANNOSE_METABOLISM	1.895	0.000	0.002
CELL_ADHESION_MOLECULES_CAMS	1.895	0.000	0.002
GLYCOSPHINGOLIPID_BIOSYNTHESIS_LACTO_AND_NEOLACTO_SERIES	1.890	0.000	0.002
ARACHIDONIC_ACID_METABOLISM	1.869	0.002	0.002
O_GLYCAN_BIOSYNTHESIS	1.863	0.002	0.002
DRUG_METABOLISM_CYTOCHROME_P450	1.843	0.000	0.003
FC_GAMMA_R_MEDIATED_PHAGOCYTOSIS	1.836	0.000	0.003
CYTOSOLIC_DNA_SENSING_PATHWAY	1.828	0.000	0.003
B_CELL_RECEPTOR_SIGNALING_PATHWAY	1.825	0.000	0.003
TOLL_LIKE_RECEPTOR_SIGNALING_PATHWAY	1.763	0.000	0.007
RETINOL_METABOLISM	1.738	0.002	0.009
CYTOKINE_CYTOKINE_RECEPTOR_INTERACTION	1.722	0.000	0.011
P53_SIGNALING_PATHWAY	1.696	0.000	0.014
NATURAL_KILLER_CELL_MEDIATED_CYTOTOXICITY	1.687	0.000	0.015
ADIPOCYTOKINE_SIGNALING_PATHWAY	1.678	0.008	0.016
FC_EPSILON_RI_SIGNALING_PATHWAY	1.676	0.000	0.015
RIG_I_LIKE_RECEPTOR_SIGNALING_PATHWAY	1.669	0.000	0.016

[a] normalized enrichment scores

Table S2. Antibodies used in the immunohistochemistry or FACS analysis

Name	Company	Catalogue No.	Dilution
Anti-E Cadherin antibody	Abcam, Cambridge, UK	ab40772	1:50
ZO-1 Polyclonal Antibody	Thermo Fisher Scientific	40-2200	1:50
Villin Antibody	Santa Cruz Biotechnology, TX, USA	sc-7672	1:50
CYP3A4 Antibody	Santa Cruz Biotechnology	sc-27639	1:50
Anti-Lysozyme antibody	Abcam, Cambridge, UK	ab36362	1:50
Mucin 2 Antibody	Abcam, Cambridge, UK	ab118964	1:50
Anti-Chromogranin A antibody	Abcam, Cambridge, UK	ab715	1:50
Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor Plus 594	Thermo Fisher Scientific	A32754	1:1000
Donkey anti-Mouse IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Thermo Fisher Scientific	A21202	1:1000
Donkey anti-Goat IgG (H+L) Cross-Adsorbed Secondary Antibody, Alexa Fluor 488	Thermo Fisher Scientific	A11055	1:1000

Table S3. Primers used in the real-time RT-PCR analysis

Gene Symbol	Primer (forward/reverse; 5' to 3')
BCRP	TGCAACATGTACTGGCGAAGA/TCTTCCACAAGCCCCAGG
CDX2	TCCGTGTACACCACTCGATATT/GGAACCTGTGCGAGTGGAT
CES1	ACCCCTGAGGTTTACTCCACC/TGCACATAGGAGGGTACGAGG
CES2	CTAGGTCCGCTGCGATTTG/TGAGGTCCTGTAGACACATGG
CLDN3	AACACCATTATCCGGGACTTCT/GCGGAGTAGACGACCTTGG
CYP2C19	ACTTGGAGCTGGGACAGAGA/CATCTGTGTAGGGCATGTGG
CYP2C9	GGACAGAGACGACAAGCACA/CATCTGTGTAGGGCATGTGG
CYP2J2	TGGCTTGCCCTTAATCAAAGAA/GGCCACTTGACATAATCAATCCA
CYP2S1	GCGCTGTATTCAGGGCTCAT/CTTCCAGCATCGCTACGGTT
CYP3A4	AAGTCGCCTCGAAGATACACA/AAGGAGAGAACACTGCTCGTG
GAPDH	GGTGGTCTCCTCTGACTTCAACA/GTGGTCGTTGAGGGCAATG
LYZ	GGCCAAATGGGAGAGTGGTTA/CCAGTAGCGGCTATTGATCTGAA
MCT1	CCGCGCATATAACGATATTT/ATCCAACTGGACCTCCAA
MDR1	GCCAAAGCCAAAATATCAGC/TTCCAATGTGTTCGGCATTA
MRP3	GTCCGCAGAATGGACTTGAT/TCACCACTTGGGGATCATTT
MUC2	GAGGGCAGAACCCGAAACC/GGCGAAGTTGTAGTCGCAGAG
OCT1	TAATGGACCACATCGCTCAA/AGCCCCTGATAGAGCACAGA
PEPT1	AATGTTCTGGGCCTTGTTTG/CATCTGATCGGGCTGAATTT
UGT1A1	CTGTCTCTGCCCACTGTATTCT/TCTGTGAAAAGGCAATGAGCAT
UGT2B17	GCTCTGGGAGTTGTGGAAAG/ATCACCTCATGACCCCTCTG
UGT2B7	GGGAAAGCTGACGTATGGCT/ACAGAAGAAAGGGCCAACGT
VIL1	AGCCAGATCACTGCTGAGGT/TGGACAGGTGTTCCTCCTTC

Supplemental References

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2. 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.
3. Negoro, R., Takayama, K., Nagamoto, Y., Sakurai, F., Tachibana, M., and Mizuguchi, H. (2016). Modeling of drug-mediated CYP3A4 induction by using human iPS cell-derived enterocyte-like cells. *Biochem. Biophys. Res. Commun.* **472**: 631–6.
4. Cui, Y., Claus, S., Schnell, D., Runge, F., and MacLean, C. (2020). In-Depth Characterization of EpiIntestinal Microtissue as a Model for Intestinal Drug Absorption and Metabolism in Human. *Pharmaceutics* **12**: 405.