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Supplemental Information

A Refined Culture System for Human Induced Pluripotent Stem Cell-Derived Intestinal Epithelial Organoids

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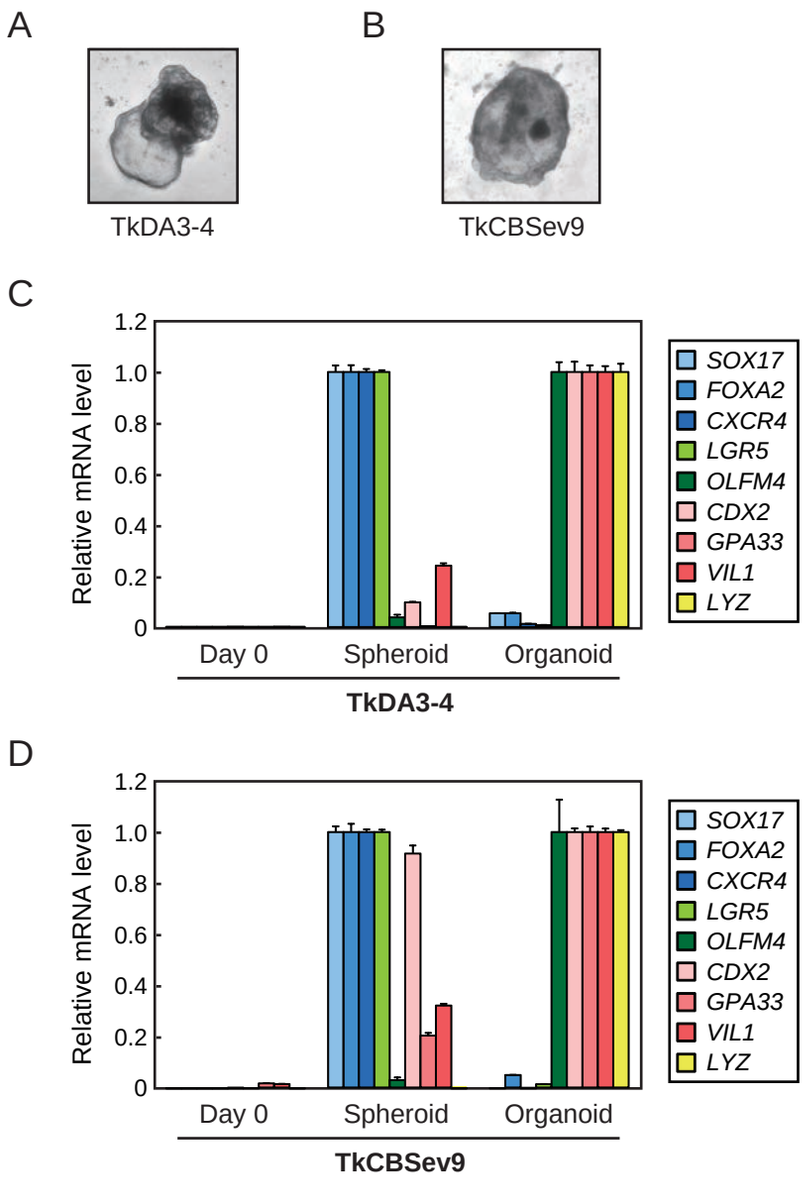

Figure S1. Differentiation of TkDA3-4 and TkCBSev9 into intestinal organoids, related to Figure 2. Human iPS cell lines, TkDA3-4 and TkCBSev9, were differentiated into spheroids and organoids with recombinant hWNT3A and hFGF2 during the initial stage of the differentiation as described in ‘‘Experimental Procedures’’. (A and B) Each magnified image of organoid-like cell clumps was taken by bright field microscopy. (C and D) The cells were harvested during the course of differentiation (days 0, 8 [spheroids], and organoids). Relative mRNA levels were determined by quantitative RT-PCR and normalized to *HPRT*. The highest expression levels of each gene in each differentiation stage were considered as ‘‘1’’. The assays were performed in three independent biological replicates. Data are presented as means ± SEM.

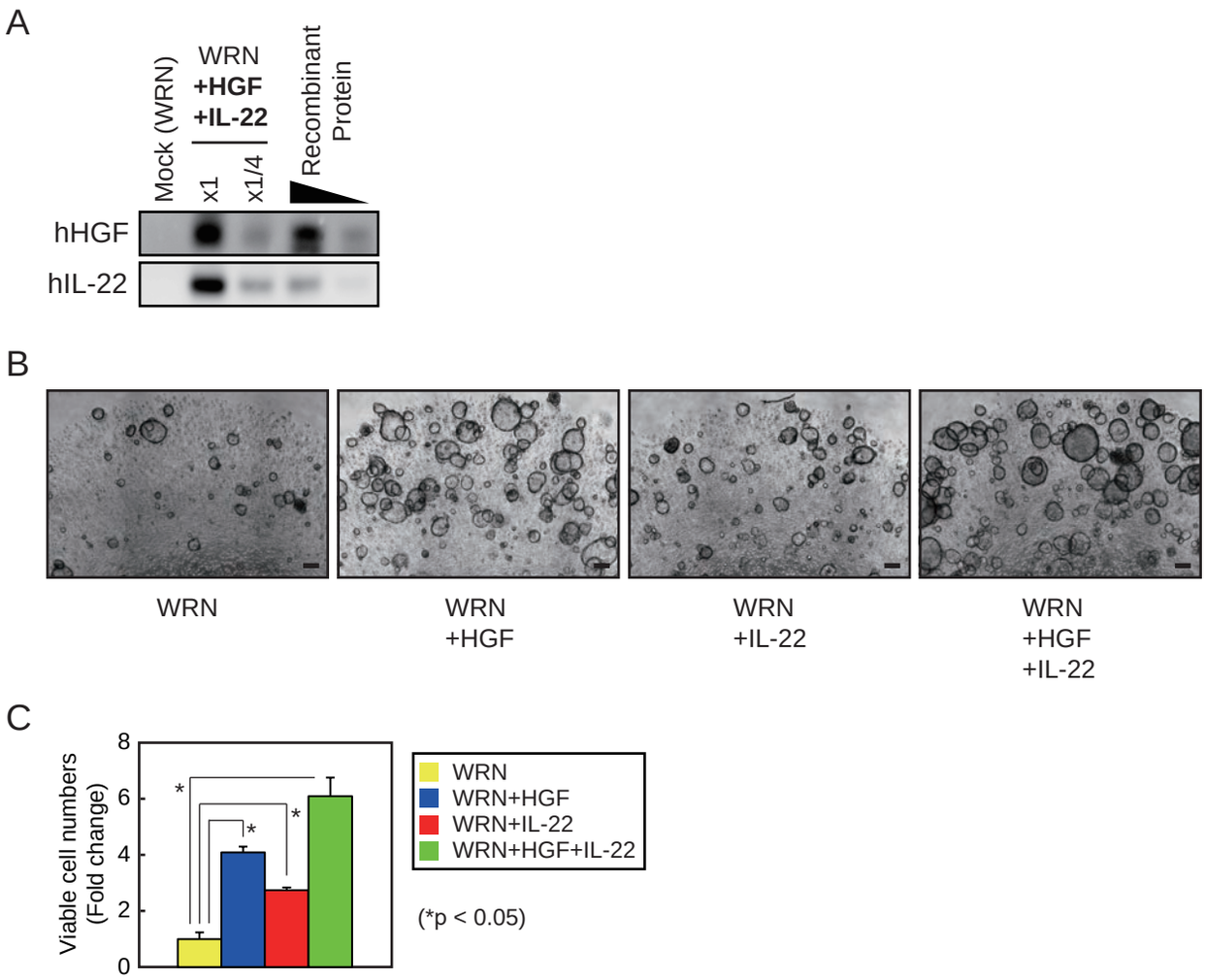

Figure S2. Preparation and characterization of WRN with HGF and/or IL-22 CM, related to Discussion. Mouse L-WRN cells established as in Figure 1 were further infected with lentiviruses for hHGF and/or hIL-22 expression. Each CM was prepared from the culture supernatants of the cells seeded at 1.4×10^6 cells/35-mm dish for 72 hr. (A) Western blot analysis of WRN+HGF+IL-22 CM undiluted (10 µL) and four-fold diluted, and the recombinant proteins HGF (1 and 0.25 ng) and IL-22 (0.2 and 0.05 ng) using anti-HGF and anti-IL-22 antibodies. (B) Bright-field images of TkDN4-M-derived organoids cultured with each conditioned medium for 7 days in Matrigel after seeded at 5,000 cells/well. Scale bars: 250 µm. (C) Viable cell numbers as in (B) were determined using the CellTiter-Glo 3D reagent. The group of WRN in the absence of HGF and IL-22 was considered as ‘‘1’’. The assay was performed in three independent biological replicates. Data are presented as mean ± SEM, *$p < 0.05$ (Dunnett’s test).

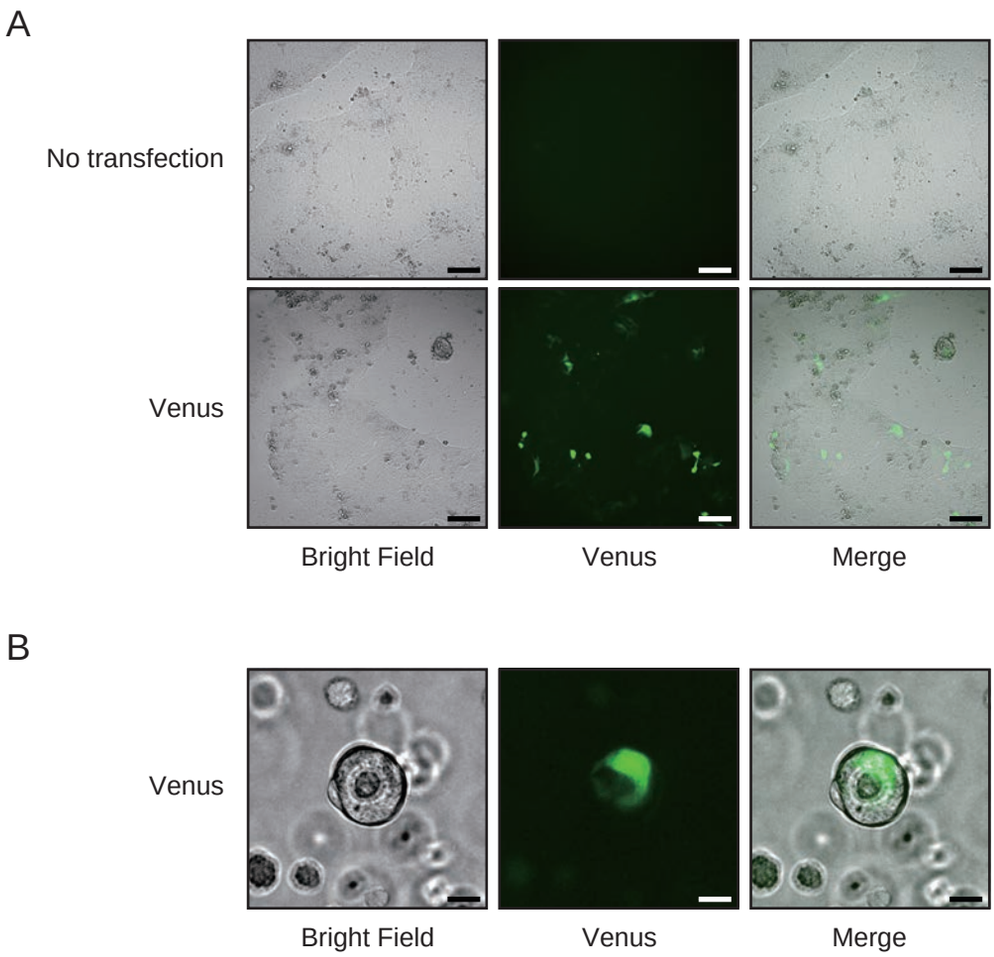

Figure S3. Lipofection-mediated gene transfection in human intestinal organoids, related to Discussion. TkDN4-M-derived organoids were disrupted by a 29G needle and seeded in a collagen I-coated 12-well plate. After a day of culture, the cells were transiently transfected with Venus expression plasmid (CSII-EF-MCS -IRES2-Venus, 1.2 μg/well) using Lipofectamine 2000 reagent (2.4 μL/well). (A) Bright-field and fluorescent images of proliferative cells were taken after 2 days of transfection. Scale bars: 100 μm. (B) Two days after transfection, the cells were trypsinized, collected, and embedded in Matrigel. After 2 days of the culture, bright-field and fluorescent images of the regenerated organoids were taken. Scale bars: 20 μm.

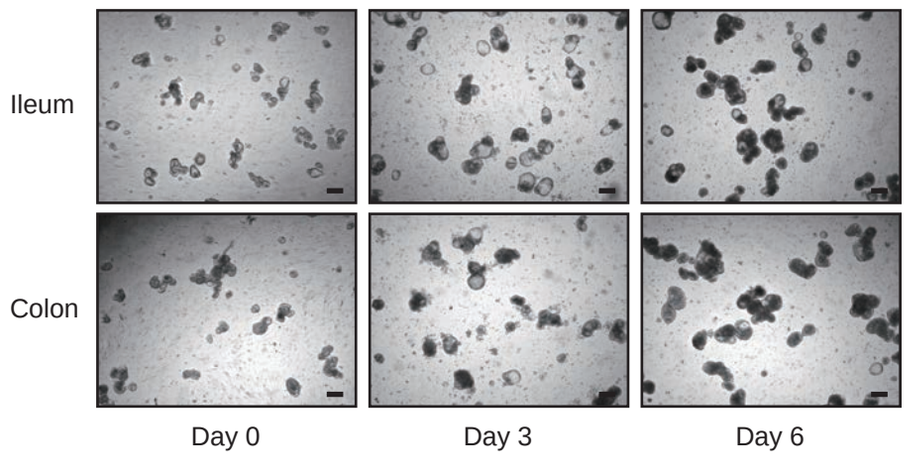

Figure S4. Growth of human primary intestinal organoids in Happy Cell ASM, related to Discussion. Human primary intestinal organoids derived from ileum or transverse colon were cultured in Happy Cell ASM supplemented with 25% WRN CM and requisite ingredients for human organoid culture (See Table S4). Bright-field images were taken during the course of the culture. Scale bars: 200 μm.

Table S3. List of materials used in this study, related to Experimental Procedures.

Antibodies	**Supplier**	**Cat. No.**
Anti-WNT3A	Abcam	ab28472
Anti-VIL1	Abcam	ab3304
Anti-MUC2	Abcam	ab11197
Anti-CHGA	Abcam	ab15160
Anti-RSPO1	Novus	NBP1-77354
Anti-NOG	Abnova	H00009241-B01
Anti-LYZ	Dako	A0099
Anti-MKI67	BD Pharmingen	550609
Anti-HGF	Cell Signaling Technology	52445
Anti-IL-22	R&D Systems	AF782

Recombinant proteins	**Supplier**	**Cat. No.**
mouse EGF	Peprotech	315-09
mouse NOG	Peprotech	250-38
mouse WNT3A	R&D Systems	1324-WN
mouse RSPO1	R&D Systems	3474-RS
human FGF4	Peprotech	100-31
human WNT3A	R&D Systems	5036-WN
human HGF	R&D Systems	294-HG
human IL-22	R&D Systems	782-IL
human NOG	R&D Systems	6057-NG
human FGF2	R&D Systems	233-FB
Activin A	R&D Systems	338-AC

Supplements for cell culture	**Supplier**	**Cat. No.**
Matrigel	Corning	354234
Collagen Gel Culture Kit	Nitta Gelatin	638-00781
Y-27632	Wako	257-00511
SB202190	Sigma	S7067
A83-01	Tocris	2939
Defined FBS	HyClone	SH30070.03
Knockout serum replacement	Gibco	10828028
Vitronectin	Gibco	A14700

Forskolin	Sigma	F6886
Polybrene	Sigma	107689
Mitomycin C	Nacalai tesque	20898-21

Table S4. Culture medium and buffer used in the study, related to Experimental Procedures.

Chelation buffer	2 mM EDTA, 5.6 mM Na_2HPO_4, 8.0 mM KH_2PO_4, 96.2 mM NaCl, 1.6 mM KCl, 43.4 mM sucrose, 54.9 mM D-sorbitol, 0.5 mM DL-dithiothreitol
iPSC maintenance medium	DMEM and Ham's nutrient mixture F-12 with 20% knockout serum replacement, 0.1 mM 2-mercaptoethanol, 2 mM L-glutamine, 1% non-essential amino acid solution, 4 ng/mL hFGF2, 100 units/mL penicillin, and 100 μg/mL streptomycin
DE1 medium	Roswell Park Memorial Institute (RPMI) 1640 with 2 mM L-glutamine, 100 ng/mL activin A, and 20 ng/mL hWNT3A
DE2 medium	RPMI 1640 with 2 mM L-glutamine, 100 ng/mL activin A, 8 ng/mL hFGF2, and 0.2% defined FBS
DE3 medium	RPMI 1640 with 2 mM L-glutamine, 100 ng/mL activin A, 8 ng/mL hFGF2, and 2% defined FBS
mid- and hindgut differentiation medium	RPMI 1640 supplemented with 2 mM L-glutamine, 2% defined FBS, 500 ng/mL hFGF4, and 500 ng/mL hWNT3A
intestine growth medium	Advanced DMEM/F12 supplemented with 15 mM HEPES (pH 7.3), 1×B-27, 1×N2, 2 mM L-Glutamine, 100 ng/mL mouse EGF (mEGF), 500 ng/mL mRSPO1, 100 ng/mL mNOG, 100 units/mL penicillin, and 100 μg/mL streptomycin
basal medium	Advanced DMEM/F12 supplemented with 10 mM HEPES (pH 7.3), 1×B-27, 1×N2, 2 mM Glutamax, 100 units/mL penicillin, and 100 μg/mL streptomycin
human organoid culture medium	Advanced DMEM/F12 with 25% WRN CM, 10 mM HEPES (pH 7.3), 1% BSA, 2 mM Glutamax, 50 ng/mL mEGF, 50 ng/mL hHGF, 10 μM Y-27632, 10 μM SB202190, 500 nM A83-01, 100 μg/mL gentamicin, 100 units/mL penicillin, and 100 μg/mL streptomycin
pre-culture medium	Advanced DMEM/F12 with 10 mM HEPES (pH 7.3), 1% BSA, 2 mM Glutamax, 50 ng/mL mEGF, 10 μM Y-27632, 10 μM SB202190, 500 nM A83-01, 100 μg/mL gentamicin, 100 units/mL penicillin, and 100 μg/mL streptomycin
pre-stimulation medium	Advanced DMEM/F12 with 20% FBS, 10 mM HEPES (pH 7.3), 2 mM Glutamax, 100 units/mL penicillin, and 100 μg/mL streptomycin
Happy Cell human organoid culture medium	1×Happy Cell (DMEM base) with 25% WRN CM, 10 mM HEPES (pH 7.3), 1% BSA, 2 mM Glutamax, 50 ng/mL mEGF, 50 ng/mL hHGF, 10 μM Y-27632, 10 μM SB202190, 500 nM A83-01, 100 μg/mL gentamicin, 100 units/mL penicillin, and 100 μg/mL streptomycin

Happy Cell pre-stimulation medium	1×Happy Cell (DMEM base) with 20% FBS, 10 mM HEPES (pH 7.3), 2 mM Glutamax, 100 units/mL penicillin, and 100 μg/mL streptomycin

Supplemental Experimental Procedures

Cell culture

HEK293T cells and L cells were obtained from ATCC. Both cell lines were cultured in DMEM with 10% FBS, 100 units/mL penicillin, and 100 μg/mL streptomycin. All cultures were incubated at 37°C in 95% humidity with 5% CO_2.

The human induced-pluripotent stem cell (iPSC) lines TkDN4-M (Takayama et al., 2010), TkCBSev9 (Yagyu et al., 2015), and TkDA3-4 (Takayama et al., 2010) were supplied by The University of Tokyo. TkDN4-M and TkCBSev9 cells were maintained as colonies in feeder-free conditions on vitronectin (Gibco)-coated plates with Essential 8 medium (Gibco); and TkDA3-4 cells were maintained on a layer of mitomycin C-treated mouse embryonic fibroblasts (feeder cells) in iPSC maintenance medium (Table S4). The cells were passaged every three to four days before reaching confluence.

Differentiation of human iPSCs into intestinal organoids

Organoids were differentiated from human iPSCs following a previously described protocol (McCracken et al., 2011), with small modifications. Briefly, 80%-90% confluent iPSCs were differentiated into definitive endoderm (DE) through treatment with DE1 medium (Table S4) for 24 hr, followed by DE2 medium (Table S4) for 24 hr, and then DE3 medium (Table S4) for a further 24 hr. For mid- and hindgut differentiation, DE cells were cultured in mid- and hindgut differentiation medium (Table S4) for up to four days. Free-floating spheroids were collected during the course of the culture, and transferred into three-dimensional cultures in Matrigel refed with intestine growth medium (Table S4), which was replaced every two to three days for 14 days. Organoids were collected after several passages, following the protocol described previously (Takahashi et al., 2017).

Lentiviral infection

HEK293T cells were transfected with each lentiviral expression plasmid together with a packaging (pCAG-HIVgp) and VSV-G- and Rev-expressing plasmid (pCMV-VSV-G-RSV-Rev) using Lipofectamine 2000 Transfection Reagent (Thermo Fisher Scientific), according to the supplier's

protocols. After 6 hr, the culture medium was replaced by fresh medium with 10 μM forskolin, and 48 hr later the medium containing lentiviruses was collected and filtered. The plasmids for lentiviral production were kindly provided by Dr. Hiroyuki Miyoshi (BioResource Center, RIKEN, Japan). Mouse L cells and organoid-derived 2D-cultured cells seeded in 6-well plates and 12-well plates, respectively, were infected with the medium supplemented with 10 μg/mL polybrene using a centrifugation method (2,500 rpm for 90 min). Following centrifugation, the cells were refed with fresh culture medium. Direct infection into organoids was conducted by incubating viral solution with recovered organoids for 90 min in 15 mL tubes.

Determination of cell viability

To measure the exact cell numbers, Matrigel-embedded TkDN4-M-derived organoids were incubated with TrypLE Express (Gibco), including 10 μM Y-27632, for 5 min at 37°C, pipetted 20–30 times, and collected by centrifugation at 440 × *g* for 5 min after the addition of five times the volume of a base medium with 10% FBS and filtration through a 20-μm nylon mesh. Five thousand cells were resuspended in Matrigel with 20% human organoid culture medium, excluding HGF, and placed into a well of a 24-well plate. After 7 days of the culture with pre-culture medium (Table S4) supplemented with each 25% CM (WRN, WRN+HGF, WRN+IL-22, WRN+HGF+IL-22), each group of organoids was collected and dispersed with TrypLE Express, including 10 μM Y-27632. After centrifugation at 440 × *g* for 5 min, cells were resuspended in 100 μL of base medium supplemented with 10% FBS, and viable cell numbers were determined using the CellTiter-Glo® 3D reagent (Promega) according to the manufacturer's protocol. The assays were performed in three independent biological replicates.

TCF reporter assay

HEK293T cells were seeded in 60-mm dishes and transfected with 1 μg TOPflash (Upstate Cell Signaling Solutions) and 1 μg pGL4.74 (hRluc/TK) (Promega) with or without 2 μg of an hR-spo1 expression plasmid (pVITRO2-hygro-mcs, InvivoGen). After 24 hr, the cells were reseeded in 96-well plates and further cultured for 8 hr. The medium was refed with WRN CM or medium containing recombinant proteins. Firefly and Renilla luciferase activities were quantified 14 hr later using a Dual-Luciferase™ reporter system (Promega), according to the manufacturer's instructions. Luciferase activities were

obtained by normalizing the firefly luciferase activities with Renilla luciferase activities. The assays were performed in three independent biological replicates.

Western blot analysis

Culture supernatants were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred onto a polyvinylidene difluoride membrane. The membrane was blocked with Blocking One (Nacalai Tesque, Japan) for 1 hr at room temperature, and then was incubated with primary antibodies against WNT3A, RSPO1, NOG, HGF, and IL-22 at a ratio of 1:1000, 1:500, 1:500, 1:1000, and 1:200, respectively. Following the incubation with HRP-conjugated secondary antibodies (1:5000, Jackson ImmunoResearch Laboratories), the immunoreactive proteins were visualized using ECL Prime Western Blotting Detection Reagent (GE Healthcare), and detected by a LAS4000mini image analyzer (GE Healthcare).

Quantitative reverse transcription polymerase chain reaction (RT-PCR)

Total cellular RNA was extracted from the cells using the RNeasy® Mini Kit (QIAGEN), and reverse transcription was performed using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems). mRNA levels were quantified by fluorescence real-time PCR on a StepOnePlus™ (Applied Biosystems) using TaqMan® Gene Expression Assays (Applied Biosystems) or PrimeTime® qPCR Assays (Integrated DNA Technologies). *GAPDH* mRNA or *HPRT1* mRNA levels were used as an internal control to normalize the mRNA levels of each gene. The assays were performed in three independent biological replicates.

Immunofluorescence staining

The organoids were fixed and permeabilized using the Cytofix/Cytoperm™ Kit (BD Biosciences). The specimens were then incubated with primary antibodies (rabbit anti-LYZ (1:400), mouse anti-MUC2 (1:250), rabbit anti-CHGA (1:100), or mouse anti-MKI67 (1:50) antibodies) at 4°C overnight. After being washed three times with Perm/Wash Buffer, the organoid specimens were further incubated with Alexa Fluor 633-conjugated Phalloidin (1:100, Invitrogen), together with Cy3-conjugated donkey anti-mouse

IgG (1:100, Jackson ImmunoResearch Laboratories), Cy3-conjugated donkey anti-rabbit IgG (1:100, Jackson ImmunoResearch Laboratories), or Alexa Fluor 488-conjugated donkey anti-rabbit IgG (1:100, Jackson ImmunoResearch Laboratories) for 3 hr at 4°C. Fluorescence staining was visualized using a Leica TCS SP2 confocal laser-scanning microscope.

Supplemental References

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