Supplementary Materials and Methods

Mouse Intestinal Crypt Isolation and Cell Dissociation

After washing 3 times with cold Hank's balanced salt solution (HBSS) without Ca^{2+}/Mg^{2+}, the isolated intestinal tissue was cut into ~3- to 5-mm pieces and incubated in 30 mmol/L EDTA in HBSS for 20 minutes in an ice bath. After removal from EDTA medium and subsequent washing with HBSS, the tube was shaken by hand for 3 to 5 minutes (2 to 3 shakes per second). The villi and mucus were removed with a 70-μm or 40-μm (the latter can get purer crypts) cell strainer (BD Biosciences). After being centrifuged at 4°C at 700 rpm for 5 minutes, the collected crypts were dissociated in 2 mL of TrypLE Express (Invitrogen), with Y-27632 (10 μmol/L) and 0.5 mmol/L *N*-acetylcysteine (Sigma), for 8 minutes at 37°C with pipetting (use the shortest incubation time based on the observation of single cell dissociation under microscope). Cell clumps and mucus were removed using a 20-μm cell strainer (BD Biosciences), and the remaining dissociated cells were washed with culture medium collected by centrifugation at 4°C at 1500 rpm for 5 minutes. For the colon, after mucosa reversion, the proximal end was stitched using surgical sutures and the intestinal tract was filled with 30 mmol/L EDTA and then the distal end was stitched too. The swollen colon tract was then incubated in 30 mmol/L EDTA HBSS for 100 minutes at 4°C, followed by shaking and crypt isolation as described above. The isolated crypts were harvested via centrifugation at 4°C at 800 rpm for 5 minutes. Single colonic epthithelial cells were dissociated as described above and centrifuged at 4 °C at 1500 rpm for 5 minutes when it was difficult to form the single cell pellets.

Mouse IEC Flow Cytometry Analysis and Cell Sorting

Single cell pellets were resuspended in staining medium (Advanced DMEM/F12, 1× penicillin-streptomycin, 2 mmol/L Glutamax, 10 mmol/L HEPES, 10 μmol/L Y27632, 0.5 mmol/L *N*-acetylcysteine) at a concentration of 5×10^6/mL (MACSquant Analyzer, Miltenyi Biotec). Cells were stained with antibodies and their corresponding isotype controls following manufacturer recommended concentrations (Supplementary Table 1) and incubated on ice for 30 minutes with gentle shaking. Following staining, cells were washed with washing buffer (Advanced DMEM/F12, 1× penicillin-streptomycin, 2 mmol/L Glutamax, 10 mmol/L HEPES, 0.5 mmol/L *N*-acetylcysteine) and centrifuged at 1500 rpm for 5 minutes. A similar process was followed for a secondary antibody staining. Cells were resuspended in staining medium at a concentration of 5×10^6/mL. 7-aminoactinomycin D and annexin V (Supplementary Table 1) were added to the staining medium 20 minutes prior to sorting. The MoFlo was set up at 12 psi with a 150-μm tip. Cells (2000–7000) were sorted into 1-mL staining buffer in BSA coated 1.5mL microcentrifuge tube (for culture) or 500 μL lysis buffer (Ambion, for RNA extraction) at a rate of approximately 4500 events per second.

Single Cell Seeding and In Vitro Culturing

Matrigel (BD Biosciences) was dissolved at 4 °C 3h before cell sorting. The cold growth factor (stock solution) was mixed carefully and completely into Matrigel with pre-cold tips. (For example: 10μL EGF, 10μL Noggin and 10μL Jagged-1 stock solution were mixed into 600uL Matrigel). Sorted cells were centrifuged at 500g for 5 minutes at 4 °C; about 900μL medium was aspirated but about 5μL of the remaining medium was removed by pipette carefully. For 4000–5000 sorted cells, 100μL of the prepared Matrigel was added and mixed gently and completely (pipetted about 20 times) with pre-cold tips to prevent the generation of bubbles. 8.5uL of Matrigel was added to the center of the well of a pre-warmed (37 °C) optical bottom 96 well plate, which was kept on a 37 °C pre-warmed metal block while the Matrigel was added. The culture plate was immediately returned to the culture incubator for about 17 minutes to polymerize the Matrigel (Polymerization time should be optimized according to the volume of Matrigel and the size of the plate). Thiazovivin (Sheng Ding Lab) and CHIR99021 stock solution was incubated at 37 °C for 10 minutes and then added to pre-warmed DMEM/F12 to a final concentration of 2.5 μmol/L, supplemented with penicillin (100U/mL)/streptomycin (100μg/mL, Sigma), Glutamax, 10 mmol/L HEPES, 1×N2, 1×B27 and 1mmol/L N-acetylcysteine. (Note that an inadequate amount of Thiazovivin and CHIR99021 will affect colony growth; whereas, adding more than 3.5 μmol/L may induce a small quantity of enteroids to spread on day4 to day8). 100uL seeding medium was added to each well. Sterilized PBS buffer was added into vacant wells on all sides of the plate to maintain humidity. Growth factors were added (Figure 4). Medium was changed by removing half of the volume and adding another half volume of fresh medium containing the required growth factors as described in the method.

Measurement of plating cells

For counting the number of seeding cells/well, 3–4 aliquots of Matrigel containing seeding cells, with each aliquot has equivalent number of cells to one well for colony assay, was spread as thin as possible into a tissue culture dish (BD Falcon, 35×10mm). The Matrigel was incubated for 15 minutes in the cell culture incubator. 4% paraformaldehyde was added to cover the Matrigel slices, which were then incubated at room temperature for 30 minutes or overnight in the fridge. The 4% paraformaldehyde was aspirated and washed 2 times with PBS. DAPI (5μg/mL) was diluted by PBS buffer (1:2000) and used to stain the fixed cells for 5 minutes. After the washing procedure, images of the DAPI-stained cells were taken under an inverted microscope using 2.5× lens and Mosaic software. All the DAPI positive cell numbers were counted.

RNA Extraction and Real-Time PCR

RNA was extracted using the RNAqueous Micro Kit (Ambion) according to the manufacturer's protocols. RNA quality was tested using an Agilent (Santa Clara ,CA) 2100 BioAnalyzer. A complementary DNA (cDNA) reverse transcription kit (Applied Biosystems) was used to synthesize cDNA.

A multiplex PCR preamplification of specific cDNA targets and endogenous control was performed using TPAMMK following the manufacturer's instructions (Applied Biosystems). The TaqMan Gene Expression Assays (Supplementary Table 2) were pooled together at 0.2× final concentration, and 14 preamplification cycles were applied. CAS 4200 and Qiagen (Valencia, CA) QIAgility qPCR setup robots were used to aliquot assays and cDNA samples into a 384-well plate. TaqMan gene expression assays were performed on triplicate samples using a 7500 real-time cycler (Applied Biosystems).

Mouse Enteroid and Colonoid Passaging

Enteroids or colonoids embedded in Matrigel were isolated and dissociated by mechanical force and then transferred to fresh Matrigel at a ratio of 1:3. Passage of enteroids or colonoids derived from single cells was performed every 5 to 8 days. Thiazovivin (2.5 μmol/L) or Y-27632 (10 μmol/L) was required on the first 2 days.

Histological Analysis of Mouse Tissue

Intestinal tissue was fixed in 1× zinc formalin (Anatech, Battle Creek, MI). The fixed tissues were embedded in paraffin and processed as previously described.[1] The primary antibodies were chick anti-GFP (1:1000, Abcam, Cambridge, MA), mouse anti–E-cadherin (1:100, BD Biosciences), mouse anti-CD24 (1:200, Biolegend, San Diego, CA), rat anti-CD44 (1:200, Biolegend), mouse anti-CD166 (1:200, R&D Systems), goat anti-CD166 (Novagen, Madison, WI 1:250), sheep anti-lysozyme (The Binding Site, San Diego, CA 1:250), rabbit anti-Lysozyme (1:500, Dako, Glostrup, Denmark), rabbit anti–chromogranin A (1:200, Santa Cruz Biotechnology, Santa Cruz, CA), and rabbit anti-sucrase (1:200, Santa Cruz Biotechnology). The secondary antibodies were Alexa 488, 564, or 647 conjugated antibodies (Invitrogen).

For whole-mount tissue staining, the muscular layer of the intestine was removed and the remaining epithelium was divided into 5- to 10-mm pieces. Staining was performed as previously described.[1] Tissues were washed with saline solution, permeabilized with 0.5% to 0.8% Triton X-100 in phosphate-buffered saline (PBS), and then blocked by 1× Universal blocking buffer (BioGenex, San Ramon, CA). Tissues were then incubated overnight at 4°C with different combinations of the following primary antibodies diluted in blocking solution: chick anti-GFP (1:500, 10 μg/mL, Abcam), rabbit anti-human lysozyme (1:200), mouse anti–E-cadherin (1:200), rabbit anti-Mucin2 (1:300), and rat anti-CD44 (1:200, Biolegend). Tissue pieces were then washed 3 times with PBS + 0.05% Tween 20. The following secondary antibodies were then added as indicated: donkey anti-chick Dylight 488 or 546 (1:500, Jackson Laboratories, West Grove, PA), donkey anti-mouse Dylight 647 (1:300, Jackson Laboratories), donkey anti-rabbit AlexaFluor 546 or 488 (1:300, Invitrogen), and donkey anti-rat Dylight 488 or 647 (1:300, Jackson Laboratories). After incubation overnight at 4°C, tissue pieces were washed 3 times with PBS + 0.05% Tween 20, stained with 4′,6-diamidino-2-phenylindole (DAPI), and imaged under a confocal microscope (LSM 510, Zeiss, Thornwood, NY).

Human Tissue Processing for Crypt Isolation

De-identified human small intestine specimens were obtained from 7 patients (3 months to 18 years old) who had surgeries related to intestinal failure or bariatrics from the Cincinnati Children's Hospital Medical Center. No patients with Crohn's disease or ulcerative colitis were used for this study. These specimens were considered "residual tissues" not relevant to pathological diagnosis.

Intestinal tissues were washed with cold HBSS (Invitrogen), and a mucosectomy was performed with surgical scissors. The mucosa was pinned flat and stretched on a Sylgard-coated glass Petri dish. Tissues were then incubated in 2 mmol/L EDTA chelation buffer for 30 minutes on ice as previously described.[2] After removal of EDTA, the mucosa was washed with cold chelation buffer and the crypts were manually picked up under a stereomicroscope. Isolated crypts were pelleted, washed with cold chelation buffer, and centrifuged at 50*g* for 5 minutes.

Culture of Human Intestinal Crypts

Crypts were embedded in Matrigel (BD Biosciences) on ice and seeded in a 48-well plate. The Matrigel was polymerized for 20 minutes at 37°C and overlaid by 250 μL of culture medium (Advanced DMEM-F12 supplemented with penicillin/streptomycin, 10 mmol/L HEPES, 2 mmol/L Glutamax, N2 and B27 supplements [Invitrogen], 1% bovine serum albumin, and 1 mmol/L *N*-acetylcysteine [Sigma]). The following growth factors and reagents were added to the culture medium: 50% Wnt3a conditioned medium (supplemented with 10 mmol/L HEPES, 2 mmol/L Glutamax, B27, N2, 1 mmol/L *N*-acetylcysteine), 100 ng/mL Noggin, 1000 ng/mL murine R-spondin 1, 50 ng/mL human recombinant epidermal growth factor (BD Biosciences), 10 nmol/L gastrin, 10 mmol/L nicotinamide, 10 μmol/L SB202190 (Sigma), and 500 nmol/L A-83-01 (Tocris, Bristol, UK). The E-cadherin stabilizer Thiazovivin (2.5 μmol/L, Stemgent) and the GSK inhibitor Chir99021 (2.5 μmol/L, Stemgent) were added to the medium for the first 2 days.

Image Analysis

The whole-mount immunostaining of enteroids was imaged by confocal microscopy (LSM710, Zeiss). Enteroids were fixed in Matrigel with 2% paraformaldehyde for 30 minutes at room temperature,

followed by permeabilization with 0.5% Triton X-100. Immunohistochemistry was performed using the following primary antibodies: mouse anti–E-cadherin (1:500), rabbit anti-Mucin2 (1:200, Santa Cruz Biotechnology), rabbit anti-lysosyme (1:200, Zymed, San Francisco, CA), rabbit anti–chromogranin A (1:200, Immunostar), and phalloidin-Tetramethylrhodamine B isothiocyanate (TRITC) (1:200, Sigma). The secondary antibodies were Alexa 488–conjugated antibodies (1:500, Invitrogen). EdU staining was performed following the manufacturer's instructions (Click-IT; Invitrogen). Counterstaining of the nuclei was performed with DAPI or Hoechst (Invitrogen).

Maintenance of Established Human Enteroids

The entire medium was changed every other day and enteroids were passaged 1:4 every week as previously described.[3] The ROCK inhibitor Y-27632 (10 μmol/L; Sigma) was added to the culture medium for the first 2 days after passaging.

Human Tissue Processing for Single IEC Analysis

Human duodenum samples were obtained from an institutional review board–exempt protocol from unidentified patients undergoing a Whipple procedure. The tissue was cleaned with PBS, and a mucosectomy was performed to isolate the mucosa. Approximately 4 g of mucosa was cut into 5-mm strips and placed into 20 mL of 2 mmol/L EDTA in PBS and incubated on ice for 30 minutes. Subsequently, the tube was lightly inverted for 10 seconds to remove villi and the supernatant was discarded. The mucosa was rinsed twice with PBS and then shaken at 2 cycles per second for 6 minutes in 10 mL of PBS to release the crypts. Ten milliliters of 2% sorbitol was added, and the remaining tissue was removed. The suspension was centrifuged at 40*g* for 3 minutes. The supernatant was removed and the pellet was resuspended in 5 mL of HBSS with 3 U of dispase, 1.45 mg of deoxyribonuclease I, and a final concentration of 10 μmol/L Y27632. The suspension was incubated at 37°C for 20 minutes and shaken at 3.5 cycles per second for 30 seconds every 2 minutes. The cells were added to 40 mL of cold Advanced DMEM/F12 medium and inverted to mix. The suspension was filtered through a 40-μm cell strainer and centrifuged at 2500 rpm for 5 minutes. The supernatant was removed and the pellet resuspended in 1 mL of staining medium (Advanced DMEM/F12, 1× penicillin-streptomycin, 2 mmol/L Glutamax, 10 mmol/L HEPES, 10 μmol/L Y27632). Cells were stained with antibodies for CD45, CD44, CD166, and CD24 and their corresponding isotype controls following the manufacturer's recommended concentrations (Supplementary Table 3) and incubated for 30 minutes. Following staining, cells were washed with Advanced DMEM/F12 and centrifuged at 1000 rpm for 5 minutes. Cells were resuspended in staining medium, and 7-aminoactinomycin D and annexin V were added to the solution shortly before sorting.

Cells were sorted on a FACSAria (BD Biosciences) cell sorter. Single stained samples were used to configure the compensation settings, and isotype controls were used to determine the positive populations. The cells were sorted following the gating scheme to select for live IECs and $CD44^{+}CD166^{+}CD24^{lo}$ cells (Supplementary Figure 15). Cells were sorted into collecting medium (Advanced DMEM/F12, 1× penicillin-streptomycin, 2 mmol/L Glutamax, 10 mmol/L HEPES, 1× N2, 1× B27, 10 μmol/L Y27632) or into RNA lysis buffer. Cell lysate was frozen immediately in dry ice. RNA was isolated using the Ambion RNAqueous Micro Kit following the manufacturer's protocol. The TaqMan Gene Expression Assays are listed in Supplementary Table 4.

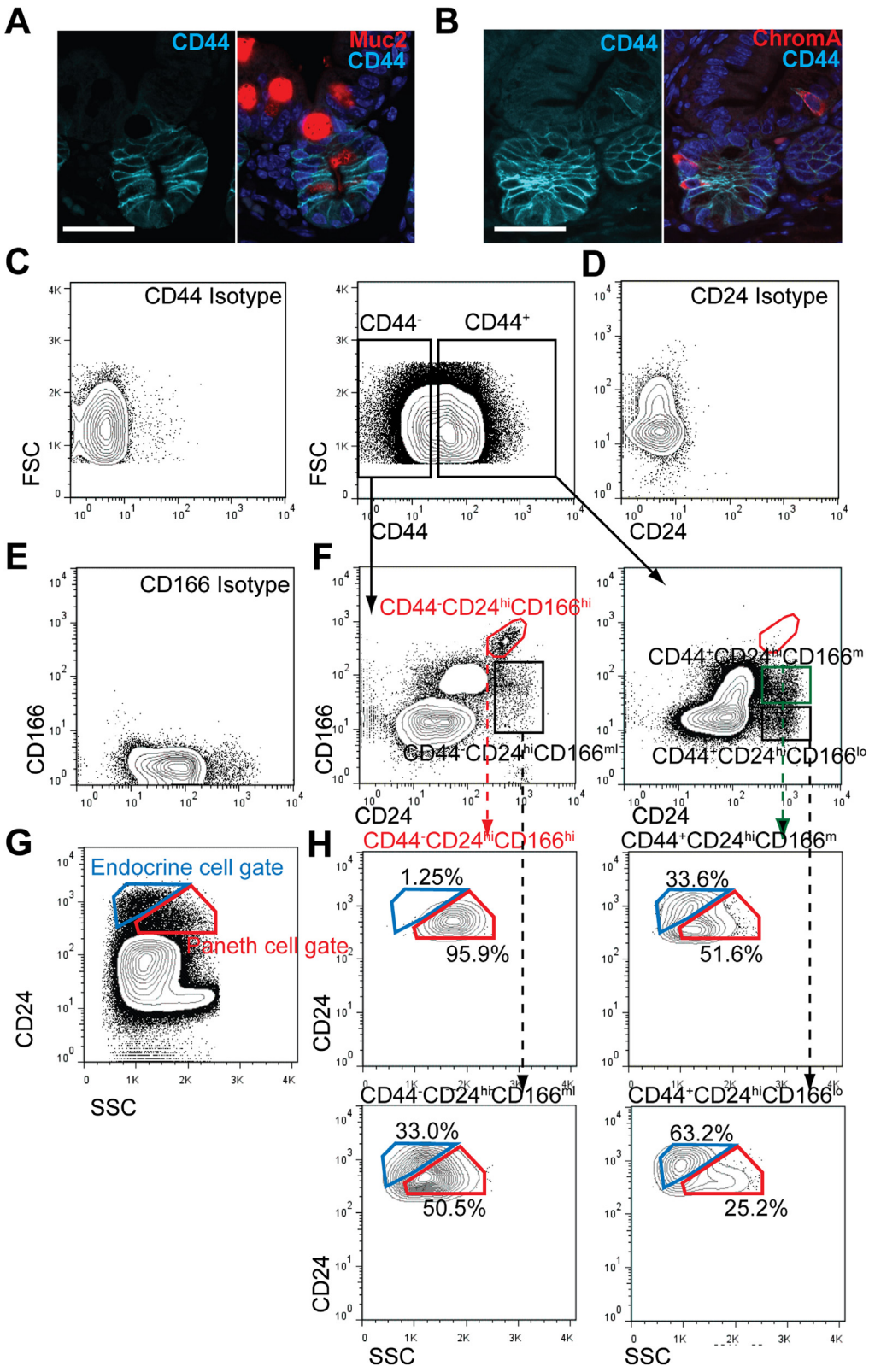
Sorted cells were centrifuged at 500*g* for 5 minutes at 4°C and resuspended in Matrigel supplemented with 750 ng/mL epidermal growth factor, 1.5 μg/mL Noggin, and 15 μmol/L Jagged-1. Cells were plated at 500 cells per 10 μL of Matrigel into a 96-well plate. Growth factors were added every other day, and the medium was changed every 4 days following the conditions outlined (Supplementary Figure 15).

Electron Microscopy

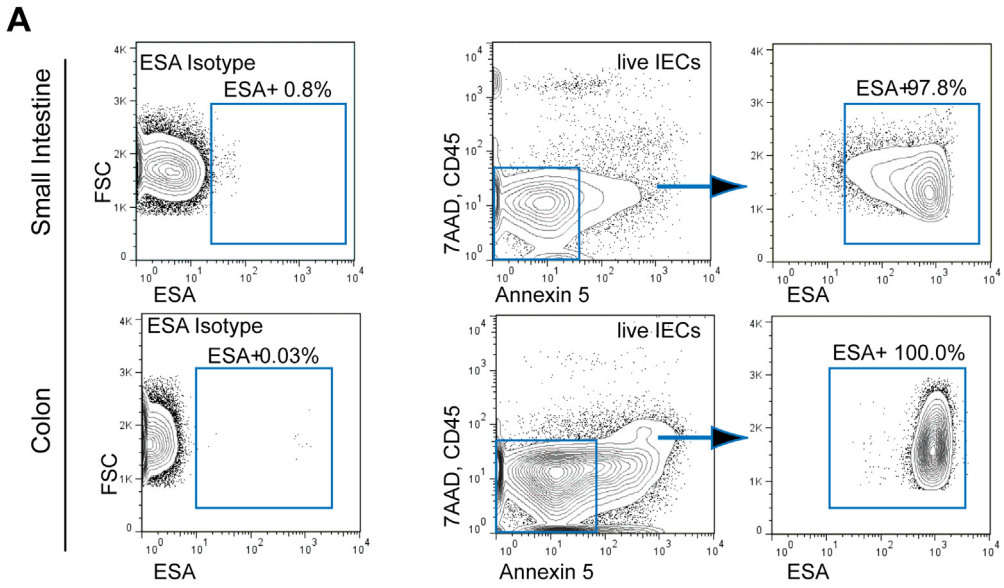
Intestinal tissues or enteroids were fixed for 2 hours with 2.5% paraformaldehyde and 2% glutaraldehyde in PBS. Fixative was removed by washing in PBS, and tissues were then treated with 1% osmium tetroxide containing 1% potassium ferricyanide for 1 hour. Samples were dehydrated through a series of dilution of ethanol up to 100%, infiltrated, and embedded in resin (epon 812). The resin was polymerized at 37°C overnight and incubated at 60°C for 24 hours. Ultrathin sections (approximately 50–70 nm) were cut on a Leica (Wetzlar, Germany) ultramicrotome with diamond knives. Sections were stained with 2% uranyl acetate and lead citrate for 10 minutes and 5 minutes, respectively, and visualized with a FEI Tecnai (Hillsboro, Oregon) electron microscope.

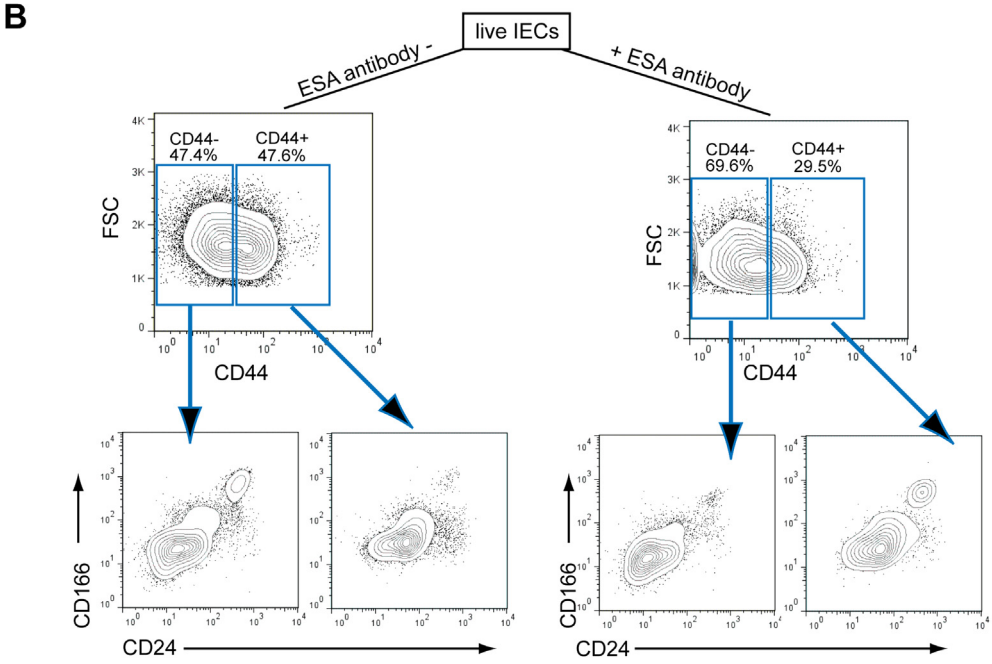
References

1. Wang F, Zou Z, Liu D, et al. Active deformation of apoptotic intestinal epithelial cells with adhesion-restricted polarity contributes to apoptotic clearance. Lab Invest 2011;91:462–471.
2. 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;141:1762–1772.
3. Jung P, Sato T, Merlos-Suarez A, et al. Isolation and in vitro expansion of human colonic stem cells. Nat Med 2011;17:1225–1227.

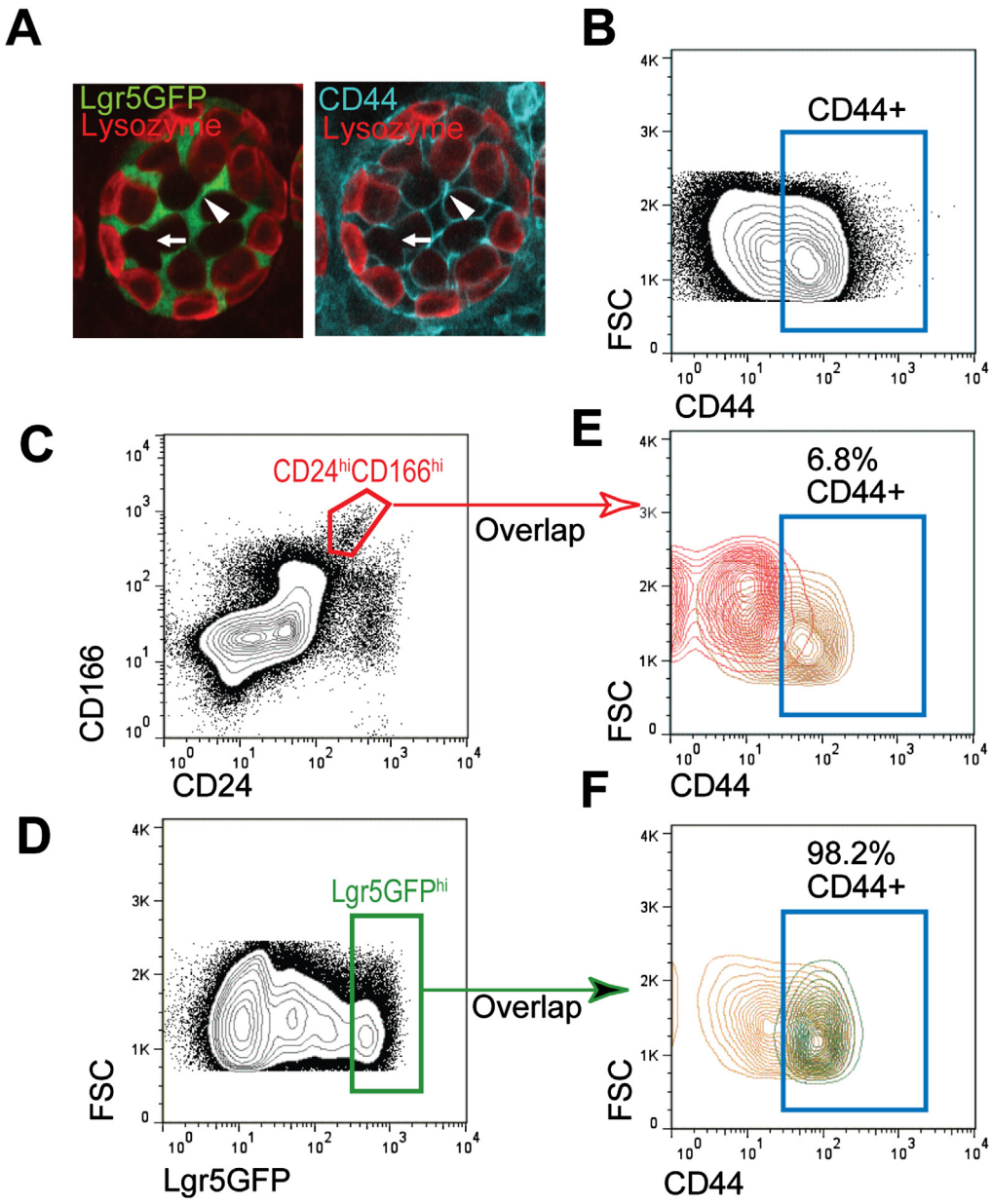


Supplementary Figure 1. CD44 expression in the small intestine and the new strategy to enrich PCs. (*A*) IHC staining shows Muc2-positive (*red*) and CD44-positive cells in crypts. (*B*) IHC staining shows chromogranin A–positive (*red*) and CD44-positive cells in crypts. (*C*) Setting up $CD44^{+}$ gate based on isotype control. (*D*) CD24 isotype control. (*E*) CD166 isotype control. (*F*) $CD44^{-}$ and $CD44^{+}$ cells were distributed into an FACS plot of CD24 versus CD166. $CD24^{hi}$ cells in $CD44^{-}$ and $CD44^{+}$ populations were divided further, depending on the CD166 expression. (*G*) The reported gates relatively enriched endocrine cells (*blue*) and PCs (*red*) in the FACS plot of CD24 versus SSC. (*H*) The subgates in *F* were analyzed in the FACS plot of CD24 versus SSC. It is noteworthy that only $CD44^{-}CD24^{hi}CD166^{hi}$ (*red polygon*) cells fell mainly into the PC gate (*red*).

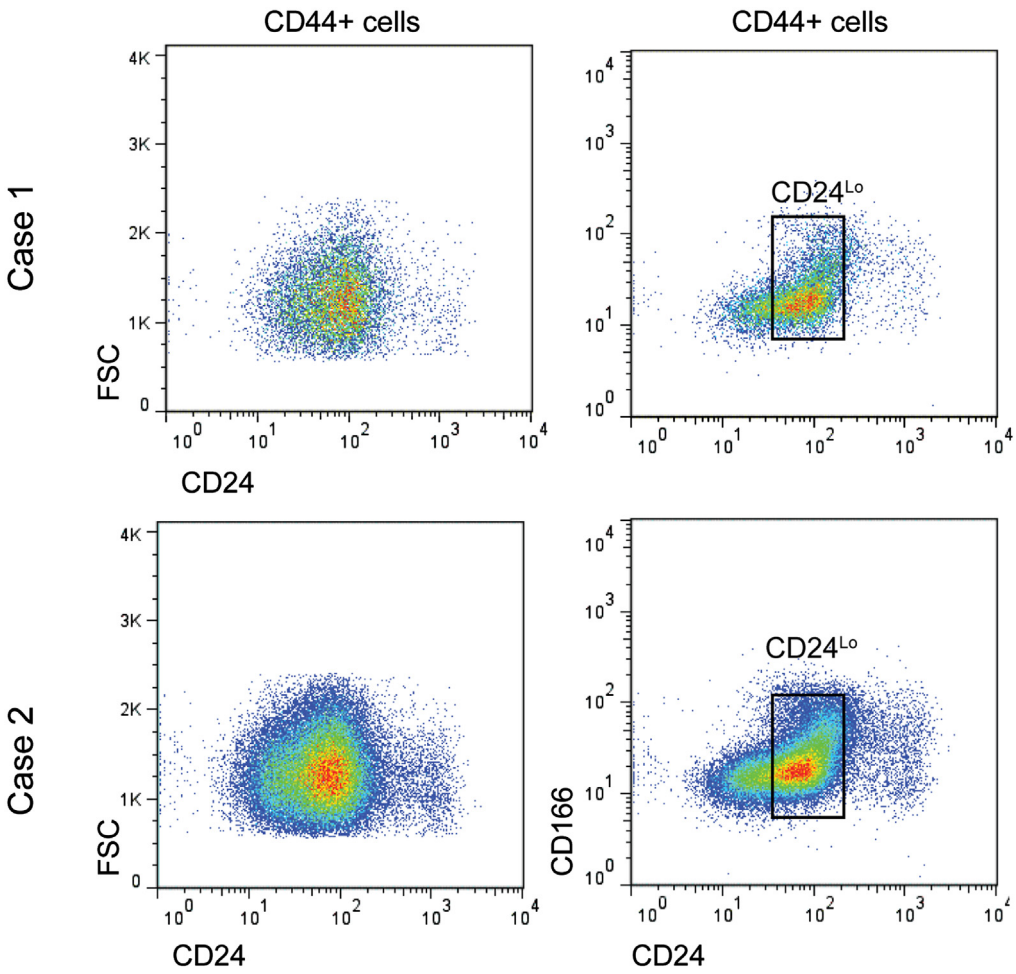




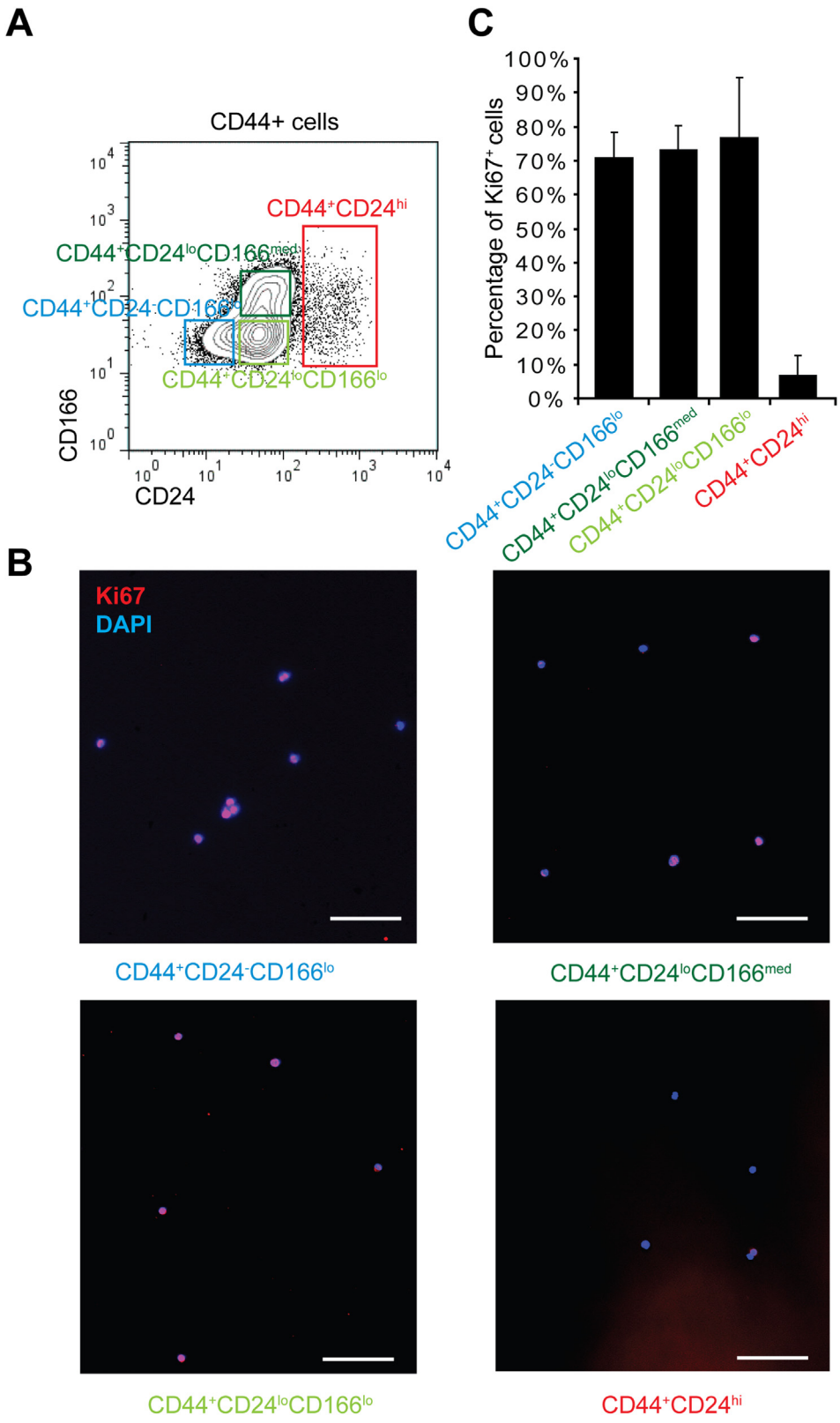
Supplementary Figure 2. ESA (EpCAM) is not required to distinguish IECs in the present FACS analysis. (*A*) Based on the isotype staining, the majority of cells in the original live IEC gate are ESA$^+$. *Upper panel,* small intestine. *Lower panel,* colon. (*B*) ESA staining can affect the other surface marker staining including CD44. The CD44$^+$ percentage and the FACS plots of CD24 versus CD166 derived from CD44$^+$ or CD44$^-$ gates were changed by adding ESA antibody.



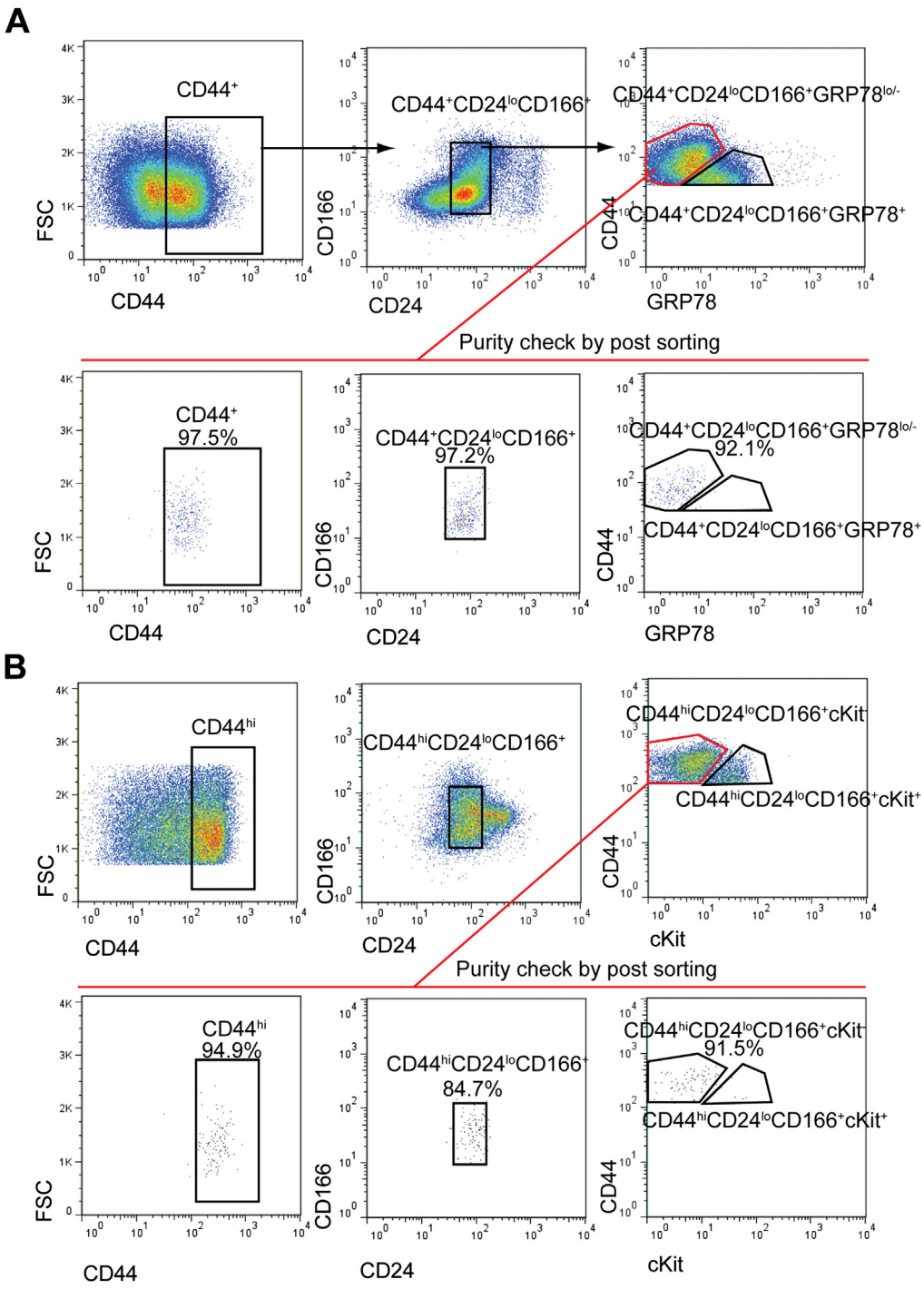
Supplementary Figure 3. Lgr5GFP^{hi} cells express a much higher level of CD44 than CD24^{hi}CD166^{hi} PCs. (*A*) Confocal image of cross sections of the crypt bottom from Figure 1, showing strong staining of CD44 (*bright blue*) in the junctions of Lgr5-GFP^{hi} stem cells (*green, arrowheads*) and weak staining between PCs (*red, arrows*). (*B*) CD44^{+} gate drawn out of total live cells. (*C*) CD24^{hi}CD166^{hi} PC gate drawn out of total live cells (see Supplementary Figure 3). (*D*) Lgr5 GFP^{hi} cell gate drawn out of total live cells. (*E*) Backgating CD24^{hi}CD166^{hi} PCs into CD44 gate, showing that the low percentage of PCs is CD44^{+}. (*F*) Backgating Lgr5 GFP^{hi} Cells into CD44 gate, showing that almost all of Lgr5 GFP^{hi} cells are CD44^{+}.



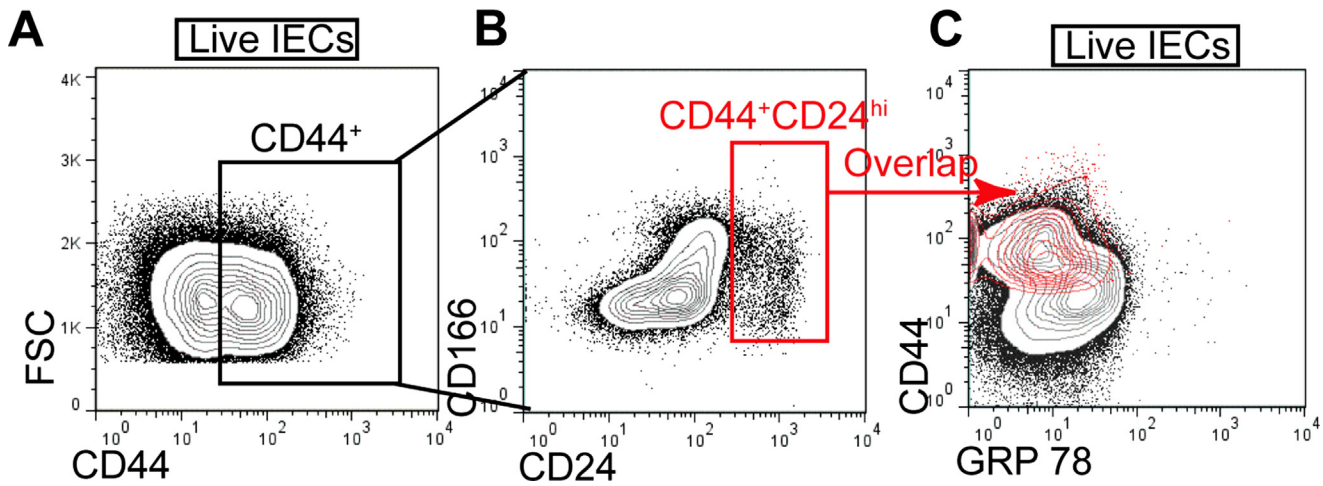
Supplementary Figure 4. CD24 versus CD166 generates a more natural FACS pattern than CD24 alone. Two representative FACS tests show the cell populations with different CD24 levels in CD24 versus FSC plots or CD24 versus CD166 plots. $CD166^{med}$ cells help define the boundary between $CD24^{neg}$ and $CD24^{lo}$.



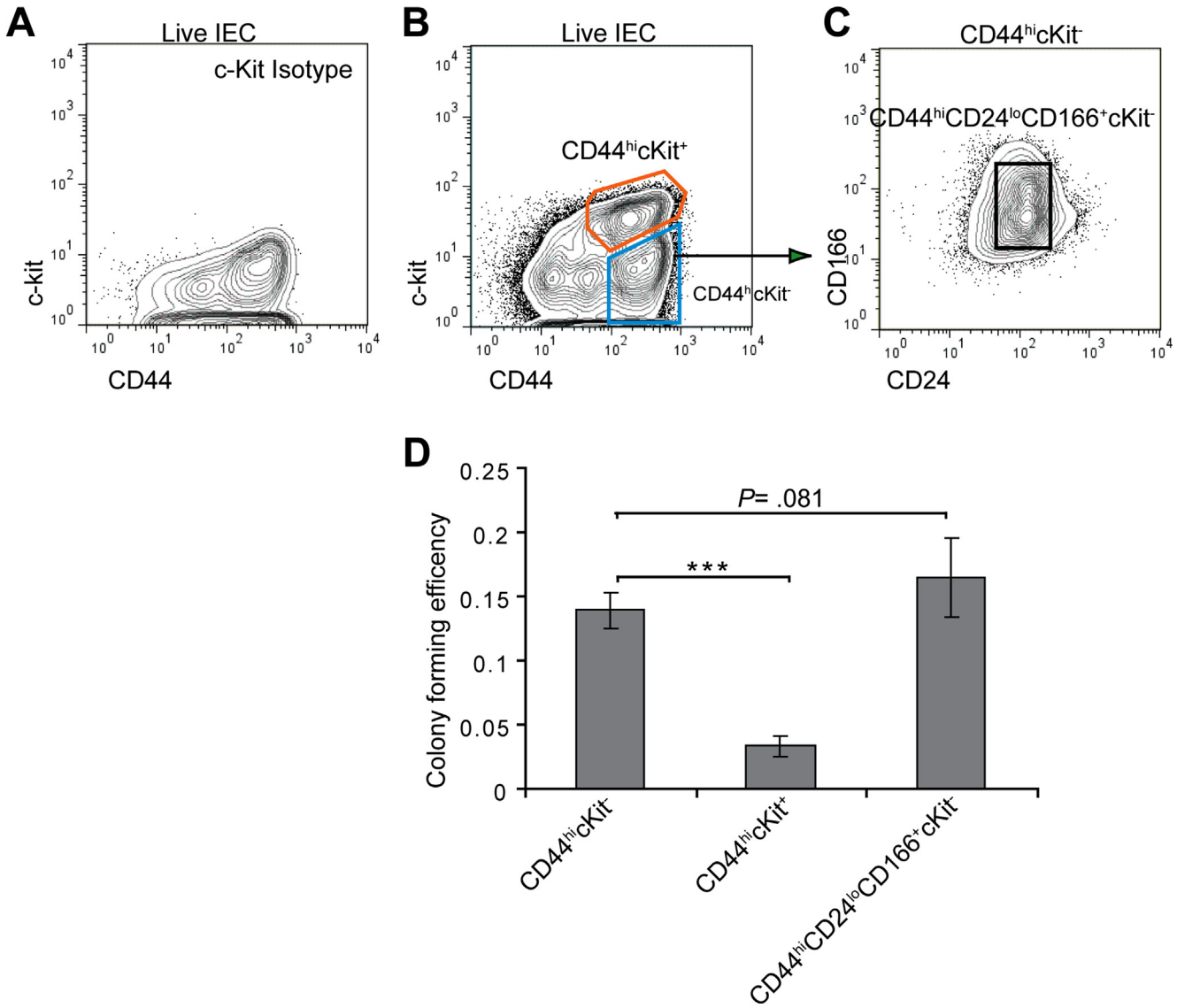
Supplementary Figure 5. Testing the Ki67 expression of 4 subpopulations in the CD24 versus CD166 plot derived from $CD44^+$ cells. (*A*) Representative FACS plot of CD24 versus CD166 derived from $CD44^+$ cells. Four subpopulations were labeled with different colors (*bright blue, deep green, bright green, red*), which is consistent with Figure 1. (*A*) Cells sorted from 4 subpopulations were stained with Ki67 (*red*) and counterstained by DAPI. *Bar* = 100 μm. (*B*) The percentage of $Ki67^+$ cells in different subpopulations (n = 3).



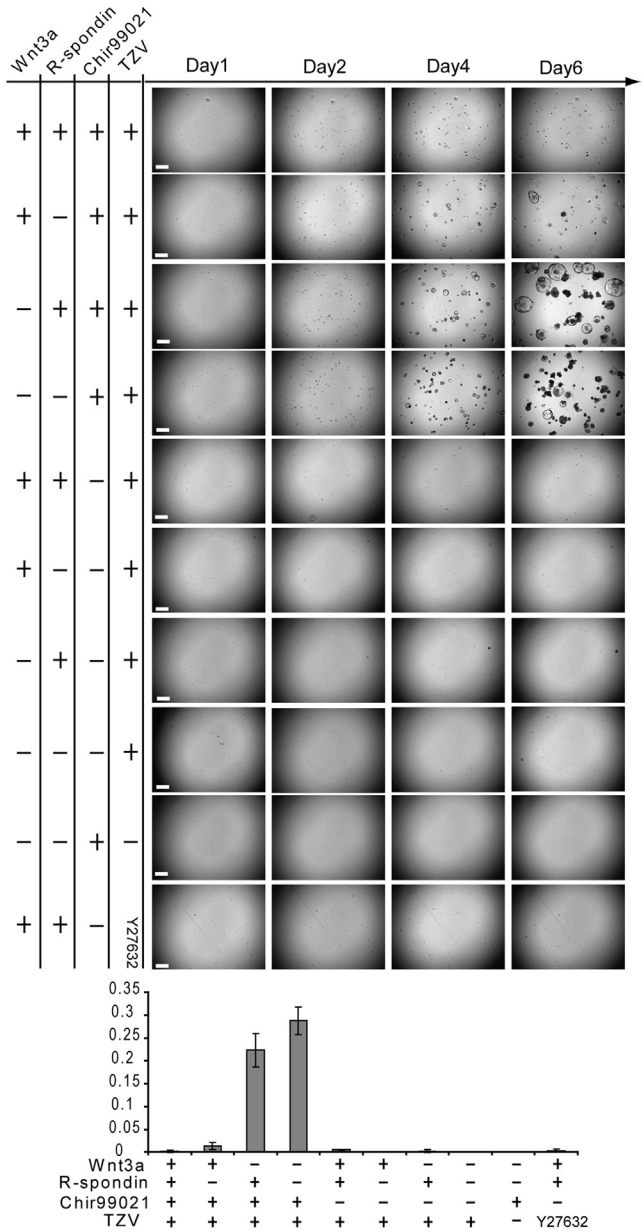
Supplementary Figure 6. Post sorting confirms the sorting purity. (*A*) The sorted $CD44^{+}$ $CD24^{lo}CD166^{+}$ $GRP78^{-/lo}$ cells from the small intestine were verified by flow cytometry, showing a high percentage (>90%) overlapping with the gates used for primary sorting. (*B*) The sorted $CD44^{+}$ $CD24^{lo}CD166^{+}$ $cKit^{-}$ cells from colon were verified by flow cytometry, showing a high percentage overlapping with the gates used for primary sorting.



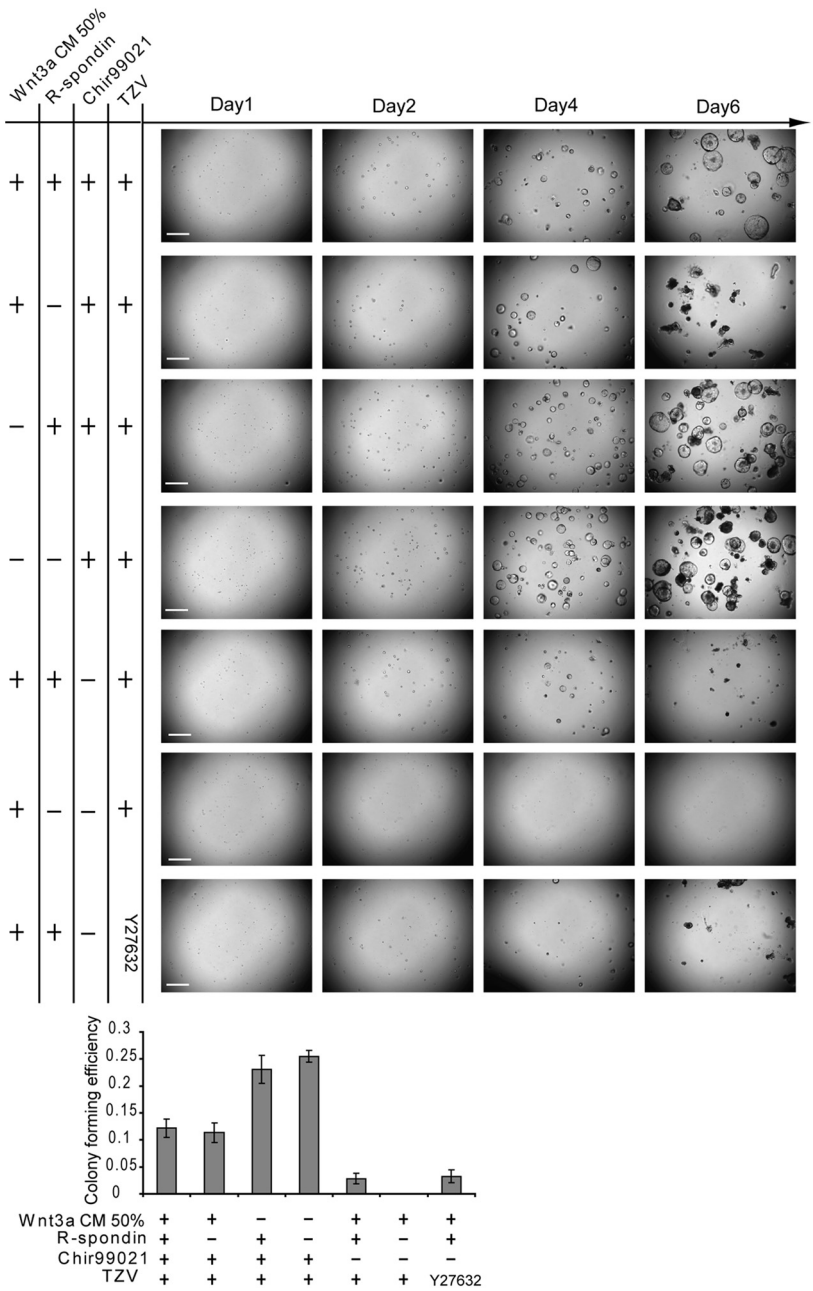
Supplementary Figure 7. $CD44^{hi}GRP78^{-/lo}$ cannot exclude the $CD44^{+}CD24^{hi}$ population. (*A*) CD44 and positive gates from a single live IEC. (*B*) $CD44^{+}$ cells distributed in an FACS plot of CD24 versus CD166. (*C*) Overlap $CD44^{+}CD24^{hi}$ cells into an FACS plot of GRP78 versus CD44 from a single live IEC.



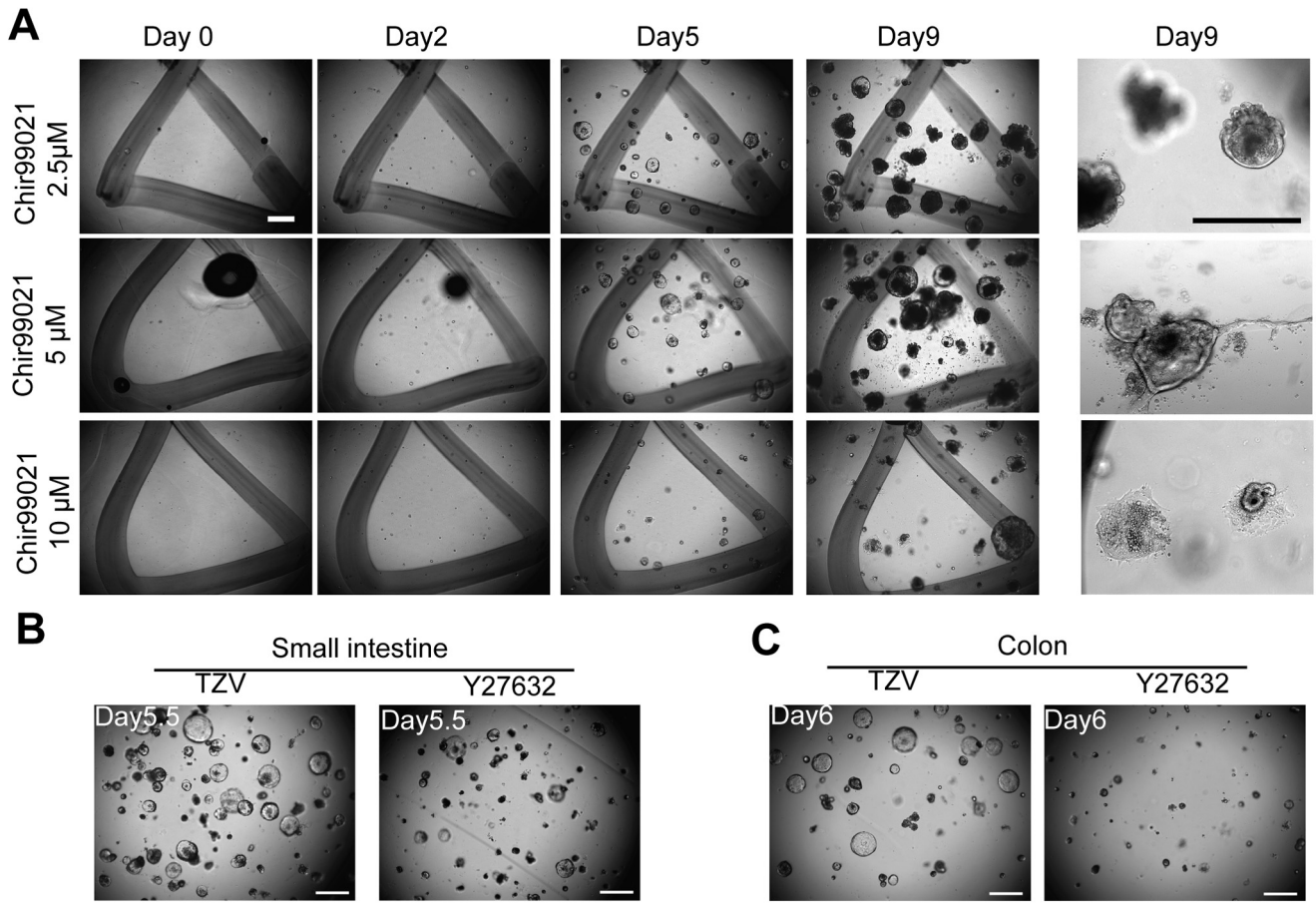
Supplementary Figure 8. The enrichment of clonogenic colonic ISCs by CD44 and c-Kit combination. (*A*) c-Kit isotype staining used to set the criterion for the c-Kit^{+} population. (*B*) Representative FACS analysis shows that the CD44^{hi} colonic IECs can be clearly separated into a c-Kit^{+} and c-Kit^{-} population. (*C*) CD44^{hi} c-Kit^{-} cells have heterogeneities for CD24 and CD166 expression and CD44^{hi} c-Kit^{-}CD24^{lo}CD166^{+} gate was drawn based on contour plot. (*D*) The CFE of CD44^{hi} c-Kit^{-}, CD44^{hi} c-Kit^{+}, CD44^{hi} c-Kit^{-}CD24^{lo}CD166^{+} cells (n = 3). ***$P < .001$.



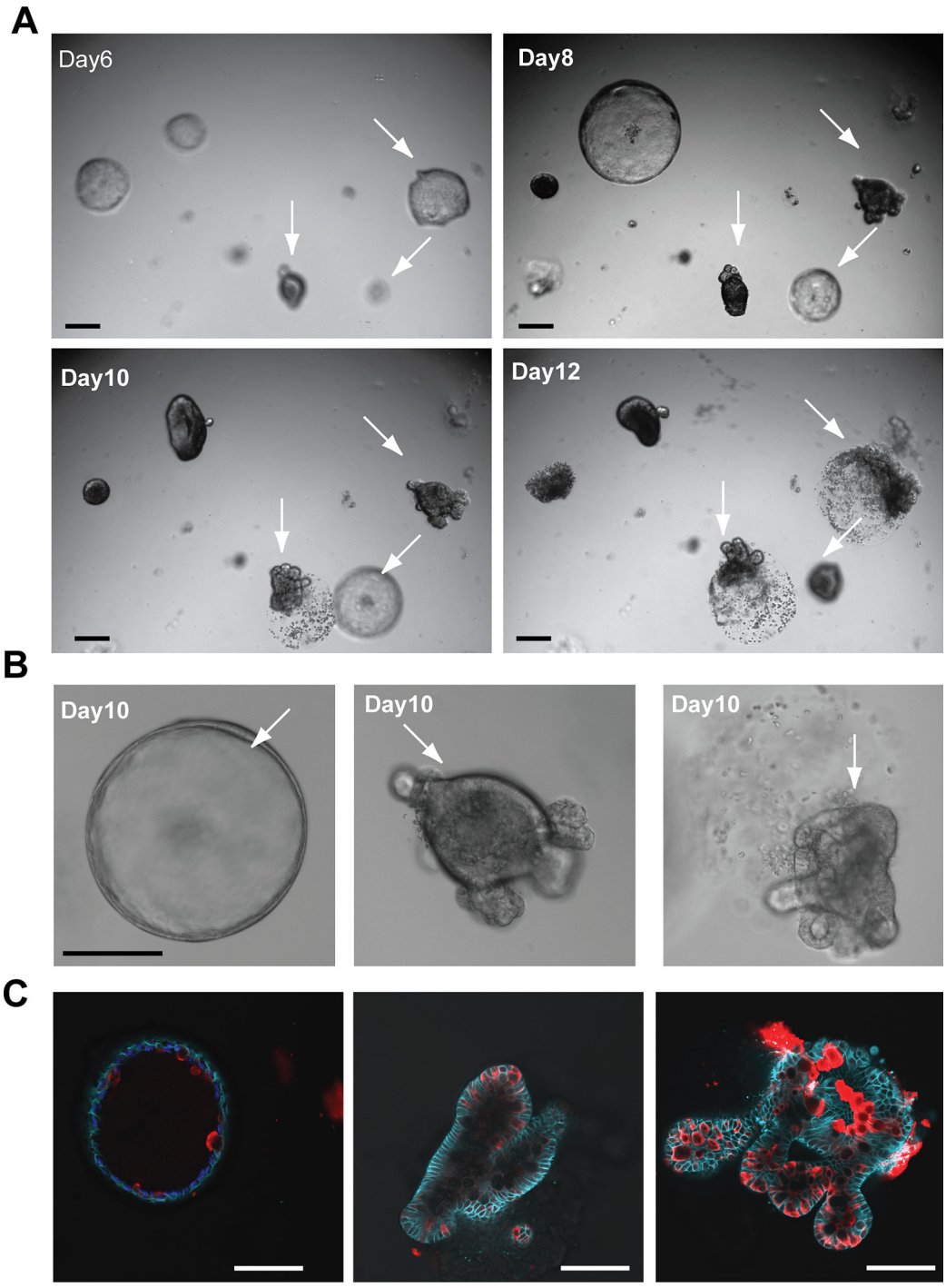
Supplementary Figure 9. Comparison of ISC colony formation induced by different combinations of Wnt3a, R-spondin, Chir99021, and Thiazovivin (TZV) on the first 2 days.Different combinations of factors and inhibitors (including Wnt3a, 100 ng/mL; R-Spondin, 1 μg/mL, Chir99021, 2.5 μmol/L; TZV, 2.5 μmol/L) were tested by adding them into culture medium on day 0. Wnt3a was added on day 2 if it was added on day 0. The high efficiency was achieved by adding Chir99021 and TZV on day 0. Adding Wnt3a, R-Spondin, and Chir99021 together inhibited colony growth, partially mimicking the effect of the high concentration of Chir99021 in Supplementary Figure 11. Y-27632 combined with Wnt3a and R-Spondin was also tested and no high CFE was observed. Other growth factors including epidermal growth factor, Noggin, and Jagged-1 were used as shown in Figure 5 in every group. n = 3 wells. Similar results were obtained in 2 independent experiments. *Bars* = 100 μm.



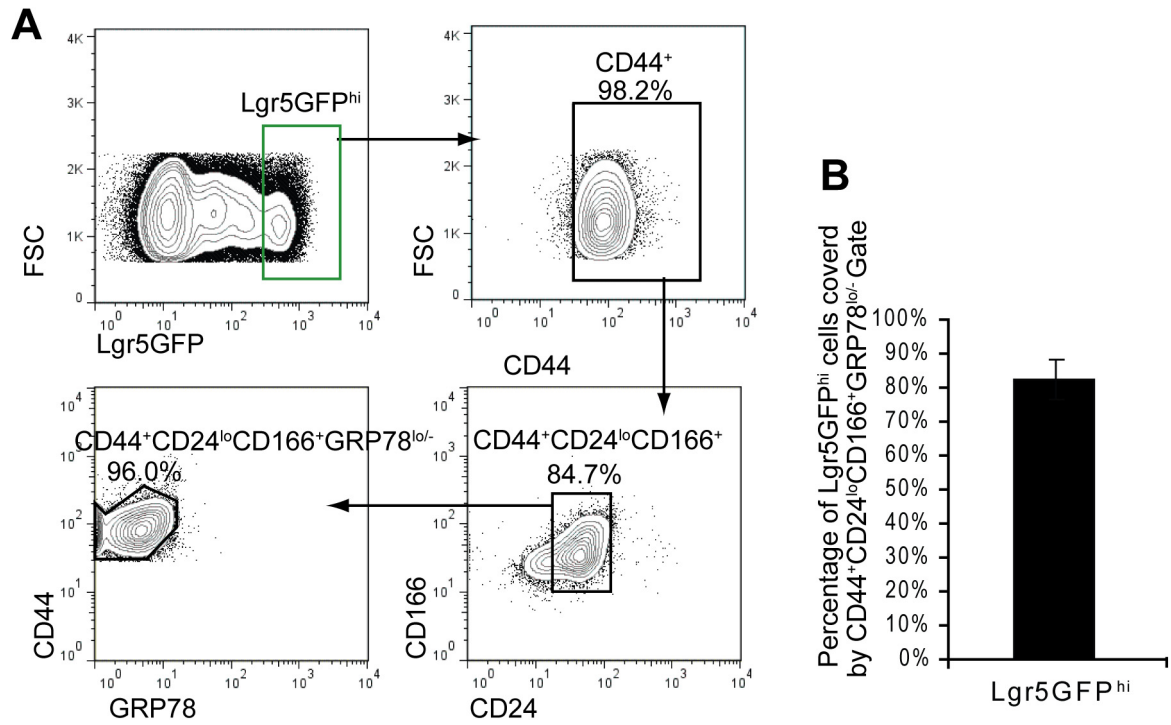
Supplementary Figure 10. Wnt3a conditional medium and R-spondin 1 cannot replace GSK inhibitor. Compared with Wnt3a, Wnt3a conditional medium has complicated components. Combination of Wnt3a conditional medium (50%), R-Spondin 1 μg/mL, Chir99021 2.5 μmol/L, and TZV 2.5 μmol/L only partially inhibits colony growth. In addition, wnt3a conditional medium combined with R-spondin, TZV, or Y27632 can lead to a CFE of $<5\%$, which is higher than commercial Wnt3a (Supplementary Figure 9). However, Chir99021 combined with TZV led to even higher CFE and much bigger colony size. Other growth factors including epidermal growth factor, Noggin, and Jagged-1 were used as shown in Figure 4 in every group. n = 3 wells. Similar results were obtained in 2 independent experiments. *Bar* = 100 μm.



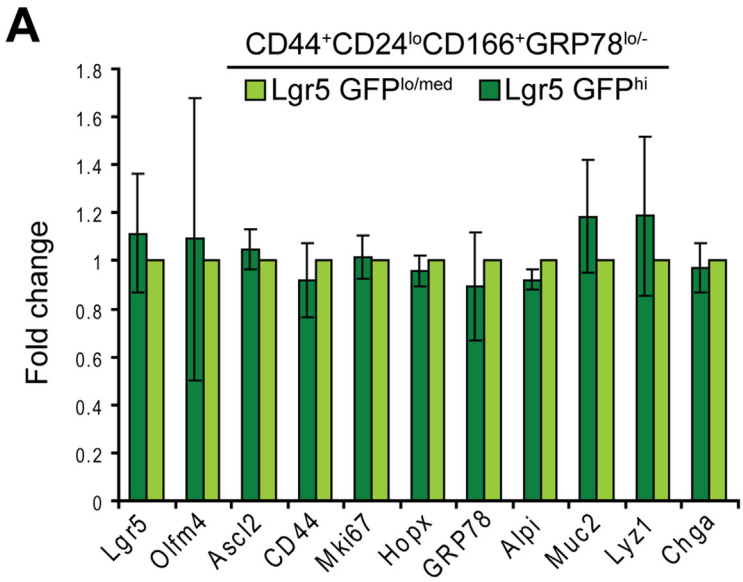
Supplementary Figure 11. The effect of Chir99021, TZV, and Y-27632 on single ISC culture. (*A*) Representative process of colony forming from single Lgr5-GFP^{hi} small intestinal cells with different concentration of Chir99021 in the medium for the first 2 days of culture. Note that the high concentration (5 μmol/L and 10 μmol/L) can cause the spreading of the colony. (*B*) Adding Y27632 (10 μmol/L) or TZV (2.5 μmol/L) into the medium on the first 2 days leads to a different size of colony derived from single Lgr5-GFP^{hi} small intestinal cells. Chir99021 and other growth factors were used in the same way in both groups. (*C*) Adding Y27632(10 μmol/L) or TZV (2.5 μmol/L) into the medium on the first 2 days led to a different size of colony derived from single Lgr5-GFP^{hi} colonic intestinal cells. Chir99021 and other growth factors were used in the same way in both groups. *Bars* = 200 μm.

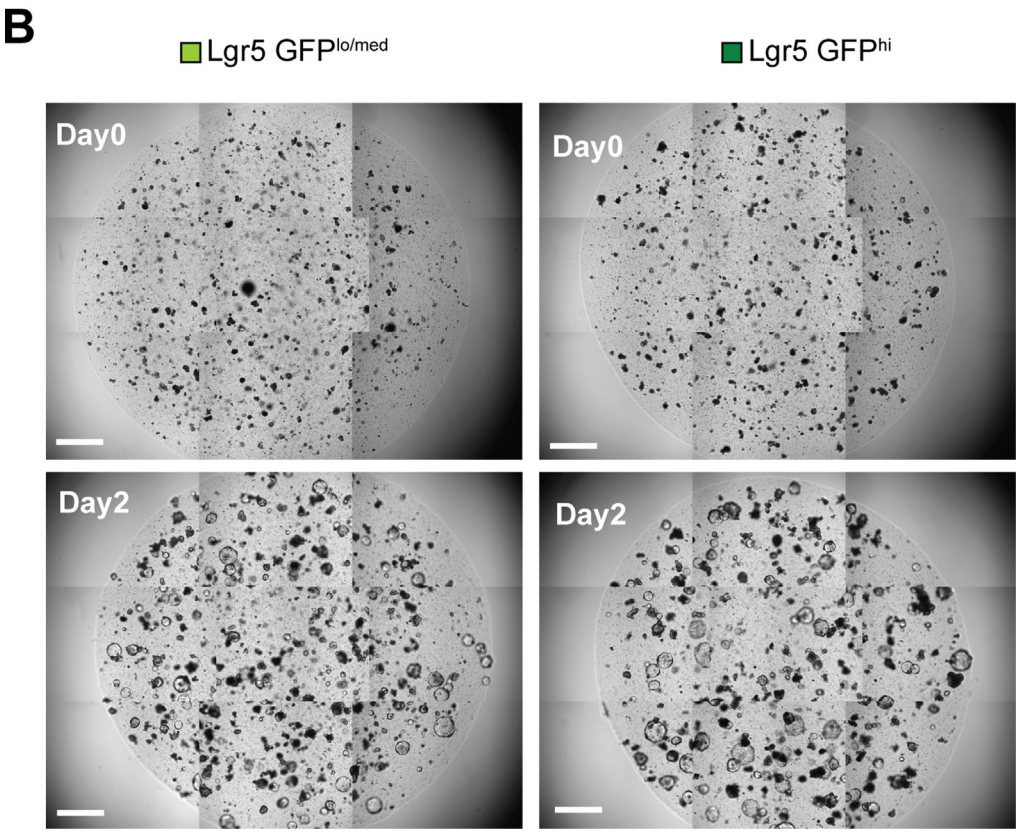


Supplementary Figure 12. The heterogeneities of morphology of colonoid derived from single Lgr5GFP^{hi} ISCs. (*A*) Representative growth process of single ISC-derived colonoid from day 6 to day 12. *White arrows* label the same colony on different days. It is noteworthy that most "closed" colonoids can become "open" along with the cell proliferation and differentiation. (*B*) High magnification of colonoids on day 10 shows different morphology (sphere, closed enteroid, open enteroid). (*C*) Representative Muc2 (*red*) and E-cadherin (*bright blue*) IHC staining shows the differentiation in colonoids with different morphology.

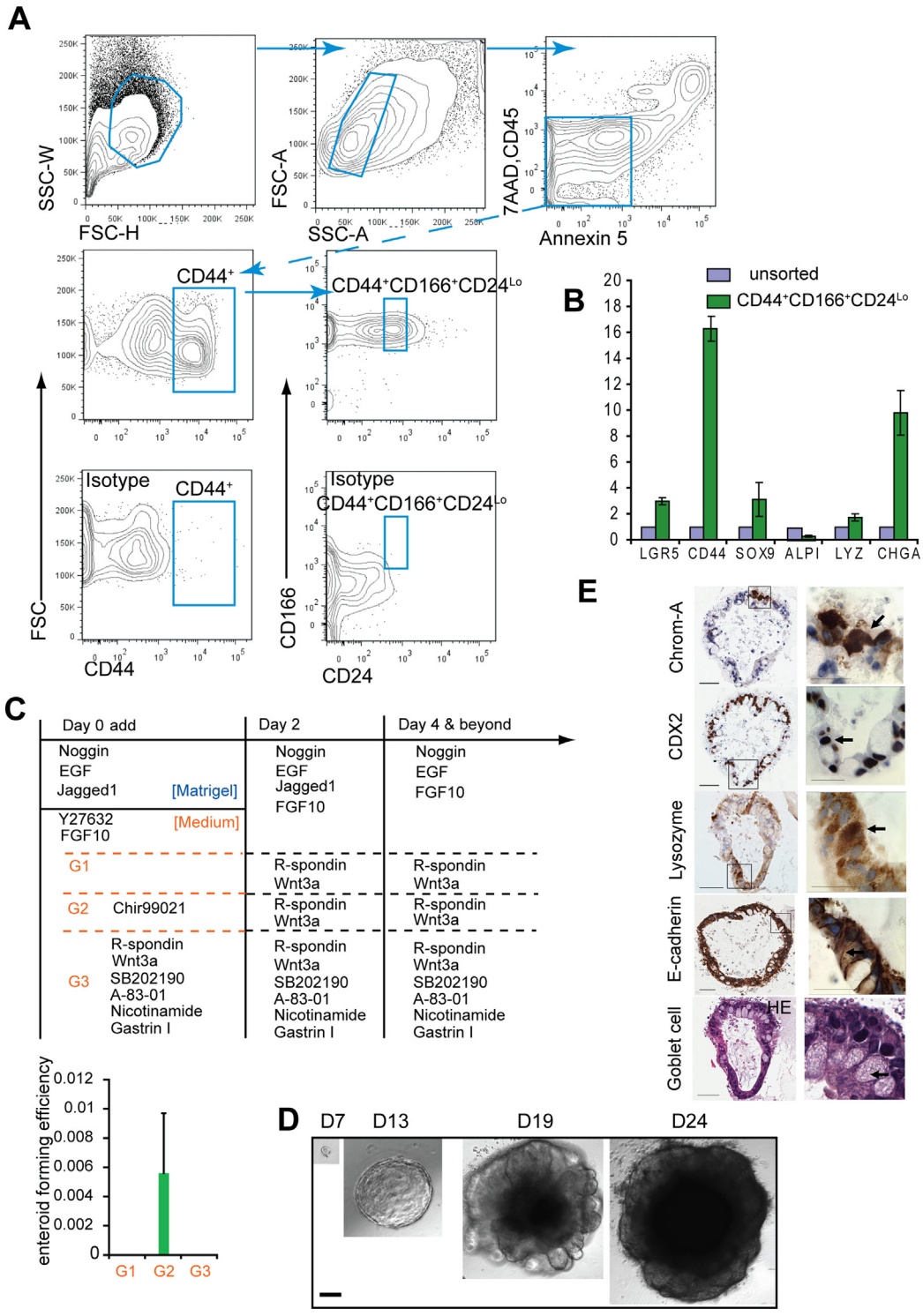


Supplementary Figure 13. $CD44^{+}$ $CD24^{lo}CD166^{+}$ $GRP78^{-/lo}$ enrich high percentage of $Lgr5GFP^{hi}$ cells. (*A*) The distribution of Lgr5GFPhi cells in $CD44^{+}$, $CD44^{+}$ $CD24^{lo}CD166^{+}$, and $CD44^{+}$ $CD24^{lo}CD166^{+}$ $GRP78^{-/lo}$ gates. (*B*) The percentage of total $Lgr5GFP^{hi}$ ISCs covered by the gate of $CD44^{+}$ $CD24^{lo}CD166^{+}$ $GRP78^{-/lo}$ (n = 3).

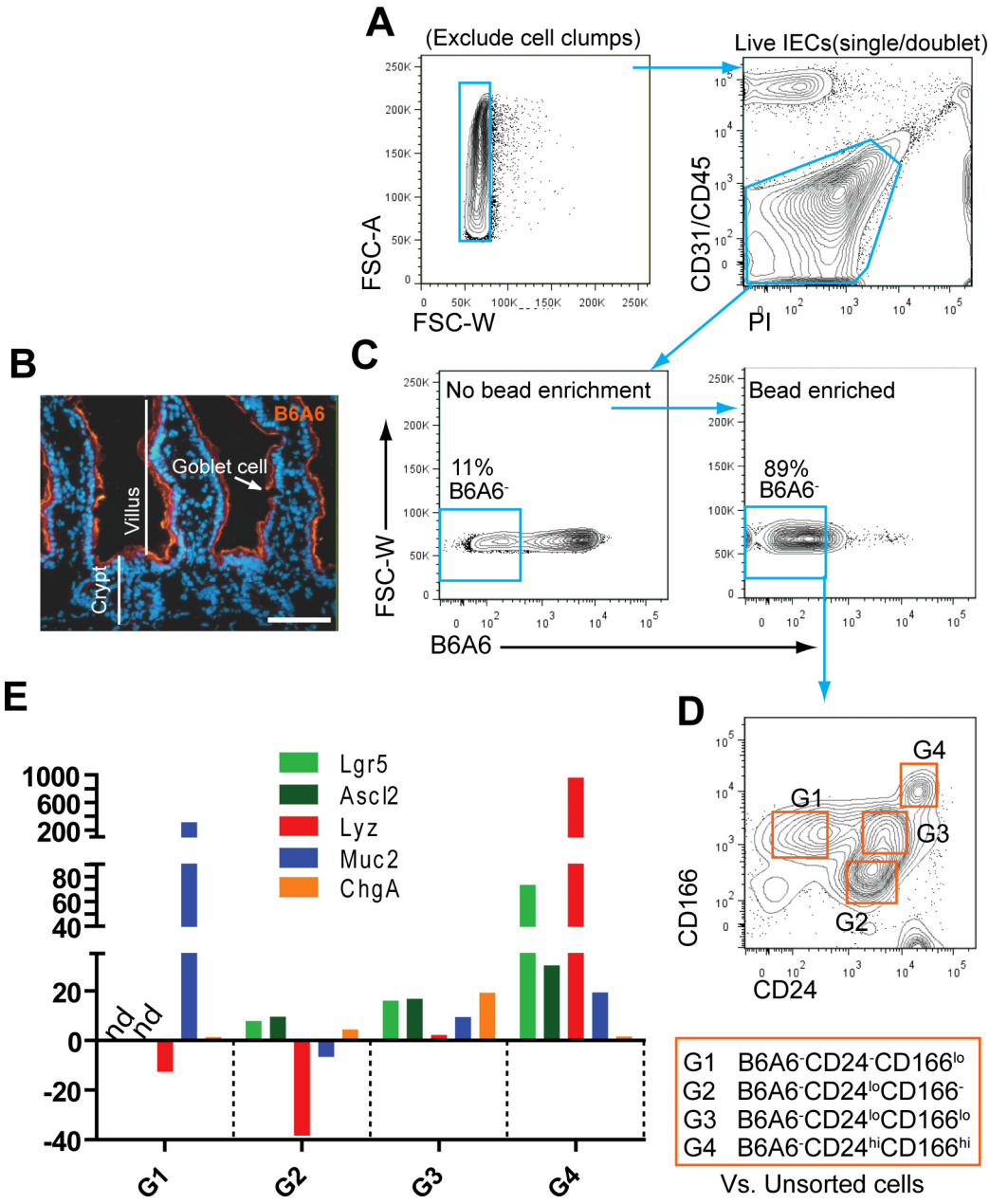




Supplementary Figure 14. The enteroids derived from different Lgr5GFP levels share similarity in gene profiling and long-term culture maintenance. (*A*) Lgr5GFP^{hi} cells or Lgr5GFP$^{med/lo}$ cells derived from CD44^{+} CD24^{lo} CD166^{+} GRP78$^{-/lo}$ population were cultured and passaged. qPCR test shows the similar expression levels of ISC related genes and differentiation associated genes in these 2 groups (n = 3). (*B*) Representative passaging image (from day 0 to day 2) showing the robust colony forming of these 2 groups, even after about 2.5 months of culture. *Bar* = 500 μm.



Supplementary Figure 15. Single human ISC isolation and culture analysis. (*A*) Experimental scheme of enriched single live ISCs. (*B*) qRT-PCR results show the gene expression in the $CD44^{+}CD166^{+}CD24^{lo}$ population relative to unsorted live IECs, indicating the enrichment of ISCs, although some differentiated cells were remained (n = 2). (*C*) Strategies to culture single $CD44^{+}CD166^{+}CD24^{lo}$ cells. Cells grew only in the group with a single dose of CHIR99021 on the first 2 days. Data are represented as mean ± SEM (n = 2). (*D*) Representative growth process of single $CD44^{+}CD166^{+}CD24^{lo}$ cells with addition of a single dose of CHIR99021. (*E*) IHC staining of $CD44^{+}CD166^{+}CD24^{lo}$ cells after 14 days of culture shows differentiation into intestinal epithelial lineages. *Arrows* in the *inset* indicate positive staining. *Scale bars* = 100 μm (*D*); 50 μm (*E*).



Supplementary Figure 16. Rat mAB-B6A6, CD24, CD166 combination to analyze ISCs and differentiated cells. (*A*) FACS plots show the exclusion of cell aggregates (*left panel*) and blood cells and dead cells (*right panel*). It is noteworthy that doublet cells cannot be efficiently removed without using Pulse width. (*B*) IHC staining shows that rat monoclonal B6A6 antibody facilitates exclusion of villus enterocytes but not some secretory cells, including goblet cells (*arrow*). (*C*) FACS pattern with FSC-W versus FSC-A to exclude cell aggregates. (*D*) FACS pattern with B6A6 versus FSC-W to show the $B6A6^{-}$ cells. FACS pattern with B6A6 versus FSC-W to show the enrichment of $B6A6^{-}$ cells by using anti-rat immunoglobulin G beads to deplete the $B6A6^{+}$ cells. FACS pattern with CD24 versus CD166 to show different cell populations in $B6A6^{-}$ cells. It is noteworthy that $B6A6^{-}$ contains some $CD44^{-}$ cells. (*E*) qRT-PCR to test ISC and lineage related gene expression in different populations (G1–G4) from *G c* compared with unsorted cells. The G4 population shows the highest lysozyme and Lgr5 expression indicating PC and CBC-PC doublets. G1 shows the highest level of Muc2 expression, indicating the enrichment of goblet cells. G3 shows the high level of Lgr5 and Ascl2, indicating $Lgr5^{hi}$ and $Lgr5^{lo/med}$ cells may be included in this population.

Supplementary Table 1. Antibodies Used in Flow Cytometry for Mouse Tissue

Purpose	Reagent name (stock)	Supplier	Working solution	Catalog no.
Dead cells	Annexin V AF647	Life Technologies	1.5:100	A23204
	7AAD 100 μg/mL(Invitrogen)	Invitrogen	1:100	A1310.
Blood and endothelial cells	PE-Cy5-anti-Mouse CD45	eBioscience	1:100	15-0451-82
	IgG2b K isotype control PE-Cy5	eBioscience	1:100	15-4031
	APC-conjugated anti-CD31	eBioscience	1:100	17-0311
	Rat IgG2a K isotype control APC	eBioscience	1:100	17-4321
CD44,	PE/Cy7-conjugated anti-CD44	Biolegend	1.5:100	103030
CD24,	PE/Cy7 rat IgG2b, κ isotype control antibody	Biolegend	1.5:100	400617
CD166	eFluor 450-conjugated anti-CD24	eBioscience	1.25:100	48-0242-82
gates	Rat IgG2b K isotype control eFluor 450	eBioscience	1.25:100	48-4031
	PE-conjugated anti-CD166 (Alcam) antibody	R&D systems	1.5:100	FAB1172p
	Normal goat IgG phycoerythrin control	R&D systems	1.5:100	IC108P
GRP78 gates	Rabbit anti-GRP78(ET-21)	Sigma	0.5:100	G9043
	Anti-rabbit IgG APC-cy7	Santa Cruz Biotechnology	1:100	sc-3847
c-kit gates	APC/Cy7-conjugated anti-c-Kit	Biolegend	1.5:100	105826
	APC/Cy7 rat IgG2b, κ isotype control antibody	Biolegend	1.5:100	400623
Epithelial cell purity check	APC/Cy7 anti-mouse CD326 (Ep-CAM)	Biolegend	2:100	118217
	APC/Cy7 rat IgG2a, κ isotype control antibody	Biolegend	2:100	400523

Supplementary Table 2. TaqMan Assay Used in qPCR for Mouse Tissue

Assay	Label
Alpi	Mm01285814_g1
Ascl2	Mm01268891_g1
CD24	PN4331348
CD44	Mm01277163_m1
ChgA	Mm00514341_m1
cKit	Mm00445212_m1
Ephb2	Mm01181021_m1
GRP78	Mm00517690_g1
GUSB	Mm01197698_m1
Hopx	Mm00558630_m1
HPRT	Mm00446968_m1
Krt20	Mm00508106_m1
Lgr5	Mm00438890_m1
Lrig-1	Mm00456116_m1
Lyz1	Mm00657323_m1
mKi67	Mm01278616_m1
Msi-1	Mm00485224_m1
mTert	Mm01352136_m1
Muc2	Mm00458299_m1
Olmf4	Mm01320260_m1
TBP2	Mm00446973_m1

Supplementary Table 3. Antibodies Used in Flow Cytometry for Human Tissue

Purpose	Reagent name	Supplier	Working solution	Catalog no.
Dead cells	Alexa Fluor 647 Annexin V	Life Technologies	2:100	A23204
	7-AAD	Life Technologies	2:100	A1310
Blood cells	PerCP Cy5.5 CD45	BD Pharmingen	2:100	552724
	PerCP Cy5.5 isotype	BD Pharmingen	2:100	552834
CD44,	PE-Cy7 CD44	BD Pharmingen	1.25 :100	560533
CD24,	PE-Cy7 isotype	BD Pharmingen	1.25 :100	560542
CD166	APC-eFluor780 CD24	eBioscience	2 : 100	47-0247
gates	APC-eFluor780 isotype	eBioscience	2 : 100	47-4714
	PE CD166	BD Pharmingen	2:100	559263
	PE isotype	BD Pharmingen	2:100	555749
Testing GRP78 gates	Rabbit anti-GRP78(ET-21)	Sigma	0.5:100	G9403
	Anti-rabbit IgG APC-cy7	Santa Cruz Biotechnology	1:100	sc-3847
Epithelial cell purity check	Alexa Fluor 488 anti-human CD326 (EpCAM) antibody	Biolegend	2:100	324210
	Alexa Fluor 488 mouse IgG2b, κ isotype control antibody	Biolegend	2:100	400329

Supplementary Table 4. TaqMan Assay Used in qPCR for Human Tissue

Assay	Label
Alpi	Hs00357579_g1
CD44	Hs01075861_m1
Chga	Hs00900373_m1
GUSB	Hs00939627_m1
HPRT	Hs02800695_m1
Lgr5	Hs00173664_m1
Lyz	Hs00426232_m1
Sox9	Hs01001343_g1
TBP2	Hs01029934_m1