Long-term Expansion of Epithelial Organoids From Human Colon, Adenoma, Adenocarcinoma, and Barrett's Epithelium

TOSHIRO SATO,* DANIEL E. STANGE,* MARC FERRANTE,*,‡,§ ROBERT G. J. VRIES,* JOHAN H. VAN ES,* STIENEKE VAN DEN BRINK,* WINAN J. VAN HOUDT,‖,¶ APOLLO PRONK,‖ JOOST VAN GORP,# PETER D. SIERSEMA,‡ and HANS CLEVERS*

*Hubrecht Institute, KNAW and University Medical Centre Utrecht, Utrecht, The Netherlands; ‡Gastrointestinal Research Unit, Department of Gastroenterology and Hepatology, and ¶Department of Surgical Oncology, University Medical Centre Utrecht, Utrecht, The Netherlands; §Department of Gastroenterology, University Hospital Gasthuisberg, Leuven, Belgium; and Departments of ‖Surgery and #Pathology, Diakonessenhuis, Utrecht, The Netherlands

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BACKGROUND & AIMS: We previously established long-term culture conditions under which single crypts or stem cells derived from mouse small intestine expand over long periods. The expanding crypts undergo multiple crypt fission events, simultaneously generating villus-like epithelial domains that contain all differentiated types of cells. We have adapted the culture conditions to grow similar epithelial organoids from mouse colon and human small intestine and colon. **METHODS:** Based on the mouse small intestinal culture system, we optimized the mouse and human colon culture systems. **RESULTS:** Addition of Wnt3A to the combination of growth factors applied to mouse colon crypts allowed them to expand indefinitely. Addition of nicotinamide, along with a small molecule inhibitor of Alk and an inhibitor of p38, were required for long-term culture of human small intestine and colon tissues. The culture system also allowed growth of mouse Apc-deficient adenomas, human colorectal cancer cells, and human metaplastic epithelia from regions of Barrett's esophagus. **CONCLUSIONS: We developed a technology that can be used to study infected, inflammatory, or neoplastic tissues from the human gastrointestinal tract. These tools might have applications in regenerative biology through ex vivo expansion of the intestinal epithelia. Studies of these cultures indicate that there is no inherent restriction in the replicative potential of adult stem cells (or a Hayflick limit) ex vivo.**

Keywords: Ex Vivo Culture Technology; Neoplasia; Signaling; Dysplasia; Hayflick Model.

Self-renewal of the small intestinal and colonic epithelium is driven by the proliferation of stem cells and their progenitors located in crypts. Although multiple culture systems have been described,[1–4] only recently have long-term culture systems become available that maintain basic crypt physiology. Two different protocols were published that allow long-term expansion of murine small intestinal epithelium. Ootani et al showed long-term growth of small fragments containing epithelial as well as stromal elements in a growth factor–independent fashion.[5] We designed a culture system for single stem cells by combining previously defined insights in the growth requirements of intestinal epithelium.[6] Wnt signaling is a pivotal requirement for crypt proliferation,[7–9] and the Wnt activator R-spondin1 induces dramatic crypt hyperplasia in vivo.[10] We found that R-spondin-1 is a ligand for Lgr5, a marker for intestinal stem cells,[11] and an essential factor to activate Wnt signal in intestinal crypts.[12] Second, epidermal growth factor (EGF) signaling is associated with intestinal proliferation.[13] Third, transgenic expression of Noggin induces expansion of crypt numbers.[14] Fourth, isolated intestinal cells undergo anoikis outside the normal tissue context.[15] Because laminin (α1 and α2) is enriched at the crypt base,[16] we explored laminin-rich Matrigel to support intestinal epithelial growth. Under this culture condition (R-spondin-1, EGF, and Noggin in Matrigel), we obtained ever-expanding small intestinal organoids, which displayed all hallmarks of the small intestinal epithelium in terms of architecture, cell type composition, and self-renewal dynamics.

Despite extensive efforts, long-term adult human intestinal epithelial cell culture has remained difficult. There have been some long-term culture models, but these techniques and cell lines have not gained wide acceptance, possibly as a result of inherent technical difficulties in extracting and maintaining viable cells.[17–24] Encouraged by the establishment of murine small intestinal culture, we aimed to adapt the culture condition to mouse and human colonic epithelium. We now report the establishment of long-term culture protocols for murine and human colonic epithelium, which can be adapted to primary colonic adenoma/adenocarcinoma and Barrett's esophagus.

Abbreviations used in this paper: DAPI, 4′,6-diamidino-2-phenylindole; DBZ, dibenzazepine; EGF, epidermal growth factor; ENR, murine epidermal growth factor, murine noggin, human R-spondin-1; g, gastrin; GFP, green fluorescent protein; HISC, human intestinal stem cell; WENR, recombinant human Wnt-3A or Wnt-3A conditioned medium plus murine epidermal growth factor, murine noggin, human R-spondin-1.

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Materials and Methods

Reagents

Reagents used in the culture experiments are shown in Supplementary Table 1.

Mice

Lgr5-EGFP-ires-creERT2 mice,[11] *APC*$^{fl/fl}$ mice,[25] *Axin2-lacZ* mice,[26] and C57B/6 wild-type mice (6–12 weeks old) were used for experiments. *Lgr5-EGFP-ires-creERT2* mice were crossed with *APC*$^{fl/fl}$ mice. Cre enzyme activity was induced by intraperitoneal injections of tamoxifen (2 mg/mouse). Murine small intestines and colons were opened longitudinally, cut in small pieces, and washed with cold phosphate-buffered saline (PBS). Regions containing intestinal adenomas were identified using a stereomicroscope, cut out with a scalpel, and washed with cold PBS.

Human Tissue Materials

Surgically resected intestinal tissues or endoscopic biopsy samples were obtained from 30 patients from the Diaconessen Hospital Utrecht or the University Medical Centre Utrecht hospital. Patient material was collected from 20 patients with colon cancer (9 cecum-ascending colon, 7 sigmoid colon, 4 rectum; 33–86 years old), 5 patients with screening colonoscopy (33–63 years old), and 5 patients with Barrett's esophagus (45–78 years old). For normal tissue, a distance of more than 3 cm to the tumors was kept. The intestinal tissues were washed and stripped of the underlying muscle layers with surgical scissors. The tissue was chopped into approximately 5-mm pieces and further washed with cold PBS. For endoscopic biopsy samples, at least 5 biopsy samples were collected. This study was approved by the ethical committee of Diaconessen Hospital Utrecht and University Medical Centre Utrecht, and all samples were obtained with informed consent.

Crypt/Adenoma Isolation and Cell Dissociation

Intestinal fragments (murine normal colon, human normal small intestine, and colon) were further washed with cold PBS until the supernatant was clear. Next, the tissue fragments were incubated in 2 mmol/L EDTA cold chelation buffer (distilled water with 5.6 mmol/L Na_2HPO_4, 8.0 mmol/L KH_2PO_4, 96.2 mmol/L NaCl, 1.6 mmol/L KCl, 43.4 mmol/L sucrose, 54.9 mmol/L D-sorbitol, 0.5 mmol/L DL-dithiothreitol) for 30 minutes on ice.[27] After removal of the EDTA buffer, tissue fragments were vigorously resuspended in cold chelation buffer using a 10-mL pipette to isolate intestinal crypts. The tissue fragments were allowed to settle down under normal gravity for 1 minute, and the supernatant was removed for inspection by inverted microscopy. The resuspension/sedimentation procedure was typically 6–8 times, and the supernatants not containing crypts were discarded. The supernatants containing crypts were collected in 50-mL Falcon tubes coated with bovine serum albumin. Isolated crypts were pelleted, washed with cold chelation buffer, and centrifuged at 150–200*g* for 3 minutes to separate crypts from single cells.

Murine colonic crypts were pelleted and resuspended with TrypLE Express (Invitrogen, Carlsbad, CA) and incubated for 15 minutes at 37°C. In this dissociation condition, colonic crypts were mildly digested, thereby physically separating colonic crypt bottoms from the top of the colon crypts.

Intestinal fragments containing adenomas from tamoxifen-induced *Lgr5-EGFP-ires-creERT2/APC*$^{fl/fl}$ mice were incubated in 2 mmol/L EDTA chelation buffer for 60 minutes on ice. Following washing with cold chelation buffer, most of the normal intestinal epithelial cells were detached, while adenoma cells remained attached to the mesenchyme. Next, the adenoma fragments were incubated in digestion buffer (Dulbecco's modified Eagle medium with 2.5% fetal bovine serum, penicillin/streptomycin [Invitrogen], 75 U/mL collagenase type IX [Sigma, St Louis, MO], 125 μg/mL dispase type II [Invitrogen]) for 30 minutes at 37°C. The adenoma fragments were allowed to settle down under normal gravity for 1 minute, and the supernatant was collected in a 50-mL Falcon tube, pelleted, and washed with PBS. Isolated adenoma cells were centrifuged at 150–200*g* for 3 minutes to separate adenoma from single cells.

Biopsy samples from Barrett's epithelium and human colon cancer samples, chopped into 5-mm pieces, were washed with PBS several times. The tissue fragments were incubated in digestion buffer for 60 minutes at 37°C. After the digestion, tissue fragments were manually picked under the microscope.

For sorting experiments, isolated crypts were dissociated with TrypLE Express (Invitrogen) including 2000 U/mL deoxyribonuclease (Sigma) for 60 minutes at 37°C. Dissociated cells were passed through a 20-μm cell strainer (Partec Cell Trics, Munster, Germany) and washed with PBS. Viable epithelial single cells were gated by forward scatter, side scatter, pulse width, and negative staining for propidium iodide or 7-Amino-Actinomycin (7-AAD) (eBioscience, San Diego, CA).

Culture of Intestinal Crypts, Adenomas, Barrett's Epithelium, and Colon Cancer

Isolated intestinal crypts, Barrett's epithelium, and colon cancer cells were counted using a hemocytometer. Crypts, fragments of epithelium, or single cells were embedded in Matrigel on ice (growth factor reduced, phenol red free; BD Biosciences) and seeded in 48-well plates (500 crypts/fragments or 1000 single cells per 25 μL of Matrigel per well). The Matrigel was polymerized for 10 minutes at 37°C, and 250 μL/well basal culture medium (advanced Dulbecco's modified Eagle medium/F12 supplemented with penicillin/streptomycin, 10 mmol/L HEPES, Glutamax, 1× N2, 1× B27 [all from Invitrogen], and 1 mmol/L *N*-acetylcysteine [Sigma]) was overlaid containing the following optimized growth factor combinations: murine EGF for murine intestinal adenomas, ENR (murine EGF, murine noggin, human R-spondin-1) for murine small intestinal crypts, WENR (recombinant human Wnt-3A or Wnt-3A conditioned medium + ENR) for murine colonic crypts, human intestinal stem cells (HISC; WENR + gastrin + nicotinamide + A83-01 + SB202190) for human small intestinal/colonic crypts, and HISC + human fibroblast growth factor 10 for Barrett's epithelium. Colon cancer cells show a heterogeneous behavior and require no addition of growth factors, murine EGF, and/or A83-01 and/or SB202190. For cell sorting experiments, Y-27632 (10 μmol/L; Sigma) was included in the medium for the first 2 days to avoid anoikis. Reagents and concentrations of each growth factor are indicated in Supplementary Table 1. An overview of the optimized combinations of growth factors and small molecule inhibitors for each organ is given in Table 1.

Image Analysis

See Supplementary Materials and Methods section.

Maintenance of Established Organoids

See Supplementary Materials and Methods.

Table 1. Optimized Culture Conditions

Species	Organ	Stem cell culture condition									Differentiation condition
		Basal culture medium	EGF	Noggin	R-spondin	Wnt3A	Nicotinamide	Gastrin	A-83-01	SB202190	Fibroblast growth factor 10	Withdraw (−) or add (+) indicating factors
Mouse	Small intestine	○	○	○	○	−	−	−	−	−	−	
	Colon	○	○	○	○	○	−	−	−	−	−	−Wnt3A
	Adenoma	○	○	−	−	−	−	−	−	−	−	
Human	Small intestine	○	+ gastrin	○	○	○	○	○	○	○	−	−Wnt3A−SB202190−nicotinamide
	Colon	○	+ gastrin	○	○	○	○	○	○	○	−	−Wnt3A−SB202190−nicotinamide
	Colon cancer	○	+ gastrin	Varied	−	−	−	−	Varied	Varied	−	
	Barrett's epithelium	○	+ gastrin	○	−	○	○	○	○	○	○	−Wnt3A−SB202190−nicotinamide+DBZ

NOTE. Basal culture medium: Advanced Dulbecco's modified Eagle medium/F12 supplemented with penicillin/streptomycin, HEPES, Glutamax, 1× N2, 1× B27, and *N*-acetylcysteine. Concentration is indicated in Supplementary Table 1.

Microarray Analysis and Real-time Polymerase Chain Reaction Analysis

The data were deposited in the GEO database under accession number GSE28907. See Supplementary Materials and Methods for detailed information.

Results

Establishment of a Mouse Colon Culture System

In an attempt to establish a mouse colon culture system, we explored our small intestinal culture condition (here termed ENR). In our experience, initial growth of colon epithelium is often observed under the ENR culture condition but is invariably abortive. Organoid formation was studied using epithelium isolated from the distal part of the mouse colon. The plating efficiency of single distal colonic crypts was much lower than that of small intestine (1%–3% vs >90%), and these organoids could not be passaged. Recently, we have shown that Paneth cells produce several Wnt ligands[28] and that the production of Wnt by these Paneth cells is essential to maintain intestinal stem cells.[29] To determine the Wnt signaling status in colon organoids, we cultured colon crypts from *Axin2-lacZ* mice (a faithful Wnt reporter)[26] or *Lgr5-GFP* knock-in mice (Lgr5 being a Wnt-dependent stem cell marker).[11]

Freshly isolated colon crypts readily expressed Axin2-LacZ or Lgr5–green fluorescent protein (GFP) at their bottoms, but they lost expression of the Wnt reporters shortly after initiation of culture (Figure 1*A* and *B* and Supplementary Figure 1). In contrast, small intestinal organoids constitutively expressed the Wnt reporters at their budding structures.[6,29] These findings suggested that colon organoids produce insufficient amounts of Wnt ligands to maintain colon stem cells. To overcome this, we added recombinant Wnt3A or Wnt3A-conditioned medium to ENR culture medium (WENR medium). This increased plating efficiency of crypts in the order of 10-fold. Colon crypts formed organoid structures with numerous Axin2-LacZ (Figure 1*A*) or Lgr5-GFP$^+$ (Figure 1*B*) buds, implying that Wnt activation was restored. Freshly isolated colon crypts contain fully mature cells in their upper parts, and we reasoned that these mature cells might interfere with organoid growth. When we mildly digested colon crypts into small clusters of cells, thus physically separating proliferative crypt bottoms from differentiated upper crypt regions, most of the fragments derived from crypt top died, yet cell clusters from colon crypt base efficiently formed organoids (Figure 1*C*).

Mouse small intestinal epithelium grown under ENR conditions generates all differentiated epithelial cell types concomitant with stem cell self-renewal. We have shown previously that the addition of Wnt3A to these cultures interferes with intestinal differentiation and yields organoids that largely consist of undifferentiated progenitors.[29] This is not unexpected given the central role of Wnt signaling in the maintenance of the undifferentiated crypt progenitor state.[30] Consistent with this observation,

colonic organoids in WENR condition failed to differentiate properly. On withdrawal of Wnt-3A, we observed differentiation along all epithelial lineages (Figure 1*D–F*). Of note, single sorted Lgr5-positive colonic epithelial stem cells can form organoids when cultured in the presence of Y-27632 for the first 2 days.

Establishment of Human Colon Culture System

Encouraged by the success of the improved mouse colon crypt culture, we applied the culture condition to human colon crypts. Although these crypts initially survived, most subsequently disintegrated within 7 days. To increase the plating efficiency of human colon crypts, we screened candidate growth factors, hormones, and vitamins (list in Supplementary Table 2). Among these, we found that gastrin and nicotinamide (precursor of oxidized nicotinamide adenine dinucleotide and found to suppress sirtuin activity[31]) improved the culture efficiency. The effect of gastrin on plating efficiency was marginal. However, the hormone did not interfere with intestinal differentiation and we decided to include gastrin (hereafter shortened to "g") in all human intestinal culture conditions. Importantly, nicotinamide (10 mmol/L) was essential for prolongation of the culture period beyond the initial 7 days (Figure 2*A*). Under this culture condition, human colonic organoids could be expanded for at least 1 month. From 1 month onward, the colonic organoids changed their morphology from budding structures into cystic structures (Figure 2*B*, *left*). Coinciding with the morphologic conversion, proliferation progressively decreased. Occasionally, cystic organoids regained their proliferative potential. However, all organoids eventually arrested growth within 3 months. A 2-phase growth arrest has been observed in other primary culture systems, such as mammary epithelial cells or keratinocytes, and has been referred to as mortality stage 1 (M1; senescence) and mortality stage 2 (M2; crisis).[32]

We assumed that growth arrest occurred because of inadequate culture conditions rather than a cell-intrinsic property of senescence/replicative aging. We therefore extended our attempts to optimize the culture condition. We screened various small molecule modulators of mitogen-activated protein kinases, of signaling molecules mutated in colon cancer, and of histone modifiers (Supplementary Table 3) under the WENRg + nicotinamide culture condition. We found that 2 small molecule inhibitors, A83-01 (Alk4/5/7 inhibitor; 500 nmol/L) and SB202190 (p38 inhibitor; 10 μmol/L), significantly improved the plating efficiency. Furthermore, the combination of the 2 compounds synergistically prolonged the culture period. We showed that all of the 10 tested samples expanded for at least 6 months with a weekly 1:5 split. Under this culture condition, the human colonic organoids displayed budding structures, rather than the cystic structures seen under the previous culture condition (Figure 2*B*). The proliferating cells were confined to the buds (Figure 2*C*). Metaphase spreads of organoids more than 3 months old consistently revealed 46 chromosomes in each cell (20 cells each tested from 3 different donors; Figure 2*D*). Human small intestinal crypts can be grown under the same culture conditions. Microarray analysis revealed that the small intestinal and colonic organoids possess comparable molecular signatures of intestinal crypts, including the expression of intestinal stem cell genes (Figure 2*E* and Supplementary Table 4). These results implied that Alk receptor and p38 signaling negatively regulate long-term maintenance of human intestinal epithelial cells. We refer to the optimized culture condition as the HISC (human intestinal stem cell culture) condition.

Human Intestinal Organoids Mimic In Vivo Differentiation

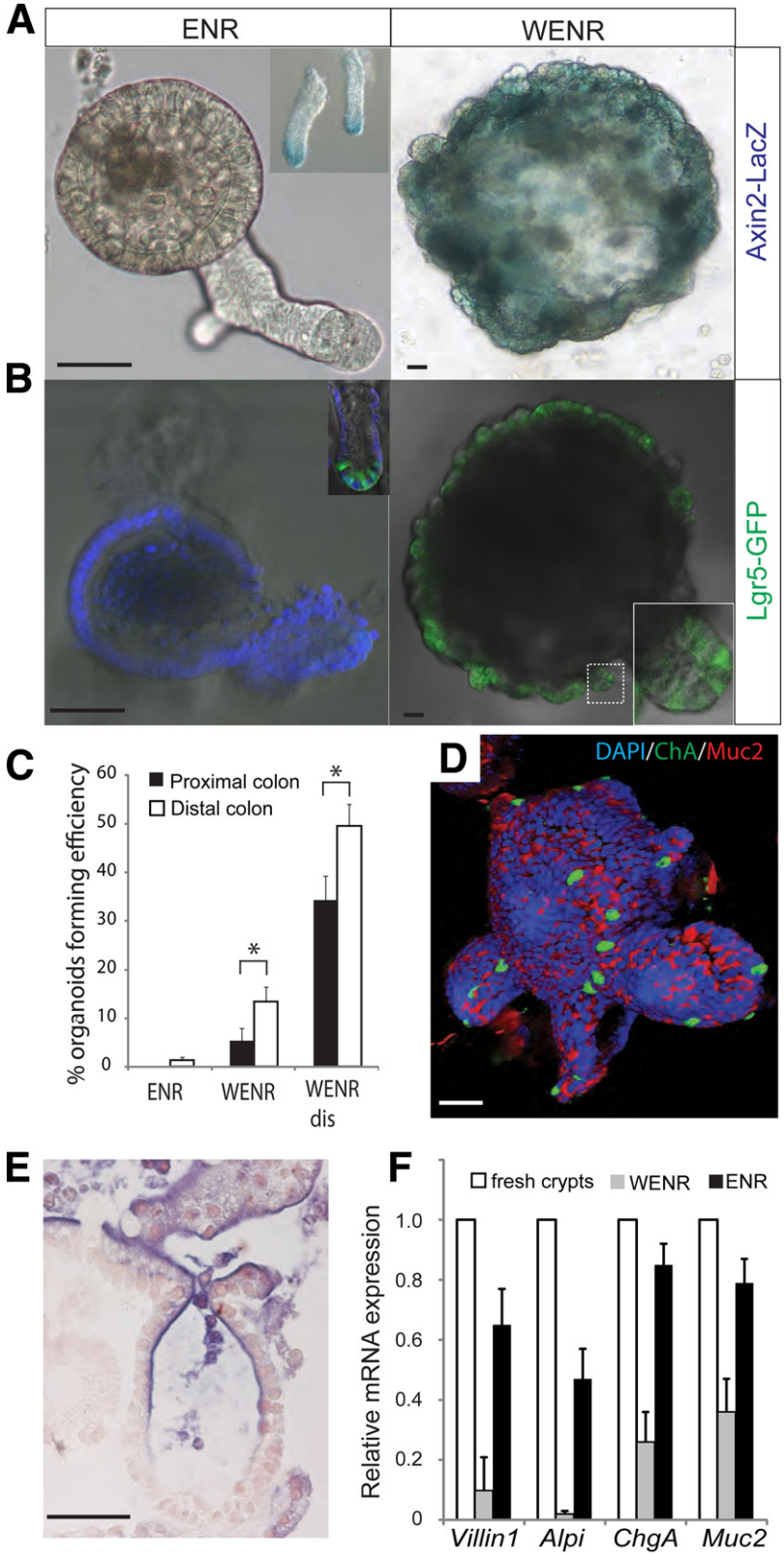
Under the HISC condition, we failed to observe differentiated cells. As was seen in the mouse colon organoids, withdrawal of Wnt was required for mature enterocyte differentiation in human colon organoids (Figure 3*A*, *top panel*, and Supplementary Figure 2). However, goblet and enteroendocrine cell differentiation remained blocked. We found that nicotinamide and SB202190 strongly inhibited this differentiation, while withdrawal of the 2 reagents enabled the organoids to produce mature goblet and enteroendocrine cells (Figure 3*A* [*middle and bottom panels*] and *B* and Supplementary Figure 2). The same differentiation inhibitory effects of Wnt, nicotinamide, and SB202190 were observed in human small intestinal organoids. Lysozyme-positive Paneth cells were observed in small intestinal organoids (Figure 3*C*) but not in colonic organoids. It has been reported that p38 inhibitor treatment in vivo inhibits goblet cell differentiation and increases intestinal epithelial proliferation.[33] Indeed, we observed the same phenotype in the p38 inhibitor–treated intestinal organoids (Figure 3*D* vs *E*).

We further examined the response of human intestinal organoids to Notch inhibition. We have previously shown that Notch inhibition with either γ-secretase inhibitors (dibenzazepine [DBZ]) or by conditional targeting of the Notch pathway transcription factor CSL depleted intestinal stem cells, terminated intestinal epithelial proliferation, and induced goblet cell hyperplasia in vivo.[34] Indeed, on treatment with DBZ (10 μmol/L), the intestinal organoids ceased their proliferation and most cells converted into goblet cells within 3 days (Figure 3*E* vs *F*).

Establishment of APC-Deficient Adenoma and Colon Adenocarcinoma

Recently, we reported efficient mouse intestinal adenoma formation from Lgr5 stem cells in *Lgr5-GFP-ires-CreERT2* × *APC*$^{fl/fl}$ mice on tamoxifen-induced Cre activation.[35] We isolated the intestinal adenomas 10 days after induction and adapted the culture condition. The adenomas efficiently formed cystic organoid structure without

budding. Because APC loss constitutively activates the Wnt pathway, we expected that R-spondin-1 would become dispensable for adenoma organoid growth. This was indeed observed. Furthermore, Noggin, which is essential for long-term culture of normal small intestine, was dispensable in adenoma organoids. Interestingly, we observed a loss of *Lgr5* but not *Axin2* in adenomatous organoids 7 days after withdrawal of Noggin (Figure 4*A* and *B*). Similar observations were made for normal intestinal organoids when grown in ER medium.[6] This indicated that Noggin,



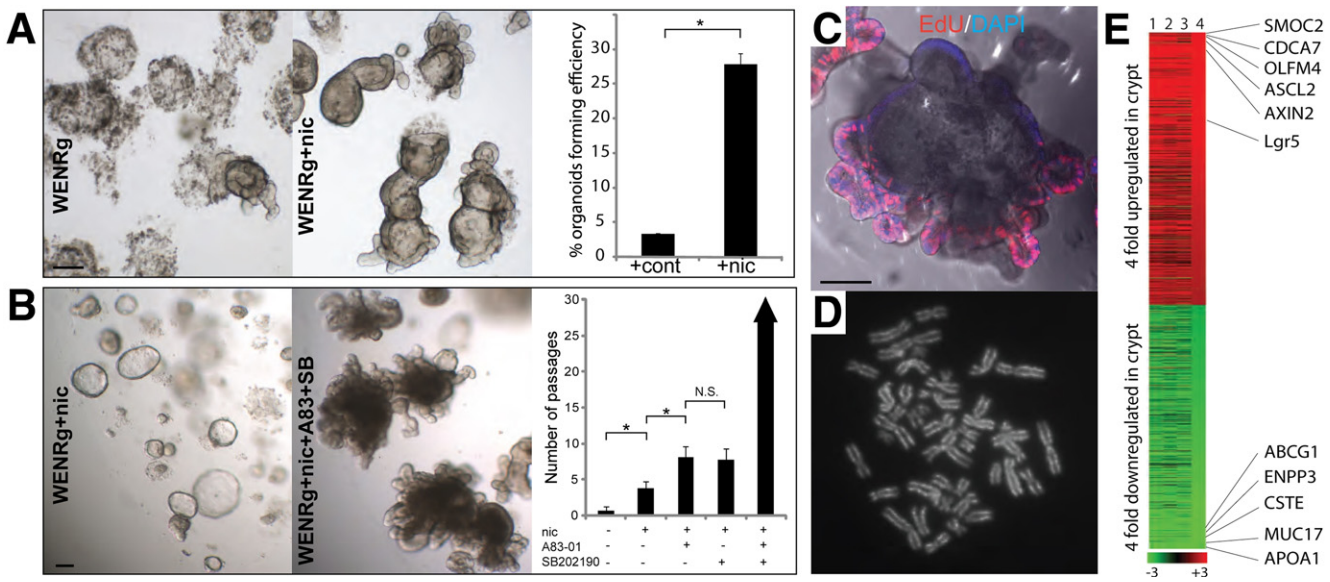


Figure 2. Human colon culture. (*A*) The effect of nicotinamide on human colon crypt organoids. The majority of human colon crypt organoids die within a few days in WENR + gastrin (WENRg) condition (*left panel*). Addition of nicotinamide (*middle panel*: WENRg + nicotinamide [nic]) improves culture efficiency and life span of human colon organoids. *$P < .001$. (*B*) The effect of small molecule inhibitor for Alk4/5/7 (A83-01) and for the mitogen-activated protein kinase p38 (SB202190) on human colon crypt organoids. (*Left panel*) Human colon organoid cultures in WENRg + nicotinamide–containing medium form cystic structures 3 to 4 weeks after culture. (*Middle panel*) Human colon organoids retain their characteristic budding structure under the HISC condition (WENRg + nicotinamide + A83-01 + SB202190). (*Right panel*) A83-01 and SB202190 synergistically increase the number of passages of the human colon organoids. *$P < .001$. N.S., not statistically significant. *Error bars* indicate SEM. n = 5. (*C*) Proliferating cells visualized by the incorporation of 5-ethynyl-2′-deoxyuridine (EdU) (*red*) are confined to the budding structures. Counterstain is DAPI (*blue*). (*D*) Representative picture of a karyotype from a 3-month-old human colon crypt organoid. *Scale* = 100 μm. (*E*) Heat map of the expression profile of cultured human intestinal organoids. Organoids cultured in vitro clearly exhibit a similar expression profile to freshly isolated small intestinal crypts and express known stem cell markers. *Lane 1*, human small intestinal organoids #1; *lane 2*, human small intestinal organoids #2; *lane 3*, human colon organoids; *lane 4*, freshly isolated human small intestinal crypts. The 4 samples are compared with human small intestinal villus.

most likely through inhibition of BMP signals, is required to maintain Lgr5 expression but is not required for expansion of adenoma organoids. Freshly isolated Lgr5^{hi} (but not Lgr5^{low}) cells isolated from intestinal crypts can initiate organoid growth in vitro.[6] To determine the existence of a similar Lgr5 hierarchy within adenomas, we isolated Lgr5-GFP^{hi}, GFP^{low}, and GFP^{-ve} cells from EN-cultured organoids and examined their organoid formation ability. After a 7-day culture, Lgr5-GFP^{hi} cells showed the highest organoid-forming efficiency, yet Lgr5-GFP^{low} or GFP^{-ve} cells also formed organoids with considerable efficiency (Figure 4*C*). Of note, sorted GFP^{-ve} adenoma cells could give rise to Lgr5-GFP^{hi} organoids (Supplementary Figure 3).

Many colorectal cancer cell lines have been isolated over the past 4 decades. Typically, such cell lines emerge as rare, clonal outgrowths after primary cultures of colon tumors enter tissue culture crisis. Currently, no robust culture system exists that allows the consistent culture of primary human colon cancer samples without culture crisis and the consequent clonal outgrowth of culture-adapted cells. As an obvious next step, we applied the intestinal adenoma culture condition to human colorectal cancer samples. As expected, colon cancer cells required

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Figure 1. Mouse colon culture. (*A*) (*Left*) Colon crypt organoids of *Axin2-LacZ* reporter mice cultured with ENR for 3 days. Absence of LacZ stain indicates no active Wnt signal is present in the colon organoids under ENR growth condition. *Inset* shows active Wnt signaling visualized by LacZ (*blue*) expression in freshly isolated colon crypts from the *Axin2-LacZ* reporter mice. (*Right*) *Axin2-LacZ* mice derived colon crypts cultured with ENR + Wnt3A (WENR) for 10 days. *Blue stain* indicates LacZ expression in these organoids. (*B*) (*Left*) *Lgr5-GFP-ires-CreER* colon crypts cultured with ENR for 3 days. Absence of GFP fluorescence indicates loss of Lgr5 expression in the colon organoids under ENR growth condition. *Inset* shows Lgr5-GFP expression in freshly isolated colon crypts from *Lgr5-GFP-ires-CreER* mice. (*Right*) *Lgr5-GFP-ires-CreER* colon crypt cultured with WENR for 10 days shows the presence of Lgr5 stem cells. (*C*) Culture efficiency is determined under 3 different conditions: ENR, WENR full crypts, and WENR crypts after mild enzymatic digestion (WENR digested). Colon crypts were isolated from proximal colon (*dark gray*) or distal colon (*light gray*). *$P < .05$. (*D* and *E*) Four days after removal of Wnt3A from the WENR, culture medium results in organoid differentiation. (*D*) Chromogranin A in enteroendocrine cells (*green*). Mucin2 in goblet cells (*red*). Counterstain is 4′,6-diamidino-2-phenylindole (DAPI) (*blue*). (*E*) Mature enterocytes are visualized by alkaline phosphatase staining (*purple*). (*F*) Relative messenger RNA expression of mature epithelial cell markers (*Villin1*, alkaline phosphatase [*Alpi*], Chromogranin A [*ChgA*], Mucin2 [*Muc2*]) are shown. WENR cultured colon crypt organoids were cultured for 4 days in WENR (*gray*) or ENR (*black*) condition. Freshly isolated colon crypts (*white*) were used for control. *Scale bar* in *A*, *B*, *D*, and *E* = 50 μm. *Error bars* indicate SEM. n = 3.

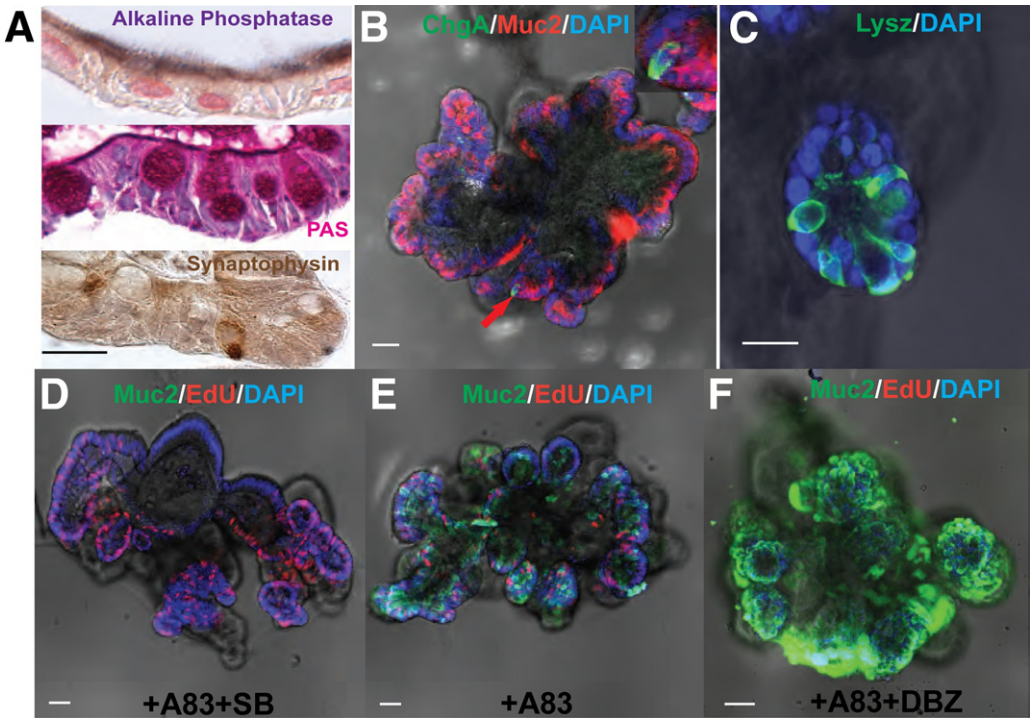

Figure 3. Human intestinal organoid cell type composition. (*A–C*) Human organoids differentiate into the different cell types of the intestine after withdrawal of nicotinamide and SB202190. Markers of the different cell types were used to show differentiation. (*A*) (*top panel*) Alkaline phosphatase (*purple*) for mature enterocytes. (*Middle panel*) Periodic acid–Schiff (PAS) staining (*purple*) for goblet cells. (*Bottom panel*) Synaptophysin (*brown*) for enteroendocrine cells. (*B*) Mucin2 (Muc2; *red*) for goblet cells and Chromogranin A (ChgA; *green*) for enteroendocrine cells (*red arrow and inset*). (*C*) Lysozyme (Lysz; *green*) for Paneth cells. (*D–F*) Goblet cell differentiation (Muc2, *green*) is blocked by SB202190 treatment of organoids (*D*), while the Notch inhibitor DBZ increases goblet cell number in the human organoids (*F*). Proliferating cells (5-ethynyl-2′-deoxyuridine [EdU] incorporation: *red*) are increased in SB202190-treated organoids (*D*) or decreased in DBZ-treated organoids (*F*). Organoids are cultured under the following conditions for 5 days: (*A, top*) ENRg + A83-01 + SB202190 + nicotinamide, (*A, middle and bottom, B, and C*) WENRg + A83-01, (*D*) WENRg + A83-01 + SB202190, (*E*) WENRg + A83-01, and (*F*) WENRg + A83-01 + DBZ. *Scale bar*: *A*, 20 μm; *B–F*, 50 μm. *A, B, and D–F*, human colon crypt organoids; *C*, human small intestinal organoids.

neither R-spondin nor Noggin. EGF was dispensable in most colon cancer organoids, while some colon cancer organoids decelerated their proliferation after withdrawal of EGF. Distinct from mouse intestinal adenoma, colorectal cancer organoids in the culture condition grew as irregular compact structures rather than as simple cystic structures (Figure 4*D*).

We examined the proliferation/differentiation status of adenoma and colon cancer organoids. As expected, most of the cells were Ki67 positive. Consistent with the strong inhibitory effect of Wnt on enterocyte differentiation (Figure 1*F* and Supplementary Figure 2), alkaline phosphatase staining was not observed in both types of organoids (Supplementary Figure 4). In contrast, we occasionally observed periodic acid–Schiff–positive goblet cells and chromogranin A–positive endocrine cells in adenoma organoids and in some colon cancer organoids (Supplementary Figure 4).

Culturing Human Metaplastic Barrett's Epithelium

Barrett's esophagus is marked by the presence of columnar epithelium in the lower esophagus, replacing the normal squamous cell epithelium as a result of metaplasia.[36] The histologic hallmark of Barrett's esophagus is the presence of intestinal goblet cells in the esophagus. Exploiting the similarity between Barrett's and intestinal epithelium, we subjected small Barrett's epithelium biopsy specimens to the HISC conditions. Under the culture conditions, normal esophageal squamous cells transiently proliferated for 1 week, but the organoids could not be passaged. Barrett's esophagus epithelium could be maintained for up to 1 month under HISC conditions (Figure 5*A*) but then formed cystic organoid structures indistinguishable from that of senescent human colon organoids and typically underwent growth arrest. Addition of fibroblast growth factor 10 (100 ng/mL) to the HISC condition enabled the Barrett's epithelium organoids to form budding structures and significantly prolonged the culture duration (>3 months) (Figure 5*B* and *C*). In contrast to human intestinal organoids, Barrett's epithelium organoids remained Ki67 positive with a minimal number of periodic acid–Schiff–positive and mucin-positive cells 4 days after withdrawal of nicotinamide and SB202190. Treatment with the γ-secretase inhibitor DBZ (10 μmol/L) for 4 days after the withdrawal blocked proliferation and induced goblet cell differentiation (Figure 5*D*). This supported our previous suggestion that local delivery of such inhibitors may represent a useful therapeutic

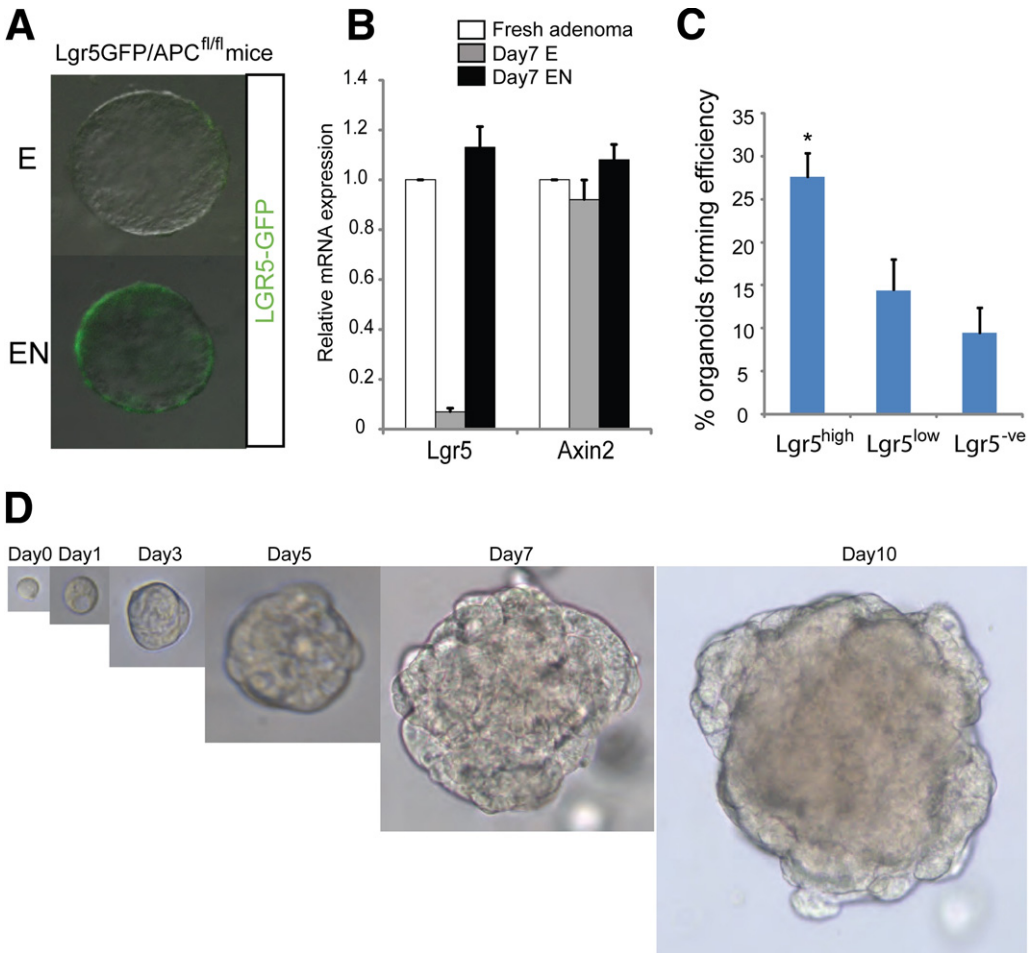

Figure 4. Adeno(carcino)ma cultures. (*A*) *Lgr5-GFP-ires-CreER/APC$^{fl/fl}$* crypts cultured with EGF (E) (*top*) or EGF + Noggin (EN) (*bottom*) for 10 days. (*B*) Relative messenger RNA expression of Lgr5 and Axin2. Freshly isolated adenoma cells (*white*) were cultured with EGF (*gray*) or EGF + Noggin (*black*). (*C*) Culture efficiency of organoids from sorted Lgr5-GFP^{hi}, Lgr5-GFP^{lo}, and Lgr5-GFP^{-ve} cells. *$P < .01$. One-way analysis of variance. *Error bars* indicate SEM. n = 3. (*D*) Time course culture of human colon adenocarcinoma cells.

strategy for the removal of Barrett's esophagus lesions by differentiation therapy.[37] Of note, we occasionally observed lysozyme-positive Paneth cells (Supplementary Figure 5), which indicates that Barrett's epithelium organoids preserve multilineage differentiation.

Discussion

The protocols developed here allow robust and long-term culture of primary human epithelial cells isolated from small intestine, colon, adeno(carcino)mas, and Barrett's esophagus. In contrast to murine small intestine, murine colonic epithelial cells require Wnt ligand in the culture medium. We have previously reported that CD24^{hi} Paneth cells produce Wnt-3/11, which are essential for stem cell maintenance in the small intestine.[29] Wnt-6 and -9b messenger RNA are expresses at the bottom of colon crypts.[28] It remains undetermined whether this local Wnt production by colon crypt base cells is sufficient to activate canonical Wnt signal in vivo or there is another source of Wnt ligand in colon mucosa. The difference between human and mouse intestinal organoid culture conditions was unexpectedly large. A83-01 inhibits ALK4/5/7, receptors that are detected in both murine and human crypts by microarray. We are currently investigating the mechanism by which ALK signal regulates human organoid growth. We have not observed cellular transformation in long-term cultures, and no chromosomal changes become obvious under the optimized culture conditions. Furthermore, the organoids can undergo a considerably higher number of cell division than reported for other adult human epithelial culture systems.[38,39] It is generally believed that somatic cells are inherently limited in their proliferative capacity, a phenomenon called replicative aging.[40] Most normal human cells are believed to count the number of times they have divided, eventually undergoing a growth arrest termed cellular senescence. This process may be triggered by the shortening of telomeres and the consequent activation of DNA damage signals (M1) or telomere attrition (M2).

In the absence of the 2 small molecule kinase inhibitors, human intestinal organoids underwent growth arrest after 10 to 20 population doublings. By contrast, the replicative capacity in the optimized culture condition was extended at least up to 100 population doublings on addition of the

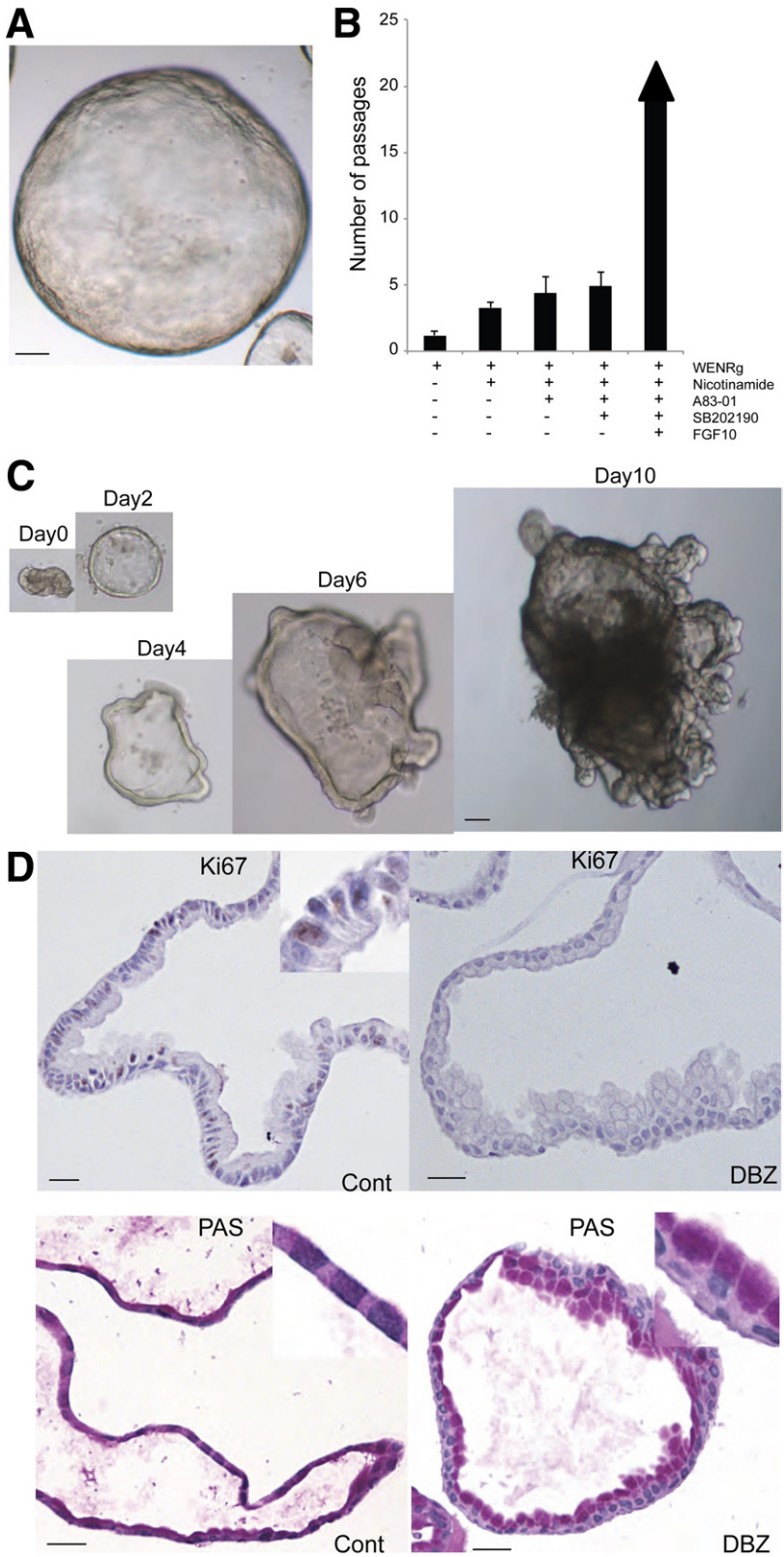

Figure 5. Culture of Barrett's esophagus and treatment with Notch inhibitor. (*A*) Isolated epithelium from Barrett's esophagus cultured with HISC condition for 7 days forms cystic structures. (*B*) Addition of fibroblast growth factor 10 (FGF10) significantly increases the number of passages for Barrett's esophagus organoids. Error bars indicate SEM. n = 3. (*C*) Representative time course of a Barrett's esophagus organoid. (*D*) Paraffin sections from Barrett's esophagus organoids. Nicotinamide and SB202190 are withdrawn for 4 days with (*right*) or without (*left*) the Notch inhibitor DBZ added to the medium. Proliferating cells (Ki67: *brown*) disappear and periodic acid–Schiff (PAS)-positive goblet cells increase with DBZ treatment.

inhibitors, which exceeded the Hayflick limit.[41] This result clearly indicates that the senescent phenotype seen in the first culture system reflects inadequate growth conditions rather than inherent replicative aging.

The culture techniques can be used to study basic aspects of stem cell biology and the control of differentiation, exemplified by depletion of stem cells and goblet cell differentiation on Notch inhibitor treatment. Moreover, the organoid culture platform may be used for pharmacologic, toxicologic, or microbiologic studies on pathologies of the intestinal tract, because the organoids represent more closely the intestinal epithelium than often-used colon cancer cell lines such as CaCo2 or DLD1. Lastly, because small biopsy specimens taken from adult donors can be expanded without any apparent limit or genetic harm, the technology may serve to generate transplantable epithelium for regenerative purposes.

Supplementary Material

Note: To access the supplementary material accompanying this article visit the online version of *Gastroenterology* at www.gastrojournal.org and at doi: 10.1053/j.gastro.2011.07.050.

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Reprint requests

Address requests for reprints to: Toshiro Sato, MD, PhD, Department of Gastroenterology, School of Medicine, Keio University, 35 Shinanomachi, Shinnjukuku, Tokyo, 160-8582, Japan; e-mail: t.sato@a7.keio.jp; and Hans Clevers, MD, PhD, Hubrecht Institute, Uppsalalaan 8, 3584CT, Utrecht, the Netherlands; e-mail: h.clevers@hubrecht.eu.

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Dr Sato's current affiliation is: Department of Gastroenterology, School of Medicine, Keio University, 35 Shinanomachi, Shinnjukuku, Tokyo, 160-8582, Japan.

Conflicts of interest

The authors disclose the following: T.S. and H.C. are inventors on several patents involving the culture system. The remaining authors disclose no conflicts.

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