Supplementary Information for:

Isolation and in vitro expansion of human colonic stem cells

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Contents:

Supplementary Data
Supplementary results

Supplementary Methods

Supplementary References

Supplementary Figures 1-11

Supplementary Tables 1-3
Supplementary Table legends
Supplementary Data

Supplementary results

ALDEFLUOR labeling and AC133 surface expression in human colonic epithelium

We examined whether EPHB2-high colon cells displayed attributes common to other adult stem cells types. Prominin-1/AC133 marks neural and hematopoietic stem and progenitor cells\(^1\) and it has been instrumental to purify tumor-initiating cells from CRCs\(^3\)\(^-\)\(^5\). AC133 surface expression in human colon crypts identified several cell populations along the EPHB2 gradient (Supplementary Fig. 4). Only a small fraction of AC133-high cells displayed high EPHB2 surface expression whereas a larger proportion of this cell population was EPHB2-low or -negative. Accordingly, we demonstrated higher levels of differentiation markers (CA1 and ANPEP) in crypt AC133-high compared to AC133-negative cells (Supplementary Fig. 4). This observation is in agreement with immunohistochemistry analysis of AC133 on human colon samples\(^6\) as well as with the patterns observed in Prominin-1 reporter mice\(^7\) both of which revealed that AC133 expression was restricted to differentiated cells of colon crypts. High aldehyde dehydrogenase (ALDH) activity has also been attributed to stem and progenitor cells of several tissues\(^8\). Indeed, the fluorescent ALDH substrate ALDEFLUOR (BODIPY-amino-acetaldehyde) stained a cell population which displayed higher LGR5 and ASCL2 expression levels and contained a large fraction of crypt EPHB2-high cells (Supplementary Fig. 5). Yet, a substantial proportion of EPHB2-negative cells were also ALDEFLUOR-high and thus we could not observe differential expression of differentiation markers amongst ALDEFLUOR-sorted populations (Supplementary Fig. 5). This result implies that high ALDH activity is not completely restricted to stem cells in human colonic crypts.

Comparative Genomic Hybridization (CGH) of human CoSC samples

To strengthen the notion that EPHB2-high cells purified from normal mucosa adjacent to CRC were not of tumoral origin, we performed genome wide DNA copy number analysis (Supplementary Fig. 9 and 10). Comparative Genomic Hybridization (CGH) demonstrated no relevant variation in DNA copy numbers between freshly isolated colonic epithelial cells and peripheral blood cells of the same individual (Supplementary
Fig. 10a,b). Importantly, by CGH analysis, we also show that the genomes of CoSCs cultures remain stable over several weeks and passages (Supplementary Fig. 10b,c).
Supplementary Methods

Colon Crypt/Single cell-isolation

Biological samples were obtained from individuals treated at the Hospital del Mar (Barcelona, Spain) or from Hospital Clinic (Barcelona, Spain) under informed consent. Experiments were approved by the ethics committee of iRB/Hospital Clinic (project ERC-208488/CRCprogramme).

Muscle layer and sub-mucosa were carefully removed from human fresh colectomy specimens and colonic mucosa was incubated with a mixture of antibiotics (Normocin (Invivogen, San Diego, California 92121, USA), Gentamycin (Invitrogen, Carlsbad, CA, USA) and Fungizone (Invitrogen)) for 15 min at room temperature (RT). Next, tissue was cut into small pieces and incubated with 10 mM Dithiotreitol (DTT) (Sigma, St. Louis, MO 63103, USA) in PBS for 2-3 times 5 min at room temperature. Samples were then transferred to 8 mM EDTA in PBS and slowly rotated for 60-75 min at 4ºC. Supernatant was replaced by fresh PBS, and vigorous shaking of the sample yielded supernatants enriched in colonic crypts. Fetal bovine serum (FBS, Sigma) was added to a final concentration of 5% and fractions were centrifuged at $40 \times g$ for 2 min in order to remove single cells. This washing procedure was repeated three times with Advanced DMEM/F12 (ADF, Invitrogen) medium supplemented with 2 mM GlutaMax (Invitrogen), 10 mM HEPES (Sigma), and 5% FBS (Sigma) (Washing buffer: WB). Purified crypts were either directly cultured (see below) or single cell suspensions were obtained as follows. Colonic crypts were re-suspended in Disaggregation Medium (ADF, Glutamax, 10 mM HEPES, N-2 media supplement (Invitrogen), B-27 media supplement without retinoic acid (Invitrogen), 10 mM Nicotinamide (Sigma), 1 mM N-Acetyl-L-cysteine (Sigma), 10 μM Y-27632 (Calbiochem, Gibbstown, NJ, USA), 2.5 μM PGE2 (Sigma), 0.1-0.5 mg ml$^{-1}$ Dispase (354235, BD Biosciences, Two Oak Park, Bedford, MA, USA), 0.4 U μL$^{-1}$ DNAse I (D5025, Sigma)) and incubated for 15-20 min at RT with occasional re-suspension using a P1000 pipette. After this, crypt suspension was gently syringed using a 1.2 mm needle (18G, BD Microlance™ 3) in order to obtain a single cell-enriched population. After disaggregation, cells were sequentially passed through 100, 70 and 40 μm mesh filters (BD Biosciences) and washed with WB.
Immunostaining of human colonic crypt cells for FACS analyses and sorting

Human colonic crypt cells were re-suspended in staining buffer (ADF, Glutamax, 10 mM HEPES, N-2, B-27 without retinoic acid, 10 mM Nicotinamide, 10 μM Y-27632, 5% FBS). 10^6 cells 250 μl were stained as follows. Mouse antibody to EPHB2 (Mab 2H9, Genentech, San Francisco, CA, USA) or isotype control antibody (IgG1 from murine myeloma, M5284, Sigma) was coupled to Allophycocyanin (APC) using the Allophycocyanin (APC) conjugation kit (PJ25K, PROzyme, Hayward, CA, USA) according to the manufacturer’s protocol. Cells were incubated with APC-coupled mouse antibody to EPHB2 or IgG1 (2 μg ml⁻¹), FITC-coupled mouse antibody to Human EpCAM/TROP1 (0.2 μg ml⁻¹, R&D Systems, Minneapolis, USA), PE-coupled mouse antibodies to CD11b, to CD31, and to CD45 (dilution 1:50 each, Miltenyi Biotec, Bergisch Gladbach, Germany) for 25 min on ice. After 2 washes with WB, propidium iodide (PI) was added (10 μg ml⁻¹, Sigma) to discriminate against dead cells and cellular debris and stained cells were sorted using a FACS Aria 2.0 (BD Biosciences, Two Oak Park, Bedford, MA, USA).

To obtain the four intestinal crypt cell populations, dead cells and debris were discarded by removing the PI⁺ subpopulation. Non-epithelial cells were excluded by removing CD11⁺ (Macrophages), CD31⁺ (Endothelial cells), and CD45⁺ (Lymphocytes) sub-populations. Epithelial cells were included only by selecting for EpCAM⁺ staining and further selected according to their differential EPHB2 surface abundance. The brightest 3.5 % EPHB2⁺ cells were sorted as the EPHB2-high fraction. The EPHB2-medium population comprised the 10-15 % EPHB2⁺ cells adjacent to the EPHB2-high. The EPHB2-low fraction was considered as the 10-15 % adjacent to the EPHB2-medium population. The EPHB2-negative subpopulation did not stain for EPHB2 as defined via the IgG₁ control staining. Finally, these four fractions were further gated according to their forward scatter (FSC-A) to avoid purification of cell aggregates.

For CD133(AC133)/Prominin-profiling, cells were stained with PE-coupled antibody to CD133/1 (AC133)(1:50, Miltenyi Biotec), APC-coupled antibody to EPHB2 (see above), FITC-coupled antibody to Human EpCAM/TROP1 (1:50, R&D Systems), and PE-Cy7-coupled antibodies to CD11b, CD45 (1:50 each, BD Biosciences), and PE-Cy7-coupled antibody to CD31 (1:50, Biolegend, San Diego, CA, USA). Similar to the gating for EPHB2 sub-populations, the brightest 3.5% AC133⁺ cells were defined and sorted as AC133-high cell fraction. Two additional AC133⁺ fractions (AC133-medium and AC133-low) were defined dependent on the staining profile. AC133-negative cells did not stain

Nature Medicine doi:10.1038/nm.2470
for AC133 compared to the negative control. Dead cells were excluded via 4',6-diamidino-2-phenylindole (DAPI) staining. Stained cells were analyzed and sorted using a FACS Aria 2.0 (BD Biosciences).

Colonic epithelial crypt cells were subjected to Aldefluor-staining (ALDEFLUOR®Kit, STEMCELL Technologies SARL, 38000 Grenoble, France) according to the manufacturer’s protocol. Directly after the Aldefluor reaction, cells were stained in Aldefluor-Reaction buffer with PerCP-eFluor®710-coupled antibody to EpCAM (eBioscience, San Diego, CA 92121, USA) or APC-coupled antibody to EPHB2 (see above) on ice for 30 min. After one washing step with Aldefluor buffer, DAPI was added to discriminate against dead cells. Stained cells were analyzed and sorted using a FACS Aria 2.0 (BD Biosciences).

For re-sorting of single cells derived from in vitro spheroids grown from single EPHB2-high cells, the enzymatically (Dispase+DNase) disaggregated cell population was re-suspended in single cell growth medium (see below) and stained with propidium iodide (PI) (10 µg ml⁻¹, Sigma) in order to discriminate against dead cells and cellular debris. To exclude sorting of cell aggregates the fraction was furthermore gated using the forward scatter (FSC-H versus FSC-W, not shown). PI-negative single cells were sorted using a FACS Aria 2.0.

**Quantitative telomere FISH analysis (qFISH)**

For quantitative telomere FISH analyses, cells sorted in single cell growth medium were directly plated on chamber slides (BD Biosciences) pre-coated with 2.5 µg ml⁻¹ Laminin (L2020, Sigma) and 5 µg ml⁻¹ Matrigel (BD Biosciences). After incubation for 20 hours at 37°C, 5% CO₂, medium was carefully removed, attached cells were washed twice using PBS, and fixation was done with Methanol/Acetic acid (3:1 Vol/Vol) three times for 30 min at RT. HT-quantitative FISH analyses (qFISH) was carried out essentially as described previously ⁹.

**Generation of human colonic crypt cell gene expression signatures**

Microarray data were normalized via quantile normalization. Expression values were summarized for the human samples via RMA¹⁰. PCA analysis showed systematic differences between individuals. Therefore, expression values for each gene were adjusted by subtracting the mean differences between individuals, as estimated by limma¹¹.
The human CoSC gene expression signature was selected by requiring the fold change EphB2-High vs. EphB2-Medium ≥ 2 and EphB2-Medium vs. EphB2-Low ≥ 1.5 consistently in the three mucosa samples (FDR<0.05). Genes with FC H vs. M ≥ 2 and M vs. L ≥ 1.5 in any of the three individuals were selected, and the expression levels were represented in a heatmap.

**Comparative Genomic Hybridization (CGH) experiments and statistical data analysis**

We collected the following samples from the same individual: blood (white blood cell-fraction), complete crypts derived from human mucosa after colectomy (for sample information see **Supplementary Table 1**), and *in vitro* organoids grown from EPHB2-high sorted cells two or six weeks after plating and serial passaging. Genomic DNA was isolated from these samples using the GenElute™ Mammalian Genomic DNA MiniPrep Kit (Sigma). For CGH, commercially available arrays (two color HG18 WG CGH Nimblegen Arrays, Roche) were used according to the manufacturer's protocol. First, we computed average log2 red to green ratios in windows of 70,000 base pairs. In a reference experiment, blood samples from a male and female individual were compared (**Supplementary Fig. 9**). The mean ratio in chrX in this experiment was 0.63. Based on this, the cut-off for significant copy number changes above normal variation was defined as 0.63 in all samples (**Supplementary Fig. 9,10**).

**In vitro culture of complete human colonic crypts and single cell-derived colonic spheres**

200-300 isolated human colonic crypt units were mixed with 50 µl matrigel and plated on pre-warmed 24-well culture dishes. After solidification (15-20 min at 37°C), crypts were overlaid with 600 µl complete crypt culture medium (Wnt3a-conditioned medium and ADF 50:50, Glutamax, 10 mM HEPES, N-2 (1×), B-27 without retinoic acid (1×), 10 mM Nicotinamide, 1 mM N-Acetyl-L-cysteine, 1 µg ml⁻¹ RSPO1 (in-house produced, see below), 50 ng ml⁻¹ human EGF (Invitrogen), 100 ng ml⁻¹ human Noggin (Peprotech, Rocky Hill, NJ, USA), 1 µg ml⁻¹ Gastrin (Sigma), 500 nM LY2157299 (Axon MedChem, Groningen, The Netherlands), 10 µM SB202190 (Sigma), and 0.01 µM PGE2 (Sigma)). This medium is referred to as “Stem” medium or “stem cell” medium. Medium was
replaced with fresh growth medium every other day. For serial passage, Matrigel-embedded organoids were released using Cell Recovery Solution (BD Biosciences). After re-suspension in HEPES-buffered ADF medium containing Glutamax and 5% FBS, single cells and debris were removed by centrifugation at 40×g. Then, organoids were incubated in Disaggregation Medium (see above) for 10-20 min at 37°C in a waterbath. Afterwards, the cell suspension was gently syringed using a 1.2 mm needle (BD Microlance™ 3). After re-plating in fresh matrigel, the culture was overlaid with single cell culture medium (see below) and medium was changed every other day. Rock inhibitor Y-27623 (10 µM) was added to the cultures the first 2-3 days after each passaging step.

In case of single cell plating, 2000-3000 sorted cells were mixed with 50 µl matrigel and plated on pre-warmed 24-well culture dishes. After solidification of the matrigel (10-15 min at 37°C), cells were overlaid with “single cell growth medium” (= complete crypt culture medium + 10 M Rock inhibitor Y-27623). Medium was replaced with fresh single cell growth medium (“Stem” medium) every other day. Rock inhibitor was included in the culture medium for seven to nine days. Note that the efficiency of organoid growth for each cell fraction refers to the number of seeded cells as automatically determined by flow cytometry (sorting events).

For differentiation of colonic spheres (5-7 days), PGE2, Nicotinamide, and SB202190 were omitted from the culture medium, Wnt3a-conditioned medium was reduced to 5%, and 10 µM gamma-secretase inhibitor DAPT (Sigma) was added. Wnt3a was completely removed from the differentiation medium 48 h prior to analyses.

**Wnt3a-conditioned medium and purification of recombinant human RSPO1**

To obtain Wnt3a-conditioned medium, L-Wnt3a cells (CRL-2647, ATCC, Middlesex TW11 0LY, UK) were seeded to 10% density and cultured in ADF medium supplemented with Glutamax and 10% FBS for 4 days. Supernatant I was replaced with fresh medium and sterile filtered. After 3 more days, Supernatant II was sterile filtered and combined with Supernatant I. Wnt3a-conditioned medium was aliquoted and stored at -20°C.

cDNA encoding for amino acids 31-293 of human RSPO1 was cloned into pOPINE-G expression vector which encodes for a N-terminal cleavable secretion leader (MGILPSPGMMPALLSLVSSSLLVLL MGCA\_ETG) and a C-terminal His-tag (KHHHHHHH)\textsuperscript{12}. After sequence verification, pOPINE-G-RSPO1-His6 was transfected into 293T cells. Human RSPO1-His6 was isolated from the cell supernatant via Immobilized Metal-
chelate Affinity Chromatography (IMAC) followed by Size-Exclusion-Chromatography (SEC). After concentration to 250-1,000 µg ml\(^{-1}\) in PBS/0.1% BSA, the recombinant human RSPO1 was aliquoted and stored at -80°C until usage.

The biological activity of Wnt3a-conditioned medium and human RSPO1 were determined by TOP/FOP assay similar to what has been published previously.\(^\text{13}\) Biological activity of in-house produced RSPO1 was validated by comparison with commercially available human RSPO1 (data not shown).

**Immuno-histochemistry, Immuno-cytochemistry and Electron-microscopy**

The immuno-staining protocol has been described previously.\(^\text{14}\) The primary antibodies used were antibody to EPHB2 (1:200, R&D Systems, AF467), antibody to MKI67 (1:500, Novocastra Meranini, Clone MM1), antibody to KRT20 (1:500, Dako, Ref.: M7019), antibody to EpCAM-Trop1 (1:50, R&D, Ref.: AF960), and antibody to Chromogranin A (rabbit, 1:2000, Abcam, Cambridge, UK).

For immuno-cytochemistry, matrigel-embedded human organoids cultured in 15 µ-Slide 8 well plates (80826, ibidi, Martinsried, Germany) were washed three times for 5 min with PBS, and HEPES-buffered 2% PFA was added for 10 min at room temperature. Cells were fixed for another 30 min with HEPES-buffered 4% PFA at RT. Next, PFA was quenched by addition of 20 mM Glycin in PBS for 10 min. After washing with PBS, cells were permeabilized using 0.2% TritonX in PBS for 30 min and pre-blocking was done with 1% BSA/0.1% TritonX in PBS for 45 min at RT. Primary antibodies in PBS/1%BSA were added overnight at 4°C. The primary antibodies used were mouse antibody to KRT20 (1:500, Dako, Ref.: M7019), mouse antibody to MKI67 (1:300, Novocastra Meranini, Clone MM1), rabbit antibody to FABP1 (1:200, Sigma, Ref.: HPA028275), rabbit antibody to Chromogranin A (1:200, Abcam, Cambridge, UK), and mouse antibody to MUC2 (kindly provided by Carmen de Bolos, Institut Municipal d'Investigació Mèdica (IMIM), Barcelona, Spain). Fluorescent Dye coupled secondary antibodies used were Donkey FITC-coupled antibody to mouse, Donkey FITC-coupled antibody to rabbit, and Donkey DyLight 649-coupled antibody to rabbit (Jackson Immunoresearch Europe, Newmarket, Suffolk, UK). After incubation with secondary antibodies for 1.5 h at RT, nuclei were stained via DNA-labelling using 4’-6-Diamidino-2-phenylindole (DAPI). After washing in PBS two times for 5 min, organoids were overlaid with VECTASHIELD Mounting Medium.
For electron microscopy, matrigel-embedded organoids were fixed in 2.5% glutaraldehyde in cacodylate buffer (0.1 M, pH 7.2) for 90 min at 4°C. The samples were embedded in Epon resin and examined with a transmission electron microscope (Tecnai Spirit Twin, FEI, Hillsboro, OR 97124, USA).

**Cell viability assay**

For the initial assessment of the number of viable cells in single cell-derived organoid cultures (For sample information see Supplementary Table 1), 500 sorted cells were embedded in 15 µl Matrigel drops in each case and seeded into 48 well tissue culture plates. For each time point analyzed, cell viability of two drops was quantified indirectly by the amount of ATP using the CellTiter-Glo® Luminescent Cell Viability Assay (G7570, Promega, Madison, WI, USA).

After 2, 6, 8, and 14 weeks of sequential organoid passaging, respectively, organoids were retrieved from matrigel using BD™ Cell Recovery Solution (BD Biosciences), washed twice with HEPES-buffered ADF medium containing Glutamax, and organoids were re-suspended in Disaggregation Medium (see above). After re-seeding of disaggregated organoids in single-cell culture medium, cell proliferation was assessed as described above. Luminescence signals were recorded using the MicroLumat Plus LB96V Luminometer (Berthold Technologies, Bad Wildbad, Germany).

**RNA isolation and RT-qPCR analysis**

RNA from sorted cells was extracted using Trizol Reagent (Invitrogen) and purified using the RNeasy® MiniKit (Qiagen). Complementary DNA (cDNA) was generated using the High-Capacity cDNA reverse transcription kit (Applied Biosystems). RT-qPCR analysis was performed using TaqMan assays (Applied Biosystems) following manufacturer’s instructions.

For isolation of small amounts of RNA coming from single organoids (250-1,000 cells per organoid) and reverse transcription, a sophisticated protocol for reliable gene expression analysis of small cell populations based on whole transcript amplification (WTA) has been applied as described recently.

Nature Medicine doi:10.1038/nm.2470
Supplementary References


Supplementary Table legends

**Supplementary Table 1. Overview of tissue samples used during this study.** For each sample, the colonic segment analyzed and the diagnosis of the individual after surgery is indicated. Furthermore, figures demonstrating the results collected from each sample and type(s) of analyses performed with the respective mucosa and/or the derived *in vitro* organoids are listed.

**Supplementary Table 2. List of genes whose expression is enriched in EPHB2-high cells.** Genes up-regulated in EPHB2-High vs. -Medium ≥ 2 fold and Medium vs. Low ≥ 1.5 fold consistently in the three independent colon mucosa samples. Average fold change in the three individuals is shown.

**Supplementary Table 3. Genome-wide expression analysis of EPHB2-high, -medium, -low and –negative cells from 3 independent colon mucosa samples.** Note that in case of sample number 3, only EPHB2-high, -medium and -low cell-derived RNA samples were processed and hybridized due to the low sample quality of the EPHB2-negative cell-derived RNA (data not shown). Colonic region of the three analyzed samples is as follows. Sample 1: Ascending colon, Sample 2: Transverse colon, Sample 3: Ascending colon. For more information on samples see **Supplementary Table 1.**
Supplementary Fig. 1. Immunohistochemical analysis of human normal colon mucosa and EpCAM profile of EPHB2-high cell population.

a. Immunohistochemical (IHC) staining for EPHB2 (upper left panel), the pan-differentiation marker KRT20 (upper right panel), the epithelial cell-specific marker EpCAM (lower left panel), and the proliferation marker MKI67 (lower right panel) are depicted.

b. To control for non-specific signal, IHC was performed using either goat anti-EPHB2 antibody (goat IgG without peroxidase-coupled secondary antibody, left panel), mouse anti-MKI67 antibody alone (mouse IgG without peroxidase-coupled secondary antibody, middle panel), or secondary antibody coupled to peroxidase alone (sec. Ab control, right panel). Specific staining is indicated by black arrows. Non-specific signals (indicated by open triangles) arise from endogenous peroxidase activity of macrophages. Serial sections of normal human colonic mucosa are shown.

c. Flow cytometry profiles demonstrating that EPHB2-high cells are exclusively EpCAM-positive (upper right sector, indicated by black arrow). For all EPHB2-sub-populations only EpCAM-positive cells were taken into account. Note that due to the colon processing and crypt isolation procedure, colon single cell samples were mainly constituted by epithelial cells. Remaining non-epithelial cells were additionally excluded via CD11b/CD31/CD45 staining.
Supplementary Fig. 2. Analysis of telomere length of the different epithelial colon cells sorted according to EPHB2 cell surface abundance

Shown are quantitative Fluorescence In Situ Hybridization (FISH) histograms indicating telomere length distribution of sorted EPHB2-high, -medium, -low, and -negative cell populations. Telomere fluorescence in arbitrary units (a.u.), average fluorescence intensity, and standard deviation (SD) are indicated. Nº, number of nuclei evaluated for different cell populations.
Supplementary Fig. 3. Comparison of the expression profiles of the different EPHB2-purified cell populations from patient biopsies.

a. Principal component analysis (PCA) of gene expression from four colon cell populations (EPHB2-high, -medium, -low and -negative) obtained from three different individuals (P1, P2 and P3).

b. Heat-map showing genes with higher expression in EPHB2-high compared to EPHB2-medium cells (FC≥2) and higher or equal expression in EPHB2-medium vs. EPHB2-low (FC≥1.5) either in individual P1, P2 or P3. Note that all genes that passed this filter in any of the three mucosa samples were included (total=348 genes).
Supplementary Fig. 4 Characterization of colonic epithelial cells purified from human mucosa samples according to their Prominin-1/AC133 surface abundance

(a-c) Three independent colonic mucosa samples were analyzed (for sample information see Supplementary Table 1). Epithelial cells (EpCAM+) showing different levels of AC133 staining (high, medium, low and negative) were gated (left panels) and sorted according to their size in order to avoid cell aggregates (not shown). Green vertical lines represent gated AC133-high population whereas blue horizontal lines indicate the gate of the EPHB2-high population (middle panels). Real-time quantitative PCR (RT-qPCR) analysis of sorted cell fractions confirmed that AC133-positive cells were enriched in differentiation marker genes CA1, ANPEP (a-c, right panels). Measurements were performed three times. Error bars indicate standard deviation.
Supplementary Fig. 5 Characterization of epithelial colon cells purified from human mucosa samples according to Aldehyde Dehydrogenase (ALDH) activity (ALDEFLUOR assay)

Single cell suspensions from human colonic crypts were subjected to ALDEFLUOR-staining (BODIPY-amino-acetaldehyde oxidization to BODIPY-amino-acetate) and analyzed by flow cytometry and RT-qPCR. (a, b) Two independent mucosal samples were analyzed (for sample information see Supplementary Table 1). Epithelial cells with different levels of Aldehyde-Dehydrogenase (ALDH)-activity (ALDEFLUOR-high, -medium, -low, and -negative) were gated (upper left panels) and sorted according to their size in order to avoid cell aggregates (not shown). ALDEFLUOR-negative cell populations were defined via the Diethylamino-benzaldehyde (DEAB)-control reaction (lower left panels) in which generation of fluorescent product by ALDH activity is blocked. RT-qPCR analysis of sorted cell fractions showed that ALDEFLUOR-high cell populations were enriched in stem cell-specific genes LGR5 and ASCL2 when compared to cell fractions with lower ALDH-activity (a, b, right panels). However, cells expressing markers of terminal differentiation (CA1, KRT20, ANPEP) were present in all four sorted Aldefluor-fractions to a similar extent (a,b, right panel). Measurements were performed three times. Error bars indicate standard deviation. c. The ALDEFLUOR-high cell pool contains all cell populations ranging from high to negative EPHB2 surface abundance.
Supplementary Fig. 6. Optimization of the *in vitro* colonic spheroid culture system.

a. Complete human colonic crypts were embedded in matrigel and cultured under different conditions. Colonic spheroid growth required supplementation of Wnt3a–conditioned medium with Nicotinamide and Prostaglandin E2 (PGE2). Spheroid growth was documented 7 days after seeding of complete single crypts by phase contrast microscopy. Original magnification: 4× objective.

b. Human colonic crypt units were isolated from fresh colectomy specimens (day 0), embedded in matrigel and cultured in complete crypt growth medium containing Wnt3a, Nicotinamide and Prostaglandin E2 (see Supplementary Methods). Organoid structure was documented at the indicated time points via microscopy. Black arrows indicate splitting/passaging of the culture and embedding of mechanically dissociated spheres into fresh matrigel. Day 0-3: 10X objective, day 5-30: 4X objective. Filled arrowheads indicate crypt bases, open arrowheads indicate crypt tops.

c. EPHB2-high human colonic single crypt cells were embedded in matrigel and cultured in single cell growth medium (see Supplementary Methods). Prostaglandine E2 (PGE2) and / or Nicotinamide were excluded from the culture media as indicated. Spheroid growth was documented after 7 days. Original magnification: 10× objective.

Nature Medicine doi:10.1038/nm.2470
Supplementary Fig. 7. Proliferation characteristics of *in vitro* organoids grown from single EPHB2-high cells

(a-c) Sorted EPHB2-high cells (for sample information see Supplementary Table 1) were embedded into matrigel and the relative amount of viable cells was measured indirectly via the amount of ATP at the time points indicated. After 5 weeks (b.) and 8 weeks (c.), disaggregated spheroids were embedded in matrigel and multiple droplets were seeded in a 48-well tissue culture plate. The relative amount of viable cells was analyzed at the indicated time points. Note that within each time window analyzed, spheroids grew exponentially with similar kinetics.

d. Summary of data obtained from the cell viability assays (a.). From the average doubling time, the number of cell doublings was calculated to approximately 45 after 9 weeks of cell propagation *in vitro*.

From the basic formula of the exponential curve $y(t) = y_0 \times e^{growth-rate \times t}$ ($y=$amount of cells, $y_0=$ amount of cells at $t = 0$ hrs, and $t=$time) we derived the growth rate (number of doubling events which occur per time unit). The cumulative number of cell doublings after different passages was calculated from the total culturing time up to this time point divided by the culture doubling time $= \ln(2) / growth\ rate$.

Note that genome-wide chromosomal stability of this sample was analyzed by Comparative Genomic Hybridization (CGH) as depicted in Supplementary Fig. 9 and 10.
Supplementary Fig. 8. Long-term in vitro culture characteristics of normal human mucosa.

a. Macroscopic images of serially passaged in vitro organoid cultures derived from single sorted EPHB2-high cells after week 14 (Passage 14) and week 15 (Passage 15). A representative 50 µl matrigel droplet is depicted for each time point.
b. Sorted EPHB2-high cells (for sample information see Supplementary Table 1) were embedded into matrigel and grown into spheroids. After 14 weeks sequentially passaged spheroids were disaggregated and embedded into fresh matrigel droplets. After this the relative amount of viable cells was analyzed at the indicated time points. Note that the long term spheroid culture grew exponentially with a doubling time of approximately 43 hours (hrs).
c. Human colonic spheroid cultures after 3 months of sequential passaging were maintained in differentiation medium for 7 days. Differentiating organoids down-modulated stem cell and proliferation genes and up-regulated differentiation markers as was analyzed by RT-qPCR. Measurements were repeated three times. Error bars indicate standard deviation.
d. Electron microscopy images of two representative organoids differentiated after a 3 months culture period and sequential passaging of organoids derived from EPHB2-high cells. Note that intermingled absorptive and secretory lineages were observed (E: enterocytes and G: goblet cells). Cells displayed the typical polarization with microvilli of enterocytes facing the spheroid lumen (L) and mucus vacuoles of goblet cells residing only in the apical cell compartment. Nuclei of differentiated cells were located at the basal part of cells, facing the collagen extracellular matrix provided by matrigel (M).
Supplementary Fig. 9. Comparative Genomic Hybridization (CGH) analyses: Reference experiment defining the ratio for copy number changes

A reference experiment was performed in order to set the cut-off for real copy number changes in our platform and hybridization conditions. Genomic DNA (gDNA, white blood cell-fraction) from male and female patients was isolated the same day in identical conditions.

a. Box plot showing ratio distributions of all probe sets for each chromosome (log2 changes). Chromosome blot of chromosome X. Shown are the ratios (log2) of probe sets covering on average a 70,000 bp window.

b. From this experiment, the cut-off to determine copy number variations in all samples was defined by the mean ratio of 0.63 in chromosome X when comparing male and female blood samples.
Supplementary Fig. 10. Comparative Genomic Hybridization (CGH) analyses of mucosal and in vitro cultured cells

Array-based CGH profiles of colonic complete crypt fraction-derived gDNA versus blood gDNA (a.), gDNA isolated from EPHB2-high cell-derived organoids after 2 weeks versus blood gDNA (b.), or gDNA isolated from serially passaged EPHB2-high cell-derived organoids after 6 weeks versus blood gDNA (c.). Note, that array-based CGH profiles of mucosal and EPHB2-high organoid samples did not display any consistent copy-number alterations (a.-c.). The only exception was one probe set located within chromosome 12p11.1 (centered at 34.405 MB) which showed a potential amplification (a. and b. only. Arrow). This potential copy number variation was not detectable when organoids which had been sequentially passaged for 6 weeks were compared against blood of the same patient (c.) indicating that it was not propagated during organoid growth. In addition, probe sets covering the 70,000 bp window regions adjacent to the detected abnormality did not indicate any significant copy number alterations (a.-c., right panel). Importantly, qPCR analysis of this region using 5 different primer sets could not confirm any significant amplification in these samples which suggest that the amplified region was a technical artifact during CGH procedure (data not shown).
Supplementary Fig. 11. A small proportion of cells from *in vitro* organoids differentiates into enteroendocrine cell lineage.

a. Human colonic spheroid cultures maintained for 7 days in differentiation medium down-modulated stem cell and proliferation genes and up-regulated differentiation markers including the enteroendocrine marker chromogranin-A (CHGA). mRNA levels were analyzed by RT-qPCR. Measurements were repeated three times. Error bars indicate standard deviation.

b. Histological sections of colon organoids differentiated *in vitro* were subjected to chromogranin A (CHGA) staining. Note the presence of sparse CHGA+ cell displaying the fusiform shape characteristic of enteroendocrine gut cells.

c. Examples of CHGA+ cells (green) from colonic spheroid cultures grown in differentiation medium. No CHGA+ cells were present in spheroids grown in stem cell medium. Original magnification: 63X objective. Counterstaining, DAPI (magenta).
<table>
<thead>
<tr>
<th>Sample No.</th>
<th>Colon segment</th>
<th>Cause of colectomy</th>
<th>Figure / Table</th>
<th>Experiment(s) performed</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Ascending colon</td>
<td>Low grade adenocarcinoma, Well differentiated</td>
<td>Supplementary Table 2, 3 Sample 1</td>
<td>Profiling of EPHB2 population via microarrays. Analyses by RT-qPCR (not shown)</td>
</tr>
<tr>
<td>2</td>
<td>Ascending colon</td>
<td>Low grade adenocarcinoma</td>
<td>Supplementary Table 2, 3 Sample 3</td>
<td>Profiling of EPHB2 population via microarrays. Analyses by RT-qPCR (not shown)</td>
</tr>
<tr>
<td>3</td>
<td>Transverse colon</td>
<td>Moderately differentiated adenocarcinoma</td>
<td>Supplementary Table 2, 3 Sample 2</td>
<td>Profiling of EPHB2 population via microarrays. Analyses by RT-qPCR (not shown)</td>
</tr>
<tr>
<td>4</td>
<td>Sigmoid colon</td>
<td>Low grade adenocarcinoma</td>
<td>Fig. 2b, sample P1</td>
<td>Organoid culture</td>
</tr>
<tr>
<td>5</td>
<td>Sigmoid colon</td>
<td>Low grade adenocarcinoma</td>
<td>Fig. 2b, sample P2 Fig. 1c, 2e</td>
<td>Profiling by RT-qPCR, organoid culture, differentiation assays (ICC)</td>
</tr>
<tr>
<td>6</td>
<td>Ascending colon</td>
<td>Tubulo-villous adenoma with low grade dysplasia</td>
<td>Fig. 2b, sample P3</td>
<td>Organoid culture</td>
</tr>
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<td>7</td>
<td>Sigmoid colon</td>
<td>Diverticulosis without diverticulitis</td>
<td>Fig. 2b, sample P4</td>
<td>Organoid culture</td>
</tr>
<tr>
<td>8</td>
<td>Sigmoid colon</td>
<td>Low grade adenocarcinoma</td>
<td>Fig. 2e, Supplementary Figure 8 and 11</td>
<td>Organoid growth, proliferation curves, differentiation assays (ICC, Electron microscopy)</td>
</tr>
<tr>
<td>9</td>
<td>Descending colon</td>
<td>Low grade adenocarcinoma</td>
<td>Fig. 2e-g</td>
<td>Organoid culture, differentiation assays (IHC, Electron microscopy)</td>
</tr>
<tr>
<td>10</td>
<td>Sigmoid colon</td>
<td>Diverticulosis with diverticulitis</td>
<td>Fig. 1d,e Supplementary Fig. 2</td>
<td>Quantitative Fluorescence in situ hybridization (telomere length analyses)</td>
</tr>
<tr>
<td>11</td>
<td>Sigmoid colon</td>
<td>Moderately differentiated adenocarcinoma in sigmoid colon</td>
<td>Fig. 2h-j Supplementary Fig. 7, 9, and 10</td>
<td>CGH analyses, proliferation curves, single cell re-sorting and 2nd generation organoid culture, differentiation assays.</td>
</tr>
<tr>
<td>12</td>
<td>Sigmoid colon</td>
<td>Low grade adenocarcinoma</td>
<td>Supplementary Fig. 4a</td>
<td>Profiling of CD133/Prominin-1 by RT-qPCR and EPHB2 status of CD133+ cells</td>
</tr>
<tr>
<td>13</td>
<td>Rectal colon</td>
<td>Tubulo-villous adenoma with high grade dysplasia</td>
<td>Supplementary Fig. 4c</td>
<td>Profiling of CD133/Prominin-1 by RT-qPCR and EPHB2 status of CD133+ cells</td>
</tr>
<tr>
<td>14</td>
<td>Sigmoid colon</td>
<td>Low grade adenocarcinoma</td>
<td>Supplementary Fig. 4b</td>
<td>Profiling of CD133/Prominin-1 by RT-qPCR and EPHB2 status of CD133+ cells</td>
</tr>
<tr>
<td>15</td>
<td>Ascending Colon</td>
<td>Moderately differentiated adenocarcinoma in cecum. Metastasis found in 2 out of 14 lymph nodes analyzed.</td>
<td>Supplementary Fig. 5a</td>
<td>Aldefluor assay (aldehyde dehydrogenase activity) and profiling of FACS-sorted sub-populations via RT-qPCR. EPHB2 status of ALDH+ cell populations.</td>
</tr>
<tr>
<td>16</td>
<td>Descending colon</td>
<td>Low grade adenocarcinoma</td>
<td>Supplementary Fig. 5b</td>
<td>Aldefluor assay (aldehyde dehydrogenase activity) and profiling of FACS-sorted sub-populations via RT-qPCR.</td>
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