

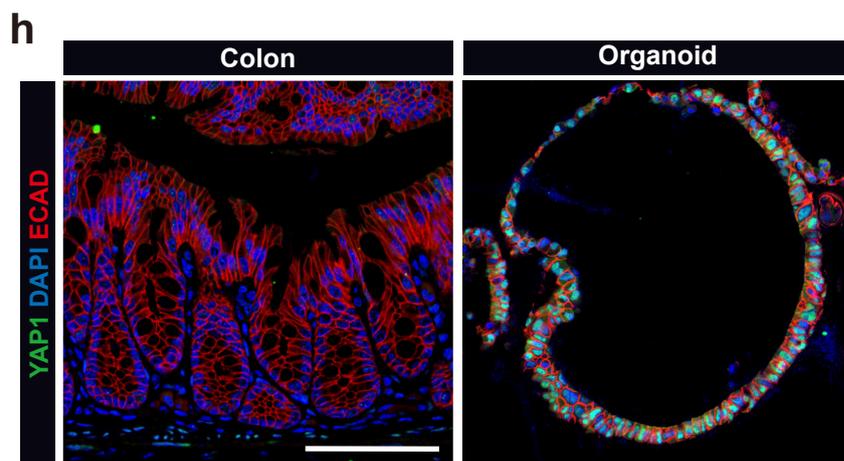
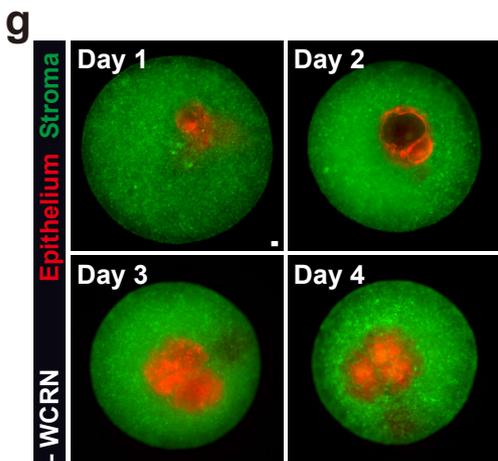
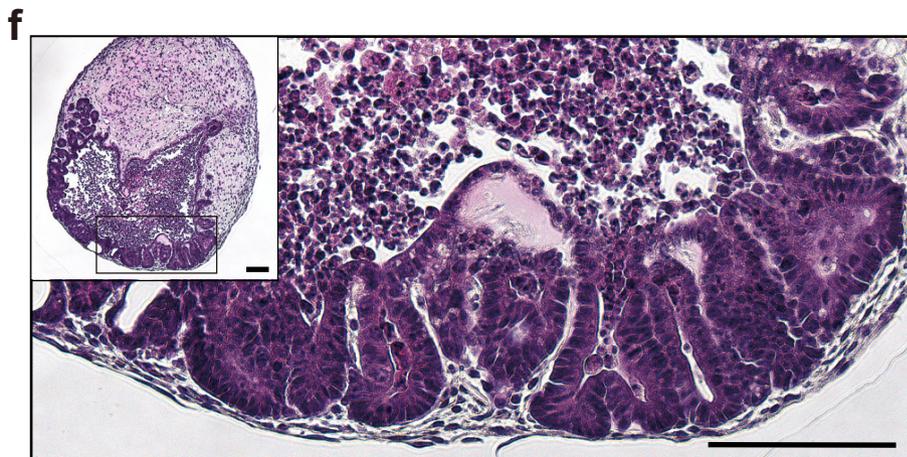
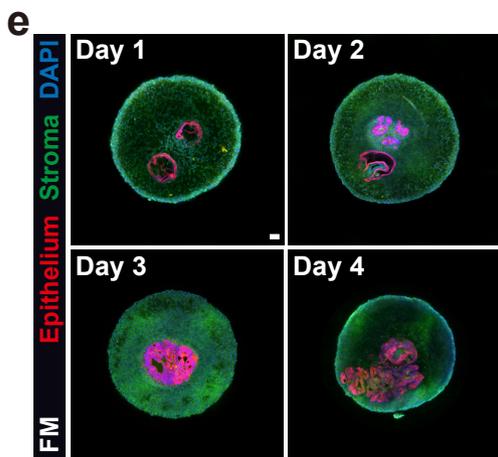
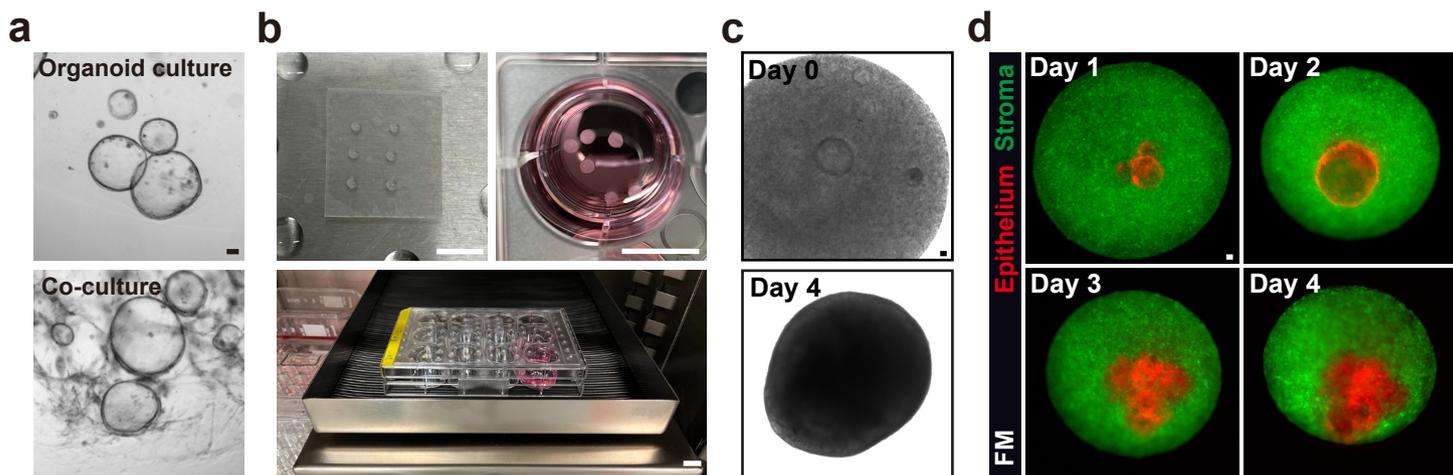
Supplementary Information

Establishment of gastrointestinal assembloids to study the interplay between epithelial crypts and their mesenchymal niche

Manqiang Lin, Kimberly Hartl, Julian Heuberger, Giulia Beccaceci, Hilmar Berger, Hao Li, Lichao Liu, Stefanie Muellerke, Thomas Conrad, Felix Heymann, Andrew Woehler, Frank Tacke, Nikolaus Rajewsky, Michael Sigal

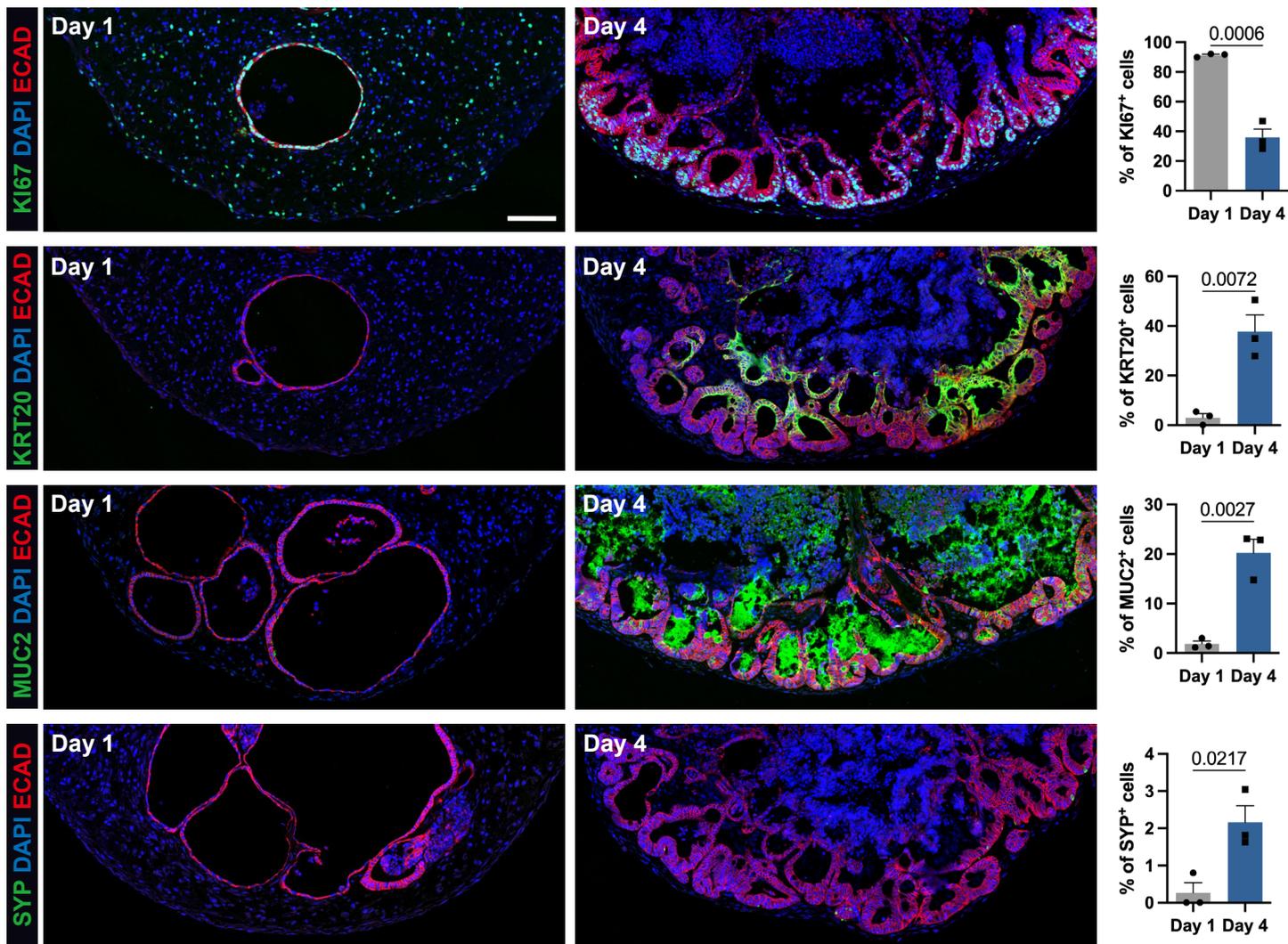
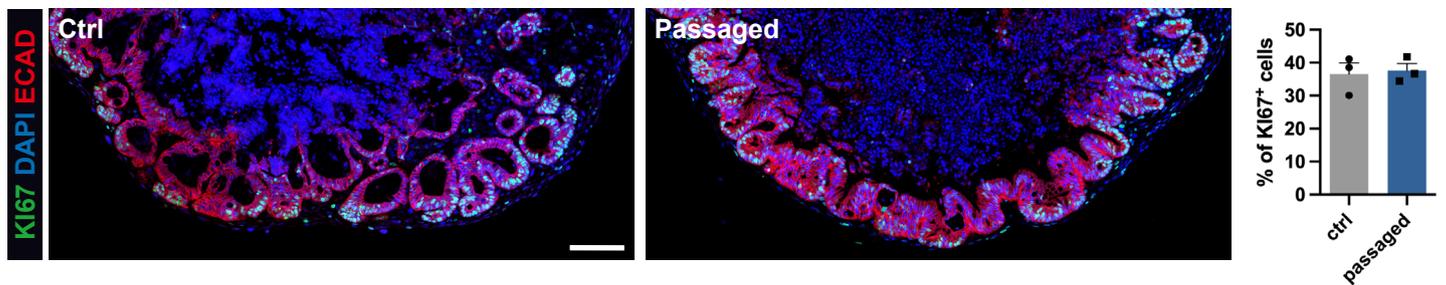
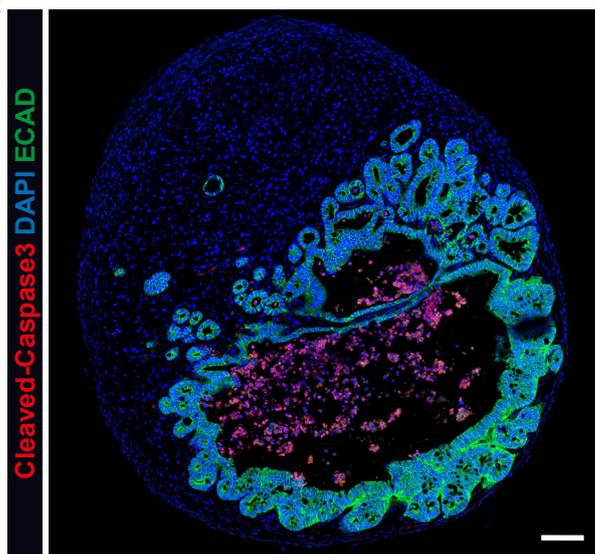
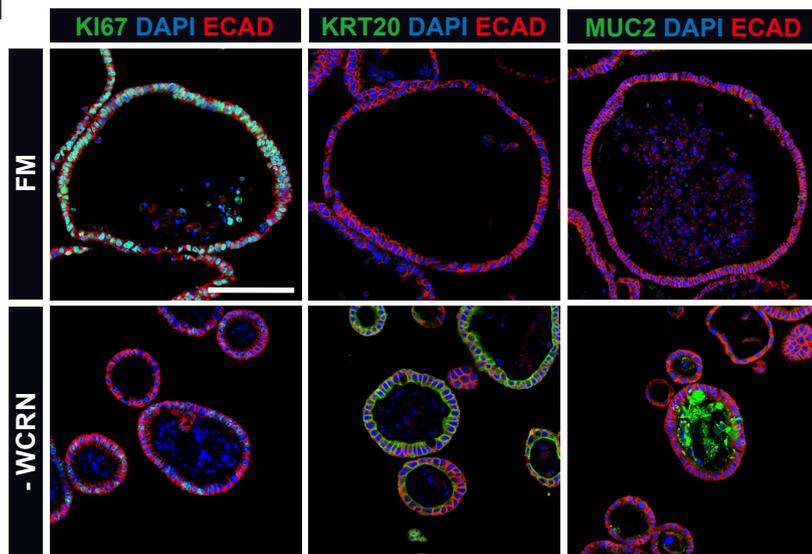
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Supplementary Figures 1-8

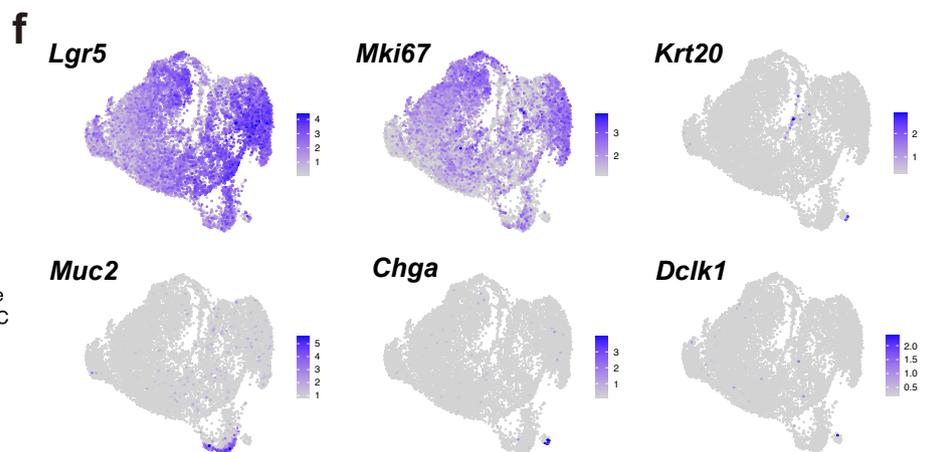
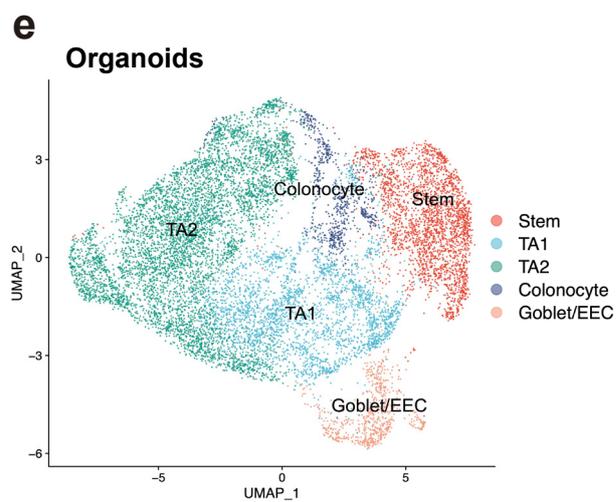
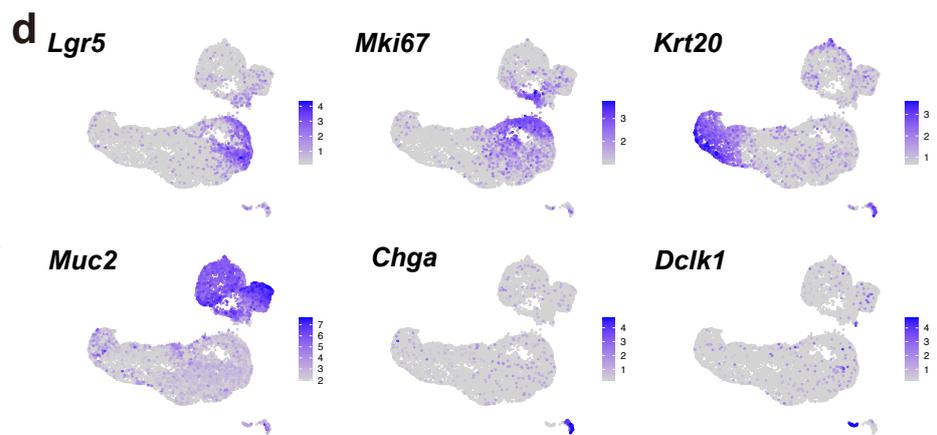
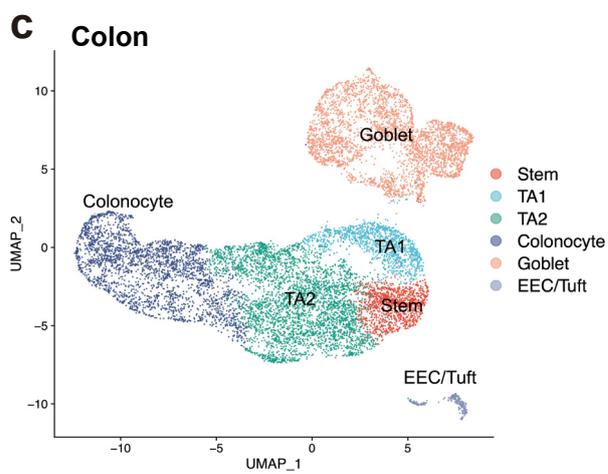
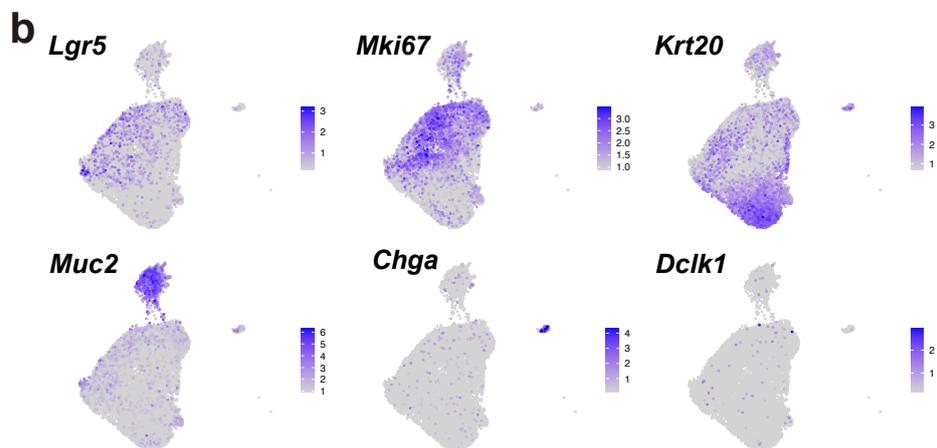
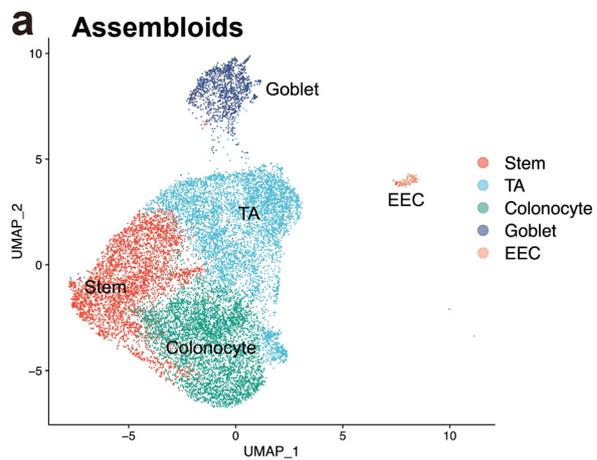


Supplementary Figure 1. Establishment of colon assembloid culture system. (a)

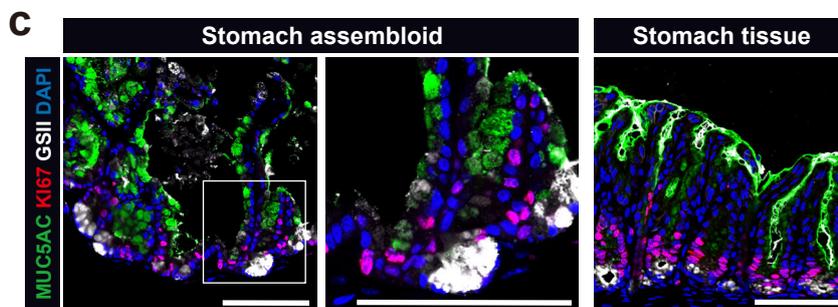
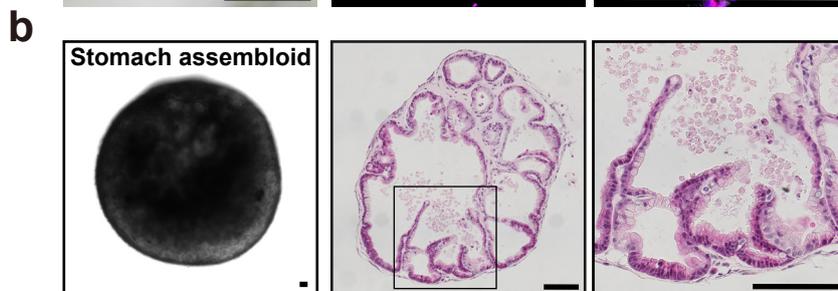
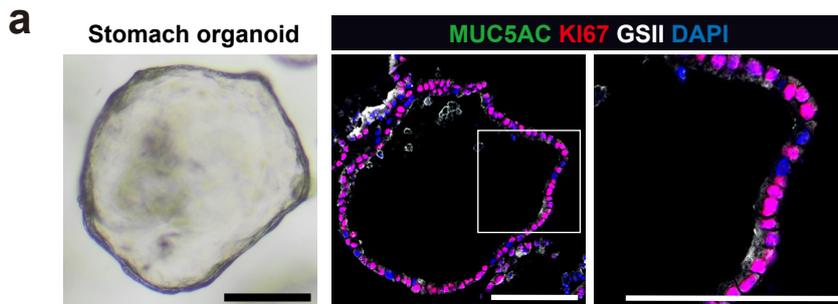
Bright-field images of conventional colon organoid culture and organoids co-cultured with primary colon stromal cells. Scale bar: 100 μm . **(b)** Upper left panel: droplets of assembloids were placed on the Parafilm. Upper right panel: assembloids were cultured in a 12-well plate. Lower panel: the culture plate was placed on an orbital shaker. Scale bars: 1 cm. **(c)** Bright-field images of assembloids on day 0 and day 4. Scale bar: 100 μm . **(d)** Live fluorescent images and **(e)** confocal microscopy images of assembloids cultured in full medium (FM) for 1 - 4 days. Epithelium was derived from *tg Act-DsRed* mice; stroma was derived from *tg Act-CFP* mice. Scale bars: 100 μm . **(f)** Hematoxylin and eosin (H&E) staining images of assembloids cultured in FM for 4 days. Scale bar: 100 μm . **(g)** Live fluorescent images of assembloids cultured in medium without sWnt, R-spondin 1, CHIR99021, or noggin (-WCRN) for 1 - 4 days. Epithelium was derived from *tg Act-DsRed* mice; stroma was derived from *tg Act-CFP* mice. Scale bar: 100 μm . **(h)** Immunofluorescence images of colon tissue and organoids stained for active YAP1. Scale bar: 100 μm . Images are representative of at least three biological replicates.

a**b****c****d**

Supplementary Figure 2. Epithelial cell organization in assembloids. (a) Immunofluorescence images and quantification of colon assembloids on days 1 and 4 labeled with markers for proliferative cells (KI67), colonocytes (KRT20), goblet cells (MUC2), and enteroendocrine cells (SYP) ($n = 3$ biological replicates per group). (b) Immunofluorescence images and quantification of control and passaged assembloids labeled for proliferative cells (KI67) ($n = 3$ biological replicates per group). (c) Immunofluorescence images of colon assembloids stained for cleaved-caspase 3. (d) Immunofluorescence images of colon organoids cultured in full medium (FM) or in medium without sWnt, R-spondin1, CHIR99021, or noggin (-WCRN), stained for KI67, KRT20, and MUC2. Scale bars: 100 μm . Data are presented as mean \pm SEM. Statistical analyses were performed using Student's *t*-test (two-tailed) for **a** and **b**. Images are representative of at least three biological replicates. Source data are provided as a Source Data file.

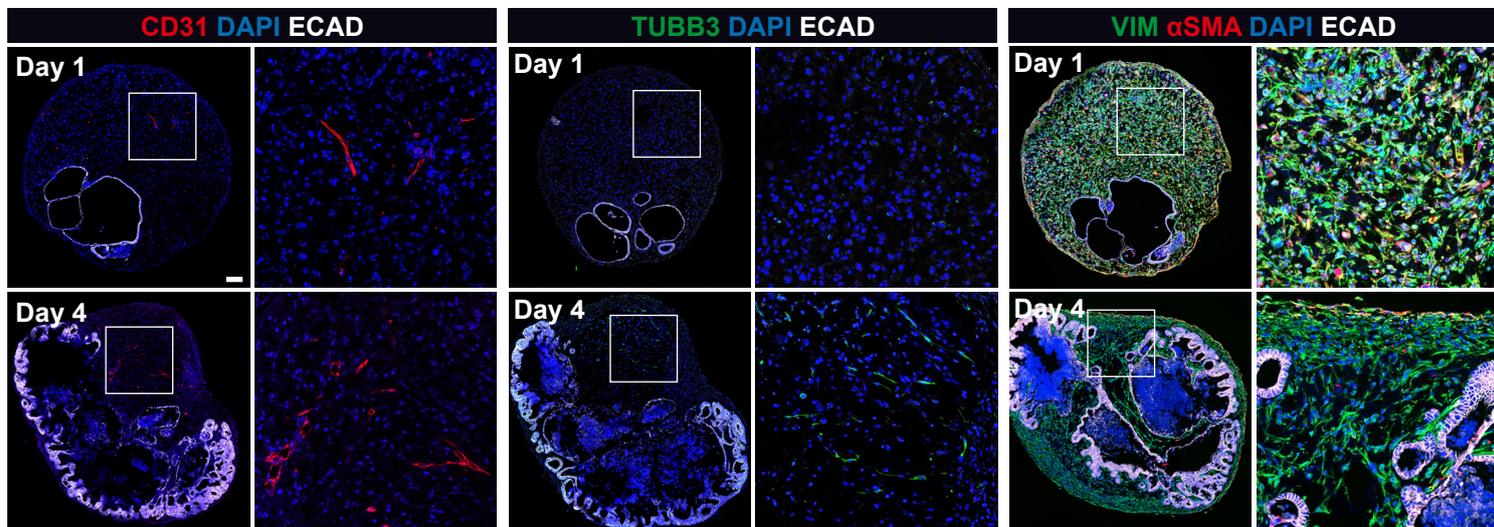
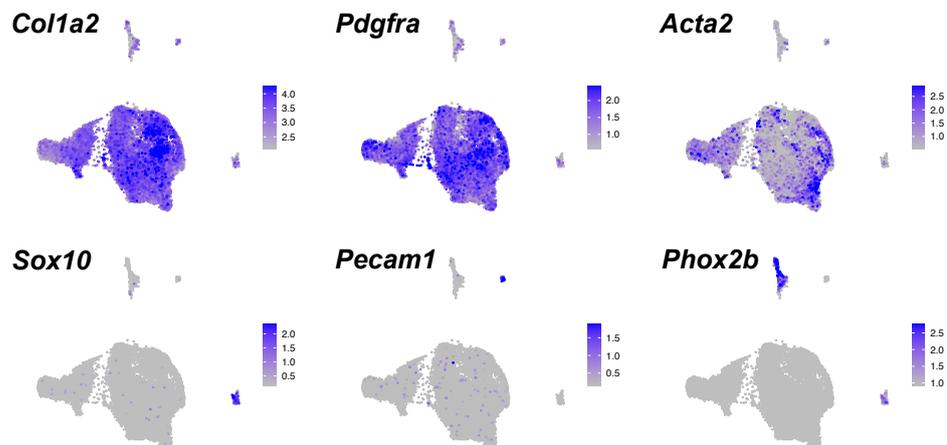
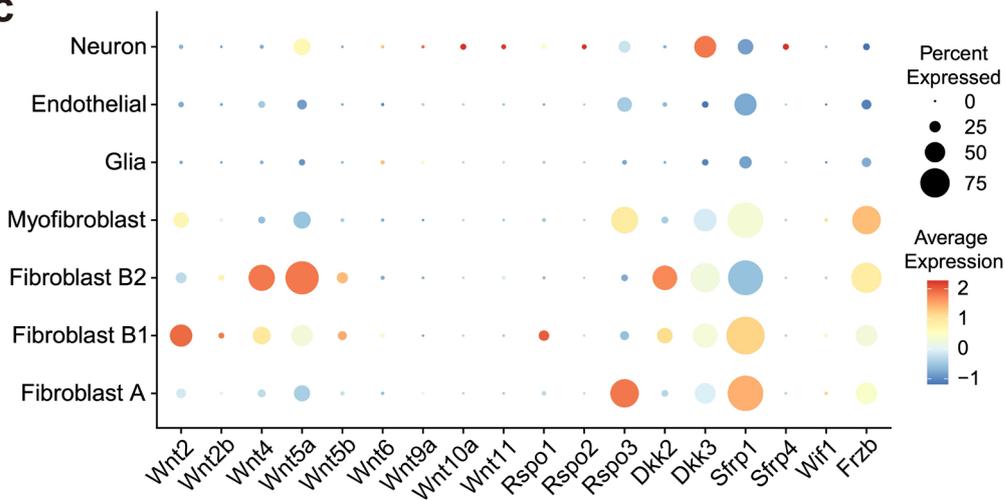


Supplementary Figure 3. Epithelial cell complexity of colon assembloids, tissue, and organoids. (a) UMAP plot of scRNA-seq dataset from epithelial cells of colon assembloids. (b) UMAP expression plots of stem cell marker gene *Lgr5*, proliferative cell marker *Mki67*, colonocyte marker *Krt20*, goblet cell marker *Muc2*, enteroendocrine cell marker *Chga*, tuft cell marker *Dclk1* in the assembloid dataset. Cells colored by normalized expression of indicated genes. (c) UMAP plot of scRNA-seq dataset from *in vivo* colon epithelial cells. (d) UMAP expression plots of marker genes of different types of epithelial cells in the dataset of colon tissue. Cells colored by normalized expression of indicated genes. (e) UMAP plot of scRNA-seq dataset from epithelial cells of colon organoids. (f) UMAP expression plots of marker genes of different types of epithelial cells in the dataset of colon organoids. Cells colored by normalized expression of indicated genes. TA, transit-amplifying cell; EEC, enteroendocrine cell. scRNA-seq data are from two biological replicates per group.



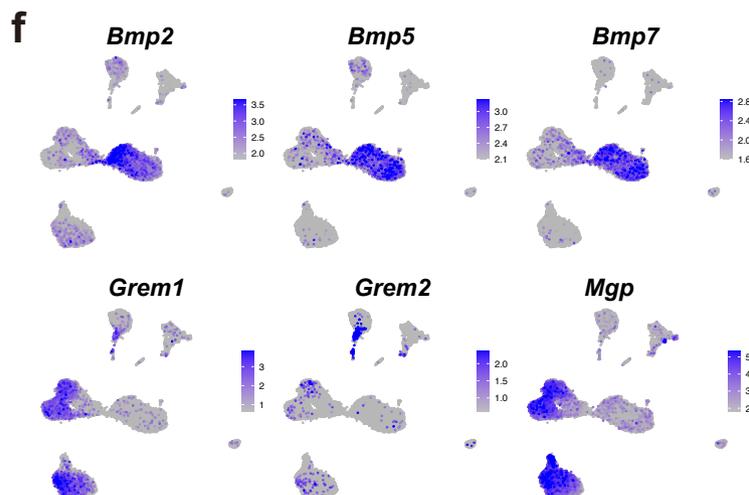
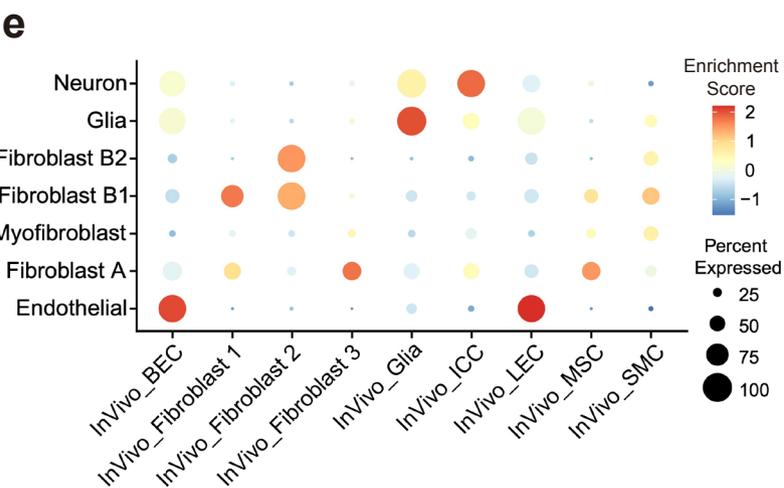
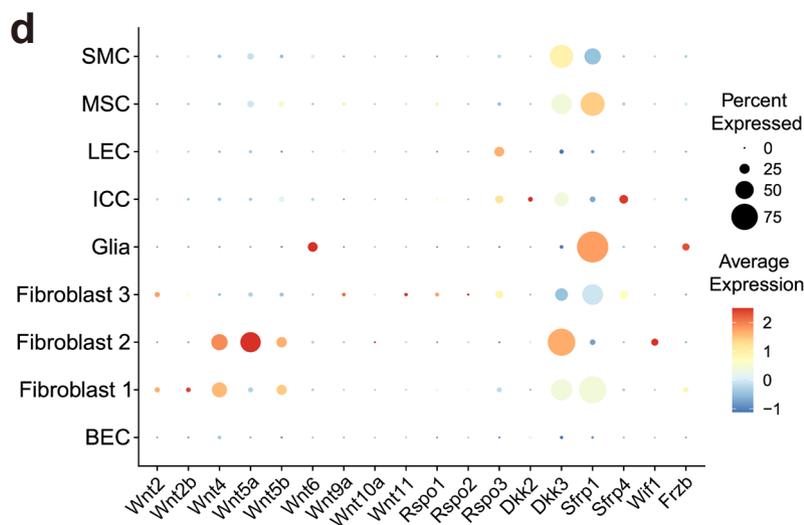
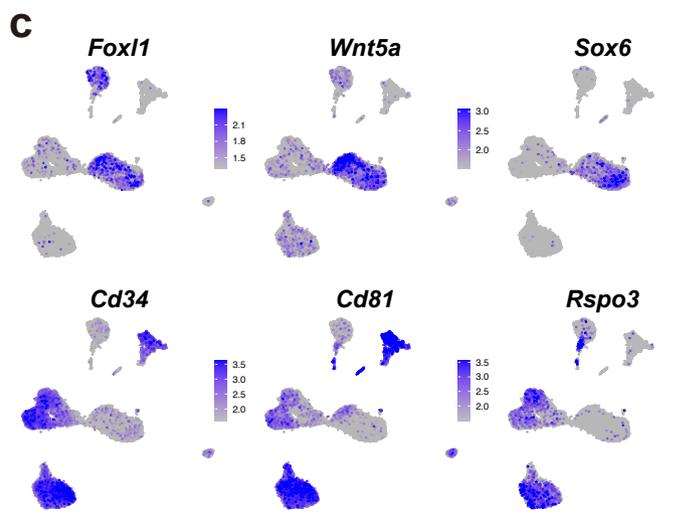
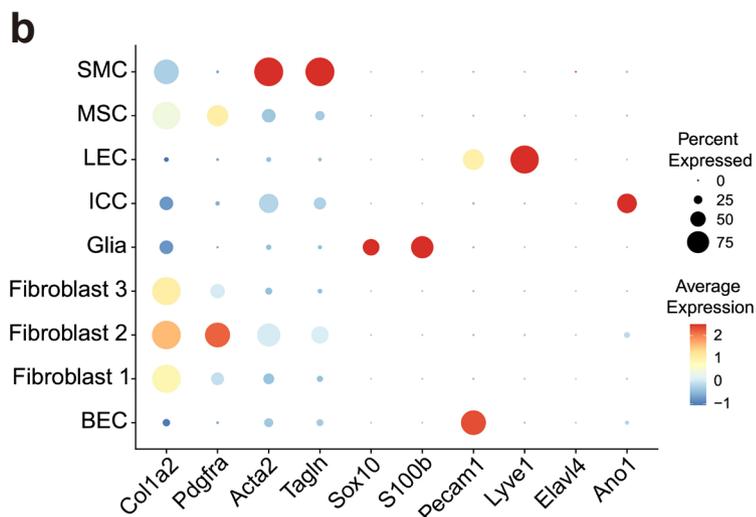
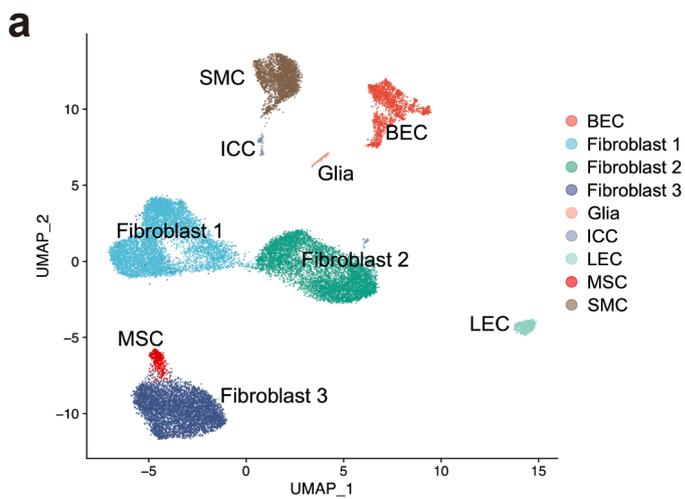
Supplementary Figure 4. Establishment of stomach assembloid culture system.

(a) Bright-field image of conventional stomach organoid culture and immunofluorescence images of stomach organoids cultured in full medium, stained for pit mucous cells (MUC5AC), proliferative cells (KI67), and base secretory cells (GSII). (b) Bright-field image and hematoxylin and eosin (H&E) staining of murine stomach assembloids (cultured for 6 days in medium without Wnt surrogate, R-spondin1, or noggin). (c) Immunofluorescence images of a stomach assembloid and stomach tissue stained for MUC5AC, KI67, and GSII. Scale bars: 100 μm . Images are representative of at least three biological replicates.

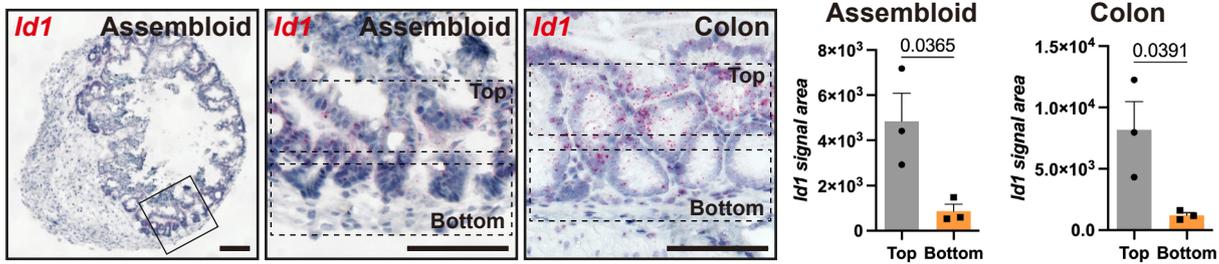
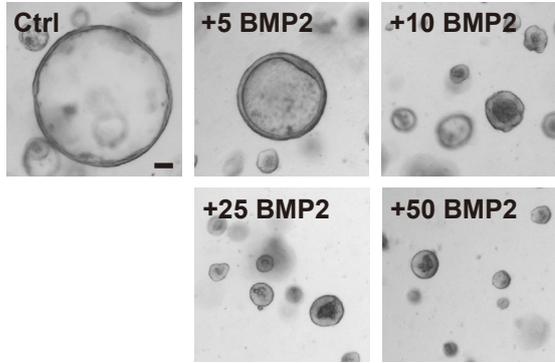
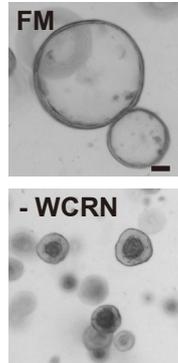
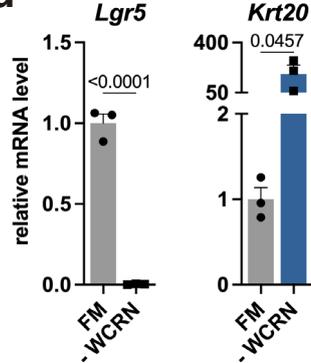
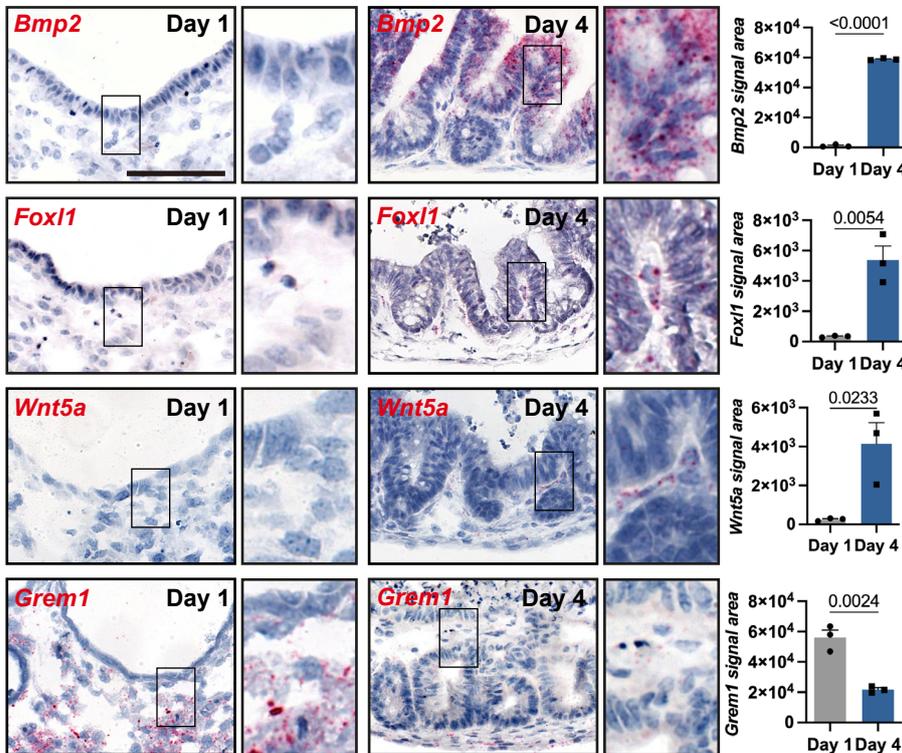
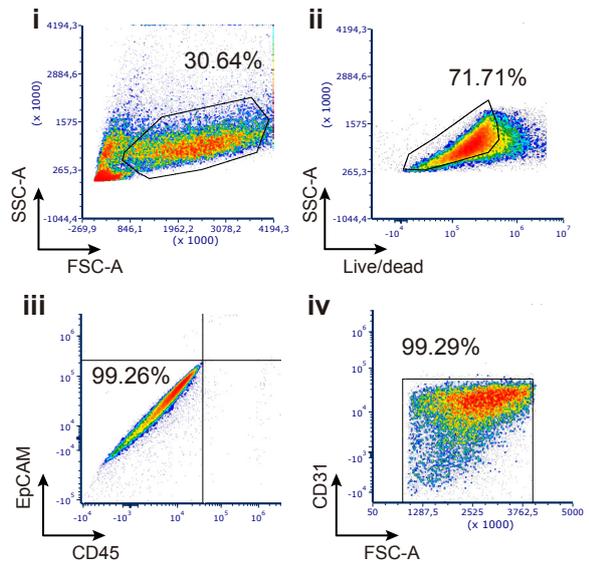
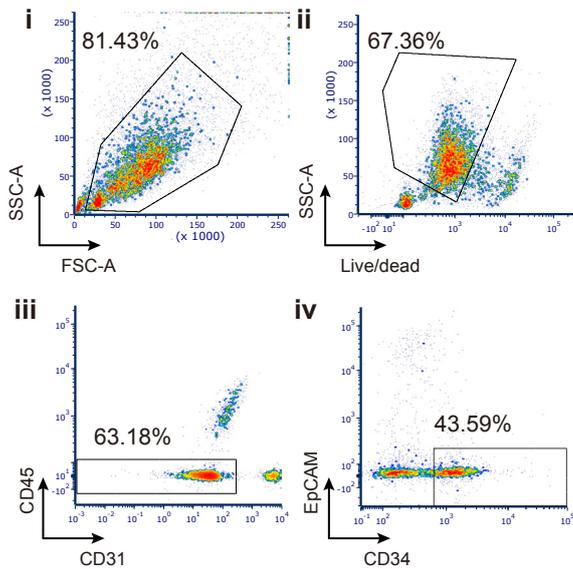
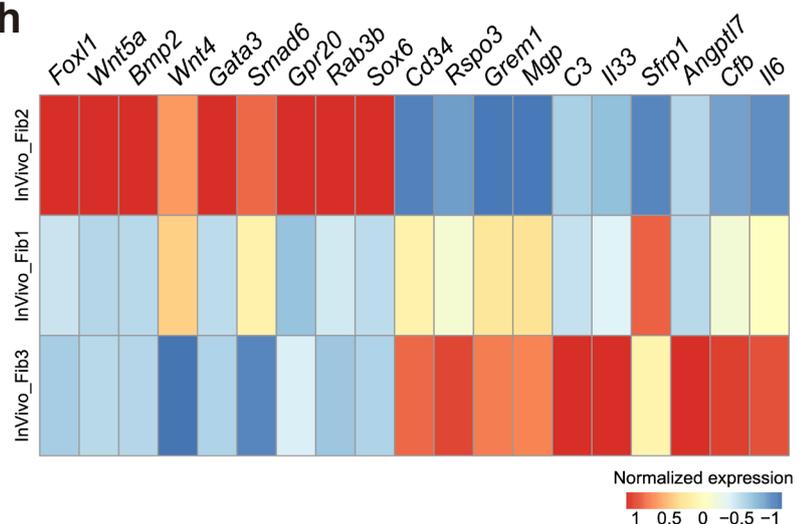
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Supplementary Figure 5. The stromal compartment in colon assembloids. (a)

Immunofluorescence images of colon assembloids (day 1 and day 4) stained for endothelial cells (CD31), neuronal cells (TUBB3), fibroblast (VIM), and myofibroblast (α SMA). Scale bar: 100 μ m. **(b)** UMAP expression plots of known marker genes against stromal cell clusters in the assembloid dataset. Cells colored by normalized expression of indicated marker genes. **(c)** Dot plot showing expression of Wnt signaling molecules in identified clusters in assembloids. Circle size represents the within-cluster probability of gene detection. Fill color represents the normalized average expression level. Images are representative of at least three biological replicates. scRNA-seq data are from two biological replicates.



Supplementary Figure 6. The stromal compartment in colon tissue. (a) UMAP plot of scRNA-seq dataset from *in vivo* colon stromal cells. (b) Dot plot showing expression of known marker genes against detected stromal clusters in colon tissue identified by scRNA-seq. Circle size represents the within-cluster probability of gene detection. Fill color represents the normalized average expression level. (c) UMAP expression plots of telocyte marker genes (*Foxl1*, *Wnt5a*, and *Sox6*) and trophocyte marker genes (*Cd34*, *Cd81*, and *Rspo3*) in the colon. Cells colored by normalized expression of indicated marker genes. (d) Dot plot showing expression of Wnt signaling molecules in identified clusters in colon tissue. Circle size represents the within-cluster probability of gene detection. Fill color represents the normalized average expression level. (e) Gene Set Variation Analysis comparing stromal cluster gene signatures between colon tissue and assembloids. Circle size represents the within-cluster probability of gene signature detection. Fill color represents the normalized average enrichment score. (f) UMAP expression plots of BMP ligand genes (*Bmp2*, *Bmp5*, and *Bmp7*) and BMP antagonist genes (*Grem1*, *Grem2*, and *Mgp*) for stromal cells in colon tissue. BEC, blood endothelial cell; ICC, interstitial cell of Cajal; LEC, lymphatic endothelial cell; MSC, mesenchymal stem cell; SMC, smooth muscle cell. scRNA-Seq data of *in vivo* mouse colon stromal cells are from published datasets GSE114374 (three biological replicates) and GSE172261 (six biological replicates).

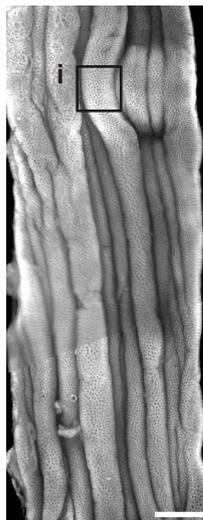
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Supplementary Figure 7. The effects of BMP signaling on colon epithelial and stromal cells.

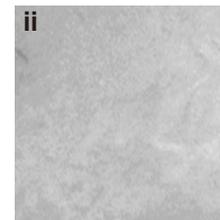
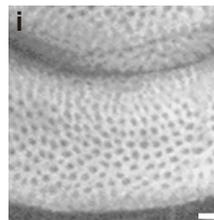
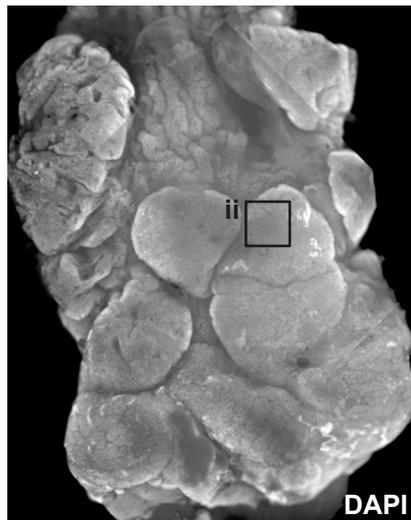
(a) Single-molecule in situ hybridization (sm-ISH) showing localization of *Id1* in assembloids and colon tissue; quantification for *Id1* signal area divided into top and bottom in the crypt ($n = 3$ biological replicates per group). (b) Bright-field images of colon organoids cultured in full medium (ctrl) or exposed to the indicated BMP2 concentrations (without noggin). (c) Bright-field images of colon organoids cultured in full medium (FM) or in medium without sWnt, R-spondin 1, CHIR99021, or noggin (-WCRN). (d) qPCR for expression of indicated genes from organoids cultured in FM or -WCRN medium ($n = 3$ biological replicates per group). (e) sm-ISH images and quantification of expression of *Bmp2*, *Foxl1*, *Wnt5a*, and *Grem1* in assembloids on days 1 and 4 ($n = 3$ biological replicates per group). (f) Representative flow cytometry gating strategies for analyses of alive (FVS450⁻) EpCAM (PE)⁻ CD45 (APC-Cy7)⁻ CD31 (APC)⁻ primary murine colonic stromal subsets. Cells were gated with forward and sideward scatter (FSV-A and SSC-A) (panel i), and dead cells were excluded by staining and gating for negative cells with FVS450 (panel ii). Immune cells and epithelial cells were excluded by gating the double negative population (CD45 (APC-Cy7)⁻/EpCAM (PE)⁻) (panel iii). Finally, endothelial cells were excluded by gating for CD31 (APC)-negative cells (panel iv). (g) Representative FACS gating strategies for sorting EpCAM⁻ CD45⁻ CD31⁻ CD34⁺ murine stromal cells. Cells were gated with forward and sideward scatter (FSV-A and SSC-A) (panel i), and dead cells were excluded by staining and gating for negative cells with FVS450 (panel ii). Immune cells and endothelial cells were excluded by gating the double negative population (CD45 (APC-Cy7)⁻/CD31 (APC)⁻) (panel iii). Finally, stromal cells were sorted by gating for the EpCAM (PE)⁻/CD34 (FITC)⁺ population (panel iv). (h) Heat map of scRNAseq data with genes differentially expressed in Fibroblast (Fib) 1, 2, and 3 of *in vivo* colon stroma.

Scale bars: 100 μm . Data are presented as mean \pm SEM. Statistical analyses were performed using Student's *t*-test (two-tailed) for **a**, **d**, and **e**. scRNA-Seq data of *in vivo* mouse colon stromal cells are from published datasets GSE114374 (three biological replicates) and GSE172261 (six biological replicates). Images are representative of at least three biological replicates. Source data are provided as a Source Data file.

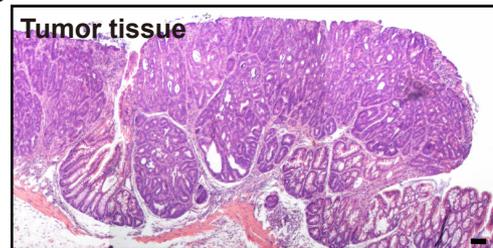
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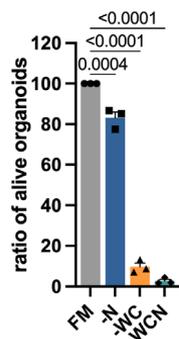
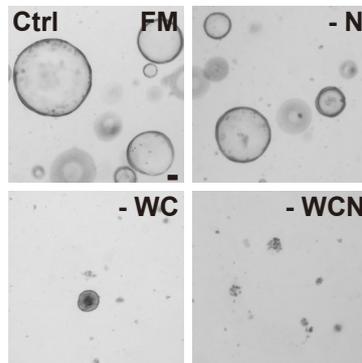
Tumor tissue



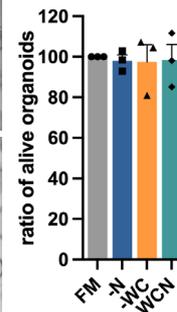
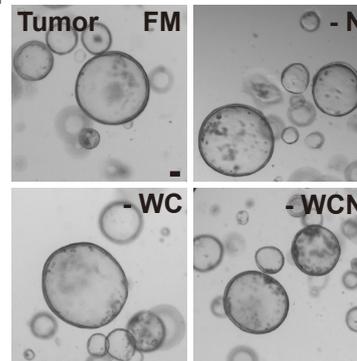
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d



Supplementary Figure 8. Establishment of AOM/DSS colitis-associated tumor model. (a) Whole-mount DAPI staining of normal colon tissue and AOM/DSS tumor tissue. Scale bars: 1 mm (high magnification: 100 μ m). (b) Hematoxylin and eosin (H&E) staining of AOM/DSS tumor tissue. Scale bar: 100 μ m. (c - d) Bright-field images and quantification of normal colon organoids (Ctrl) and colon tumor organoids cultured in full medium (FM), noggin-free (-N), sWnt-CHIR99021-free (-WC), or sWnt-CHIR99021-noggin-free (-WCN) media ($n = 3$ biological replicates per group). Scale bar: 100 μ m. Data are presented as mean \pm SEM. Statistical analyses were performed using Student's *t*-test (two-tailed) for c and d. Images are representative of at least three biological replicates. Source data are provided as a Source Data file.