



Editor's choice
Scan to access more
free content

ORIGINAL ARTICLE

Development of an enhanced human gastrointestinal epithelial culture system to facilitate patient-based assays

Kelli L VanDussen,¹ Jeffrey M Marinshaw,² Nurmohammad Shaikh,³ Hiroyuki Miyoshi,¹ Clara Moon,¹ Phillip I Tarr,^{3,4} Matthew A Ciorba,² Thaddeus S Stappenbeck¹

► Additional material is published online only. To view please visit the journal online (<http://dx.doi.org/10.1136/gutjnl-2013-306651>).

For numbered affiliations see end of article.

Correspondence to

Dr Thaddeus S Stappenbeck, Department of Pathology and Immunology, Washington University School of Medicine, 660 S. Euclid, Box 8118, St. Louis, MO 63110, USA; stappenb@pathology.wustl.edu and Dr Matthew A Ciorba, Division of Gastroenterology, Department of Internal Medicine, Washington University School of Medicine, 660 S. Euclid, Box 8124, St. Louis, MO 63110, USA; mciorba@dom.wustl.edu

Received 19 December 2013

Revised 22 May 2014

Accepted 6 June 2014

Published Online First

9 July 2014



► <http://dx.doi.org/10.1136/gutjnl-2014-307809>



CrossMark

To cite: VanDussen KL, Marinshaw JM, Shaikh N, et al. *Gut* 2015;**64**:911–920.

ABSTRACT

Objective The technology for the growth of human intestinal epithelial cells is rapidly progressing. An exciting possibility is that this system could serve as a platform for individualised medicine and research. However, to achieve this goal, human epithelial culture must be enhanced so that biopsies from individuals can be used to reproducibly generate cell lines in a short time frame so that multiple, functional assays can be performed (ie, barrier function and host–microbial interactions).

Design We created a large panel of human gastrointestinal epithelial cell lines (n=65) from patient biopsies taken during routine upper and lower endoscopy procedures. Proliferative stem/progenitor cells were rapidly expanded using a high concentration of conditioned media containing the factors critical for growth (Wnt3a, R-spondin and Noggin). A combination of lower conditioned media concentration and Notch inhibition was used to differentiate these cells for additional assays.

Results We obtained epithelial lines from all accessible tissue sites within 2 weeks of culture. The intestinal cell lines were enriched for stem cell markers and rapidly grew as spheroids that required passage at 1:3–1:4 every 3 days. Under differentiation conditions, intestinal epithelial spheroids showed region-specific development of mature epithelial lineages. These cells formed functional, polarised monolayers covered by a secreted mucus layer when grown on Transwell membranes. Using two-dimensional culture, these cells also demonstrated novel adherence phenotypes with various strains of pathogenic *Escherichia coli*.

Conclusions This culture system will facilitate the study of interindividual, functional studies of human intestinal epithelial cells, including host–microbial interactions.

INTRODUCTION

The intestinal epithelium is uniquely adapted to perform a variety of homeostatic functions, including barrier formation and maintenance, digestion and absorption of nutrients, modulation of the microbiome and participation in host immune responses.¹ Perturbation of these functions is implicated in gastrointestinal disorders, including infections and IBD. It is difficult to dissect epithelial-intrinsic roles using in vivo models due to

Significance of this study

What is already known on this subject?

- Conditioned medium containing Wnt3a, R-spondin 3 and Noggin supports the growth of mouse intestinal epithelial spheroids that are enriched for stem cells.
- Proof-of-principle methods have demonstrated growth of human intestinal epithelial cells as organoids.
- There is great interest in adapting human intestinal epithelial spheroid culture methods for the study of individual patients.

What are the new findings?

- Spheroid lines can be reproducibly developed from biopsy material from healthy patients as well as from patients with inflammatory diseases in as little as 2 weeks.
- The protocol for generation of epithelial lines is the same for all regions and donors.
- All cultures showed robust growth and region-specific differentiation.
- Adherence of specific pathogens to primary colonic epithelial cells was unexpected and enhanced compared to immortalised cells typically used as a system for host–pathogen interactions.

How might it impact on clinical practice in the foreseeable future?

- This robust and reproducible culture system for human intestinal epithelial cells offers a novel platform to create individual-specific assays and to interrogate host–pathogen interactions.

the complex interactions between epithelial and neighbouring cells, including stromal and haematopoietic cells as well as resident and transient intestinal microbes. To improve our understanding of epithelial cell function in health and disease, methods to study non-transformed epithelial cells in vitro are critical.

Recently, there have been substantial advances in mouse and human intestinal epithelial cell culture methods.^{2–16} These methods can be generally

categorised as those that start cultures with embryonic stem cells or induced pluripotent stem (iPS) cells (induced organoid culture)^{6,13} or with dissociated crypts/stem cells from intestinal tissue (organoid or spheroid culture).^{2–5,7–9,11,12,14–16} While iPS-derived organoids are valuable for investigating embryonic development, the length of time that is required to generate mature, differentiated intestinal epithelial cells may limit their utility for patient-directed experimental approaches. In contrast, spheroid culture is particularly suited for individual-based medicine due to a relatively rapid differentiation rate. Accordingly, there is great interest in developing spheroid culture methods for the study of interindividual variation (attributed to genetics, age, gender, etc.) in human intestinal epithelial cell function. This would permit the use of an individual's own intestinal epithelial cells to screen for novel therapies, define host–microbial/host–pathogen interactions and perform clinical testing to determine the efficacy of a particular therapy.^{17,18} Remaining challenges for spheroid culture include the ability to reproducibly isolate and culture material from endoscopic biopsies, a reduction in the technical complexity and cost associated with current methods and the ability to grow these cells in a robust and timely manner commensurate with patient care.

Both iPS cell-based and spheroid-based culture methods employ growth media that contain canonical Wnt ligand, R-spondin and Noggin, the critical factors that support intestinal epithelial stem cell growth.¹² Because the cost of adding these components to the culture media as recombinant factors is very high, alternative strategies to deliver these factors have been employed, such as subepithelial myofibroblast feeder cells⁵ or conditioned media.^{7,8} We engineered an L-cell line to secrete Wnt3a, R-spondin 3 and Noggin (L-WRN), thereby creating a cost-effective conditioned medium (L-WRN CM) that can be collected and used to efficiently deliver these critical growth factors to cultured epithelial cells.^{7,8} The L-WRN CM supported the robust growth of mouse intestinal epithelial spheroids that were highly enriched for stem cells.⁷ Here, we overcame challenges associated with developing human intestinal spheroid culture protocols for patient-based assays by adapting our mouse method to the culture of human gastrointestinal epithelial cells.

MATERIALS AND METHODS

Collection of human tissue samples and spheroid culture

Biopsy tissues were obtained from 47 adults during routine endoscopy at the Washington University School of Medicine in collaboration with the Washington University Digestive Diseases Research Core Center (DDRCC) BioSpecimens Core. Biopsies were collected using standard endoscopic biopsy forceps (Radial Jaw 4 Large Capacity Jaw O.D 2.4 mm, Boston Scientific, Natick, Massachusetts, USA) and immediately placed in 5 mL of ice-cold washing medium (DMEM/F12 with HEPES (Sigma D6421) supplemented with 10% fetal bovine serum (Sigma), 2 mM L-glutamine, 100 units/mL penicillin and 0.1 mg/mL streptomycin (Sigma)). The study was approved by the Institutional Review Board of Washington University School of Medicine. Written informed consent was obtained from all donors.

Procedures for establishing and maintaining human spheroid cultures are based on our previous publications^{7,8,19,20} with additional details provided in online supplementary methods. In brief, to isolate crypt/gland units, biopsies were minced with fine scissors and digested in 1 mL of collagenase solution (2 mg/mL collagenase type I (Invitrogen, Grand Island, New York, USA) and 50 µg/mL gentamicin (Invitrogen) in washing media) for ~20–30 min with pipetting every 5–10 min. Crypts/glands

were filtered through a 70 µm strainer, incubated with 9 mL of ice-cold washing media and pelleted by centrifugation at 20–50 g for 5 min. Frequently, a second incubation with 5 mL washing medium followed by centrifugation at 50–150 g for 5 min was performed. Pelleted crypts/glands were then suspended in 1 mL washing medium, transferred to a 1.5 mL tube and pelleted by centrifugation at 200 g for 5 min. The supernatant was carefully removed by pipette, and the pellet was suspended in Matrigel (BD Biosciences, San Jose, California, USA; 15 µL/well). The Matrigel mixture was plated into 24-well tissue culture plates on ice. Plates were flipped upside down (prevents epithelial cells from attaching to the plastic) and incubated at 37°C to polymerise the Matrigel and 400 µL of 50% L-WRN CM,^{7,8} a 50/50 mix of L-WRN CM and fresh primary culture media (Advanced DMEM/F-12 (Invitrogen) supplemented with 20% fetal bovine serum, 2 mM L-glutamine, 100 units/mL penicillin and 0.1 mg/mL streptomycin), supplemented with 10 µM Y-27632 (ROCK inhibitor; Tocris Bioscience, R&D Systems, Minneapolis, Minnesota, USA) and 10 µM SB 431542 (TGFR1 inhibitor; Tocris Bioscience, R&D Systems) was added to each well. Lower percentages of L-WRN CM were obtained by further dilution with primary culture medium. Differentiation media (with or without 5 µM DAPT (EMD Millipore)) included Y-27632, but not SB 431542. Experiments used the spheroid lines bolded in online supplementary table S1 at passages 6–40. Live spheroids were imaged with a Pupil Cam (Ken-a-Vision, Kansas City, Missouri, USA) fixed to a phase microscope (Fisher Scientific). Cell growth was measured with the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) Cell Proliferation Assay Kit (ATCC, Manassas, Virginia, USA).

Gene expression analysis

RNA was isolated with the NucleoSpin RNA II kit (Macherey-Nagel, Bethlehem, Pennsylvania, USA), which includes DNase treatment. Reverse transcriptase reactions (50 µL) used 1 µg RNA and the Iscript cDNA synthesis kit (Bio-Rad, Hercules, California, USA). Eukaryotic quantitative PCR (qPCR) reactions were performed with SYBR Advantage qPCR Premix (Clontech, Mountain View, California, USA) and the primers in online supplementary table S2. Expression levels were determined with triplicate assays per sample and normalised to the expression of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*), which was expressed at similar levels in all samples.

Analysis of primary epithelial cell monolayers

Transwell culture methods were adapted from our recently published method for mouse colonic spheroids.¹⁹ Human spheroids (~1 well of a 24-well plate per Transwell) were dissociated, strained through a 40 µm filter, seeded onto Transwell membranes (Fisher Scientific, CoStar 3470) coated with 0.1% gelatin (earlier experiments) or Matrigel diluted 1:40 in phosphate buffered saline (PBS) (later experiments) and provided 5% L-WRN CM (10 µM Y-27632 was included O/N and then removed during daily media changes). Transepithelial resistance (TER) measurements¹⁹ and mucus layer analyses²¹ were performed as previously described. Z-stack images (1.1 µm, with an optimal interval of 0.55 µm) were generated with a Zeiss LSM510 Meta laser scanning confocal microscope (Carl Zeiss Inc, Thornwood, New York, USA) equipped with Argon (Ex. 488 Em. BP 505–530) and HeNe1 (Ex. 543 Em. BP 560–615) lasers, a 63×, 1.4 numerical aperture Zeiss Plan Apochromat oil objective and LSM software. Rectal and ileal spheroid lines were infected with recombinant lentiviruses expressing an enhanced green

fluorescent protein (EGFP) under the *hPGK* promoter^{7,8} using a described protocol.⁸

Bacterial adherence assays

Epithelial cells grown on glass chamber slides (Lab-Tek; coated with 1:40 Matrigel diluted in PBS) were washed in antibiotic-free medium followed by incubation with 0.3 mL of *Escherichia coli*-containing media ($\sim 10^9$ colony forming units/mL) for 1 h (37°C, 5% CO₂), washing three times with media to remove non-adherent bacteria and returned to the incubator. After 3 h, cells were washed five times with ice-cold PBS (to further remove non-adherent bacteria) and either fixed/stained (cold methanol/0.5% crystal violet) or lysed for gDNA isolation (DNeasy Tissue kit; Qiagen). Qualitative adherence assays were performed in duplicate (n=2–4 assays/human cell line). qPCR was performed using human *GAPDH* and bacterial *malB* primers (see online supplementary table S2) (n=4 assays/*E. coli* strain/human cell line). The relative adherence index (RAI) for each wild-type *E. coli* is the ratio of *malB* to *GAPDH* divided by this ratio for the non-adherent strain ORN172.

Statistical analyses

A Student's t test or one-way analysis of variance (ANOVA) followed by a Dunnett's multiple comparisons or Tukey post-test were performed as indicated in the figure legends with Prism GraphPad V6 software. p Value < 0.05 was considered to be significant.

RESULTS

Establishment of gastrointestinal epithelial spheroid cultures from endoscopic biopsies

To reproducibly grow intestinal epithelial cells from individual patients, we adapted a system developed for mouse intestinal epithelial cells. To initiate cultures, intestinal crypts were isolated by collagenase digestion from biopsy specimens (4–18 mm² each; 2–3 biopsies per culture) collected during routine endoscopy (figure 1A). Isolated crypts were embedded in basement membrane matrix (Matrigel) and incubated in a 50/50 mix of L-WRN CM and fresh primary culture media (referred to herein as 50% L-WRN CM). We found that ROCK (Y-27632) and TGFBR1 (SB 431542) inhibitors were required to maintain the human intestinal epithelial cell cultures for >3 passages (see online supplementary figure S1), which is a modification from our mouse culture system. Within 24 h of plating, small spheroids formed (figure 1A). Typically, the cultures could be sufficiently expanded to prepare frozen stocks (10 vials/line) within 4–6 passages (15–25 days) (see online supplementary figure S2).

This method to establish human intestinal spheroid lines was successful for all endoscopically accessible sites of the gastrointestinal tract including individuals who were either healthy or had inflammatory disease (ie, Barrett's oesophagus and IBD; figure 1B). We also established epithelial lines in patients with prior intestinal surgery from tissue sites that included neo-terminal ileum (post-bowel resection surgery) and the ileal pouch portion of an ileal pouch-anal anastomosis. To date, we generated 65 human spheroid lines from 47 individuals, including 25 patients with IBD (see figure 1B and online

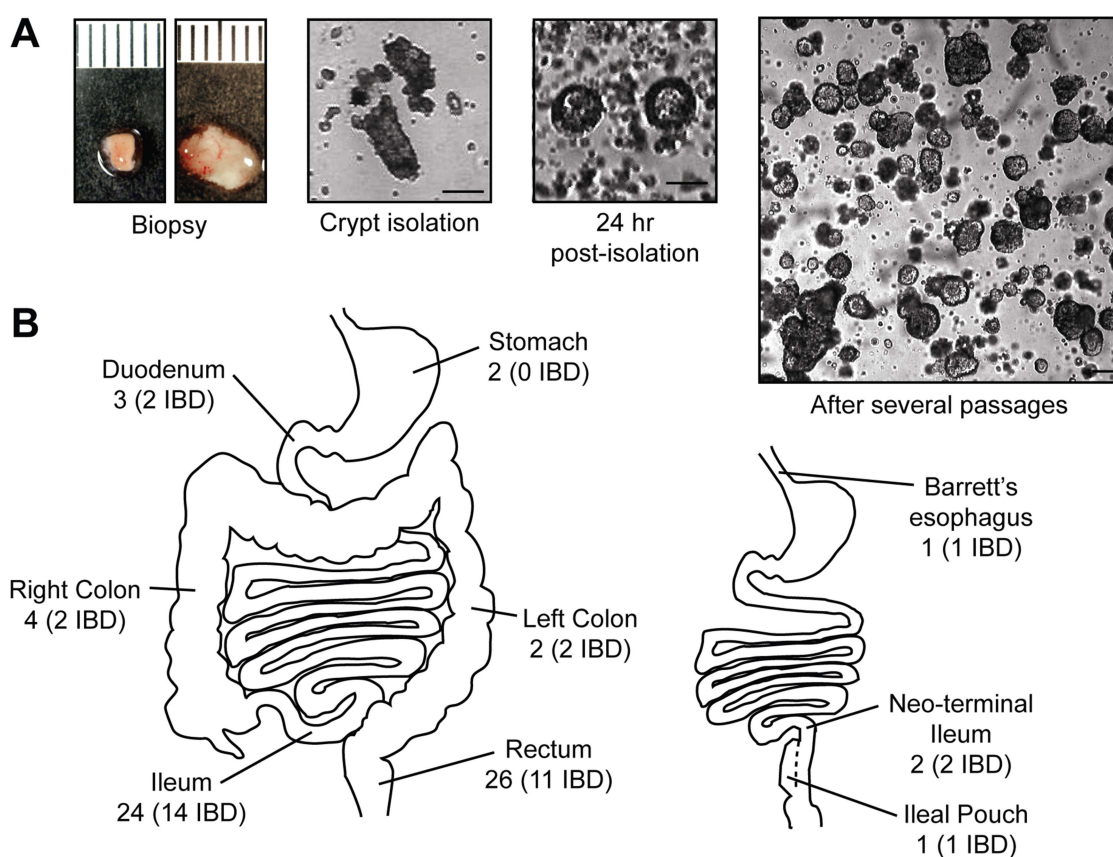


Figure 1 Establishment of human epithelial spheroid cultures from multiple regions of the gastrointestinal tract. (A) Representative images showing the procedure for establishment of intestinal spheroid culture. Ruler lines above biopsy samples indicate 1 mm. Bars, 100 μ m. (B) Diagram illustrating the numbers of spheroid and gastric lines established to date. The first number reports the total number of lines established from the indicated region, and the number in parenthesis indicates the number of those lines established from patients with IBD.

supplementary table S1). Thus, this culture system can support growth of epithelial cells from multiple sites of the gastrointestinal tract and from patients with or without disease.

Robust growth of epithelial spheroids from human rectum and ileum

Rectal and ileal spheroids, regardless of donor status (healthy control or IBD), grew rapidly in 50% L-WRN CM (figure 2A,B). Over a 2-day period, the rectal and ileal spheroids exhibited a robust increase in cell number (2.4-fold and 2.9-fold increase, respectively; figure 2B). This result suggested that the spheroids grown in 50% L-WRN CM were enriched for a highly proliferative stem and/or progenitor cell population. Accordingly, rectal and ileal cell lines abundantly expressed leucine-rich repeat-containing G protein-coupled receptor 5 (*LGR5*), a well-established stem cell marker in mouse intestine²³ (figure 2C). The spheroid lines expressed similar levels of villin 1 (*VIL1*), demonstrating that they retained their intestinal epithelial identity

(figure 2C). Expression of vimentin (*VIM*) was not detected in any line after passage 7 (the earliest passage analysed for this marker), confirming that mesenchymal cells were eliminated during initial passages (figure 2C). Thus, in contrast to other culture techniques that contain cells of mesenchymal origin,^{5 10 13 24} this system allows for the study of isolated epithelial cells.

Human spheroids retain a region-specific differentiation programme

Because the spheroid cultures appear to be enriched for stem cells, it is important to identify conditions that permit differentiation. Wnt signalling is a well-established pathway that contributes to intestinal stem cell activity and maintenance in vivo.^{25–29} We have previously shown that the 50% L-WRN CM is the major source of Wnt stimulation in our culture system and that its dilution results in diminished Wnt signalling activity in mouse intestinal epithelial spheroids.⁷ Similar results were produced using the human spheroids (figure 3A). Moreover, addition of the Notch inhibitor N-[N-(3,5-Difluorophenacetyl-L-alanyl)]-(S)-phenylglycine t-butyl ester (DAPT), which promotes the differentiation of mouse and human spheroids,^{4 10 11 19 20} rapidly reduced the proportion of cells in S-phase in rectal and ileal lines (see figure 3B and online supplementary figure S3). Thus, by downregulating Wnt and Notch signalling, cell cycle exit was quickly induced.

We next examined mRNA expression of differentiated cell markers in rectal, ileal and duodenal spheroids grown in 50% L-WRN CM or 5% L-WRN CM with or without DAPT. As expected, *LGR5* expression was greatly decreased and expression of differentiated cell markers was generally increased in spheroids cultured in 5% L-WRN CM with or without DAPT as compared with 50% L-WRN CM (see figure 4 and online supplementary figures S4 and S5). In accordance with their in vivo expression patterns, carbonic anhydrase 1 (*CA1*) was specifically induced in rectal spheroids and sucrase-isomaltase (*SI*) was specifically induced in ileal spheroids when grown in 5% L-WRN CM with DAPT (figure 4A, B). The addition of DAPT was important for inducing expression of secretory cell markers, including transcription factors atonal homologue 1 (*ATOH1*) and neurogenin 3 (*NEUROG3*), goblet cell markers mucin 2 (*MUC2*) and trefoil factor 3 (*TFF3*) and endocrine cell marker chromogranin A (*CHGA*) (see figure 4C and online supplementary figures S4 and S5). Expression of the Paneth cell marker α -defensin 5 (*DEFA5*) (see figure 4D and online supplementary figure S5) was strongly induced only in small bowel spheroids when grown in 50% L-WRN CM with DAPT, in accordance with the in vivo distribution and the critical role of Wnt signalling in the formation of Paneth cells.^{30 31} Region-specific induction of small bowel markers was also observed. Transient receptor potential cation channel, subfamily V, member 6 (*TRPV6*; calcium channel) and solute carrier family 10, member 2 (*SLC10A2*; bile acid transporter), were preferentially expressed in duodenal and ileal spheroids, respectively (figure 4E, F). These data suggest that regional specification of epithelial cells is, at least in part, cell-intrinsic, as previously suggested.³²

Development of a Transwell system for human primary intestinal epithelial cells

The growth of intestinal epithelial cell monolayers is necessary for in vitro studies of physiological processes, such as analyses of cellular transport or secretion or for co-culture experiments that will likely be used in patient-based assays. Transwell assays have been difficult to perform using other culture methods for

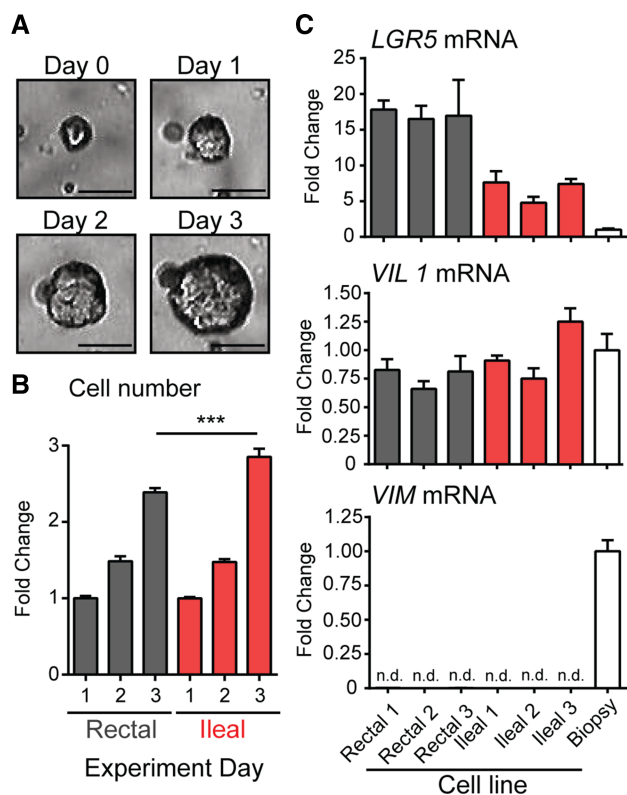


Figure 2 Robust growth of cultured intestinal spheroids in conditioned medium containing Wnt3a, R-spondin 3 and Noggin. (A) Representative images demonstrating the qualitative growth of an individual spheroid imaged daily over a 3-day period. Bars, 100 μ m. (B) Spheroids were seeded (day 0) into 96-well format and growth was assessed at 24 h intervals by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay on days 1, 2 and 3. The reduction of MTT (yellow colour) to formazan (purple colour) by metabolically active cells was measured by absorbance at 595 nm with a plate reader. The background subtracted results for three rectal (gray bars) and three ileal (red bars) cell lines are presented as fold change (mean \pm SEM) compared with day 1 for each region (n=9 per line at each time point). ***p=0.004 by Student's t test. (C) qPCR gene expression analysis for (A) *LGR5*, (B) *VIL1* and (C) *VIM* in rectal and ileal epithelial cell cultures. Fresh biopsy samples were used as a positive control (white bars). Data are presented as fold change relative to the biopsy samples (mean \pm SEM; n=3 samples per bar). n.d., not detected.

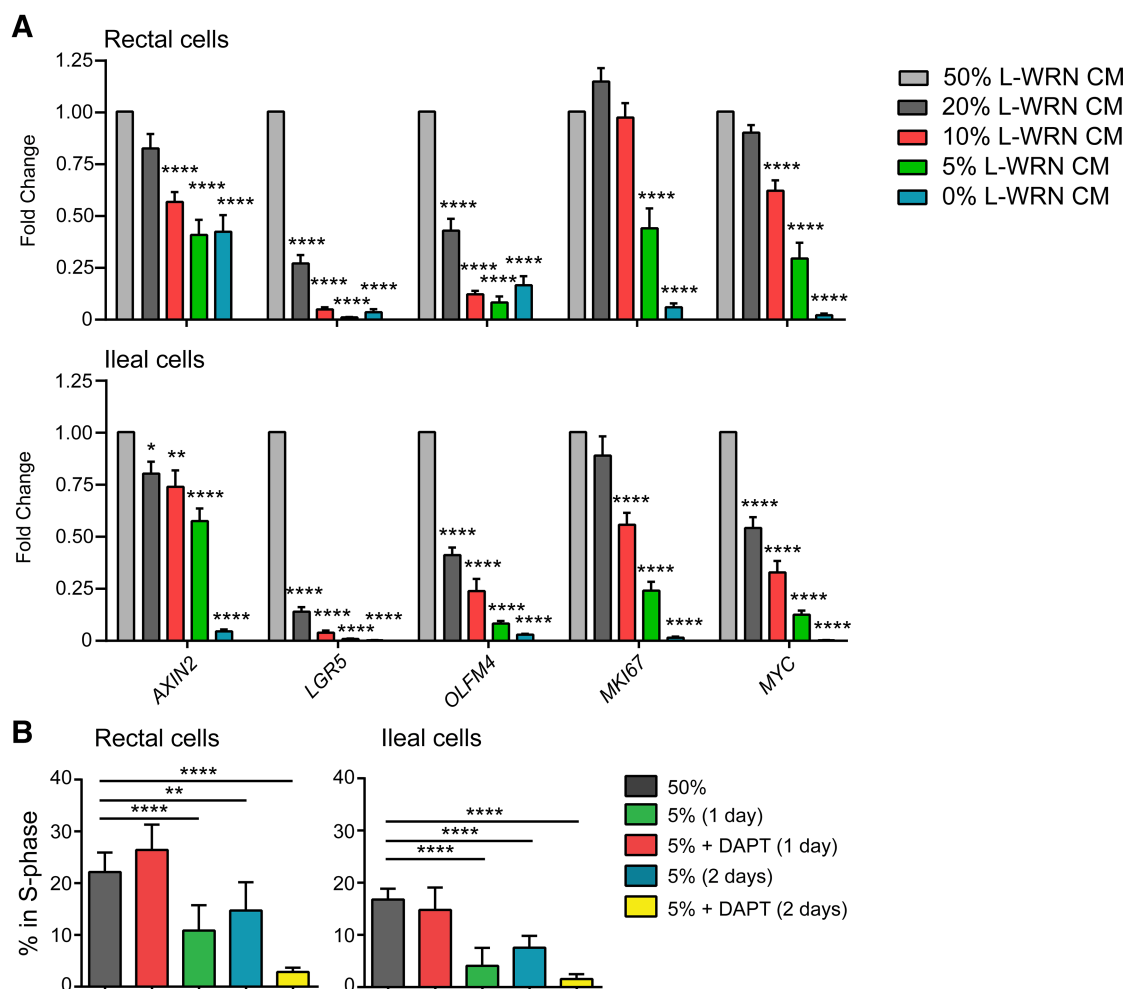


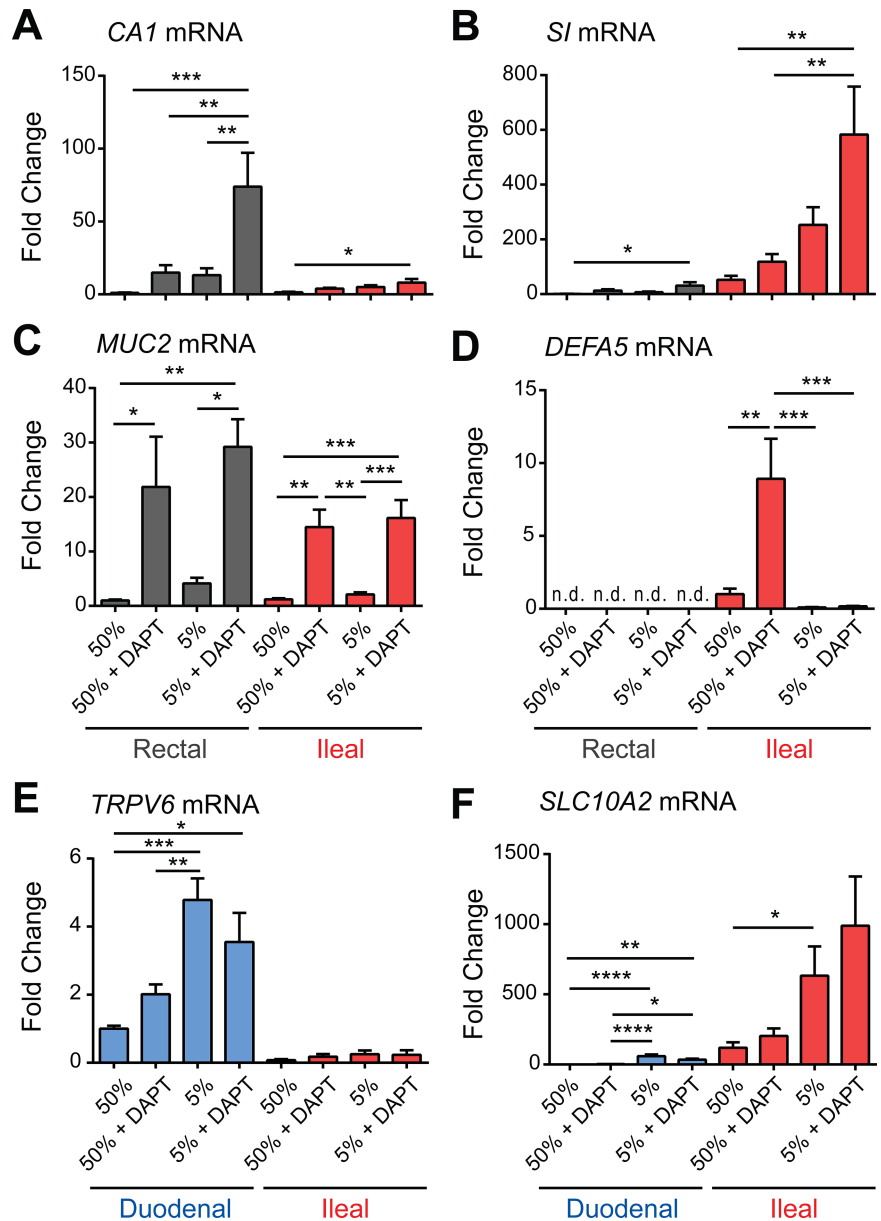
Figure 3 Human intestinal epithelial spheroids grown in low percentages of conditioned medium containing Wnt3a, R-spondin 3 and Noggin (L-WRN CM) exhibit decreased Wnt signalling activity. (A) Human rectal and ileal spheroids ($n=3$ lines per region) were cultured for 2 days in 50%, 20%, 10%, 5% or 0% L-WRN CM. Gene expression of *AXIN2*, *LGR5*, *OLFM4*, *MKI67* and *MYC* was determined by qPCR ($n=3$ experiments per line). A dose-dependent decrease was observed for *AXIN2* (canonical Wnt target), mouse intestinal stem cell markers *LGR5* and olfactomedin 4 (*OLFM4*), and proliferative markers *MKI67* and *MYC*. Both 0% and 5% L-WRN CM showed a significant decrease for all of these markers compared with 50% L-WRN CM. As some assays required cells with some potential to divide, we used cells grown in 5% L-WRN CM for downstream applications. Data were presented as fold change (mean \pm SEM) compared with 50% L-WRN CM for each experiment. * $p<0.05$; ** $p<0.01$; **** $p<0.0001$ by one-way analysis of variance (ANOVA) followed by a Dunnett's post-test with 50% L-WRN CM set as the control group: $F=17.58$, $p<0.0001$ (A, *AXIN2*); $F=449.60$, $p<0.0001$ (A, *LGR5*); $F=113.70$, $p<0.0001$ (A, *OLFM4*); $F=56.13$, $p<0.0001$ (A, *MKI67*); $F=87.09$, $p<0.0001$ (A, *MYC*); $F=49.48$, $p<0.0001$ (B, *AXIN2*); $F=1570.00$, $p<0.0001$ (B, *LGR5*); $F=156.70$, $p<0.0001$ (B, *OLFM4*); $F=63.81$, $p<0.0001$ (B, *MKI67*); $F=125.20$, $p<0.0001$ (B, *MYC*). (B) Spheroids were passaged (day 0) and allowed to recover overnight in 50% L-WRN CM. Media was replaced daily, with some cultures changed to 5% or 5% L-WRN CM plus 5 μ M DAPT for the indicated number of days. On day 3, the spheroids were treated with EdU for 2 h, dissociated, stained and sorted by flow cytometry based on EdU incorporation and SYTOX AAD (DNA content marker). An average of 22.2% of rectal cells and 16.8% of ileal cells were in S-phase when grown in 50% L-WRN CM. One day of culture in 5% L-WRN CM was not sufficient to reduce the proportion of cells in S-phase; however, after 2 days, a decrease in the proportion of cells in S-phase was observed for both rectal and ileal lines. Addition of 5 μ M DAPT to the 5% L-WRN CM rapidly decreased the proportion of S-phase cells within 1 day and led to greatly diminished proportions of S-phase cells (1.6–2.9%) after 2 days. ** $p<0.01$; *** $p<0.001$; **** $p<0.0001$ by one-way ANOVA followed by a Dunnett's post-test with 50% L-WRN CM set as the control: $F=43.01$, $p<0.0001$ (rectal); $F=54.13$, $p<0.0001$ (ileal).

primary intestinal epithelial cells, likely due to the inability to generate sufficient numbers of cells. We recently developed a method to form functional epithelial monolayers with mouse colonic epithelial cells.¹⁹ We adapted this method to grow human intestinal epithelial cells on Transwell membranes (figure 5). Within 3 days of seeding, both rectal and ileal epithelial cells formed polarised monolayers, as evidenced by H&E staining and co-immunostaining for the apical brush border protein VILLIN 1 and the tight junction protein ZO-1 (figure 5A, B). Goblet (MUC2-positive) and endocrine (CHGA-positive) cell populations (figure 5A, B) were present in proportions similar to those

observed in colonic surface or ileal villus epithelium, with goblet cells accounting for 8.4% and 8.7% and endocrine cells accounting for 0.4% and 1.0% of total epithelial cells in the rectal and ileal monolayers, respectively. UEA-1 lectin can be used to distinguish both mouse and human Paneth cells³³ (see online supplementary figure S6). We observed rare UEA-1 reactive cells in ileal, but not in rectal, monolayers (figure 5B and data not shown).

We tested the barrier function of the epithelial cells grown on Transwells. Rectal and ileal monolayers exhibited an average TER of 395 and 400 Ω cm², respectively, indicating barrier integrity (figure 5C). We also demonstrated a mucus layer using

Figure 4 Region-specific differentiation of rectal and ileal spheroids. (A–D) qPCR gene expression analysis for (A) *CA1*, (B) sucrase-isomaltase (*SI*), (C) *MUC2*, (D) *DEFA5*, (E) *TRPV6* and (F) *SLC10A2* in rectal (gray bars), ileal (red bars) or duodenal (blue bars) spheroids (n=3 lines per region). RNA was collected from spheroids that had been passaged, allowed to recover overnight in 50% conditioned medium containing Wnt3a, R-spondin 3 and Noggin (L-WRN CM) and then treated with 50% or 5% L-WRN CM with or without the addition of 5 μ M DAPT for an additional 2 days, with media replaced daily. Data are presented as fold change relative to rectal or duodenal 50% L-WRN CM, except for *DEFA5*, which is presented as fold change relative to ileal 50% L-WRN CM (mean \pm SEM; n=3 per line). n.d., not detected. *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001 by one-way analysis of variance followed by a Tukey's post-test: F=7.23, p=0.0008 (A, rect); F=3.73, p=0.0210 (A, ile); F=3.53, p=0.0256 (B, rect); F=6.24, p=0.0019 (B, ile); F=4.99, p=0.0060 (C, rect); F=6.16, p=0.0020 (C, ile); F=9.42, p=0.0001 (D, ile); F=9.05, p=0.0002 (E, duod); F=0.67, p=0.5777 (E, ile); F=17.33, p<0.0001 (F, duod); F=4.39, p=0.0151 (F, ile).



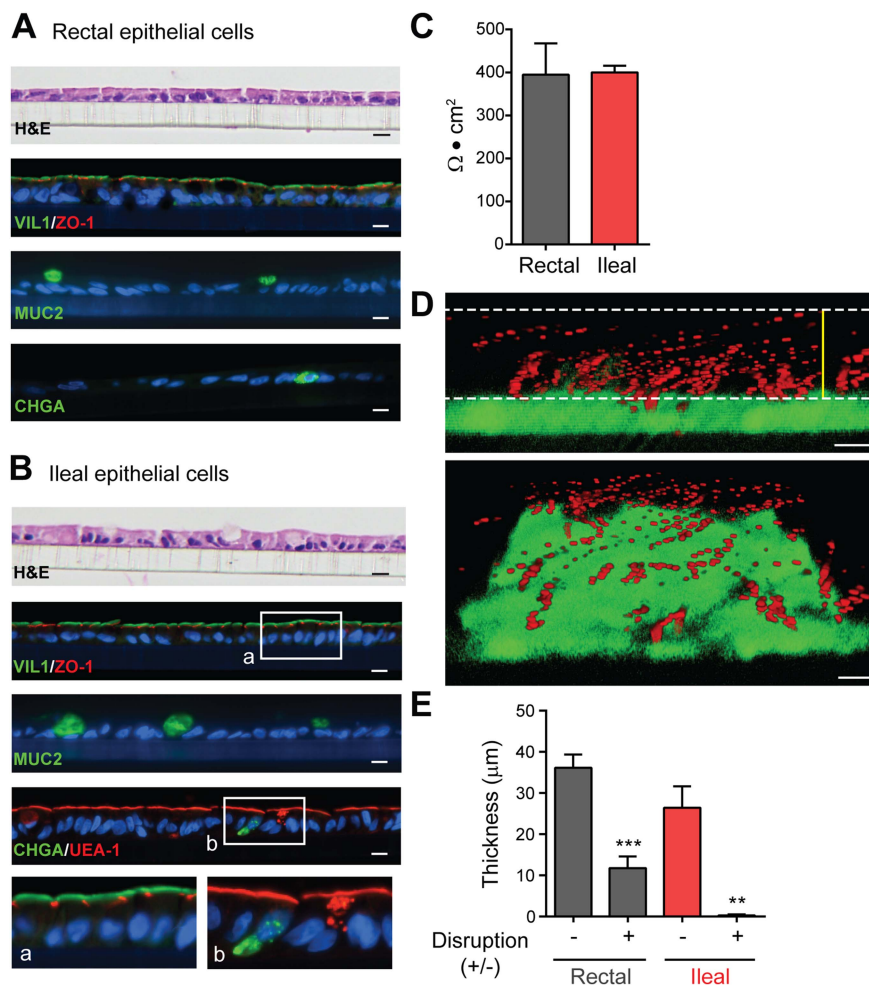
a recently reported method for mouse tissue explants.²¹ For these experiments, spheroid cells labelled with EGFP by lentiviral infection (see online supplementary figure S7) were used to generate epithelial monolayers. Red fluorescent beads (1- μ m diameter) were allowed to sediment into the mucus layer. The distance between the apical surface of the epithelial cells and the beads, which become suspended in the mucus, was determined using Z-stack imaging by confocal microscopy (figure 5D). We observed a mucus layer reproducibly covering the apical surface of the epithelial monolayers (36 and 26 μ m thick in rectal and ileal monolayers, respectively; figure 5E). Aspiration by pipetting was reported to remove the outer mucus layer in mouse tissue explants.^{21 34–36} After aspiration by pipetting, we found a significant decrease in mucus thickness in both ileal and rectal lines (figure 5E), suggesting that the mucus layers were disrupted by mechanical dissociation.

E. coli adherence to human primary intestinal epithelial cells

As a proof of concept that host-based assays with cultured intestinal epithelial cells are feasible and offer data not already

known from standard cell line assays, we compared the adherence of various diarrheagenic *E. coli* strains to primary intestinal epithelial lines and HeLa cells (a conventional immortalised cell line used currently for this assay³⁷). We first performed adherence assays using wild-type strains with enteroaggregative (EAggEC; 042 and UD792)^{38 39} or enteropathogenic (EPEC; B171 and E2348/69)^{40 41} *E. coli* phenotypes previously demonstrated with HeLa cells (figure 6). These strains adhered robustly to both rectal and ileal epithelial cells with patterns similar to those observed with HeLa cells (ie, more diffuse adherence for EAggEC vs dense microcolonies of localised adherence for EPEC). Similar adherence patterns were observed with epithelial lines derived from different individuals (n=3 per region). The non-adherent laboratory strain *E. coli* ORN172,⁴² as expected, did not adhere to any cell line by visual assessment (figure 6A). A quantitative assay to determine the RAI (see legend) for each bacterial strain corroborated the visual assessment of EPEC and EAggEC adherence, with RAI values for rectal and ileal epithelial cells exceeding those of ORN172 by up to two orders of magnitude (figure 6B). These data also confirmed that the EAggEC UD792 strain adhered more robustly to rectal cells as compared with ileal cells.

Figure 5 Dissociated spheroid cells form polarised monolayers on Transwell membranes. (A and B) Spheroids were dissociated and seeded onto coated Transwell membranes to form epithelial monolayers. After 3 days, monolayers were used for measurement of transepithelial resistance (TER) and/or fixed and paraffin-embedded. Paraffin sections from rectal (A) and ileal (B) epithelial monolayers were stained for H&E or immunostained for VIL1 and ZO-1, MUC2 or chromogranin A (CHGA) (with UEA-1 lectin for ileal cells) to visualise differentiated cell types. (a) and (b) denote insets. Bars, 10 μm . (C) TER measurements are shown as the resistance \times area product ($\Omega \text{ cm}^2$) for three lines per intestinal region (mean \pm SEM; $n=4$ Transwells per line). (D) Representative confocal Z-stack images of ileal monolayer (most epithelial cells are GFP-positive, green) and fluorescent beads (red). The top image is a transverse view with white dashed lines denoting the apical surface of the epithelial layer and range of suspended beads and a yellow solid line indicating the measured mucus thickness. The lower image shows a tilted view of the transverse image. Bars, 10 μm . (E) Graph of mucus thickness measurements in rectal and ileal monolayers before (–) and after (+) disruption of the mucus layer by pipetting (mean \pm SEM; $n=4$ –7 Transwells per bar). ** $p<0.01$; *** $p<0.001$ by Student's t-test comparing mucus thickness before and after disruption from the same region.



We next assessed adherence of the enterohemorrhagic *E. coli* O157:H7 strain 86–24 (figure 6).^{43 44} Whereas exceptionally rare HeLa cells exhibited any adherent bacteria (see figure 6 and online supplementary figure S8), diffuse adherence of *E. coli* O157:H7 was readily observed in the rectal and ileal epithelial cells (figure 6A). These visual observations were supported by our quantitative assay, where significantly greater RAI values were observed for rectal and ileal epithelial cells than for HeLa cells (figure 6B).

DISCUSSION

Here, we describe a culture system for human gastrointestinal epithelial cells with great potential for use in patient-specific assays. Key advantages of this system include use of endoscopic biopsy tissue as starting material and the rapid expansion of the spheroids, which allows for line establishment from an individual patient within a time frame that is commensurate with patient care (~2–3 weeks). By eliminating the requirement for surgical specimens, spheroid cultures from a wide array of patient populations can be analysed, including healthy controls or patients preselected based on age, genotype, diagnosis, disease features, etc.

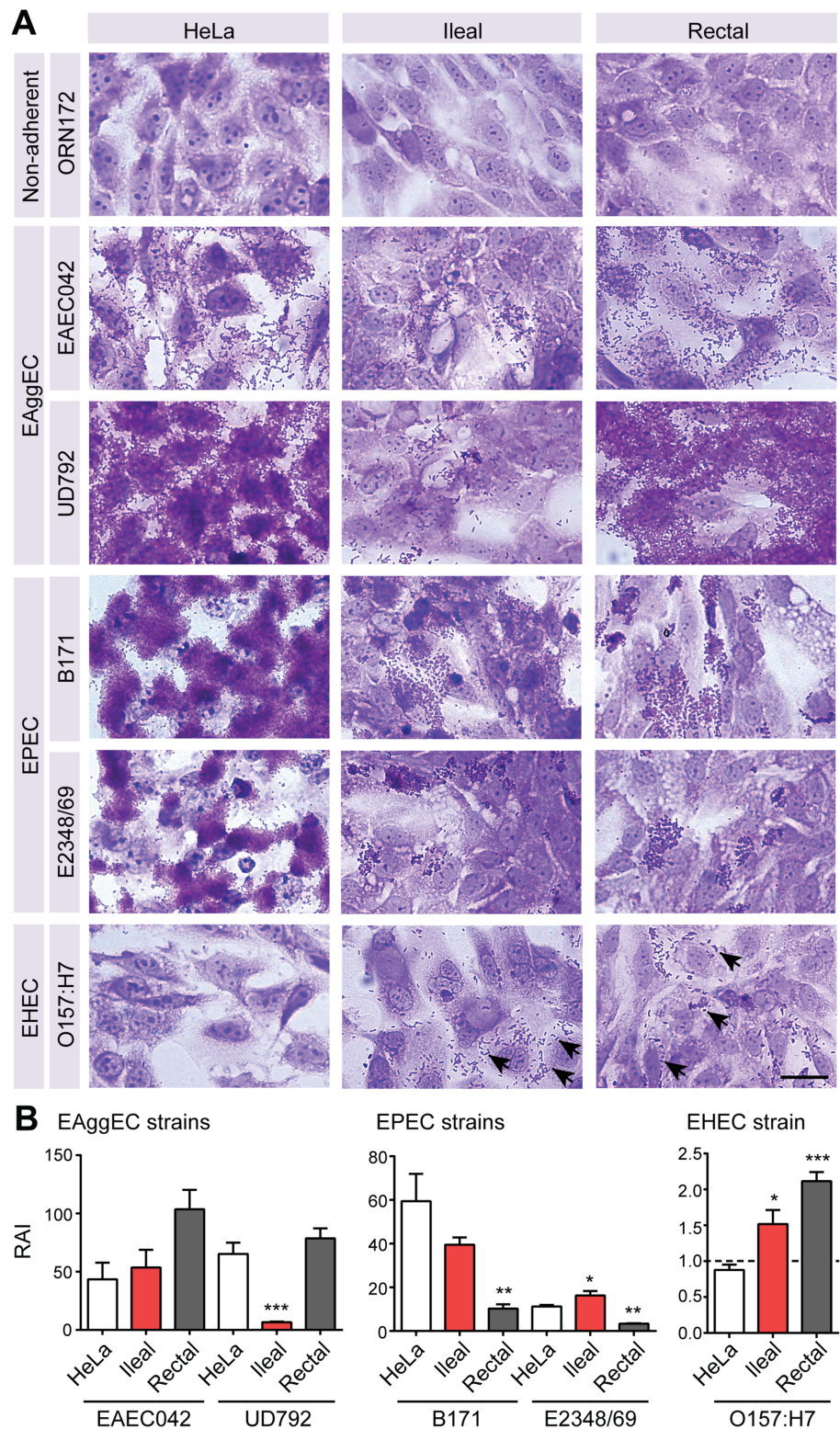
Another advantage of this spheroid culture system is that the protocol is relatively simple. The same protocol can be used to

establish and maintain spheroid lines from any accessible gastrointestinal site and there is no requirement for cell sorting schemes, such as those described by other protocols.^{4 11 15} We have had near 100% success in establishing cell lines from particular patients as long as the biopsy contains a sufficient number of crypts. This high level of reproducibility is important because spheroid lines will need to be produced following a single endoscopic procedure in order to perform individualised assessments.

Biopsy material has also been successfully applied in explant/organ culture systems.^{24 45–48} Cultured explants enable study of interactions between epithelial cells and neighbouring cells, such as resident immune cells, whereas spheroid culture enables study of epithelial-specific responses. An important advantage of the spheroid culture system is the ability to establish and store lines from specific patients, such that multiple experiments can be performed with the same cell line over time. Indeed, we observed that experiments repeated over time with the same lines produced similar results. In addition, the rapid growth and differentiation capacity of the cultured spheroids allowed us to obtain the critical cell mass needed to perform a variety of functional assays to assess the characteristics of stem/progenitor cells or differentiated cells. Such assays could easily be adapted for the analysis of other epithelial functions, in vitro screening and other patient-based assays.

Figure 6 Development of a bacterial adherence assay for human intestinal spheroid cells. Rectal and ileal spheroids were grown on glass chamber slides for 3 days to promote two-dimensional cell growth prior to assessment, which allows for the visualisation of adherent bacteria. For adherence assays, rectal and ileal spheroid-derived epithelial cells were incubated with the indicated strains for 1 h followed by several washes to remove non-adherent *Escherichia coli*. Similarly treated HeLa cells served as a positive control for adherence.

(A) Visual assessment of adherence phenotype was assessed by crystal violet staining (adherent bacteria are dark purple). Clustered and scattered adherence of the O157:H7 strain was observed with spheroid-derived epithelial cells (arrows). Bar, 30 μ m. (B) qPCR was used to determine the relative adherence index (RAI) for the enteroaggregative *E. coli* (EAggEC), enteropathogenic *E. coli* (EPEC) and enterohemorrhagic (EHEC) strains (mean \pm SEM; n=4 per strain per cell type). The RAI for each wild-type *E. coli* is the ratio of *malB* to glyceraldehyde 3-phosphate dehydrogenase divided by this ratio for ORN172 (represented by dashed line in EHEC strain graph), which is a non-adherent control. *p<0.05; **p<0.01; ***p<0.001 compared with HeLa cells by one-way analysis of variance followed by a Tukey's post-test: F=4.41, p=0.0463 (EAEC042); F=26.73, p=0.0002 (UD792); F=10.66, p=0.0042 (B171); F=27.35, p=0.0001 (E2348/69); F=19.04, p=0.0006 (O157:H7).



The mucus layer is a critical component of the physical barrier separating the host from the luminal environment, thus providing additional protection from potential pathogens.⁴⁹ We found that the spheroid-derived intestinal epithelial cells produced a mucus layer that could be mechanically dissociated. This disruption was more prominent with ileal mucus than rectal mucus, a finding in agreement with in vivo studies that show a single loose mucus layer in the ileum that can be mostly removed by pipetting and a more complex and resistant mucus layer in the colon.^{21 34–36}

The adherence of EPEC to rectal epithelial cells was unexpected as these organisms are generally considered to be small bowel pathogens based on extensive cell biology that has focused on the attaching and effacing lesions that result when they adhere to microvilli⁵⁰ and the classic association of EPEC with non-bloody diarrhoea. However, some reports have described patients infected with EPEC or EAggEC who have bloody diarrhoea,^{38 51} and EPEC have been noted to adhere to the colon during human infections,^{52 53} suggesting that there may be a role for these bacteria in colonic pathology.

Additionally, *E. coli* O157:H7 strain 86–24 adhered well to both rectal and ileal epithelial cells, which contrasts with the extremely sparse adherence to HeLa cells,^{43–44} a phenotype we confirmed. These data suggest that primary human epithelial cells are more appropriate for studying the adherence phenotypes of diarrheagenic *E. coli* than immortalised cell lines. These phenotypes also suggest new lines of investigation into intestinal, and especially colonic, receptors for bacterial adhesins. Interestingly, even though there is considerable colonic pathology in *E. coli* O157:H7 human infections,⁵⁴ there is little direct evidence of the ability of this pathogen to adhere to the mucosa lining this organ⁵⁵ and a lack of clarity regarding direct bacterial–colon interaction.⁵⁶ We also present a new way to quantify *E. coli* adherence to epithelial cells using unified technology (single locus copy number of bacteria and host cells by DNA amplification) to normalise bacterial adherence to eukaryotic cells. This technique extends prior work using lung epithelial cells⁵⁷ and qPCR of enteropathogens adherent to manually enumerated intestinal cell lines.⁵⁸

The microbiome plays an important role in the pathogenesis of Crohn's disease (CD).^{59–61} The CD microbiome is enriched for Enterobacteriaceae (including *E. coli*),^{62–64} with several studies reporting higher proportions of adherent and invasive *E. coli*.^{65–68} These findings suggest that overgrowth of specific bacteria, such as adherent-invasive *E. coli*, may be a driver of inflammation in CD.⁶² An advantage of the spheroid culture system is that it allows for preselection of donors with particular genotype and, thus, would permit the study of potential CD susceptibility genotype–phenotype relationships that have been hypothesised to affect the sensing of and response to microbes by intestinal epithelial cells.⁶⁹ Thus, we propose that the primary intestinal epithelial cell culture system described here will provide an informative experimental platform to test potential inter-host variations that might affect the colonisation potential of these bacteria.

Author affiliations

¹Department of Pathology and Immunology, Washington University School of Medicine, St. Louis, Missouri, USA

²Division of Gastroenterology, Department of Internal Medicine, Washington University School of Medicine, St. Louis, Missouri, USA

³Division of Gastroenterology, Hepatology and Nutrition, Department of Pediatrics, Washington University School of Medicine, St. Louis, Missouri, USA

⁴Department of Molecular Microbiology, Washington University School of Medicine, St. Louis, Missouri, USA

Acknowledgements The authors thank Dr Bill Stenson for comments on the manuscript and Drs Chien-Huan Chen, Themistocles Dassopoulos, Dayna Early, Alexandra Gutierrez, Kevin Korenblat, Deborah Rubin, Shelby Sullivan, Sandeep Tripathy and Jean Wang for collection of biopsy tissues. We thank Kelly Monroe for assisting with patient recruitment and consent, Wandy Beatty for assistance with confocal imaging and Dr. James Nataro for providing strain EAEC042.

Contributors KLV, HM, CM, NS, PIT, MAC and TSS contributed to the study concept and design. JMM and MAC contributed to the review of endoscopy procedures and clinical records. KLV and JMM contributed to establishment of cell lines. KLV performed epithelial cell experiments. NS performed *E. coli* adherence assays. KLV, HM, NS, PIT, MAC and TSS contributed to the analysis and interpretation of the data. KLV and TSS drafted the manuscript. All authors contributed to the critical revision of the manuscript. MAC and TSS obtained funding and supervised the study.

Funding This work was supported by the NIH (grants DK071619, DK089016, DK100737, DK90251 and DK102557; Mucosal Immunology Studies Team Translational Pilot Project and Young Investigator Pilot Award, Parent Grant AI095550) and the Crohn's and Colitis Foundation of America (CCFA) Genetics and Microbiome Consortia. KLV was supported by an NIH training grant (T32 AI007163) and a CCFA Research Fellowship Award. The Washington University Digestive Disease Research Core Center is supported by a grant from the National Institute of Diabetes and Digestive and Kidney Disease (NIDDK) (P30DK052574).

Competing interests None.

Ethics approval Institutional Review Board of Washington University School of Medicine.

Provenance and peer review Not commissioned; externally peer reviewed.

REFERENCES

- 1 Maloy KJ, Powrie F. Intestinal homeostasis and its breakdown in inflammatory bowel disease. *Nature* 2011;474:298–306.
- 2 Cao L, Kuratnik A, Xu W, et al. Development of intestinal organoids as tissue surrogates: cell composition and the Epigenetic control of differentiation. *Mol Carcinog* Published Online First: 21 Sept 2013. doi:10.1002/mc.22089
- 3 Fordham RP, Yui S, Hannan NR, et al. Transplantation of expanded fetal intestinal progenitors contributes to colon regeneration after injury. *Cell Stem Cell* 2013;13:734–44.
- 4 Jung P, Sato T, Merlos-Suarez A, et al. Isolation and in vitro expansion of human colonic stem cells. *Nat Med* 2011;17:1225–7.
- 5 Lahar N, Lei NY, Wang J, et al. Intestinal subepithelial myofibroblasts support in vitro and in vivo growth of human small intestinal epithelium. *PLoS ONE* 2011;6:e26898.
- 6 McCracken KW, Howell JC, Wells JM, et al. Generating human intestinal tissue from pluripotent stem cells in vitro. *Nat Protoc* 2011;6:1920–8.
- 7 Miyoshi H, Ajima R, Luo CT, et al. Wnt5a potentiates TGF-beta signaling to promote colonic crypt regeneration after tissue injury. *Science* 2012;338:108–13.
- 8 Miyoshi H, Stappenbeck TS. In vitro expansion and genetic modification of gastrointestinal stem cells in spheroid culture. *Nat Protoc* 2013;8:2471–82.
- 9 Mustata RC, Vasile G, Fernandez-Vallone V, et al. Identification of Lgr5-independent spheroid-generating progenitors of the mouse fetal intestinal epithelium. *Cell Rep* 2013;5:421–32.
- 10 Ootani A, Li X, Sangiorgi E, et al. Sustained in vitro intestinal epithelial culture within a Wnt-dependent stem cell niche. *Nat Med* 2009;15:701–6.
- 11 Sato T, Stange DE, Ferrante M, et al. Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. *Gastroenterology* 2011;141:1762–72.
- 12 Sato T, Vries RG, Snippert HJ, et al. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature* 2009;459:262–5.
- 13 Spence JR, Mayhew CN, Rankin SA, et al. Directed differentiation of human pluripotent stem cells into intestinal tissue in vitro. *Nature* 2011;470:105–9.
- 14 von Furstenberg RJ, Gulati AS, Baxi A, et al. Sorting mouse jejunal epithelial cells with CD24 yields a population with characteristics of intestinal stem cells. *Am J Physiol Gastrointest Liver Physiol* 2011;300:G409–17.
- 15 Wang F, Scoville D, He XC, et al. Isolation and characterization of intestinal stem cells based on surface marker combinations and colony-formation assay. *Gastroenterology* 2013;145:383–95.
- 16 Yin X, Farin HF, van Es JH, et al. Niche-independent high-purity cultures of Lgr5+ intestinal stem cells and their progeny. *Nat Methods* 2014;11:106–12.
- 17 Klotz C, Aebischer T, Seeber F. Stem cell-derived cell cultures and organoids for protozoan parasite propagation and studying host-parasite interaction. *Int J Med Microbiol* 2012;302:203–9.
- 18 Kuratnik A, Giardina C. Intestinal organoids as tissue surrogates for toxicological and pharmacological studies. *Biochem Pharmacol* 2013;85:1721–6.
- 19 Moon C, Vandussen KL, Miyoshi H, et al. Development of a primary mouse intestinal epithelial cell monolayer culture system to evaluate factors that modulate IgA transcytosis. *Mucosal Immunol* 2014;7:818–28.
- 20 Patel KK, Miyoshi H, Beatty WL, et al. Autophagy proteins control goblet cell function by potentiating reactive oxygen species production. *EMBO J* 2013;32:3130–44.
- 21 Ermund A, Schutte A, Johansson ME, et al. Studies of mucus in mouse stomach, small intestine, and colon. I. Gastrointestinal mucus layers have different properties depending on location as well as over the Peyer's patches. *Am J Physiol Gastrointest Liver Physiol* 2013;305:G341–7.
- 22 Stelzner M, Helmrich M, Dunn JC, et al. A nomenclature for intestinal in vitro cultures. *Am J Physiol Gastrointest Liver Physiol* 2012;302:G1359–63.
- 23 Barker N, van Es JH, Kuipers J, et al. Identification of stem cells in small intestine and colon by marker gene Lgr5. *Nature* 2007;449:1003–7.
- 24 Tsilingiri K, Barbosa T, Penna G, et al. Probiotic and postbiotic activity in health and disease: comparison on a novel polarised ex-vivo organ culture model. *Gut* 2012;61:1007–15.
- 25 Kim KA, Kakitani M, Zhao J, et al. Mitogenic influence of human R-spondin1 on the intestinal epithelium. *Science* 2005;309:1256–9.
- 26 Korinek V, Barker N, Moerer P, et al. Depletion of epithelial stem-cell compartments in the small intestine of mice lacking Tcf-4. *Nat Genet* 1998;19:379–83.
- 27 Kuhnert F, Davis CR, Wang HT, et al. Essential requirement for Wnt signaling in proliferation of adult small intestine and colon revealed by adenoviral expression of Dickkopf-1. *Proc Natl Acad Sci USA* 2004;101:266–71.
- 28 Pinto D, Gregorieff A, Begthel H, et al. Canonical Wnt signals are essential for homeostasis of the intestinal epithelium. *Genes Dev* 2003;17:1709–13.

- 29 van de Wetering M, Sancho E, Verweij C, *et al.* The beta-catenin/TCF-4 complex imposes a crypt progenitor phenotype on colorectal cancer cells. *Cell* 2002;111:241–50.
- 30 Noah TK, Donahue B, Shroyer NF. Intestinal development and differentiation. *Exp Cell Res* 2011;317:2702–10.
- 31 Stappenbeck TS. Paneth cell development, differentiation, and function: new molecular cues. *Gastroenterology* 2009;137:30–3.
- 32 Middendorp S, Schneeberger K, Wiegerinck CL, *et al.* Adult stem cells in the small intestine are intrinsically programmed with their location-specific function. *Stem Cells* 2014;32:1083–91.
- 33 Falk P, Roth KA, Gordon JI. Lectins are sensitive tools for defining the differentiation programs of mouse gut epithelial cell lineages. *Am J Physiol* 1994;266:G987–1003.
- 34 Atuma C, Strugala V, Allen A, *et al.* The adherent gastrointestinal mucus gel layer: thickness and physical state in vivo. *Am J Physiol Gastrointest Liver Physiol* 2001;280:G922–9.
- 35 Johansson ME, Larsson JM, Hansson GC. The two mucus layers of colon are organized by the MUC2 mucin, whereas the outer layer is a legislator of host-microbial interactions. *Proc Natl Acad Sci USA* 2011;108(Suppl 1):4659–65.
- 36 Johansson ME, Phillipson M, Petersson J, *et al.* The inner of the two Muc2 mucin-dependent mucus layers in colon is devoid of bacteria. *Proc Natl Acad Sci USA* 2008;105:15064–9.
- 37 Nataro JP, Kaper JB. Diarrheagenic *Escherichia coli*. *Clin Microbiol Rev* 1998;11:142–201.
- 38 Denno DM, Shaikh N, Stapp JR, *et al.* Diarrhea etiology in a pediatric emergency department: a case control study. *Clin Infect Dis* 2012;55:897–904.
- 39 Nataro JP, Deng Y, Cookson S, *et al.* Heterogeneity of enteroaggregative *Escherichia coli* virulence demonstrated in volunteers. *J Infect Dis* 1995;171:465–8.
- 40 Levine MM, Bergquist EJ, Nalin DR, *et al.* *Escherichia coli* strains that cause diarrhoea but do not produce heat-labile or heat-stable enterotoxins and are non-invasive. *Lancet* 1978;1:1119–22.
- 41 Riley LW, Junio LN, Libaek LB, *et al.* Plasmid-encoded expression of lipopolysaccharide O-antigenic polysaccharide in enteropathogenic *Escherichia coli*. *Infect Immun* 1987;55:2052–6.
- 42 Woodall LD, Russell PW, Harris SL, *et al.* Rapid, synchronous, and stable induction of type 1 piliation in *Escherichia coli* by using a chromosomal lacUV5 promoter. *J Bacteriol* 1993;175:2770–8.
- 43 Bilge SS, Vary JC Jr, Dowell SF, *et al.* Role of the *Escherichia coli* O157:H7 O side chain in adherence and analysis of an rfb locus. *Infect Immun* 1996;64:4795–801.
- 44 Tarr PI, Bilge SS, Vary JC Jr, *et al.* Iha: a novel *Escherichia coli* O157:H7 adherence-conferring molecule encoded on a recently acquired chromosomal island of conserved structure. *Infect Immun* 2000;68:1400–7.
- 45 Abner SR, Guenther PC, Guarner J, *et al.* A human colorectal explant culture to evaluate topical microbicides for the prevention of HIV infection. *J Infect Dis* 2005;192:1545–56.
- 46 Autrup H, Barrett LA, Jackson FE, *et al.* Explant culture of human colon. *Gastroenterology* 1978;74:1248–57.
- 47 Losonsky GA, Fantry GT, Reymann M, *et al.* Validation of a gastrointestinal explant system for measurement of mucosal antibody production. *Clin Diagn Lab Immunol* 1999;6:803–7.
- 48 Nicotra LL, Vu M, Harvey BS, *et al.* Prostaglandin ethanolamides attenuate damage in a human explant colitis model. *Prostaglandins Other Lipid Mediat* 2013;100:101:22–9.
- 49 McGuckin MA, Linden SK, Sutton P, *et al.* Mucin dynamics and enteric pathogens. *Nat Rev Microbiol* 2011;9:265–78.
- 50 Dean P, Kenny B. The effector repertoire of enteropathogenic *E. coli*: ganging up on the host cell. *Curr Opin Microbiol* 2009;12:101–9.
- 51 Bokete TN, Whittam TS, Wilson RA, *et al.* Genetic and phenotypic analysis of *Escherichia coli* with enteropathogenic characteristics isolated from Seattle children. *J Infect Dis* 1997;175:1382–9.
- 52 Rothbaum R, McAdams AJ, Giannella R, *et al.* A clinicopathologic study of enterocyte-adherent *Escherichia coli*: a cause of protracted diarrhea in infants. *Gastroenterology* 1982;83:441–54.
- 53 Rothbaum RJ, Partin JC, Saalfeld K, *et al.* An ultrastructural study of enteropathogenic *Escherichia coli* infection in human infants. *Ultrastruct Pathol* 1983;4:291–304.
- 54 Griffin PM, Olmstead LC, Petras RE. *Escherichia coli* O157:H7-associated colitis. A clinical and histological study of 11 cases. *Gastroenterology* 1990;99:142–9.
- 55 Kelly J, Oryshak A, Wenetsek M, *et al.* The colonic pathology of *Escherichia coli* O157:H7 infection. *Am J Surg Pathol* 1990;14:87–92.
- 56 Phillips AD, Navabpour S, Hicks S, *et al.* Enterohaemorrhagic *Escherichia coli* O157:H7 target Peyer's patches in humans and cause attaching/effacing lesions in both human and bovine intestine. *Gut* 2000;47:377–81.
- 57 Wright C, Herbert G, Pilkington R, *et al.* Real-time PCR method for the quantification of *Burkholderia cepacia* complex attached to lung epithelial cells and inhibition of that attachment. *Lett Appl Microbiol* 2010;50:500–6.
- 58 Candela M, Seibold G, Vitali B, *et al.* Real-time PCR quantification of bacterial adhesion to Caco-2 cells: competition between bifidobacteria and enteropathogens. *Res Microbiol* 2005;156:887–95.
- 59 Darfeuille-Michaud A. Adherent-invasive *Escherichia coli*: a putative new *E. coli* pathotype associated with Crohn's disease. *Int J Med Microbiol* 2002;292:185–93.
- 60 Kaser A, Zeissig S, Blumberg RS. Inflammatory bowel disease. *Annu Rev Immunol* 2010;28:573–621.
- 61 Marteau P. Bacterial flora in inflammatory bowel disease. *Dig Dis* 2009;27(Suppl 1):99–103.
- 62 Baumgart M, Dogan B, Rishniw M, *et al.* Culture independent analysis of ileal mucosa reveals a selective increase in invasive *Escherichia coli* of novel phylogeny relative to depletion of Clostridiales in Crohn's disease involving the ileum. *ISME J* 2007;1:403–18.
- 63 Frank DN, St Amand AL, Feldman RA, *et al.* Molecular-phylogenetic characterization of microbial community imbalances in human inflammatory bowel diseases. *Proc Natl Acad Sci USA* 2007;104:13780–5.
- 64 Martinez-Medina M, Aldeguer X, Gonzalez-Huix F, *et al.* Abnormal microbiota composition in the ileocolonic mucosa of Crohn's disease patients as revealed by polymerase chain reaction-denaturing gradient gel electrophoresis. *Inflamm Bowel Dis* 2006;12:1136–45.
- 65 Boudeau J, Glasser AL, Masseret E, *et al.* Invasive ability of an *Escherichia coli* strain isolated from the ileal mucosa of a patient with Crohn's disease. *Infect Immun* 1999;67:4499–509.
- 66 Burke DA, Axon AT. Adhesive *Escherichia coli* in inflammatory bowel disease and infective diarrhoea. *BMJ* 1988;297:102–4.
- 67 Darfeuille-Michaud A, Neut C, Barnich N, *et al.* Presence of adherent *Escherichia coli* strains in ileal mucosa of patients with Crohn's disease. *Gastroenterology* 1998;115:1405–13.
- 68 Gaffer MH, Holdsworth CD, Duerden BI. Virulence properties of *Escherichia coli* strains isolated from patients with inflammatory bowel disease. *Gut* 1992;33:646–50.
- 69 Khor B, Gardet A, Xavier RJ. Genetics and pathogenesis of inflammatory bowel disease. *Nature* 2011;474:307–17.

SUPPLEMENTARY MATERIALS AND METHODS

Establishment of human spheroid cultures

Crypt/gland units were isolated and plated in Matrigel as described in the main text. At the end of the isolation procedure, the cell/Matrigel mixture was plated into 2-4 wells of a 24-well tissue culture plate (plating into multiple wells helped to dilute out the effects of cell types that die because their growth is not supported by our media and to ensure line recovery if yeast contamination occurred in a well). Cells were grown in an incubator at 37°C with 5% CO₂.

Initial passages after isolation were performed every 3-5 days at a 1:1-1:2 ratio (Supplementary Figure 2). Generally, passaging was performed every 3 days if >10 spheroids per well were present and every 5 days if there were <10 spheroids per well present. Additionally, spheroids were typically not expanded into more than 2 wells until at least 20 spheroids were present in each well. During the first several passages, the human spheroids were incubated in trypsin-EDTA solution for ~45-60 seconds followed by vigorous pipetting 10-15 times (see below for passaging method). For later passages, spheroids were typically incubated in trypsin-EDTA solution for 60-90 seconds followed by vigorous pipetting 20-30 times. Within the first several passages, the spheroid density typically was sufficient to perform subsequent passages every 3 days at a 1:3-1:4 ratio, with media replenished every other day.

One important difference from our mouse culture system is that the human intestinal spheroids grow better when cultures are maintained as a denser spheroid population (see image in Fig. 1). A second difference was that the addition of two inhibitors to the media was required to maintain the epithelial cell cultures for >3 passages (Supplementary Figure 1): the ROCK inhibitor is required because isolated intestinal epithelial cells are sensitive to anoikis¹, and the TGFBR inhibitor is required to repress TGF- β activity in serum to maximize epithelial cell proliferation^{2,3}.

Maintenance of spheroid cultures

Spheroid cultures were maintained in 24-well tissue culture plates, and the medium was replaced every other day. Spheroids were passaged 1:3-1:4 every 3 days. For each passage, the spheroid/Matrigel

was scratched into 500 μ L of PBS containing 0.5 mM EDTA (PBS-EDTA) with a 1000 μ L pipette and transferred to a 15 mL conical tube. Spheroids were pelleted by centrifugation at 200 g for 5 min, and the supernatant was discarded. Spheroids were dissociated by incubating in 200 μ L of 0.25% trypsin in PBS-EDTA for 60-90 sec (or 2 min if passaging for Transwell culture) in a 37°C water bath, followed by addition of 500 μ L of washing medium and mechanical dissociation by vigorous pipetting (~20-30 times if passaging for maintenance or three-dimensional culture, ~40-50 times if passaging for Transwell culture). An additional 5 mL of washing medium was then added to inactivate the trypsin. Spheroids were pelleted by centrifugation at 200 g for 5 min and the supernatant was aspirated, leaving ~200 μ L. Spheroids were transferred to a 1.5 mL microfuge tube in 1 mL washing medium and pelleted by centrifugation at 200 g for 5 min. The supernatant was carefully removed, and the pellet was re-suspended in Matrigel (15 μ L/well). Following polymerization of the Matrigel, 50% L-WRN CM supplemented with 10 μ M Y-27632 and 10 μ M SB 431542 was added.

Additional notes:

1. The human intestinal spheroids do not survive long exposure to PBS-EDTA or trypsin/PBS-EDTA. Work at a deliberate pace and/or do not work with too many cell lines/wells at once to avoid loss of culture density due to excessive cell death.
2. We prefer a particular trypsin reagent for human intestinal spheroid culture: 10X Trypsin Solution (Sigma T4549) is diluted to 1X with PBS-EDTA, aliquoted and stored at -20°C. For a working stock, single 1X aliquots are thawed and stored at 4°C.
3. Human spheroids grow and perform best in functional assays when they are a small, uniform size. If spheroids become very large, it may be necessary to trypsinize for a longer time period (up to 2 min at 37°C), pipette more vigorously during mechanical dissociation and/or strain through a 40- μ m filter after trypsinization.
4. For differentiation experiments, only ROCK inhibitor was included in the media; TGFBR1 inhibitor was not included because it was not required for terminal experiments and because

active TGF- β signaling has been shown to be important for the suppression of proliferation and promotion of differentiation in intestinal epithelial cells²⁻⁴.

Storage and recovery of spheroid cultures

For frozen storage, the spheroid/Matrigel mixture was removed from the well by scratching with a 1000 μ L pipette and transferred to a 15 mL conical tube containing 5 mL of washing medium. Cells were pelleted by centrifugation at 200 g for 5 min, and the supernatant was removed by aspiration. The cell pellet was re-suspended in freezing medium (primary culture medium plus 10% DMSO; 500 μ L/well) and transferred to 1-mL cryotubes (500 μ L/cryotube). Cells were frozen slowly overnight at -80°C and then transferred to liquid nitrogen storage. To recover spheroids, frozen stocks were quickly thawed at 37°C, transferred to a 15 mL conical tube containing 5 mL washing medium and pelleted by centrifugation at 200 g for 5 min. The supernatant was removed by aspiration. Spheroids were transferred to a 1.5 mL microfuge tube in 1 mL washing medium, pelleted and re-suspended in Matrigel as described above.

Cell cycle analysis

Cell cycle analysis was assessed using the Click-iT EdU Cell Proliferation AlexaFluor488 kit (Invitrogen) according to the manufacturer's directions. Spheroids were treated for 2 hours with 10 μ M EdU and then dissociated into single cells by trypsinization and vigorous pipetting. A Click-iT staining reaction was performed following fixation and permeabilization. SYTOX AAD (Invitrogen) was used as a DNA content marker. Flow cytometry was performed using a FACSCalibur flow cytometer (BD Biosciences). Data analysis was performed with FloJo software.

Histological analysis of human epithelial cell monolayers

For histological analysis (Day 3), cells were fixed in 10% formalin for 10 min, washed 3 times in 70% ethanol and processed for paraffin embedding. Transverse sections (5 μ m) were stained with

hematoxylin and eosin (visualized with an Olympus BX51 light microscope with a DP70 digital camera) or used for immunostaining. For immunostaining, sections were de-paraffinized and hydrated, boiled in Unmasking Solution (Vector Laboratories, Burlingame, CA) or Trilogy (Cell Marque, Rocklin, California) for 10-20 min, rinsed in PBS, blocked in 1% bovine serum albumin/0.1% Triton-X100 for 30 minutes and incubated with primary antibody at 4°C overnight. Primary antibodies included rabbit anti-ChgA (1:2000, Abcam, Cambridge, MA), rabbit anti-Muc2 (1:200, Santa Cruz Biotechnology, Inc., Dallas, Texas), goat anti-lysozyme (1:100; Santa Cruz Biotechnology, Inc.), mouse anti-chicken Villin 1 (1:100; AbD Serotec, Raleigh, NC) and rabbit anti-ZO-1 (1:100; Invitrogen). Slides were rinsed 3 times with PBS and incubated for 30-60 min at room temperature with species specific secondary antibodies (1:500; Invitrogen) conjugated to AlexaFluor488 or AlexaFluor546. UEA-1 lectin conjugated to TRITC (1:500, Sigma) was applied during secondary antibody incubation. Slides were washed 3 times in PBS and stained with bis-benzamide (Hoescht 33258, Invitrogen) to visualize nuclei and mounted with Fluoromount-G (Southern Biotech). Staining was visualized with either a Zeiss (Oberkochen, Germany) Axiovert 200 microscope with an AxioCam MRM digital camera.

Additional methods for mucus layer analysis

For mucus thickness measurements, medium containing fluorescent beads (FluoSpheres, Invitrogen) was gently added to the apical chamber of each Transwell (1:500 final dilution) and allowed to sediment for 40 min followed by confocal imaging. To disrupt mucus layers, media were aspirated and then monolayers were washed with vigorous pipetting followed by addition of fresh medium containing fluorescent beads, which were allowed to sediment for 40 min prior to re-imaging.

Preparation of epithelial and bacterial cells for adherence assays

For two-dimensional culture, human intestinal spheroids from ileum or rectum were dissociated and seeded onto 8-well glass chamber slides (Lab-Tek; 1-2 wells of a 24-well plate were used to seed one chamber slide) coated with Matrigel diluted 1:40 in PBS. Spheroids were allowed to recover overnight in

50% L-WRN CM with 10 μ M Y-27632. The following day, the medium was replaced with 5% L-WRN CM with 10 μ M Y-27632 and changed daily. Prior to the adherence assay (Day 3), the epithelial cells were washed three times with antibiotic-free primary culture media containing only 5% FBS.

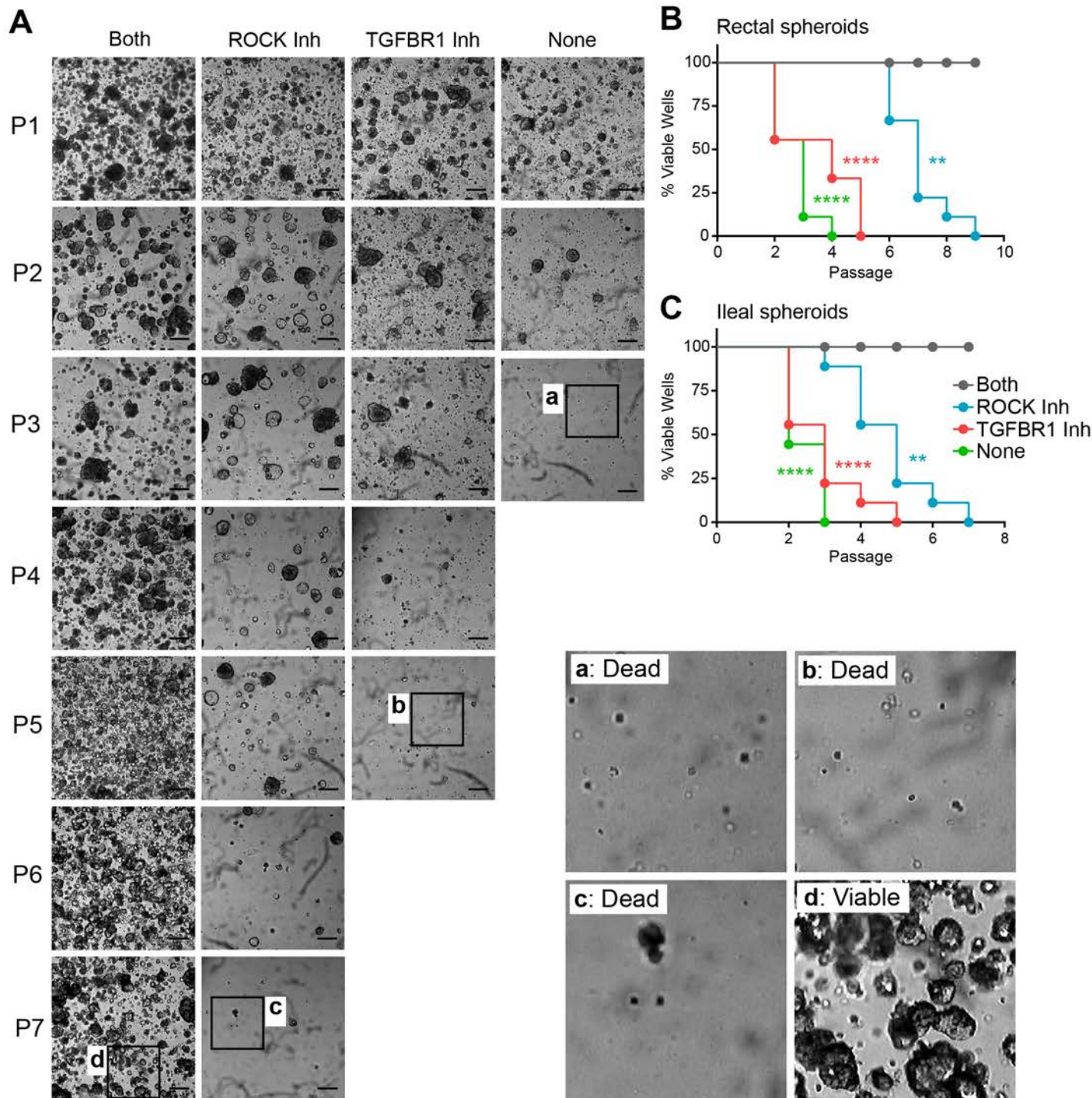
To prepare the bacterial stocks, *E. coli* strains were streaked from frozen stocks to MacConkey agar and grown overnight at 37°C. A single colony was used to inoculate 5 ml of LB broth and incubated overnight at 37°C without agitation. Cultures were pelleted and re-suspended in antibiotic-free DMEM with FCS and 0.5% D-mannose to a concentration of $\sim 10^9$ colony forming units/mL, confirmed by OD₆₀₀ and bacterial titration.

Supplemental statistical analyses

Statistical analyses were performed with Prism GraphPad v6 software. For analysis of the survival curve serial passaging experiment (percent well viability), a log-rank test was used to determine statistical significance. For all other analyses, a Student's t test or 1-way ANOVA followed by a Dunnett's multiple comparisons or Tukey post test was used. $P < 0.05$ was considered to be significant.

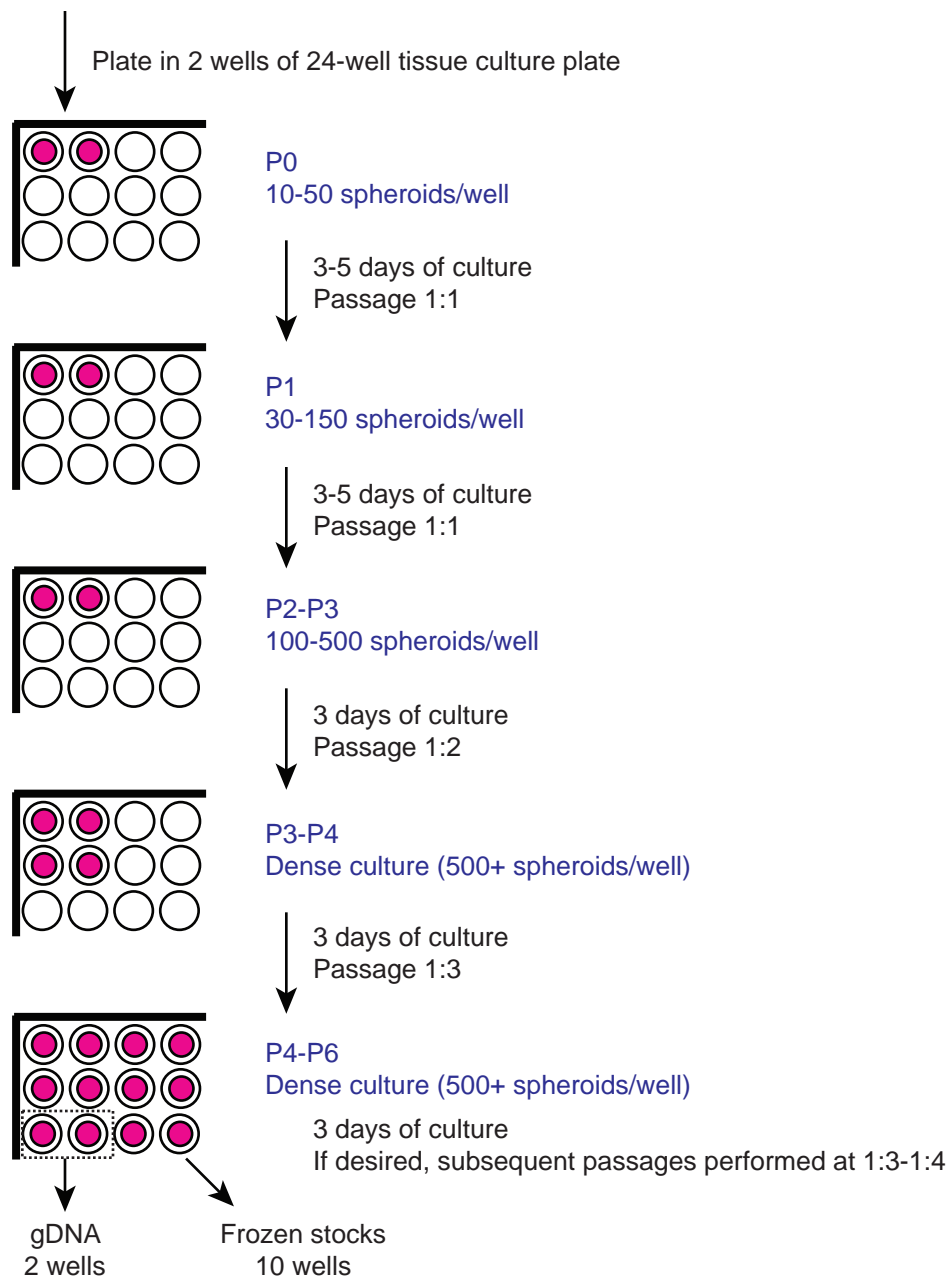
SUPPLEMENTAL REFERENCES

1. Grossmann J, Maxson JM, Whitacre CM, et al. New isolation technique to study apoptosis in human intestinal epithelial cells. *Am J Pathol* 1998;153:53-62.
2. Bach SP, Renehan AG, Potten CS. Stem cells: the intestinal stem cell as a paradigm. *Carcinogenesis* 2000;21:469-76.
3. Moses HL, Yang EY, Pietenpol JA. TGF-beta stimulation and inhibition of cell proliferation: new mechanistic insights. *Cell* 1990;63:245-7.
4. Miyoshi H, Ajima R, Luo CT, et al. Wnt5a potentiates TGF-beta signaling to promote colonic crypt regeneration after tissue injury. *Science* 2012;338:108-13.

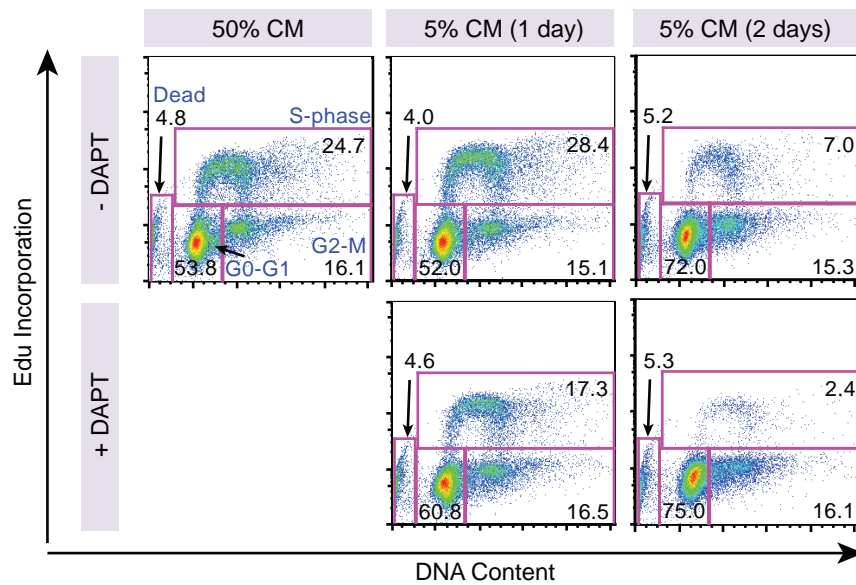


Supplementary Figure 1. Addition of ROCK and TGFBR inhibitors to the 50% L-WRN CM is required for long-term passage of spheroid lines. (A-C) Human rectal (n = 3 lines) and ileal (n = 3 lines) spheroids were cultured in 50% L-WRN CM plus Y-27632 (ROCK Inh), SB 431542 (TGFBR1 Inh), both inhibitors (Both) or without inhibitors (None). Spheroids were serially passaged 1:3 every 3 days with images taken just prior to each passage. (A) Images for one representative experiment with the relative passage number (P1-P7) shown on the left. (B, C) Summaries of serial passage experiments showing percent well viability at each passage for rectal (B) and ileal (C) cultures (n = 3 experiments per line). **, P<0.01; ***, P<0.001; ****, P<0.0001 by log-rank test compared to “Both”: $\chi^2 = 10.72$, P = 0.0011 (B, ROCK); $\chi^2 = 17.95$, P<0.0001 (B, TGRBR1); $\chi^2 = 18.48$, P<0.0001 (B, None); $\chi^2 = 10.21$, P = 0.0014 (C, ROCK); $\chi^2 = 18.14$, P<0.0001 (C, TGFBR1); $\chi^2 = 17.41$, P<0.0001 (C, None). Inh, inhibitor. Bars, 200 μ m.

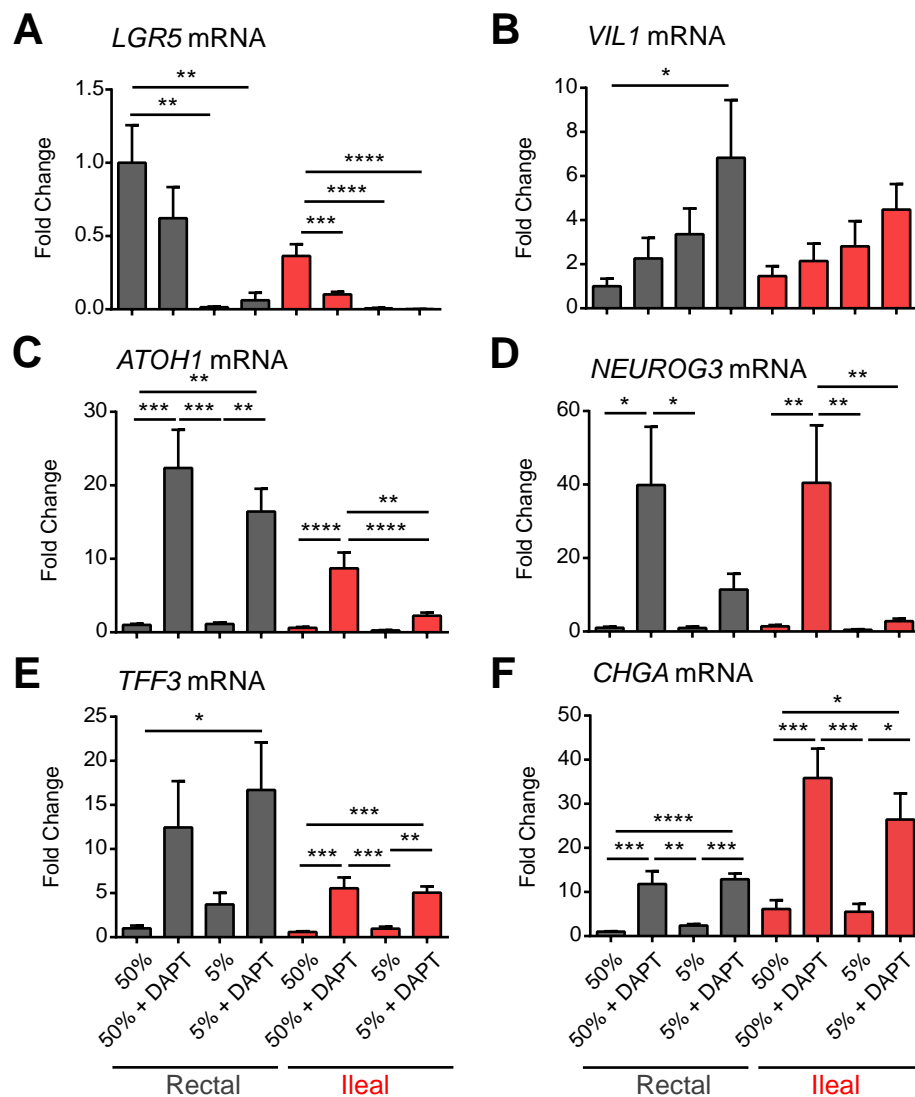
Isolated crypt/gland units from 2-3 biopsies
~200-300 crypt/gland units



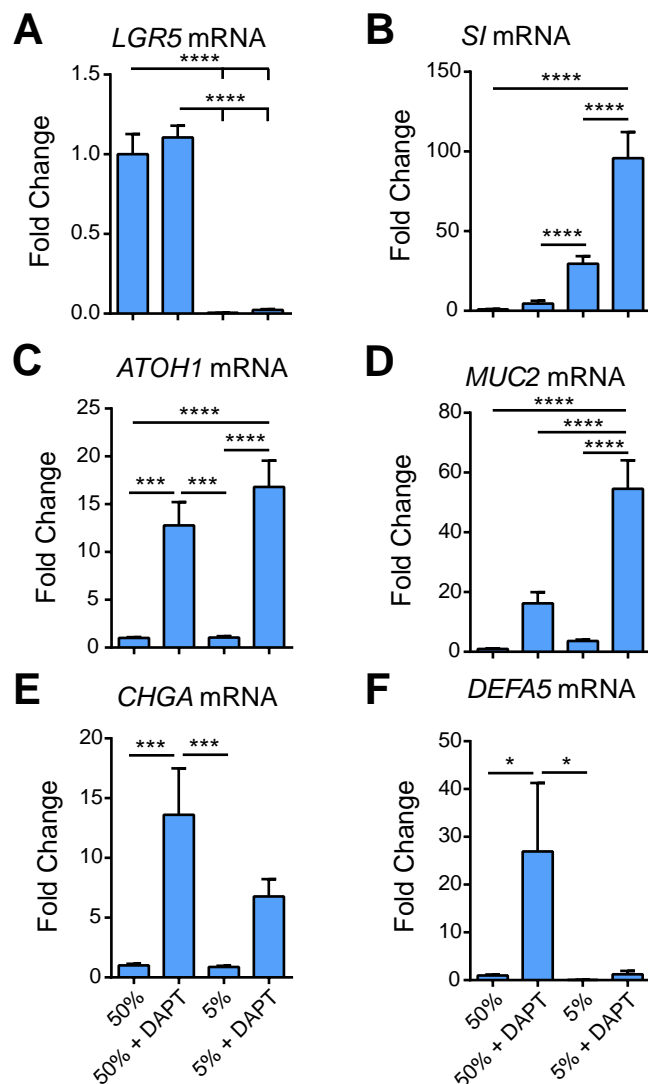
Supplementary Figure 2. Work flow for establishing gastrointestinal epithelial cell cultures from endoscopic biopsies. Isolated crypt/gland units were embedded in Matrigel and plated into 2-wells of a 24-well tissue culture plate (occupied wells denoted by pink circles). Generally, a culture could be expanded to 12 densely seeded wells within 4-6 passages (15-25 days). The typical passage scheme and approximate number of spheroids per well is provided. gDNA, genomic DNA.



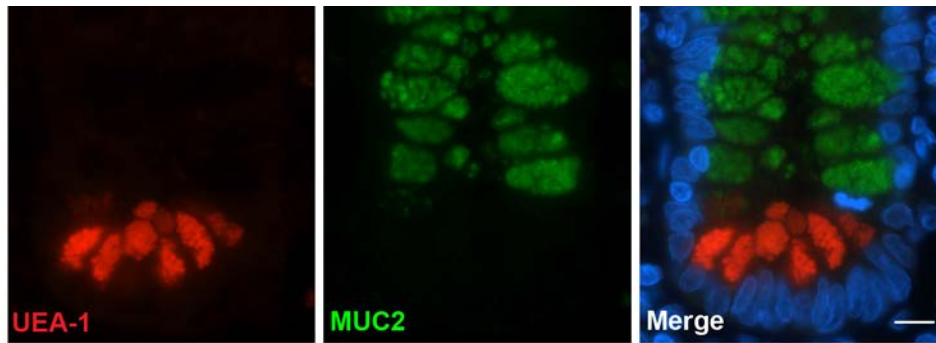
Supplementary Figure 3. Reduced L-WRN CM concentration results in cell cycle exit. Representative dot plots from one cell cycle analysis experiment (rectal line). For these experiments, spheroids were passaged (Day 0) and allowed to recover overnight in 50% L-WRN CM. Media was replaced daily, with some cultures changed to 5% or 5% L-WRN CM plus 5 μ M DAPT for the indicated number of days. On Day 3, the spheroids were treated with EdU for 2 hr, dissociated, stained and sorted by flow cytometry based on EdU incorporation and SYTOX AAD (DNA content marker). Fluorescence-activated cell sorting (FACS) was performed with a FACSCalibur (BD Biosciences), and data were analyzed with FloJo software. For each sample, 30,000 events were collected.



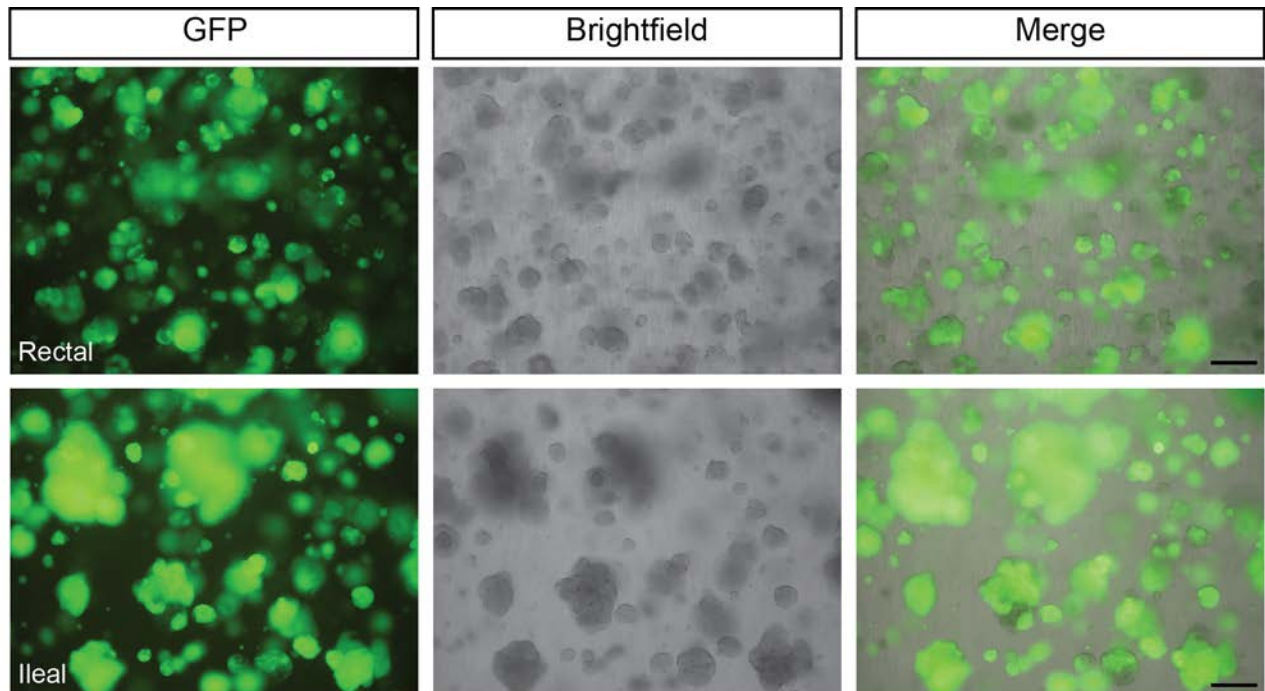
Supplementary Figure 4. Rectal and ileal spheroids retain a site-specific program of differentiation. (A-F) Human rectal (n = 3 lines) and ileal (n = 3 lines) spheroids were passaged (Day 0) and cultured in 50% L-WRN CM overnight. The following day (Day 1), medium was replaced to either 50% L-WRN CM, 50% L-WRN CM + 5 μ M DAPT, 5% L-WRN CM or 5% L-WRN CM + 5 μ M DAPT (all with ROCK inhibitor). Media were replaced daily, and cell lysates were collected for RNA extraction on Day 3. Gene expression levels of *LGR5* (A), *VIL1* (B), *ATOH1* (C), *NEUROG3* (D), *TFF3* (E) and *CHGA* (F) were analyzed by qRT-PCR. Data are presented as fold change relative to rectal 50% L-WRN CM (mean \pm s.e.m.; n = 3 per line). *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; ****, $P < 0.0001$ by 1-way ANOVA followed by a Tukey's post-test compared to 50% L-WRN CM of the same region: F = 7.86, $P = 0.0005$ (A, Rect); F = 17.28, $P < 0.0001$ (A, Ile); F = 2.73, $P = 0.0599$ (B, Rect); F = 1.94, $P = 0.1429$ (B, Ile); F = 12.82, $P < 0.0001$ (C, Rect); F = 12.73, $P < 0.0001$ (C, Ile); F = 6.62, $P = 0.0013$ (D, Rect); F = 11.83, $P < 0.0001$ (D, Ile); F = 3.70, $P = 0.0215$ (E, Rect); F = 13.21, $P < 0.0001$ (E, Ile); F = 14.57, $P < 0.0001$ (F, Rect); F = 10.60, $P < 0.0001$ (F, Ile).



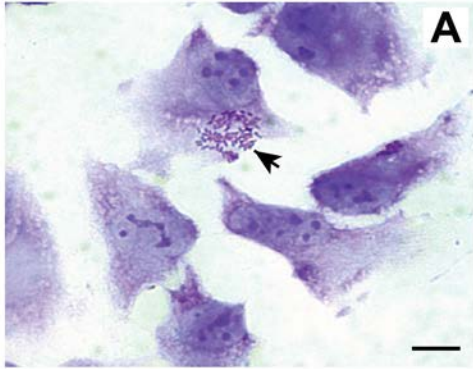
Supplementary Figure 5. Differentiation of human duodenal spheroid epithelial cells. (A-F) Human duodenal spheroids ($n = 3$ lines) were passaged (Day 0) and cultured in 50% L-WRN CM overnight. The following day (Day 1), medium was replaced to either 50% L-WRN CM, 50% L-WRN CM + 5 μ M DAPT, 5% L-WRN CM or 5% L-WRN CM + 5 μ M DAPT (all with ROCK inhibitor). Media were replaced daily, and cell lysates were collected for RNA extraction on Day 3. Gene expression levels of *LGR5* (A), *SI* (B), *ATOH1* (C), *MUC2* (D), *CHGA* (E) and *DEFA5* (F) were analyzed by qRT-PCR. Data are presented as fold change relative to 50% L-WRN CM (mean \pm s.e.m.; $n = 3$ per line). *, $P < 0.05$; ***, $P < 0.001$; ****, $P < 0.0001$ by 1-way ANOVA followed by a Tukey's post-test compared to 50% L-WRN CM condition: $F = 62.82$, $P < 0.0001$ (A); $F = 27.97$, $P < 0.0001$ (B); $F = 22.09$, $P < 0.0001$ (C); $F = 25.65$, $P < 0.0001$ (D); $F = 9.46$, $P = 0.0001$ (E); $F = 3.64$, $P = 0.0239$ (F).



Supplementary Figure 6. UEA-1 lectin localizes to human Paneth cells. Formalin-fixed paraffin-embedded tissue from the human small intestine was immunostained for UEA-1 lectin (red) and MUC2 (green). A single, representative crypt base is shown. Bar, 10 μ m.



Supplementary Figure 7. Generation of GFP-expressing human intestinal spheroid lines via lentivirus infection for mucus layer analysis. Rectal and ileal human intestinal spheroids were infected with a lentiviral construct that contained a non-specific, scrambled shRNA and a GFP expression cassette. Very high infection efficiency was observed, with nearly 100% of the epithelial cells in both the rectal and ileal lines expressing the GFP marker within 3 days after infection (data not shown). The high infection efficiency resulted in the persistence of mostly pure GFP-expressing cell populations for several passages without the requirement for selection of positive clones (images are from the fourth passage post-infection). Bar, 200 μ m.



Supplementary Figure 8. Sparse adherence of E. coli O157:H7 strain 86-24 to HeLa cells. HeLa cells (A) were incubated with enterohemorrhagic *E. coli* O157:H7 strain 86-24 for 1 hr followed by several washes and staining with crystal violet to visualize adherent bacteria (dark purple). Sparse adherence was noted for the O157:H7 strain with HeLa cells; a single bacterial colony was observed in a single field (arrow). Other fields of view had no adherent cells. Bar, 20 μ m.

Supplementary Table 1 List of established epithelial cell lines from regions of the gastrointestinal tract.

Patient	Tissue Region	Patient Age (years)	Diagnosis* (Non-IBD/CD/UC)
1	Rectum	52	Non-IBD
2	Neo-terminal ileum	39	UC
2	Ileal pouch	39	UC
3	Neo-terminal ileum	29	CD
4	Rectum	25	CD
5	Rectum	62	Non-IBD
6	Rectum	60	Non-IBD
7	Left colon	23	UC
7	Right colon	23	UC
8	Rectum	37	CD
9	Rectum	55	Non-IBD
10	Rectum	42	Non-IBD
11	Rectum	76	Non-IBD
11	Right colon	76	Non-IBD
12	Stomach (body)	58	Non-IBD
12	Stomach (fundus)	58	Non-IBD
13	Right colon	61	CD
14	Rectum	44	CD
15	Rectum	57	CD
16	Rectum	47	Non-IBD
16	Terminal ileum	47	Non-IBD
17	Rectum	71	CD

17	Right colon	71	CD
18	Rectum	19	UC
18	Terminal ileum	19	UC
19	Rectum	47	UC
19	Esophagus (distal)	47	UC
20	Rectum	49	Non-IBD
21	Left colon	56	UC
22	Duodenum	35	CD
23	Duodenum	54	Non-IBD
24	Duodenum	69	Non-IBD
25	Rectum	40	Non-IBD
25	Terminal ileum	40	Non-IBD
26	Terminal ileum	44	UC
27	Rectum	60	Non-IBD
28	Rectum	28	UC
28	Terminal ileum	28	UC
29	Rectum	57	Non-IBD
29	Terminal ileum	57	Non-IBD
30	Rectum	51	Non-IBD
30	Terminal ileum	51	Non-IBD
31	Terminal ileum	69	Non-IBD
32	Rectum	34	UC
32	Terminal ileum	34	UC
33	Terminal ileum	50	Non-IBD
34	Rectum	34	Non-IBD
34	Terminal ileum	34	Non-IBD

35	Rectum	31	UC
35	Terminal ileum	31	UC
36	Terminal ileum	23	CD
37	Terminal ileum	29	CD
38	Rectum	30	CD
38	Terminal ileum	30	CD
39	Terminal ileum	33	CD
40	Rectum	56	UC
40	Terminal ileum	56	UC
41	Terminal ileum	37	CD
42	Rectum	32	Non-IBD
42	Terminal ileum	32	Non-IBD
43	Terminal ileum	25	CD
44	Terminal ileum	60	Non-IBD
45	Terminal ileum	50	Non-IBD
46	Terminal ileum	29	CD
47	Terminal ileum	23	CD

Bold indicates lines used to generate the data presented in this study.

*All cell lines from IBD patients were established from regions of non-active inflammatory disease.

CD, Crohn's disease; IBD, inflammatory bowel disease; UC, ulcerative colitis.

n.a., not available.

Supplementary Table 2 Oligonucleotide sequences used for quantitative reverse transcriptase PCR.

<i>Gene</i>	<i>Forward Primer (5' to 3')</i>	<i>Reverse Primer (5' to 3')</i>
<i>ATOH1</i>	GCAAAAGAATTTGTCTCCTTCTCT	ATCCGAGTCACTGTAATGGGAAT
<i>AXIN2</i>	CATGACGGACAGCAGTGTAGA	AACTCCAGCTTCAGCTTTTCC
<i>CA1</i>	ACAACGATAACCGATCAGTGC	CTTTGCAGAATTCCAGTGAGC
<i>CHGA</i>	AGAATTTACTGAAGGAGCTCCAAG	TCCTCTCTTTTCTCCATAACATCC
<i>DEFA5</i>	AGGAAATGGACTCTCTGCTCTTAG	TTGCACTGCTTTGGTTTCTATCTA
<i>GAPDH</i>	GACCTGCCGTCTAGAAAAACC	GCTGTAGCCAAATTCGTTGTC
<i>KI67</i>	AAGAGAGTGTCTATCAGCCGAAGT	GTGGCCTGTACTAAATTGACTGTG
<i>LGR5</i>	CCTTCATAAGAAAGATGCTGGAAT	GTTTAATGGGGGAAATGTACAGAG
<i>LYZ</i>	GGTTACAACACACGAGCTACAAAC	AGTTACACTCCACAACCTTGAACA
<i>MUC2</i>	AGGATCTGAAGAAGTGTGTCACTG	TAATGGAACAGATGTTGAAGTGCT
<i>MYC</i>	GGCACTTTGCACTGGAACCTTA	GCAGTAGAAATACGGCTGCAC
<i>NEUROG3</i>	CCCATTCTCTCTTCTTTTCTCCTT	GTTGAGGCGTCATCCTTTCTAC
<i>OLFM4</i>	TTCTCCTAGCCCTTCTGTTCTTCC	TTCCAAGCGTCCACTCTGTCC
<i>SI</i>	CTGCATTTGAAAGAGGACAGC	ACTCTGCTGTGGAAGTCCTGA
<i>SLC10A2</i>	GGGTTACTCCCTGGGGTTTC	CCATGACATTTCTTGTATGCCACA
<i>TFF3</i>	AGCTCTGCTGAGGAGTACGTG	ACAGAAAAGCTGAGATGAACAGTG
<i>TRPV6</i>	GACAGGAGACGGGACCTCTA	TTGGCAGCTAGAAGGAGAGGA
<i>VIL1</i>	ACTACTGGATTGGCCAGGACT	TGGACGTCATAGGAGTTGGTC
<i>VIM</i>	CAGGCTCAGATTCAGGAACAG	CAGAGAGGTCAGCAAACCTTGG
<i>GAPDH</i> (DNA assay)	TGACAACGAATTTGGCTACAGC	TGATGGTACATGACAAGGTGC
<i>malB</i> (DNA assay)	AACAGTGTTTCCAGACTACCG	TTCCCAGTCATTCTGTTGTGC