Supporting Information

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SI Materials and Methods

Tumor Cell Preparation. A total of 117 colorectal cancer samples were obtained from 107 patients at the Osaka Medical Center for Cancer and Cardiovascular Diseases (Table S1). The study protocol was approved by the local ethics committee. Surgical specimens and endoscopic biopsy specimens were minced into 2-mm cubes using forceps or razor blades and washed several times with HBSS. Tumor fragments were digested with 0.26 U/mL Liberase DH solution (Roche Diagnostics) at 37 °C for 2 h. The partially digested tissue was filtered through a 500-μm mesh metal filter and a 100-μm mesh filter (Becton Dickinson). The filtrate was refiltered through a 40-μm cell strainer. The tumor tissue retained in the strainer was collected, washed twice with HBSS, and transferred to stem cell medium [StemPro hESC medium (Invitrogen) supplemented with 8 ng/mL bFGF (Invitrogen)]. The detailed method is described herein.

CTOS Culture. CTOSs were cultured in suspension in stem cell medium. For 3D culture, the CTOSs were embedded in Cellmatrix type I-A (Nitta Gelatin) droplets on untreated tissue culture dishes and overlaid with stem cell medium. The medium was changed two to three times a week. For further analysis or passage, CTOSs were released from the Cellmatrix after 1–2 wk of cultivation by incubation with 0.2 mg/mL collagenase type 4 (Worthington). For expansion, CTOSs were cut into cell clusters using 23-gauge needles, and the cell clusters were transferred to fresh StemPro medium. The next day, the newly formed CTOSs were again embedded in Cellmatrix droplets, and culture was repeated. The serum-containing medium was DMEM (Invitrogen) supplemented with 10% FBS (Gibco). The detailed method is described herein.

Animal Studies. Animal studies were performed in compliance with the guidelines of our institutional animal studies committee. For primary colon cancer transplantation, tissue specimens were minced with scissors into ∼2-mm cubes and implanted s.c. in the flanks of NOD/SCID mice (Charles River Laboratories). For implantation, CTOSs were injected s.c. in the flanks of the mice. Recipient mice were anesthetized by i.p. injection of pentobarbital before implantation or injection. Mice were observed for up to 6 mo and killed when tumors reached 10 mm in diameter. The number of sites with tumors is shown as a fraction of the number of inoculated sites (Table S2). CTOS preparation from xenograft tumors was performed as described for primary human tumors.

Cell Lines. HCT116 and WiDr cells were obtained from ATCC. HCT116 spheroids were generated by suspension culture. Cells were harvested by tryptic dissociation, and suspended in serum containing medium on nontreated dish. For implantation, HCT116 or WiDr cells were injected s.c. in the flanks of the mice.

Flow Cytometry Analysis. CTOSs were washed with PBS and treated briefly with 0.25% trypsin/EDTA. Dispersed cells were washed with HBSS containing 7 mg/mL trypsin inhibitor (Sigma). Antibodies used were EpCAM, CD133 (Miltenyi), CD45, CD31, and mouse IgG isotype control (BD Pharmingen). Cells were incubated in HBSS containing 0.5% BSA with fluorescence-conjugated primary antibodies at 4 °C in the dark for 30 min. Isotype-matched mouse Ig served as the control. Samples were analyzed using a FACSCalibur flow cytometer (BD Biosciences) and FlowJo software (Tree Star).

Apoptosis Detection. Cell-surface annexin-V expression and propidium iodide (PI) uptake were detected by flow cytometry or fluorescence microscopy (Olympus) using the Annexin V-FITC Apoptosis Detection Kit (Biovision). Western blotting was performed as described previously (1). The antibodies used were caspase-3, cleaved caspase-3, Akt, pAkt(473), Erk, pErk (Cell Signaling), and PARP (BD Pharmingen). The ApopTag Red in Situ Apoptosis Detection Kit (Chemicon) was used for TUNEL staining according to the manufacturer's instructions.

Immunohistochemistry. Immunohistochemistry was performed as described previously (2). The primary antibodies used for immunohistochemistry were E-cadherin (Takara), β-catenin (BD Bioscience), α-SMA (Sigma), CD68 (DAKO), villin (Santa Cruz), and TP-53 (Novocastra). Antigen retrieval was performed on formalin-fixed paraffin-embedded samples by microwaving samples in 10 mM citrate buffer (pH 6.0). After incubation with secondary antibodies and treatment with the Vectastain ABC Kit (Vector Laboratories), peroxidase activity was visualized with Vector NovaRED (Vector Laboratories). The sections were counterstained with hematoxylin. Frozen tissue samples were fixed with acetone/methanol. After incubation with Alexa-488– or Alexa-594–conjugated secondary antibody (Molecular Probes), the sections were examined by fluorescence microscopy.

Cell–Cell Contact Inhibition. For enforced enzymatic disperse, CTOSs were treated briefly with 0.25% trypsin/EDTA. Cells were then cultured in StemPro medium and collected at the indicated time points. For inhibition assays of E-cadherin–dependent cell–cell contact, CTOSs were precultured in Cellmatrix gel. CTOSs were removed from gel by treatment with 0.1 mg/mL collagenase type 4 (Worthington) for 1 h and washed with PBS. Then CTOSs were suspended in fresh medium containing anti–E-cadherin neutralizing antibody (clone SHE 78-7; Takara) or mouse IgG isotype control (Sigma), and collected at the indicated time points. Collected cells or CTOSs were washed once with cold PBS and analyzed by Western blotting.

Evaluation of CTOS Growth and Passage. Growth was evaluated after CTOSs were cultured in Cellmatrix type I-A for 1 wk. The CTOS growth ratio was calculated as follows: (major axis length) × (minor axis length) after cultivation/(major axis length) × (minor axis length) before cultivation. Successful growth was achieved if the CTOS growth ratio was >2. Successful passage was achieved when the CTOS formed a secondary CTOS after mechanical dissociation, and this secondary CTOS achieved successful growth. Table 1 summarizes the CTOS formation, growth, and passage results.

Genomic Analysis. Mutations in exon 2 of *KRAS* and exon 13 of *BRAF* were detected by direct sequencing. DNA was extracted from CTOSs or paraffin-embedded samples of parental tumors using the DNeasy Blood and Tissue Kit (QIAGEN). The region spanning the mutation sites was amplified by PCR using the following primers: *KRAS*: 5′-gcctgctgaaaatgactgaa-3′ and 5′-agaatggtcctgcaccagtaa-3′; and *BRAF*: 5′-tgcttgctctgataggaaaatg-3′ and 5′-ccacaaaatggatccagaca-3′. Sequencing was performed using the BigDye Terminators v1.1 Cycle Sequencing Kit and ABI PRIZM 3100 Genetic Analyzer (Applied Biosystems). Detection of a human- or mouse-specific genome was performed by PCR using the following primers: human *B2M*: 5′-accccactgaaaaagatga-3′ and 5′-ccctgacaatcccaatatgc-3′; and mouse *B2M*: 5′ -caccggagaatgggaagccgaa-3′ and 5′- tccacacagatggagcgtccag-3′.

Inhibition of the PI3K/AKT Signaling Pathway. To evaluate the influence of PI3K inhibition on growth, CTOSs were cultured in the presence or absence of LY294002 (PI3K inhibitor; Calbiochem) at the indicated concentrations. After 7 d of cultivation, growth ratios were calculated. For assessing intracellular pathway inhibition by LY294002, CTOSs were cultured overnight in DMEM/F12 with 1% BSA, incubated with or without LY294002 at the indicated concentrations for 1 h, and then incubated with or without StemPro supplementation for another 1 h. The CTOS lysates were analyzed by Western blotting using the primary antibodies indicated above.

Protocol for CTOS Preparation.

i) Place the tissue sample in 20 mL of DMEM (11965-092; Gibco) supplemented with 100 units/mL penicillin (15140-122; Gibco) and 100 μg/mL streptomycin (15140-122; Gibco) in a 50-mL centrifuge tube on ice (2345-050; Iwaki) immediately after tumor resection or biopsy.
ii) Store the specimen at 4 °C until ready to proceed. It is critical to start the following steps as soon as possible.
iii) Discard the storage medium.
iv) Wash the samples with 20 mL HBSS (14025-092; Gibco) by inverting the tube.
v) Discard the HBSS wash solution.
vi) Add 20 mL HBSS.
vii) Transfer the medium and the samples to a 10-cm tissue culture dish (3020-100; Iwaki).
viii) Remove necrotic tissue using forceps or razor blades.
ix) Transfer the samples to 30 mL HBSS in a new 10-cm tissue culture dish.
x) Mince the tissue with forceps or razor blades into small (1–2 mm) pieces.
xi) Transfer the medium and the minced tissue to a 50-mL centrifuge tube.
xii) Centrifuge at 1,000 rpm (200 × *g*) at 4 °C for 5 min.
xiii) Discard the medium.
xiv) Wash the samples with 20 mL HBSS by inverting the tube.
xv) Repeat steps 12–14.
xvi) Centrifuge at 1,000 rpm (200 × *g*) at 4 °C for 5 min.
xvii) Discard the HBSS wash solution.
xviii) Resuspend the pellets in 20 mL DMEM supplemented with 100 units/mL penicillin, 100 μg/mL streptomycin, and 0.28 units/mL Liberase DH (05401054; Roche).
xix) Transfer the digestion mixture to a 100-mL sterile conical flask with a magnet bar (Fig. S6*A*).
xx) Digest the samples for 2 h in a 37 °C water bath with constant stirring [Immersible Magnetic Stirrer and Controller from Scinics (MS-101, MC-303)] (Fig. S6*B*).
xxi) Transfer the digestion medium to a 50-mL centrifuge tube.
xxii) Centrifuge at 1,000 rpm (200 × *g*) at 4 °C for 5 min.
xxiii) Discard the medium.
xxiv) Wash the samples with 20 mL HBSS by inverting the tube.
xxv) Filter the samples with a stainless steel wire mesh (hole size 500 μm; Fig. S6*C*).
xxvi) Transfer the filtrate to a 50-mL centrifuge tube.
xxvii) Filter the samples with a 40-μm cell strainer (352340; BD Biosciences).
xxviii) Dip the bottom of the cell strainer in 30 mL HBSS in a 10-cm tissue culture dish; swirl it gently to remove the debris, the single cells, and the cell clumps with diameters <40 μm (Fig. S6*D*).
xxix) Transfer the cell strainer to a new 10-cm tissue culture dish containing 30 mL HBSS.
xxx) Collect the organoids that remain in the cell strainer using a Pipetman (F123602; Gilson; Fig. S6*E*).
xxxi) Centrifuge at 1,000 rpm (200 × *g*) at 4 °C for 5 min.
xxxii) Discard the medium.
xxxiii) Wash the samples with 20 mL HBSS by inverting the tube.
xxxiv) Centrifuge at 1,000 rpm (200 × *g*) at 4 °C for 5 min.
xxxv) Discard the HBSS wash solution.
xxxvi) Add 4 mL serum-free stem cell medium [StemPro hESC SFM (A10007-01; Gibco) supplemented with 8 ng/mL bFGF (13256-029; Invitrogen), 0.1 mM 2-mercaptoethanol (137-06862; Wako), 100 units/mL penicillin, 100 μg/mL streptomycin, and 25 μg/mL amphotericin B (541-01961; Wako)].
xxxvii) Transfer the organoids and medium to a 6-cm nontreated dish [(AG2000) Eiken Chemical or (1010-060) Iwaki].
xxxviii) Incubate in a 5% CO_2-humidified chamber at 37 °C for 24 h.
xxxix) View under a phase contrast microscope; CTOSs appear as bright, smooth spheres.

Note: Use stainless steel wire mesh (hole size 250 μm) or a 100-μm cell strainer (352360; BD Biosciences), depending on the desired CTOS size (Fig. S6*F*).

Protocol for CTOS Expansion.

i) Prepare a collagen solution by mixing the following: A, Cellmatrix type I-A (Nitta Gelatin); B, 5× DMEM (12100-038; Gibco); C, reconstitution buffer (50 mM NaOH, 260 mM NaHCO3, 200 mM Hepes). A:B:C = 7:2:1. After mixing A and B well, add C and again mix well. Keep the reconstituted collagen solution on ice to prevent gel formation.
ii) Pour 100 μL reconstituted collagen solution onto a 3.5-cm nontreated dish (1000-035; Iwaki) to create a gel base (Fig. S6*G*).
iii) Allow the gel to solidify at 37 °C for 30 min.
iv) After gel formation, place the dish on ice.
v) Using 30 μL reconstituted collagen solution, add a second (upper) gel layer to the gel base prepared in steps 3 and 4 (Fig. S6*H*).
vi) Using a microscope for observation, gently pick up CTOSs (40–100 μm in diameter) using a Pipetman (F144801; Gilson). Typically, a 2-μL aliquot of culture medium will contain 1–10 CTOSs.
vii) Put the CTOSs into the upper gel layer, pipetting gently to disperse the CTOSs evenly.
viii) Allow the gel to solidify at 37 °C for 30 min.
ix) Add 3 mL serum-free stem cell medium [StemPro hESC SFM (A10007-01; Gibco) supplemented with 8 ng/mL bFGF (13256-029; Invitrogen), 0.1 mM 2-mercaptoethanol (137-06862; Wako), 100 units/mL penicillin, 100 μg/mL streptomycin, and 25 μg/mL amphotericin B (541-01961; Wako)].
x) Incubate in a 5% CO_2-humidified chamber at 37 °C for 2–3 wk until the diameters of the CTOSs are ∼250 μm. Change the medium every 3 d.
xi) Discard the medium.
xii) Digest the gels with 3 mL DMEM (11965-092; Gibco) supplemented with 0.2 mg/mL collagenase type 4 (4186; Worthington) at 37 °C for 1 h.
xiii) Release the CTOSs from the digested gel by pipetting up and down.
xiv) Transfer the CTOS suspension to a 15-mL centrifuge tube.
xv) Add 10 mL PBS and mix gently.
xvi) Centrifuge at 1,000 rpm (200 × *g*) for 2 min.
xvii) Discard the supernatant.
xviii) Add 5 mL HBSS containing 1% BSA and resuspend the CTOSs by pipetting; transfer them to a 60-mm nontreated culture dish (1010-060; Iwaki).
xix) Using microscopic observation, tear CTOSs using two sterile 23-gauge needles. One needle (held with the nondominant hand) holds the CTOS steady while a needle in the dominant hand tears the CTOS. Tear each CTOS into two to four pieces.

xx) Add 3 mL serum-free stem cell medium to a new 3.5-cm nontreated dish.

xxi) Pick up the CTOS fragments with a pipette and transfer them to the dish prepared in step 20.

xxii) Incubate in a 5% CO_2-humidified chamber at 37 °C overnight.

xxiii) Use a phase contrast microscope to view the CTOSs, which appear as bright, smooth spheres.

1. Kusama T, et al. (2001) Inhibition of epidermal growth factor-induced RhoA translocation and invasion of human pancreatic cancer cells by 3-hydroxy-3-methylglutaryl-coenzyme a reductase inhibitors. *Cancer Res* 61:4885–4891.

2. Ohue M, et al. (1994) A frequent alteration of p53 gene in carcinoma in adenoma of colon. *Cancer Res* 54:4798–4804.

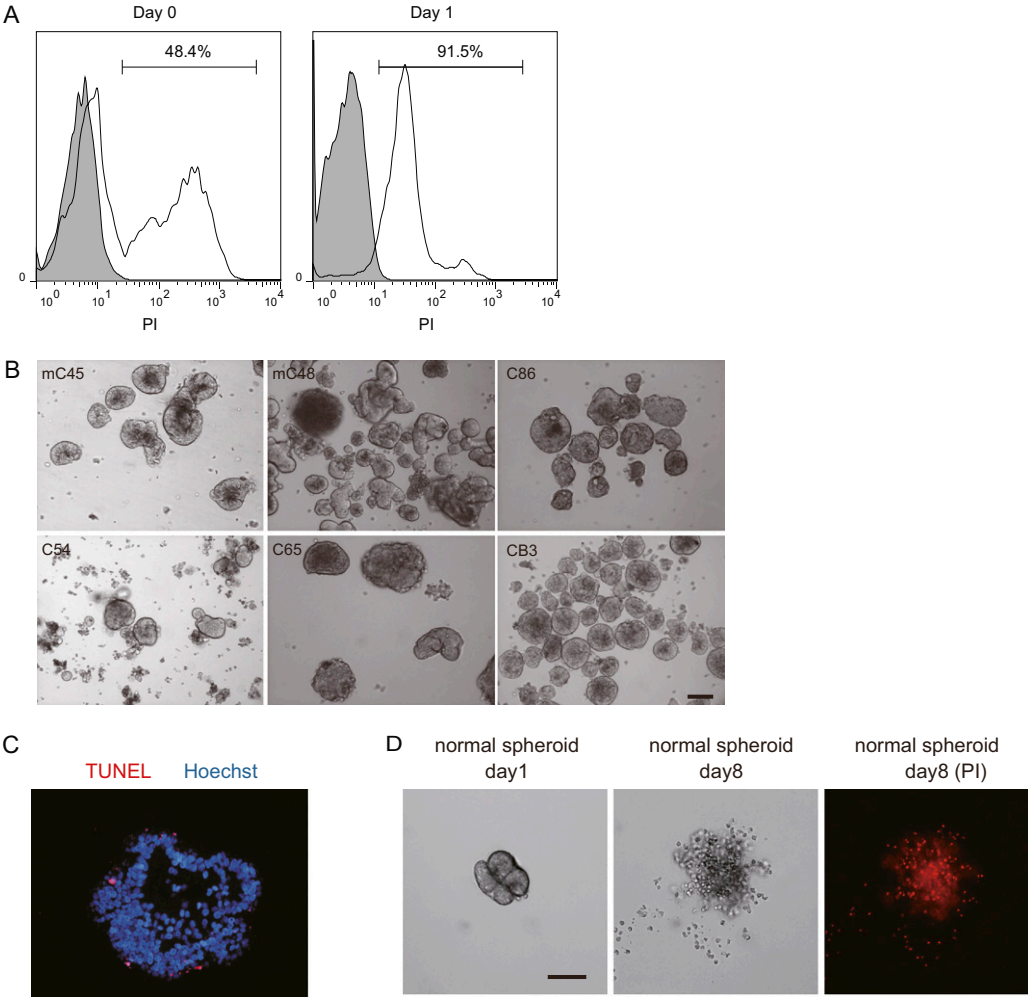


Fig. S1. Preparation of CTOSs and FT cells. (*A*) Low viability of EpCAM+ cells in the FT under suspension culture conditions. PI stainability among EpCAM-gated cells was evaluated by flow cytometry. The results of PI-treated cells are indicated with a line curve, and those of control cells are with a filled curve. (*B*) Formation of CTOSs from colorectal cancers from the indicated patients and from the endoscopic biopsy specimen CB3. Phase contrast images of CTOSs on day 1 are shown. (Scale bar: 100 μm.) (*C*) TUNEL (red) and Hoechst 33342 (blue) staining of a CTOS. (*D*) Phase contrast picture of a spheroid prepared from normal colon mucosa at day 1 (*Left*). The spheroid was cultured under the CTOS culture conditions. The images of the same spheroid at day 8 after preparation: phase contrast (*Center*) and PI staining (*Right*). (Scale bar: 100 μm.)

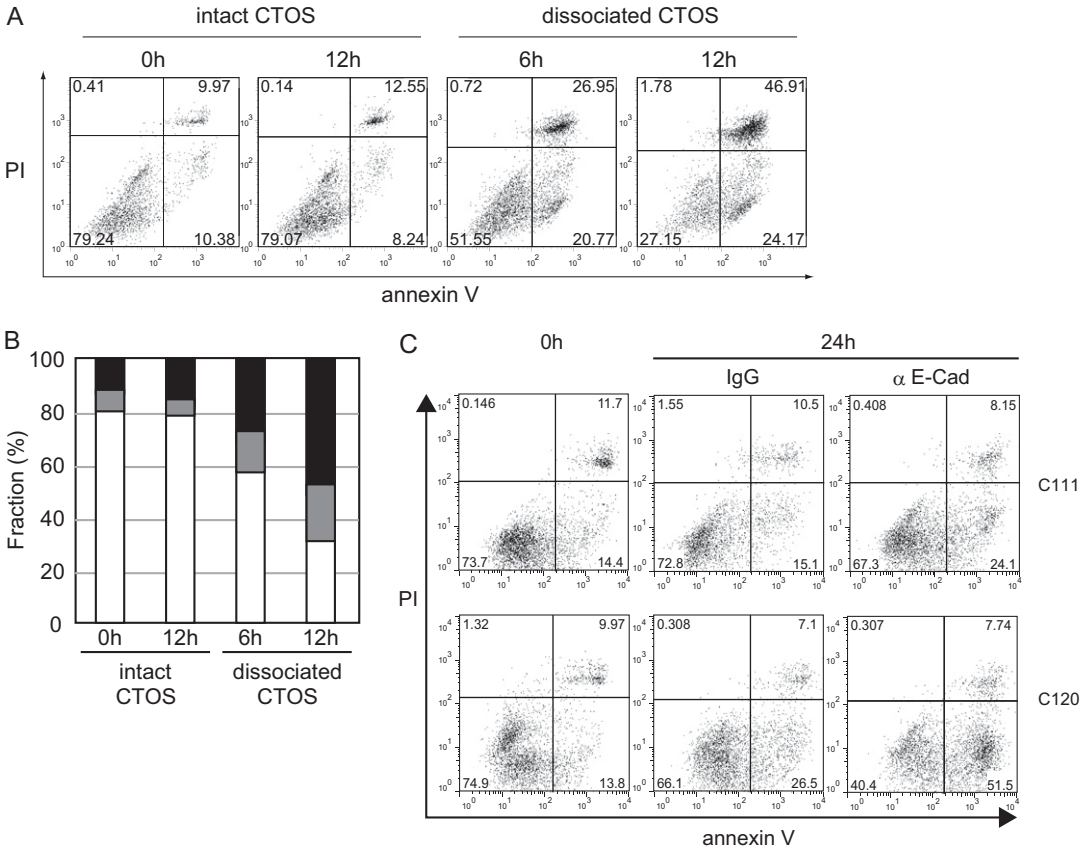
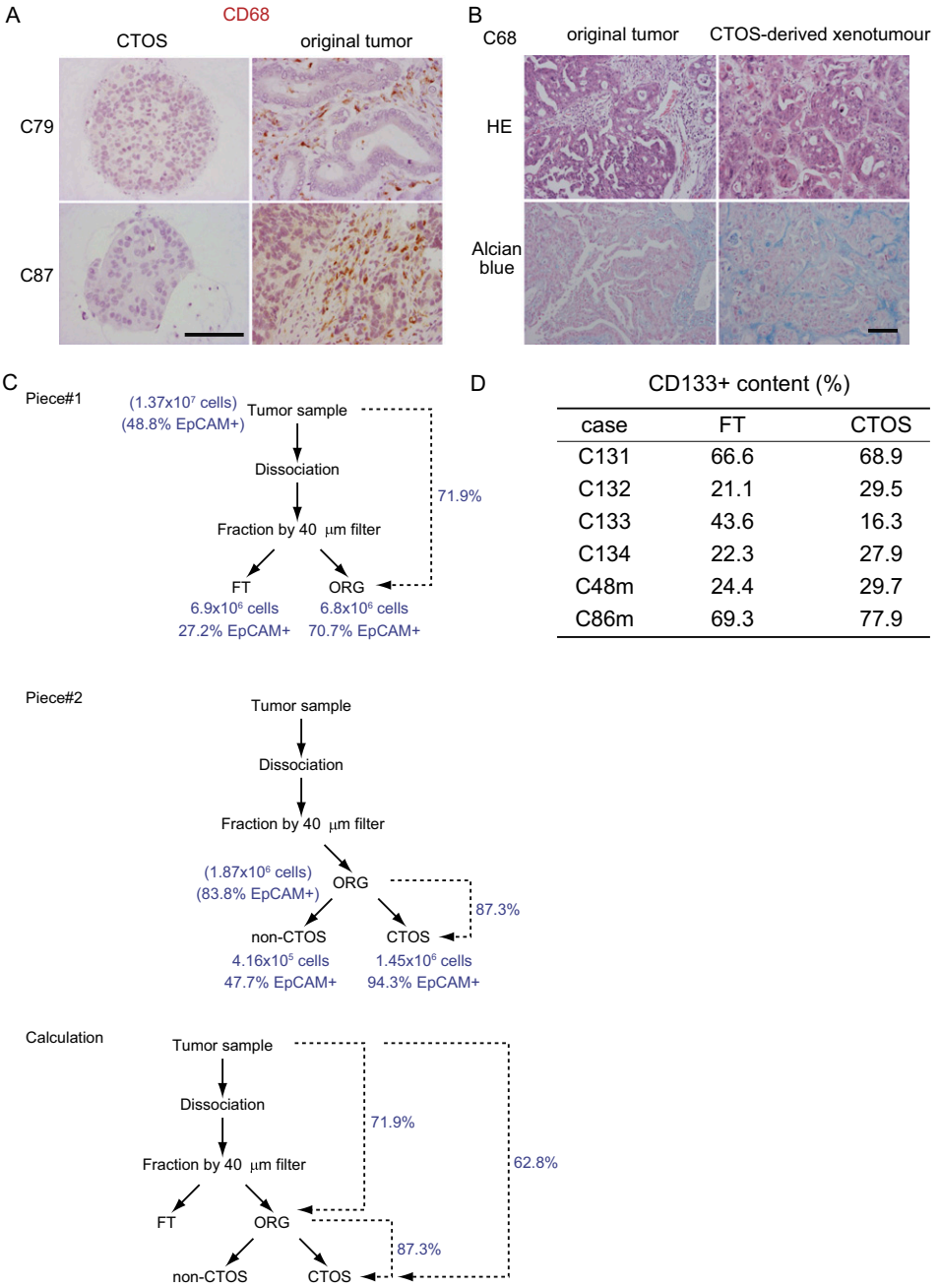


Fig. S2. CTOSs retaining cell–cell contact exhibit high viability. (*A* and *B*) Flow cytometric analysis of double-stained cells (PI and annexin V). The experiments were performed with the CTOSs from five different patients, and the representative data (C75) is shown. (*A*) Intact CTOSs were cultured for the indicated time and analyzed right after dissociation. Alternatively, CTOSs were dissociated and single cells were analyzed after culture as indicated. (*B*) Proportion of cells in *A* that were positive for both annexin V and PI (black), positive for annexin V only (gray), and negative for both (white). (*C*) Flow cytometric analysis of the indicated CTOSs treated with the E-cadherin neutralizing antibody. Percentages of the cell population are shown in each quadrant.

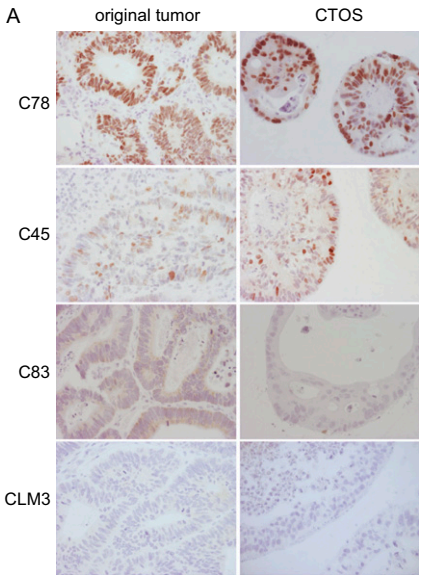


D

CD133+ content (%)

case	FT	CTOS
C131	66.6	68.9
C132	21.1	29.5
C133	43.6	16.3
C134	22.3	27.9
C48m	24.4	29.7
C86m	69.3	77.9

Fig. S3. CTOSs preserve characteristics of parental tumors. (*A*) CD68 staining of tumors and derived CTOSs from two patients. (Scale bar, 100 μm.) (*B*) H&E staining and Alcian blue staining of tumors from the indicated patients (*Left*) and the derived xenotumor (*Right*). (C) Schematic illustration of the process for assessing the recovery rate of EpCAM+ cells in CTOSs. As an example, the results from the C86 xenograft tumor are presented. Tumor specimens were divided into two pieces. Both pieces were mechanically and enzymatically digested, as described in *SI Materials and Methods*, and fractionated with a 40-μm mesh filter. Piece 1: Organoids were dispersed with 0.25% trypsin/EDTA. The cell number of each fraction, FT or ORG, was determined using a hemocytometer. Total cell number was calculated as a sum of the number of FT and ORG cells. The rate of EpCAM+ cells in the FT or ORG was evaluated by FACS analysis. Then the recovery rate of EpCAM+ cells in the ORG from the original tumor was calculated. Piece 2: The ORG was cultured for 1 d in serum-free stem cell medium. The cells draping the CTOSs were detached by pipetting. A mixture of the draping cells and CTOSs was suspended in 15 mL of PBS and centrifuged at 100 × *g* for 30 s to collect CTOSs as a pellet. The supernatants were centrifuged again at 400 × *g* for 5 min to collect non-CTOS cells in a pellet. After dispersion with trypsin, the cell numbers of each fraction, non-CTOSs or CTOSs, were counted. The total cell number in the ORG was calculated as a sum of the number of non-CTOS cells and CTOS cells. The rate of EpCAM+ cells in non-CTOSs or CTOSs was evaluated by FACS analysis, and the recovery rate of EpCAM+ cells in CTOSs from the ORG was then calculated. Together, the recovery rate of EpCAM+ cells in CTOSs from the original tumor was calculated. (*D*) CD133+ fraction of EpCAM+ gated cells in FT cells and CTOSs.



B

	KRAS		
	original	CTOS	
C45	wild	G12D	←
C48	wild	wild	
C51	wild	wild	
C59	wild	wild	
C61	G13D	G13D	
C62	wild	wild	
C65	wild	wild	
C66	G12V	G12V	
C68	G12D	G12D	
C69	ND	G12D	←
C71	G12D	G12D	
C73	wild	wild	
C75	wild	G13D	←
C76	G12D	wild	←
C77	wild	wild	
C78	wild	wild	
C79	wild	wild	
C80	G12V	G12V	
C81	G12D	G12D	
C84	wild	G13D	←
C85	G13D	G13D	
C93	wild	wild	
C111	wild	wild	
CB4	wild	wild	
CB8	wild	wild	

Fig. S4. CTOSs preserve characteristics of parental tumors. (*A*) TP53 staining of patients' tumors and CTOSs. (*B*) *KRAS* mutation status of FFPE samples of original tumors and CTOSs. Blue arrows indicate cases with a detected *KRAS* mutation only in the CTOSs; red arrow indicates a case in which the *KRAS* mutation was detected only in the FFPE sample. ND, not determined due to low-quality sequencing results.

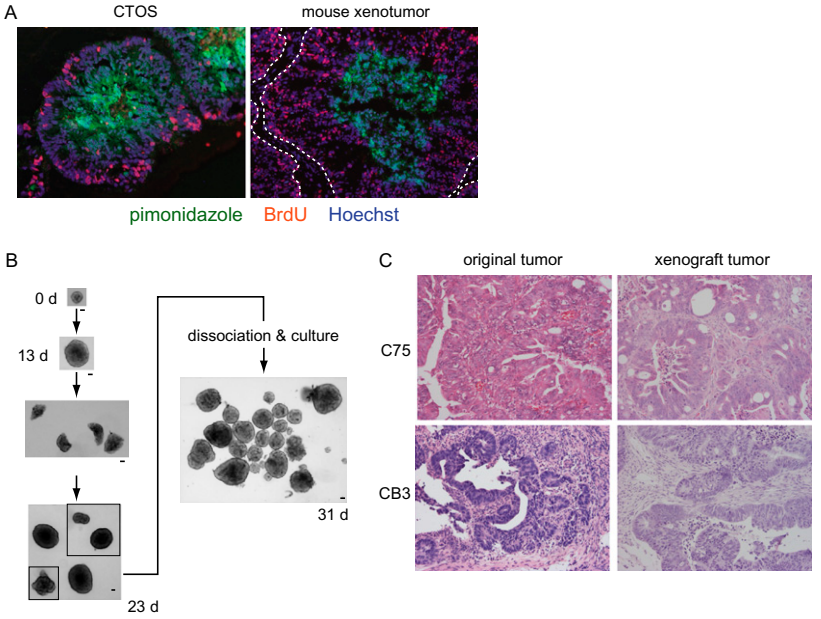


Fig. S5. (*A*) Proliferation of cancer cells in CTOSs and a CTOS-derived xenograft tumor. BrdU (red) and pimonidazole (green) staining of the CTOSs (*Left*) and the CTOS-derived xenograft tumor (*Right*). Cell nuclei were stained with Hoechst 33342 (blue). CTOSs were treated with BrdU (Roche) and Hypoxyprobe-1 (pimonidazole; Natural Pharmacia International) for 2 h. Tumor-bearing mice were i.p. injected 2 h before sacrifice. (Scale bar: 100 μm.) (*B*) Expansion of a CTOS (C45) by mechanical dissociation and culture. (*C*) H&E staining images of the original tumors, and the xenograft tumors generated from the indicated CTOSs after long-term culture.

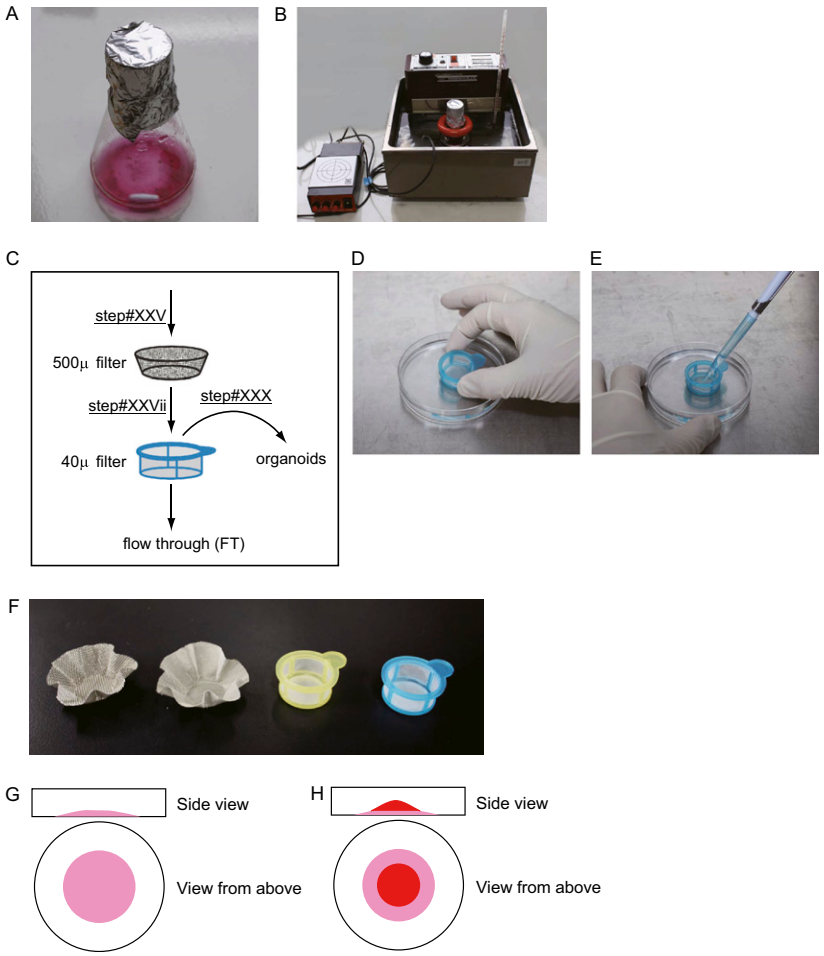


Fig. S6. Supporting figures for the protocol for CTOS preparation and expansion. See also *SI Materials and Methods* for the text information of the protocol for CTOS preparation and expansion.

Table S1. Case description, mutation status, and CTOS characteristics of colorectal cancer specimen

Sample ID	Patient age	Patient sex	Tumor location	Tumor histology	TNM stage	Mutation				CTOS		
						KRAS		BRAF				
						Original	CTOS	Original	CTOS	Formation	Growth	Passage
C45	60	M	Ra	Mod	IV	Wild	G12D	ND	Wild	Yes	Yes	Yes
C48	83	M	Rab	Mod	IIB	Wild	Wild	Wild	Wild	Yes	Yes	No
C49	68	M	Rs	Well	IIIB					Yes	No	
C50	77	M	Ce	Mod	I					Yes		
C51	65	M	T	Mod	IV	Wild	Wild	Wild	Wild	Yes	Yes	
C52	51	M	S	Poor	IV					Yes		
C53	60	F	Ce	Muc	IIB					Yes	Yes	
C54	63	M	Rs	Well	IIA					Yes		
C56	61	F	T	Poor	IIIC					No		
C58	56	F	Rs	Mod	IIIC					Yes		
C59	57	F	Rab	Mod	IIIB	Wild	Wild	Wild	Wild	Yes	Yes	
C60	63	M	S	Mod	IIA					Yes		
C61	64	F	Rs	Mod	IIB	G13D	G13D	ND	Wild	Yes	Yes	
C62	74	F	A	Mod	IIIC	Wild	Wild	Wild	Wild	Yes	Yes	Yes
C63	79	F	D	Mod	IIA					Yes		
C64	69	M	S	Mod	IV					Yes		
C65	68	M	A	Poor	I	Wild	Wild	Wild	Wild	Yes	Yes	Yes
C66	77	F	Ce	Well	IIIB	G12V	G12V		Wild	Yes	Yes	Yes
C67	69	F	A	Mod	IIIB					Yes	No	
C68	60	M	Rs	Mod	IV	G12D	G12D	Wild	Wild	Yes		
C69	56	M	A	Mod	IIIB	ND	G12D	ND	Wild	Yes		
C70	71	M	Rs	Mod	IIIB					Yes	Yes	
C71	85	F	Ce	Mod	IIA	G12D	G12D	Wild	Wild	Yes	Yes	
C72	58	M	S	Mod	IIB					Yes	Yes	
C73	38	M	S	Mod	IIB	Wild	Wild	Wild	Wild	Yes	No	
C74	64	F	S	Mod	IIA					Yes		
C75	46	F	Ra	Mod	IV	wild	G13D	ND	Wild	Yes	Yes	Yes
C76	65	F	A	Endocrine cell	IIA	G12D	Wild	Wild	Wild	Yes	No	
C77	75	F	Rs	Well	IIIB	Wild	Wild	Wild	Wild	Yes	Yes	No
C78	82	M	DS	Mod	IIA	Wild	Wild	Wild	Wild	Yes	No	
C79	78	M	T	Well	IIA	Wild	Wild	Wild	Wild	Yes	Yes	No
C80	73	M	T	Muc	IIA	G12V	G12V	Wild	Wild	Yes	Yes	Yes
C81	61	M	D	Muc	IIB	G12D	G12D	Wild	Wild	Yes	Yes	Yes
C82	66	M	Ra	Poor	IIIC	Wild		Wild		Yes	Yes	
C83	56	M	Rs	Mod	IIIB	G12C		Wild		Yes	No	
C84	76	F	Ce	Mod	IIIB	Wild	G13D		Wild	Yes	Yes	No
C85	64	M	Rs	Mod	IIA	G13D	G13D	Wild	Wild	Yes	Yes	
C86	72	M	S	Mod	IV		G12V		Wild	Yes	Yes	Yes
C87	53	F	D	Mod	IV					Yes		
C88	48	M	Rb	Muc	IIIB		G12D		Wild	Yes	Yes	
C89	60	F	Rb	Mod	I					Yes		
C90	82	M	T	Well	IIIB					Yes		
C91	61	M	Ra	Mod	IIIB					Yes		
C92	69	M	Rb	Mod	IV					Yes		
C93	71	F	Rs	Mod	IIIB	Wild	Wild	Wild	Wild	Yes	Yes	No
C94	67	F	Ce	Well	IIA	G12D		Wild		Yes	Yes	Yes
C95	75	F	A	Well	IV		G12D		Wild	Yes	No	No
C96	73	F	Ce	Well	IV		G13D		Wild	Yes	No	No
C97	71	F	Rb	Mod	IIIC		G13D		Wild	Yes	Yes	Yes
C98	65	M	Ce	Well	IIIA		Wild		Wild	Yes	Yes	Yes
C99	71	M	A	Mod	IIIB		Wild		Wild	Yes	No	
C100	79	M	T	Well	IIIC		Wild		Wild	Yes	Yes	
C101	66	F	Rs	Mod	IIIC					Yes		
C102	77	F	S	Mod	IV		G13D		Wild	Yes	Yes	
C103	71	M	Rs	Mod	IIB		G13D		Wild	Yes	Yes	
C104	59	F	S	Mod	IIIC					Yes	Yes	Yes
C105	65	F	A	Well	IIIB					Yes		
C106	75	F	A	Well	IIA					Yes		

Table S1. Cont.

Sample ID	Patient age	Patient sex	Tumor location	Tumor histology	TNM stage	Mutation				CTOS		
						KRAS		BRAF				
						Original	CTOS	Original	CTOS	Formation	Growth	Passage
C107	67	M	Ra	Mod	IIA					Yes		
C108	72	M	S	Mod	IIIB					Yes	Yes	
C109	20	F	T	Well	IV					Yes		
C110	77	M	Rb	Mod	I					Yes		
C111	71	F	Rs	Mod	IV	Wild	Wild	Wild	Wild	Yes	Yes	Yes
C112	62	M	A	Mod	IIIB	Wild		Wild		Yes		
C113	59	F	Rb	Mod	IIA	G12D		Wild		Yes		
C114	63	M	A	Mod	I					Yes	Yes	No
C115	61	F	S	Mod	IIIB		Wild		Wild	Yes		
C116	68	F	A	Mod	I					Yes		
C117	60	M	Rb	Well	I					Yes	Yes	
C118	74	F	A	Poor	IIIC					Yes		
C119	81	M	T	Mod	IIIB					Yes		
C120	73	M	T	Mod	IIA					Yes	Yes	Yes
C121	76	M	Rb	Well	I					Yes	Yes	No
C122	62	M	Ra	Well	I					Yes		
C123	65	M	T	Mod	IV					Yes		
C124	59	M	Rs	Mod	IV					Yes		
C125	66	M	Rb	Mod	IV					Yes		
C127	79	F	Rb	Muc	IIIB					Yes		
C131	56	F	S	Well	IIIB					Yes		
C132	73	F	T	Mod	IIIB					Yes	Yes	Yes
C133	76	M	D	Mod	IIIB					Yes		
C134	65	M	A	Mod	IIIB					Yes		
CLM2	50	F	lung		IV	G12D	G12D	wild	Wild	Yes	Yes	Yes
CLM3	79	M	lung		IV	Wild	Wild	D593G	D593G	Yes	Yes	Yes
CLM4	72	M	lung		IV					Yes		
CB1	61	M	Rb	Well	IV					Yes	Yes	Yes
CB2	32	F	Rb	Well	IIIC					Yes	Yes	
CB3	76	F	A	Well	IIIB		G12V		Wild	Yes	Yes	Yes
CB4	78	M	T	Well	IIA	Wild	Wild	Wild	Wild	Yes	Yes	Yes
CB5	61	M	D	Mod	IIB					Yes		
CB6	62	M	D	Poor	IV					No		
CB7	64	M	Rs	Well	IIA					Yes	No	
CB8	45	M	Rb	Mod	IIB	Wild	Wild	Wild	Wild	Yes	No	
CB9	60	F	Rb	Mod	I					Yes		
CB10	61	M	Ra	Mod	IIIC					No		
CB11	58	F	S	Mod	IV					Yes		
CB12	73	M	S	Mod	IV					Yes	No	
CB14	77	F	A	Well	IV		G12C		Wild	Yes	Yes	Yes
CB16	69	M	Rb	Well	IV					Yes	Yes	No
CB17	79	M	Ra	Well	IIIA		G12D		Wild	Yes	Yes	No
CB18	77	M	Rs	Well	I		G13D		Wild	Yes	No	
CB19	80	F	S	Mod	IIA					Yes		
CB20	42	F	Ras	Mod	IIIA					Yes		
CB21	62	M	Rb	Mod	IIIA					Yes		
CB22	57	M	Rb	Mod	I					Yes	Yes	Yes
CB23	63	F	Rb	Well	I					Yes		
CB24	75	F	A	Group4	IV					Yes	No	
CB25	71	F	Ra	Mod	I					Yes		
CB26	72	F	Ce	Mod	IIA					Yes		
CB27	55	F	Rb	Mod	IIB					Yes	Yes	Yes
CB28	73	F	Ce	Well	IV					Yes	No	
CB29	68	F	Ra	Well	IV					Yes	No	
CB30	47	M	Rb	Well	IIIB					No		
CB31	81	F	T,S	Mod	IIIB					Yes	Yes	Yes
CB32	67	M	Ra	Well	I					Yes	No	
CB33	72	M	T	Well	I					Yes	No	
CB34	57	F	S	Well	I					Yes	Yes	

CLM, lung metastasis samples; CB, endoscopic biopsy samples. Tumor location: Ce, cecum; A, ascending colon; T, transverse colon; D, descending colon; S, sigmoid colon, Rs, rectosigmoid; Ra, upper rectum; Rb, lower rectum; Muc, mucinous carcinoma; ND, not determined; blank, not assessed.

Table S2. Number of sites that generated s.c. tumor by CTOS transplantation

	No. of CTOSs injected			
Case	1,000	100	50	10
C68	–	1/3	–	–
C69	–	3/4	–	–
C86	1/2	0/2	–	–
C75	1/2	0/2	–	–
C88	1/2	0/2	–	–
C81	2/2	2/2	–	–
C97	–	2/2	–	–
CB1	–	2/3	–	–
CB3	1/2	1/2	–	–
mC45	3/4	2/2	2/2	1/2
mC48	6/6	–	–	–
mC61	8/8	0/2	–	–

CB: CTOSs derived from endoscopic biopsy samples.

Table S3. Reported success rates of establishing colon cancer cell lines by standard technique

Success rate	Ref.
17% (6/35)	(1)
29% (13/45)	(2)
38% (9/24)	(3)
11% (11/99)	(4)

1. Kirkland SC, Bailey IG (1986) Establishment and characterization of six human colorectal adenocarcinoma cell lines. *Br J Cancer* 53:779–785.
2. Willson JKV, et al. (1987) Cell culture of human colon adenomas and carcinomas. *Cancer Res* 47:2704–2713.
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4. Leibovitz A, et al. (1976) Classification of human colorectal adenocarcinoma cell lines. *Cancer Res* 36:4562–4569.