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Colonoscopy-based colorectal cancer modeling in mice with CRISPR-Cas9 genome editing and organoid transplantation

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Abstract

Most genetically engineered mouse models of colorectal cancer are limited by tumor formation in the small intestine, a high tumor burden that limits metastasis, and the need to generate and cross mutant mice. Cell line or organoid transplantation models generally produce tumors in ectopic locations, such as the subcutaneous space, kidney capsule, or cecal wall, that do not reflect the native stromal environment of the colon mucosa. Here, we describe detailed protocols to rapidly and efficiently induce site-directed tumors in the distal colon of mice that are based on colonoscopy-guided mucosal injection. These techniques can be adapted to deliver viral vectors carrying Cre recombinase, CRISPR-Cas9 components, CRISPR-engineered mouse tumor organoids, or human cancer organoids to mice to model the adenoma-carcinoma-metastasis sequence of tumor progression. The colonoscopy injection procedure takes approximately 15 minutes, including preparation. In our experience, anyone with reasonable hand-eye coordination can become proficient with basic mouse colonoscopy with a few hours of practice. These approaches are ideal for a wide range of applications, including assessment of gene function in tumorigenesis, examination of tumor-stroma interactions, studies of cancer metastasis, and translational research with patient-derived cancers.

Editorial summary

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Author Contributions

J.R. designed and performed all experiments. T.T. assisted in design, construction, and production of lentiviral vectors. A.A., M.A., and S.S. assisted with mucosal injection experiments. T.J. participated in the interpretation of results. Ö.H.Y. supervised the experiments.

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Colonoscopy-guided mucosal injection is used to induce site-directed tumors and to transplant tumors in the distal colons of mice. Tumors for engraftment are obtained from cancer organoids derived from mouse or human tissue and can be genetically modified prior to use.

Introduction

Colorectal cancer (CRC) is the third most commonly diagnosed cancer in the United States, despite CRC screening programs and risk factor changes such as decreased smoking rates and increased use of aspirin. While incidence in adults > 50 years has decreased by 32% since 2000, incidence in younger adults has unexpectedly increased by 22% in the same time period¹. Observational studies in humans provide limited insights into disease biology. *In vitro* studies using mouse and human cultures of cancer cells and three-dimensional organoids have greatly advanced our understanding of the mechanisms of colorectal carcinogenesis. However, unlike cell culture or organoid systems, mouse models offer the ability to study tumor initiation, tumor-stromal interactions, immune regulation of cancer, angiogenesis, and metastasis.

Modelling colon cancer in the mouse

The most commonly used mouse models of CRC are based on deletion of the *Apc* tumor suppressor gene^{2,3}. Loss of *Apc* results in stabilization and nuclear translocation of β -catenin, which drives constitutive expression of Wnt target genes that induce dysregulated intestinal stem cell proliferation and tumor formation. These models recapitulate the histology of human adenomas, or cancer precursor lesions. Germline loss of a single *Apc* allele, followed by spontaneous somatic deletion of the second *Apc* allele (e.g., the *Apc*^{Min} mouse), results in approximately 30-100 adenomas in the small intestine in mice between 4 and 6 months of age^{4,5}. However, sporadic CRC in humans usually presents with a single cancer in the colon; small intestinal cancer is rare. Investigators have therefore selected promoters to drive Cre recombinase expression in the colons of *Apc*^{fl/fl} mice, with varying tumor numbers in the colon vs. the small intestine³. Alternatively, adenoviral Cre recombinase can be delivered to the distal colons of *Apc*^{fl/fl} mice with rectal enema (which requires abdominal surgery)⁶ or a specially designed sponge⁷. A significant limitation of most genetically engineered mouse models (GEMMs) of CRC is the need to generate, maintain, and cross mice with germline mutations to assess gene function in tumorigenesis.

Transplantation models offer advantages over GEMMs, including lower cost and the ability to manipulate cell lines or three-dimensional organoid cultures *in vitro*. While cell lines poorly reproduce the histology of human cancer *in vivo*, engrafted organoids and patient-derived xenografts (PDXs) closely mimic key histopathological features of human cancer.⁸ Mouse tumor organoids with multiple cancer-associated mutations (i.e., *Apc*, *Trp53*, *Kras* and/or *Smad4*) have been transplanted into the mouse flank, rectal mucosa, and inflamed colon to model CRC and liver metastasis^{9,10}. Normal human organoids have been engineered with key cancer-associated mutations using the CRISPR-Cas9 system, and then engrafted into the mouse flank, kidney capsule, and cecal wall¹¹⁻¹⁴. Furthermore, patient-derived primary CRCs can be cultured with close to 100% efficiency as organoid lines, and then transplanted into the mouse flank, kidney capsule, and spleen (to model liver

seeding)¹⁵. However, transplantation into these ectopic sites does not permit study of tumor invasion of the muscularis propria or extravasation through the colon serosa into the circulation. Other groups have transplanted human CRC cell lines into the rectal mucosa or patient-derived tumor organoids into the colon (following induction of colitis) to model primary cancer and liver metastasis^{10,16,17}. GEMMs and transplantation models of CRC that produce tumors in the colon are described in Table 1.

Use of colonoscopy in the establishment of mouse models of colorectal cancer

Colonoscopy is the examination of the colon using an instrument containing a light source, camera, and a working channel for tissue sampling or intervention. Colonoscopy is commonly used in clinical medicine for the detection and removal of pre-cancerous adenomas to prevent CRC in susceptible individuals. The first reports of white-light colonoscopy in mice were by Huang et al. and Witz et al. in 2002^{18,19}. Since then, mouse colonoscopy has been applied for near-infrared imaging^{20–23}, optical coherence tomography^{24,25}, chromoendoscopy (i.e., methylene blue painting of the mucosa)²⁶, fluorescence^{27,28}, narrow band imaging²⁹, and confocal microscopy²⁹. These technologies are used to improve on white light for detection of small tumors³⁰. Tumor biopsy with specialized forceps has also been described^{30,31}.

The use of colonoscopy-guided mucosal injection for CRC modeling was first reported by Zigmond et al. in 2011, who orthotopically transplanted mouse CRC cell lines with a 30-gauge injection needle and showed that the cells successfully formed tumors in the colon³². Since then, another group has applied this approach to model liver metastasis from orthotopically engrafted human CRC cell lines³³.

We recently developed mouse models of CRC and metastasis based on colonoscopy-guided mucosal injection of viral vectors or tumor organoids³⁴. We improved on existing mucosal injection methods by using a 33-gauge needle and a 100 µl syringe, which offers precise site-directed injection and the formation of a mucosal “bubble”, with minimal risk of colon perforation. We applied this technique to model CRC with CRISPR-Cas9 gene editing of the colon epithelium and with orthotopic transplantation of mouse and human organoids³⁴. Furthermore, we showed that these systems can be used quickly (i.e., within 2–3 months) to study putative tumor-associated genes, model the entire adenoma-carcinoma-metastasis sequence, sequentially mutate genes in established adenomas, assess tumor stem cell function, study patient-derived cancers in the native colon environment, and model normal organoid function. Our transplantation model is also uniquely suited to studying the effects of epithelial-stromal interactions in colorectal tumorigenesis.

Comparison with other methods

Cancer researchers have many mouse models of CRC to consider for their studies. The selection of a mouse model is based on many factors, including cost, time, and the details of the hypothesis being tested. In general, studies that do not require multiple gene mutations may be easily performed in GEMMs with germline *Apc* mutations, with or without additional cancer associated mutations. Many of these mouse strains are readily available from vendors such as Jackson Laboratories or the National Cancer Institute. We developed a

colonoscopy-based approach to induce Cre-mediated recombination in the distal colons of *Apc^{fl/fl}* mice to model CRC. An important advantage of this model is that tumors form in defined locations in the distal colon, can be longitudinally monitored or biopsied with colonoscopy, and do not require time-consuming surgery⁶ or a specialized rectal sponge⁷. If additional gene mutations are desired, germline GEMMs are not ideal because of the time, labor, and expense required to generate mutant mice.

Orthotopic transplantation models offer the ability to rapidly assess gene function in CRC, model metastasis, and study patient-derived tissues *in vivo*. In these systems, tumor organoids are engineered with desired mutations using CRISPR-Cas9 editing, which is much faster than generating germline GEMMs. Fluorescent markers and other desired features can also be easily introduced into organoids. In our models, engineered tumor organoids are engrafted into the colon mucosa via colonoscopy guided injection. Tumors with advanced mutations spontaneously invade into the local vasculature and metastasize to the liver³⁴. Other groups have reported cancer organoid transplantation without colonoscopy, either by injection into prolapsed rectal mucosa⁹ or by rectal enema into mice with colitis^{10,17}. Neither of these models permit control of tumor number or location in the colon, and they require the introduction of trauma or inflammation, respectively, both of which are potential confounding factors. Both models (like our model) produce cancers in the native colon environment that invade to form liver metastases. An alternative orthotopic transplantation model is based on surgical insertion of CRISPR-Cas9-engineered human cancer organoids into the cecal wall. The resulting tumors cannot be monitored with colonoscopy, but model liver and lung metastasis in the setting of advanced mutations^{13,14}.

We developed techniques to edit cancer-associated genes in the colon epithelium using colonoscopy-guided injection of CRISPR-Cas9 components in lentiviral constructs.³⁴ Our group previously used a similar somatic gene editing system to model lung cancer in mice³⁵. An important advantage of this model is that tumors with multiple mutations can be quickly and efficiently induced in wild-type mice from any background. These tumors are histologically similar to tumors found in traditional GEMMs. In addition, our *in situ* gene editing model offers the versatility of modeling spatially defined tumors with customizable lentiviral vectors that contain multiple desired components (e.g., Cre recombinase, fluorescent markers, Cas9, multiple sgRNAs). These features are difficult to model in germline GEMMs. A limitation of this model is that reproducible lentiviral infection of colon stem cells *in situ* generally requires viral titers of at least 10,000 TU/ μ l. There are currently no other published *in vivo* genome editing models of CRC.

Our mouse models require a mouse colonoscopy system and at least a few hours of training for mouse colonoscopy and the mucosal injection technique. Once the user knows how to operate the equipment, the injection procedure, including set up, takes approximately 15 minutes. Advantages and disadvantages of commonly used GEMMs and transplantation models of CRC, including the models discussed in this paper, are outlined in Table 1.

Experimental Design

In this article, we provide step-by-step protocols for mouse intestinal organoid generation (modified from Refs ^{36,37}) lentiviral infection (modified from a retroviral transfection

protocol in Ref ³⁸), human CRC organoid generation (modified from Refs ^{15,39}), and colonoscopy-guided mucosal injection (modified from Ref ³² and previously described in Refs ^{34,40}) for CRC modeling. Mouse or human intestinal organoid infection with viral vectors can be substituted with transfection³⁸ or electroporation of plasmid DNA vectors⁴¹.

Considerations in mouse selection.—Ideal recipient mice for colonoscopy-guided mucosal injection are 6-8 weeks old. Young mice have colons that are smaller in diameter and less elastic than those of older mice, which makes the mucosal injection process more efficient. Female mice are preferred to male mice because the colons are smaller in diameter. However, we also regularly perform mucosal injection experiments in males or in mice > 8 weeks of age without difficulty. Mouse strains are chosen based on the experimental design. Colon-specific *Apc* / tumors are generated in *Apc^{fl/fl}* mice or *Apc^{fl/fl}; Villin^{CreER}* mice with mucosal injection of viral Cre recombinase or 4-hydroxytamoxifen, respectively. *In situ* gene editing is performed in wild-type mice or mice of any background using lentiviral constructs expressing a short guide RNA targeting exon 16 of *Apc* (sgApc)⁴² and Cas9 (e.g., U6::sgApc-EFS::Cas9-P2A-GFP). Alternatively, lentiviral delivery of sgApc (with Cre recombinase) is sufficient to induce tumor formation in *Rosa26^{LSL}-Cas9-eGFP/+* gene-targeted mice. Epithelial-specific gene editing is achieved by delivering CRISPR-Cas9 components to mice with Cas9 activity limited to Villin-expressing epithelial cells (i.e., tamoxifen-treated *Rosa26^{LSL}-Cas9-eGFP/+; Villin^{CreER}* mice). Orthotopic engraftment of tumor organoids is generally performed in NOD scid gamma (NSG) or *Rag2* / mice. Transplantation in syngeneic hosts is limited by smaller tumor size and lower engraftment rate compared to transplantation in immunodeficient hosts, most likely due to an immune response to the mucosal injection that may be overcome with multiple cancer-associated mutations (i.e., *Apc* / ; *Kras^{G12D/+}*; *Trp53* /). Human organoids are transplanted into NSG mice or *Rag2* / mice. Separate colonies of control mice are not required; the same cohort of mice can be randomized to experimental or control lentivirus or transplanted with organoids from either study group.

Plasmid selection.—Plasmids selection is based on the goals of the study. Tumor organoids can be generated by infection of mouse intestinal or colon organoids with U6::sgApc-EFS::Cas9-P2A-GFP lentivirus. If desired, additional genes can be edited using plasmids expressing specific sgRNAs. Alternatively, tumor organoids can be created from *Rosa26^{LSL}-Cas9-eGFP/+* organoids by infecting with lentiviral constructs expressing sgApc and Cre recombinase. These plasmids are also used for *in vivo* gene editing models of CRC.

Materials

REAGENTS

Tamoxifen

- Tamoxifen (Sigma Aldrich, catalog # T5648) CAUTION: Tamoxifen is a hazardous substance. Local regulations on tamoxifen use in animals should be followed. Wear appropriate personal protective equipment and consider housing mice in separate cages or a separate room for the duration of injections.
- 100% ethanol (Sigma Aldrich, catalog # E7023)

- Sunflower seed oil (Spectrum Chemical, catalog # S1929)

Activated tamoxifen

- 4-hydroxytamoxifen (Calbiochem, catalog # 579002) CAUTION: Tamoxifen is a hazardous substance. Local regulations on tamoxifen use in animals should be followed. Wear appropriate personal protective equipment and consider housing mice in separate cages or a separate room for the duration of injections.
- 100% ethanol (Sigma Aldrich, catalog # E7023)

Adenoviral Cre recombinase

- Ad5CMV::Cre (University of Iowa Viral Vector Core Facility)

Lentiviral plasmids available from Omer Yilmaz Laboratory:

- PGK::Cre
- U6::sgApc-EFS::Cas9-P2A-GFP
- U6::sgApc-CMV::Cre
- U6::sgApc-EFS::turboRFP
- U6::sgTrp53-CMV::Cre

Lentiviral Plasmids available from Addgene:

- pSpax2 packaging plasmid (Addgene catalog # 12260)
- pMD2.G packaging plasmid (Addgene catalog # 12259)

lentiviral production

- HEK293T cells (ATCC catalog # CRL-3216)
- TransIT-LT1 Transfection Reagent (Mirus Bio, catalog # MIR 2304)
- DMEM (Life Technologies, catalog # 11965084)
- OptiMEM serum free media (Invitrogen, catalog # 31985-070)
- GreenGo Cre reporter cells (available from Tyler Jacks Laboratory)³⁵
- Bleach

Mouse intestinal organoid culture and infection

- Phosphate buffered saline without magnesium and calcium, 10× (i.e., PBS^{−/−}) diluted with distilled water to 1× PBS (ThermoFisher Scientific, catalog # 70011044)
- Ethylenediaminetetraacetic acid (EDTA), 0.5 M (Ambion, catalog # am9261)
- 50% L-WRN conditioned media generated from L-WRN cells (ATCC catalog # CRL-3276).

- Growth factor-reduced Matrigel (Corning, catalog # 356231)
- Y-27632 dihydrochloride monohydrate (APExBIO, catalog # A3008)
- Nicotinamide (Sigma Aldrich, catalog # N3376)
- TrypLE Express Enzyme, no phenol red (ThermoFisher Scientific, catalog # 12604013)
- Polybrene, 10mg/ml (Sigma Aldrich, catalog # TR-1003)
- Advanced DMEM/F12 (Life Technologies, catalog # 12634-028)
- Penicillin, 5,000 units/ml, and Streptomycin, 5,000 µg/ml (ThermoFisher Scientific, catalog # 15070063)
- L-glutamine, 200 mM (ThermoFisher Scientific, catalog # 25030081)
- Hygromycin, 50 mg/ml (Invitrogen, catalog # 10687-010)
- G418, 50 mg/ml (Sigma-Aldrich, catalog # G8168)
- N2 Supplement, 100× (ThermoFisher Scientific, catalog # 17502048)
- B27 Supplement, 50× (ThermoFisher Scientific, catalog # 17504044)
- Fetal bovine serum (FBS) (Corning, 35-010-CV)

Human colorectal cancer organoid culture and passage

- Collagenase Type 1 (Worthington, catalog # LS004194)
- 50% L-WRN conditioned media generated from L-WRN cells
- Advanced DMEM/F12 (Life Technologies, catalog # 12634-028)
- N2 Supplement, 100× (ThermoFisher Scientific, catalog # 17502048)
- B27 Supplement, 50× (ThermoFisher Scientific, catalog # 17504044)
- Penicillin, 5,000 units/ml, and Streptomycin, 5,000 µg/ml (ThermoFisher Scientific, catalog # 15070063)
- L-glutamine, 200 mM (ThermoFisher Scientific, catalog # 25030081)
- EGF (PeproTech, catalog # 315-09)
- SB202190 (Sigma Aldrich, catalog # S7067)
- A83-01 (Tocris, catalog # 2939)
- Y-27632 dihydrochloride monohydrate (Sigma-Aldrich, catalog # Y0503)
- Nicotinamide (Sigma-Aldrich, catalog # 47865-U)
- Primocin (InvivoGen, catalog # ant-pm-1)
- Trypsin (Sigma-Aldrich, catalog # T4549)
- Normocin (InvivoGen, catalog # ant-nr-1)

- Gentamycin (Amresco, catalog # E737)
- Fungin Antifungal Reagent (InvivoGen, catalog # ant-fn-1)
- SMEM (Life Technologies, catalog # 11380-037)
- Fetal bovine serum (FBS) (Corning, 35-010-CV)
- Cell Recovery Solution (Corning catalog # 354253)
- DMSO (Sigma-Aldrich, catalog # D2650)

CAUTION: DMSO is harmful if inhaled or absorbed through the skin. It is flammable.

MICE

- *Rosa26^{LSL-Cas9-eGFP/+}* (Jackson Laboratory, Stock Number 026175)⁴³
- *Villin^{CreER}* (available from laboratory of Sylvia Robine)⁴⁴
- *Apc^{fl/fl}* (NCI Mouse Repository, Strain Number 01XAA)⁴⁵
- NOD scid gamma (NSG; Jackson Laboratory, Stock Number 005557)

CAUTION: Experiments involving mice must conform to relevant institutional and national regulations. The protocol described here was used in experiments that were approved by the MIT Institutional Animal Care and Use Committee.

HUMAN COLORECTAL CANCER SPECIMENS

- We obtain human CRC samples from Tufts Medical Center, Massachusetts General Hospital, and Brigham and Women's Hospital / Dana Farber Cancer Institute. CRC samples should be obtained from viable tissue (typically pink-appearing, as determined by the clinical pathologist) and be at least 1 mm³ in size.

CAUTION: Research subject consent and human tissue collection procedures must confirm to relevant institutional and national regulations. The respective Institutional Review Board committees and the Massachusetts Institute of Technology Committee on the Use of Humans as Experimental Subjects approved the study protocols we used in our research.

CRITICAL: Place the tumor sample into ice-cold PBS as soon as possible, preferably in the operating room. This will preserve viability of the tumor cells. Once placed into cold PBS, tumor tissue is viable for many hours. It is also important to distinguish viable tumor tissue (often pink) from necrotic tumor tissue (often yellow or white). Necrotic tumor tissue will not typically grow as organoids.

EQUIPMENT

Common equipment

- Glass microscope slide
- 50 ml conical tubes
- Pipettes, 10, 200, and 1000 μ l (Eppendorf)
- Pipette tips, 10, 200, and 1000 μ l
- Easypet 3 pipette (Eppendorf)
- Pipettes, 5, 10 and 25 ml
- Tubes, 1.5 ml
- Tissue culture plates, 100 mm and 150 mm
- Parafilm (Bemis)
- 24-well tissue culture plates (Olympus, catalog # 25–107)
- Cell culture incubator with 5% CO₂, 37 degrees C
- Biological hood
- Centrifuge 5424 table-top centrifuge (Eppendorf, catalog # 022620401)
- Centrifuge 5810R (Eppendorf, catalog # 022625101)
- Inverted phase microscope
- Water bath, 32 degrees C
- Thermomixer C heating block, 37 degrees C (Eppendorf, catalog # 5382000015)

Lentiviral production

- PES vacuum-driven membrane filter, 0.45 μ m pore size (VWR, catalog # 10040–438)
- Ultracentrifuge (Beckman Coulter)
- Ultracentrifuge tubes (Beckman Coulter, catalog # 344058)

Mouse intestinal organoid culture and infection

- Extra Fine Bonn Scissors (Fine Science Tools, catalog # 14085–08)
- Mayo scissors (Fine Science Tools, catalog # 14010–15)
- Dissecting forceps, fine tip, curved (VWR, catalog # 82027–406)
- 70 μ m cell strainer (Corning, catalog # 352350)
- PES vacuum-driven membrane filter, 0.2 μ m pore size (VWR, catalog # 10040–436)
- Compact Digital Rocker, (ThermoFisher Scientific, catalog # 88880020)

Human colorectal cancer organoid culture

- 0.2 µm pore size syringe filter (Acrodisc Syringe Filter, Protein LowBind, Pall Life sciences, catalog # PN 4612)
- Syringes, 5 ml (VWR, catalog # BD309646) and 10 ml (VWR, catalog # BD309695)
- 100 µm cell strainer (Corning, catalog # 431752)

Colonoscopy-guided mucosal injection

- Oral gavage needle (Roboz, catalog # FN 7905)
- Optical and fluorescence colonoscopy system: Image 1 H3-Z Spies HD Camera System (part TH100), Image 1 HUB CCU (parts TC200, TC300), 175 Watt D-Light Cold Light Source (part 20133701-1), AIDA HD capture system, and fluorescent filters in the tdTomato (emission 554 nm) and GFP channels (emission 509 nm) (all from Karl Storz)
- Hopkins Telescope (Karl Storz, part 64301AA) with examination sheath (Karl Storz, part 64301BA) and operating sheath (Karl Storz, part 64301AA).

CRITICAL: This endoscope is commonly used for murine colonoscopy.³⁰ We use it with the examination sheath for tumor imaging, particularly for fluorescence. Alternatively, it can also be used with the operating sheath for mucosal injection.

- Colonoscope with integrated working channel (Richard Wolf 1.9 mm/9.5 French pediatric urethroscope, part number 8626.431)

CRITICAL: We prefer this endoscope for mucosal injection. This endoscope offers two advantages over the Hopkins telescope from Karl Storz: 1) a 45-degree angle of the light channel, which is more comfortable to hold; and 2) an integrated working channel to facilitate mucosal injection.

- Mucosal injection needle: (Hamilton, 33 gauge, small Hub RN NDL, 16 inches long, point 4, 45-degree bevel, like part number 7803-05)

CRITICAL: The injection needle is custom made by Hamilton. If desired, the needle can be made shorter (less resistance to flow, Operator and Assistant seated closer together), or longer (more resistance to flow, Operator and Assistant seated further apart). If using the Karl Storz endoscope instead of the Richard Wolf endoscope for mucosal injection, use of a shorter injection needle is possible. Other research groups have used 30 gauge needles³², which have less resistance to flow but are more difficult to insert into the mucosa and are more likely to cause perforation. We have not experimented with different bevel angles.

- Mucosal injection syringe (Hamilton Inc., part number 7656-01)
- Transfer needle (Hamilton Inc., part number 7770-02)

- Extra screw cap (Hamilton Inc., part number 30902)
- Isoflurane (available through animal facility)

REAGENT SETUP

Tamoxifen

- Mix 100 mg of tamoxifen into 1 ml of 100% ethanol, and then add 9 ml sunflower seed oil. Tamoxifen does not readily go into suspension. Shake on orbital rocker for 1 hour at room temperature to reconstitute, or shake at 55 degrees C to accelerate resuspension. Protect from ambient light by wrapping in aluminum foil. Inject *Rosa26^{LSL-Cas9-eGFP/+}*; *Villin^{CreER}* mice i.p. with 250 µl per 25 g of body weight for three consecutive days. Reconstituted tamoxifen can be stored at –20 degrees C for at least three months.

Activated tamoxifen

- Reconstitute 4-hydroxytamoxifen in 100% ethanol to 5 mM. Then, dilute 5 mM stock to 100 µM in PBS. Store at –20 degrees C, then thaw on ice immediately before injection. Reconstituted tamoxifen can be stored at –20 degrees C for at least three months.

Y27632 stock solution

- Make a 10 mM stock solution in water. Aliquots can be stored at –20 °C for three months.

SB202190 stock solution

- Make a 10 mM stock solution in DMSO. Aliquots can be stored at –20 °C for three months.

Nicotinamide stock solution

- Make a 10 mM stock solution in 1× PBS. Aliquots can be stored at –20 °C for three months.

Collagenase Type 1 working solution

- Mix 20 mg of Collagenase Type 1 per 1 ml of 1× PBS to make a working solution of 2,500 units/ml. Filter the solution with a 0.2 µm pore size syringe filter. 250 µl aliquots can be stored at –20 °C for three months.

EGF working solution

- Make a X stock solution in 1× PBS. Aliquots can be stored at –20 °C for three months.

A83–01 working solution

- Make a 500 µM stock solution in 1× PBS. Aliquots can be stored at –20 °C for three months.

Matrigel

- Thaw Matrigel on ice overnight, then divide on ice into 500 μ l or 1 ml aliquots. Aliquots can then be thawed on ice in ~ 1 hour. Always keep on ice prior to use. Multiple freeze/thaw cycles do not appear to affect Matrigel performance. Avoid direct handling as much as possible, as the Matrigel will polymerize with body heat. We have observed occasional lot-to-lot variability in Matrigel viscosity. Therefore, we suggest ordering Matrigel in bulk from the same lot that you have tested.

Adenoviral Cre recombinase

- Thaw Ad5CMV::Cre on ice, then dilute to the desired concentration in PBS. The virus titer is provided with purchase and is usually 10^7 TU/ μ l. We suggest diluting to 100,000–300,000 TU/ μ l for rapid tumor formation within 2–4 weeks.

CAUTION: Adenovirus is required to be handled at Biosafety Level 2 (BSL2). For animal studies, adenoviral vectors require ABL2 containment. Ethanol cleaning does not inactivate adenovirus; 10% bleach (0.5% sodium hypochlorite) should be used instead. Institutional approval is typically required for use of viral vectors in research animals. Confirm these requirements with your institution's biosafety officer.

CRITICAL: Adenovirus should be stored at -80 degrees C. Thaw only on ice, not at room temperature. According to the manufacturer, adenoviral titer is stable for approximately three freeze/thaw cycles. We recommend making small working aliquots of approximately 250 μ l per mouse after the initial thaw.

Plasmid cloning for lentivirus production

- Cloning of lentiviral shuttle vectors is performed using modular “Gibson” assembly, as previously described.⁴⁶

50% L-WRN conditioned media

- Detailed procedures for producing 50% L-WRN conditioned media are described in Ref ⁴⁷. Briefly, grow L-WRN cells in DMEM supplemented with 10% FBS, 50 units/ml penicillin, 50 μ g/ml streptomycin, 2 mM L-glutamine, 500 μ g/ml hygromycin, and 500 μ g/ml G418. Wash cells with PBS, then passage without hygromycin and G418 into the desired number of tissue culture plates. When the cells are 90% confluent, replace media with ADMEM/F12 supplemented with 20% FBS, 50 units/ml penicillin, 50 μ g/ml streptomycin, and 2 mM L-glutamine. Harvest media every day for 6 days, centrifuge at 2000 g for 15 minutes at room temperature to remove cells, and pass the supernatant through a 0.2 μ m vacuum filter. Finally, dilute media 1:1 with ADMEM/F12 supplemented with 20% FBS, 500 units/ml penicillin, 500 μ g/ml streptomycin, and 2 mM L-glutamine to produce 50% L-WRN conditioned media. Store the media at 4 degrees C or -20 degrees C for up to 3 months.

CRITICAL: The quality of L-WRN conditioned media may vary depending on the source of FBS. Test different supplies and lots of FBS for L-WRN conditioned media efficacy. Media may be tested by organoid-forming assay with mouse intestinal crypts, or with a Wnt reporter Topflash assay, as we have previously described.⁴⁸

Infection media

- 50% L-WRN conditioned media + 10 μ M Y-27632 + 10 mM Nicotinamide + 8 μ g/ml polybrene

CRITICAL: Prepare fresh for each use.

Infection culture media

- 50% L-WRN conditioned media, 10 μ M Y-27632, and 10 mM Nicotinamide

CRITICAL: Prepare fresh for each use.

Mouse minimal media

- Advanced DMEM/F12, 50 units/ml penicillin, 50 μ g/ml streptomycin, 2 mM L-glutamine, 1 $\times$ N2, and 1 $\times$ B27. Store the media at 4 degrees C or -20 degrees C for up to 3 months.

Human antibiotic solution

- 1 $\times$ PBS, 50 units/ml penicillin, 50 μ g/ml streptomycin, 50 mg/ml normocin, 50 mg/ml gentamicin, and 10 μ g/ml Fungin Antifungal Reagent. Prepare fresh for each use.

Human growth media

- 50% L-WRN conditioned media, 1 $\times$ N2, 1 $\times$ B27, 40 ng/ml EGF, 3 μ M SB202190, 500 nM A83-01, 10 μ M Y-27632 dihydrochloride monohydrate, 1 μ M N-acetyl-L-cysteine, 10 mM nicotinamide, 10 nM human gastrin I, 100 μ g/ml Primocin, and 10 μ g/ml Fungin Antifungal Reagent. Store the media at 4 degrees C or -20 degrees C for up to 3 months.

Human minimal media

- Advanced DMEM/F12, 50 units/ml penicillin, 50 μ g/ml streptomycin, 2 mM L-glutamine, 1 $\times$ N2, 1 $\times$ B27, and 100 μ g/ml Primocin. Store the media at 4 degrees C or -20 degrees C for up to 3 months.

Human trypsin

- Dilute 10 $\times$ Trypsin (Sigma-Aldrich) with PBS to make 1 $\times$ human trypsin. Store at 4 degrees C for up to 3 months.

PROCEDURES

Lentivirus preparation—(TIMING: 5 days)

CAUTION: Lentivirus must be handled at Biosafety Level 2 (BSL2). For animal studies, lentiviral vectors typically require Animal Biosafety Level 1 containment. Specific biosafety requirements may depend on the specific vector used (e.g., the use of CRISPR-Cas9 components to target tumor suppressor genes). Institutional approval is typically required for use of viral vectors in research animals. All virus-producing cells, syringes, culture plates, and filters should be treated with 10% bleach for 15 minutes before discarding. Lentivirus work areas should be decontaminated with 10% bleach following preparation. Confirm these requirements with your institution's biosafety officer.

CRITICAL: If lentivirus will be used for mucosal injection experiments within 48 hours, we recommend storing the lentivirus on ice in a 4 degrees C cold room. If lentivirus will be used a few days or more after generation, then we suggest freezing in small working aliquots, in addition to a small aliquot for titering. Lentivirus should be stored at −80 degrees C. Thaw on ice. Avoid freeze-thaw cycles.

CRITICAL: Alternatives to mouse or human organoid infection with viral vectors include transfection³⁸ or electroporation³⁹ of plasmid DNA vectors.

1. Generate lentivirus using a second-generation lentiviral assembly platform involving a lentivirus shuttle vector (generated in the pLV1–5 backbone⁴⁶), a packaging plasmid (psPAX2) and a VSV-G-expressing envelope plasmid (pMD2.G). Transfect these three components into HEK293T cells using Mirus TransIT-LT1 transfection reagent, according to the manufacturer's protocol^{49,50}.
2. Prepare 150 mm tissue culture plates with HEK293T cells in DMEM 10% FBS. The cells should be at approximately 70% confluence at the time of transfection.

CRITICAL: Transfection efficiency can be affected by the source of FBS or lot. Compare FBS sources to find one that is suitable for your experiments. The health of the HEK293T cells is also critical to obtaining a high viral titer. Use low passage cells (less than passage 15 relative to your frozen stock) and split them 3 times per week.

3. Add 7.5 µg psPAX2, 2.5 µg pMD2.G, and 10 µg plasmid DNA to 2 ml of OptiMEM. Then, add 60 µl Mirus TransIT-LT1 transfection reagent dropwise to the DNA mixture while gently flicking the tube.
4. Incubate for 20 minutes at room temperature.
5. Slowly add the transfection mixture dropwise to the plate of HEK293T cells.
6. 18 hours later, remove media and replace with fresh media.
7. The next day (i.e., approximately 48 hours after transfection), collect the supernatant containing virus. If cells have lifted from the plate, centrifuge at 200 g for 5 minutes to remove cells, and then collect the supernate.

8. Filter through a 0.45 μm PES filter.
9. Concentrate using an ultracentrifuge (120,000 g at 4 degrees C for 2 hours) and then discard the supernate into 10% bleach. Absorb excess supernate by inverting on paper towels for 2–3 minutes. Resuspend the pellet overnight in OptiMEM (50–200 μl per ultracentrifuge tube, depending on desired concentration).

CRITICAL: Concentration of lentivirus by ultracentrifugation is possible only for lentiviruses that have a VSV-G coat.

CRITICAL: Avoid excessive pipetting of virus back and forth as this will shear virus and reduce titer.
10. If desired, harvest virus a second day (i.e., 72 hours after transfection). Viral titer is generally lower for the second harvest.
11. PAUSEPOINT. Store virus at 4 degrees C for a few days or aliquot and store at –80 degrees C for up to 1 year.

Lentiviral titer measurement (TIMING: 3 days)—CRITICAL: Fresh virus offers the highest possible concentration for immediate application, with the disadvantage that the viral concentration will not be known for three days. If using fresh virus within the next 1–2 days for experiments, we suggest titering fresh virus. If using frozen virus for future experiments, we recommend titering from a frozen aliquot of virus.

CRITICAL: Viral titer of approximately 1,000–10,000 TU/ μl is desired for organoid infection. If virus concentration is significantly higher (i.e., greater than 100,000 TU/ μl), then organoid viability may be reduced.

CRITICAL Cre-expressing lentivirus is titered using GreenGo reporter cells based on Cre activity, as previously described.^{35,46} Infection of these cells with viral Cre recombinase results in expression of GFP. GFP-expressing lentivirus is titered directly on GFP fluorescence in GreenGo or other cell lines.

12. Seed GreenGo cells into 96 well tissue culture plates at a density of 5000 cells per well.
13. Infect with serial dilutions of lentivirus, and then assess infection efficiency by FACS analysis of GFP⁺ cells three days later.
14. Calculate viral titer (TU/ μl) for each dilution as $[-\ln(\text{proportion uninfected cells}) \times \text{total number cells}] / \text{volume of virus } (\mu\text{l})$.

CRITICAL STEP Lentiviral titer is inversely proportional to the insert size between the long terminal repeats (LTRs).

Organoid culture

15. If using mouse intestinal or colon organoids, follow option A. If using human organoids, follow option B.

A) Isolation and culture of murine intestinal crypts (TIMING: 2 hours)

- i. Begin with a mouse with the desired genotype, either: an *Apc*^{-/-} mouse (e.g., tamoxifen-treated *Apc*^{fl/fl}; *Villin*^{CreER}), *Apc*^{fl/fl} mouse (for subsequent infection with adenovirus or lentivirus expressing Cre recombinase); or a mouse of any desired background (for subsequent infection with lentivirus expressing CRISPR-Cas9 components to edit *Apc* and other cancer-associated genes).
- ii. Euthanize the animal according to the requirements of your animal facility. We expose the mouse to CO₂ for 1 minute, followed by confirmatory cervical dislocation. Following euthanasia, use dissecting scissors and forceps to remove the small intestine and/or the colon and place into 50 ml of ice-cold PBS. We typically do not include the cecum for crypt culture.
- iii. Flush the small intestine and/or the colon with 50 ml of PBS using an oral gavage needle. Repeat flushing until the intestinal effluent is clear.
- iv. With the small intestine or the colon on the gavage needle, gently lateralize the tissue by stripping over the needle, and then lay flat in a dish containing 50 ice-cold PBS, so that the tissue is entirely covered with PBS.
- v. Gently wipe off mucus and lumen contents with fingers.
- vi. If isolating small intestinal epithelium, cut into 3–4 roughly equal-sized fragments with Mayo scissors. The precise size of the fragments is not critical. Place small intestinal pieces or colon into a 50 ml conical tube containing 30 ml cold PBS.
- vii. Invert the conical tube 5 times. Decant fluid and refill tube to 30 ml with cold PBS. Repeat this process up to 5 times, until PBS is clean of debris.
- viii. Fill tube with cold PBS to 50 ml. Add 1ml of 0.5 M EDTA for final concentration of 10 mM EDTA.
- ix. Incubate on orbital rocker for ~45 minutes at 4 degrees C.
- x. Gently decant PBS/EDTA and replace with 50 ml fresh cold PBS.

CRITICAL STEP: It is important to remove the intestine from PBS/EDTA after approximately 45 minutes to 1 hour. Excess exposure to EDTA will break up the crypts and reduce viability.
- xi. Transfer intestine, colon, and PBS into a 15 cm dish on ice.
- xii. Scrape the luminal surface of the tissue with a glass microscope slide to remove both villi and crypts (if small intestine) or crypts (if colon). If you do not see any tissue lift off, you are most likely scraping the serosal side. In this case, flip the intestine over and scrape again. Alternatively, firmly shake the 50 ml conical tube 5 times to dissociate crypts from the intestine.
- xiii. If using the small intestine, filter the PBS/villi/crypts through a 70 µm mesh. Villi will be caught in the mesh and crypts will pass through. Colon crypts do not need to be filtered.

- xiv. Centrifuge crypts at 300 g x 5 minutes at 4 degrees C.
- xv. Gently resuspend crypts in 50% L-WRN conditioned media supplemented with 10 μ M Y-27632, and then count the number of crypts in 10 μ l. If the sample is too concentrated, dilute with 50% L-WRN conditioned media supplemented with 10 μ M Y-27632 until you count approximately 300 crypts. The crypts should be close together but not overlapping.
- xvi. Mix resuspended crypts with Matrigel in a 1:3 ratio, then plate 300 crypts per well in a 48 well plate (i.e., 10 μ l of resuspended crypts with 30 μ l of Matrigel per well) or 600 crypts per well in a 24 well plate (i.e., 20 μ l of resuspended crypts with 60 μ l of Matrigel per well).
- xvii. Incubate plate on the bench for 2 minutes, and then in a 37 degree tissue culture incubator for 15 minutes to allow Matrigel to polymerize.
- xviii. Add 300 μ l of 50% L-WRN conditioned media supplemented with 10 μ M Y-27632 per well in a 48 well plate and 650 μ l per well in a 24 well plate.
- xix. Grow crypts in 50% L-WRN conditioned media supplemented with 10 μ M Y-27632 for 3–4 days to form organoids in an undifferentiated, stem-like state. You will need at least 1–2 wells with large, cystic organoids from a 24 well plate. Approximately 40% of crypts should form organoids.
- xx. Change media for fresh 50% L-WRN conditioned media supplemented with 10 mM nicotinamide 24 hours prior to viral infection. The ideal organoids for infection are cystic and with few dead cells in the lumen.

B) Human colorectal cancer organoid culture

TIMING: 2 hours

- i. Place fresh CRC sample into ice-cold PBS.
- ii. Wash the sample 3 times with PBS, and then transfer to a dish on ice.
- iii. Dice the tissue into small pieces using a blade or scissors. Transfer sample into a 15 ml tube.
- iv. Incubate in human antibiotic solution for 15 minutes.
- v. Wash with PBS once.
- vi. Decant PBS, and then resuspend with 250 μ l of collagenase type 1 working solution. Dilute the sample with 4.75 ml SMEM for a final concentration of 125 units/ml.
- vii. Incubate at 32 degrees C for 10–30 minutes until the tissue is dissociated into small clumps. Aggressively shake the sample every 5 minutes.

CRITICAL: Examine the sample every 5 minutes under a microscope. Tumor tissue should ideally be in small aggregates, not single cells, to maximize viability in the organoid assay. The dissociation time must be tailored to the tissue.

- viii. Filter through 100 μ m filter.
- ix. Block the collagenase in the sample on ice using 5 ml of media containing at least 10% FBS (i.e., 50% L-WRN conditioned media) for 1 minute.
- x. Centrifuge using the “short spin” or equivalent setting for 8 seconds, or alternatively, spin at 300 g for 5 minutes at 4 degrees C.
- xi. Decant and wash the cells twice with PBS.
- xii. Resuspend the pellet in human growth media.
- xiii. Mix resuspended CRC tissue with Matrigel in a 1:3 ratio. We suggest seeding 20 μ l of resuspended tumor tissue with 60 μ l of Matrigel per well in a 24 well plate.

CRITICAL: Tumor aggregates should be seeded densely (almost touching each other) to maximize organoid formation.

- xiv. Incubate plate at room temperature for 2 minutes, followed by 37 degrees for 15 minutes to allow Matrigel to polymerize, and then add 650 μ l of human growth media per well in a 24 well plate. Organoids should form within 3 days.
- xv. To separate tumors organoids from dead cells, mechanically dissociate Matrigel with a 1000 μ l pipette tip, centrifuge (short spin for 8 seconds), decant, and then chemically dissociate Matrigel with 500 μ l Cell Recovery Solution for 30 minutes on ice. Centrifuge (short spin for 8 seconds), resuspend with cold PBS, centrifuge again, and then seed in Matrigel and human minimal media in a 3:1 ratio. Culture in human minimal media.
- xvi. To passage CRC organoids, mechanically dissociate Matrigel, and then incubate with 500 μ l of human trypsin in a 37 degree C heating block for 1–2 minutes. Continuously agitate the sample with a 200 μ l pipette tip. Organoids should be dissociated into small clumps of 5–10 cells. Block trypsin with media containing 10% FBS, centrifuge (i.e., short spin for 8 seconds), wash with cold PBS, centrifuge again, and then resuspend in human minimal media. Plate with Matrigel as previously described. Culture in human minimal media. Unlike mouse tumor organoids, human CRC organoids should be maintained densely. We passage in a 1:1 ratio for 2–3 passages, and then 1:2 for further passages.

Infection of mouse intestinal organoids

TIMING: 5 hours

- 16. Thaw virus on ice, and then collect 1–2 wells of mouse organoids from a 24 well plate (see step 1) in 1 $\times$ PBS. Mechanically dissociate the organoids with a 1000 μ l pipette tip. Vigorously dissociate the organoids by pipetting up and down for 1 minute. Look at the tube periodically under a microscope to see if the organoids are broken up. The goal is to get small clumps of organoids of approximately 5–10 cells. Healthy organoids, as defined by minimal or no dead cells in the lumen, dissociate readily. Colon organoids generally require longer and more vigorous dissociation (i.e., 2 minutes) than small intestinal organoids

as well as 2–3 minutes of chemical dissociation with TrypLE Express at 32 degrees.

CRITICAL STEP: Thaw viral reagents on ice one hour prior to organoid infection. Avoid freeze/thaw cycles.

17. Resuspend organoids in 500 μ l of infection media using a 1000 μ l pipette tip.
18. Add approximately 50,000–100,000 TU of virus to the dissociated organoids.
19. Cover with Parafilm and balance tubes ready for centrifugation.
20. Centrifuge for 1 hour at 600 g at 32 degrees C.
21. Incubate at 37 degrees in a tissue culture incubator for approximately 4 hours. For viral titers of approximately 10,000 TU/ μ l, a shorter incubation step of 2 hours is sufficient and increases viability of dissociated organoid cells.
22. Plate infected organoids in a 1:3 ratio in Matrigel and infection culture media. For example, 1 well of infected organoids should be plated into 3 wells to allow space for dissociated organoid cells to grow.
23. Matrigel polymerization usually requires 2 minutes at room temperature, followed by 15 minutes at 37 degrees in a tissue culture incubator. Then, add 650 ml of infection culture media to each well in the 24 well plate and culture in a tissue culture incubator.

Selection of infected organoids (TIMING: 5 days)

24. The following day, change media for fresh 50% L-WRN conditioned media.
25. If necessary, split organoids three days after infection. Change media for fresh 50% L-WRN conditioned media every two days.
26. Select *Apc*-deficient organoids 5 days after infection to allow time for Cas9 to edit the *Apc* tumor suppressor gene. Earlier selection may result in poor organoid survival. To do this, vigorously break up Matrigel with a 1000 μ l pipette tip, collect organoids, wash with 1 $\times$ PBS, chemically dissociate (as described in step 3), and seed in Matrigel with minimal media. Only *Apc*-deficient organoids will survive without Wnt3a and Rspodin-1 supplementation.

Colonoscopy-guided mucosal injection

27. To prepare mouse tumor organoids for colonoscopy, follow option A. For human organoids follow option B.

A) Preparation of mouse tumor organoids (TIMING: 30 minutes):

- i) Vigorously dissociate mouse tumor organoids with a 1000 μ l pipette tip for at least 1 minute or enzymatically with 500 μ l TrypLE Express for 1 minute (small intestinal organoids) or 3 minutes (colon organoids) in a 1.5 ml tube in a 32 degrees C water bath. Organoids should be monitored with an inverted

microscope during dissociation. Organoids should be dissociated into aggregates of partially broken organoids (i.e., approximately 10–20 cells) to facilitate passage through the 33-gauge needle.

- ii) Dilute the dissociated organoids with 500 μ l of 50% L-WRN conditioned media, centrifuge with a table-top centrifuge with the fast-spin setting for 8 seconds at room temperature, decant the supernatant, wash the organoid pellet with 1 $\times$ PBS, and decant again.
- iii) Resuspend the organoid pellet in mouse minimal media (approximately 70 μ l per well of organoids) with 10% Matrigel. Count the aggregates before injection. We typically deliver 40–50 mouse organoids (counted before dissociation) or ~ 150 organoid aggregates (counted after dissociation) per injection, or roughly 1 well of organoids (60 μ l) from a 24 well plate. This corresponds to approximately 20–25 organoid aggregates per 10 μ l drop.

B) Preparation of human CRC organoids (TIMING: 30 minutes):

- i) Dissociate human CRC organoids with a 1000 μ l pipette tip or enzymatically with human trypsin at 37 degrees C in a heating block. Pipette the sample every 10 seconds with a 200 μ l pipette tip. Human organoids are usually smaller than mouse organoids, and therefore require less dissociation to pass through the injection needle.
- ii) Dilute the dissociated organoids with 500 μ l of 50% L-WRN conditioned media, centrifuge with a table-top centrifuge with the fast-spin setting for 8 seconds at room temperature, decant the supernatant, wash the organoid pellet with 1 $\times$ PBS, and decant again.
- iii) Resuspend the organoid pellet in human minimal media (approximately 70 μ l per well of organoids) with 10% Matrigel. Count the aggregates before injection. We typically deliver ~ 150 organoid aggregates (counted after dissociation) per injection, or roughly 1 well of organoids (60 μ l) from a 24 well plate. This corresponds to approximately 20–25 organoid aggregates per 10 μ l drop.

Preparation of mice (TIMING: 10 minutes)

- 28. Anesthetize the mice in 2% isoflurane. Mice should be kept warm with a heating pad if necessary. Adjust isoflurane level so that the mouse is breathing approximately 1 breath per second. Large mice and males typically require greater amounts of anesthesia than smaller mice and females.
- 29. Place the mouse in a supine position. Clean the colons of the anesthetized mice with a rapid enema of tap water (or sterile water for immunodeficient mice) using an oral gavage needle. Use gentle abdominal pressure to force stool pellets out. The water has an added benefit of lubricating the colonoscope to facilitate colonoscopy.

Colonoscopy and preparation of equipment (TIMING: 10 minutes; can be concurrent with mouse preparation): CRITICAL: Two people are required for the

mucosal injection procedure. The Operator is defined as the person performing the colonoscopy and directing the injection needle. The Assistant is defined as the person drawing up the sample into the injection syringe (using the transfer needle) and performing sample injection. The Assistant should be seated close to the Operator. The length of the injection needle can be customized according to the desired positions of the Operator and Assistant. In the following steps, it is indicated who should perform each step.

30. Our colonoscopy set up is shown in Figure 1a. Required components are an air pump, air pump tubing, isoflurane, anesthesia delivery hose, anesthesia chambers, oxygen tank, image processor, image capture device, light source, light source cable, and colonoscope. Attach the air pump, light source cable, and camera head to the colonoscope. Place the mouse in a supine position under continuous anesthesia. Insert the colonoscope into the rectum. The colon will insufflate with air. Inspect the distal colon (approximately the distal 3 cm in an adult mouse) to verify that the colon lumen is free of stool pellets and excess water. Colonoscopy in mice has been previously described in detail³⁰ and this reference can be consulted for further guidance.

CRITICAL STEP: We use a pediatric urethroscope by Richard Wolf for colonoscopy and for mucosal injections (Figure 1b; see equipment section above).

31. Set up the injection equipment as outlined in Supplementary Video 1.
32. Operator: Place the injection needle through a screw cap (Video 1).
33. Assistant: Place the transfer needle onto the injection syringe and screw it tightly with a second screw cap (Video 1).

Colonoscopy-guided mucosal injection (TIMING: 5 minutes or less per mouse)

—**CRITICAL:** In our experience, anyone with reasonable hand-eye coordination can become proficient with basic mouse colonoscopy with a few hours of practice.

34. Operator: Insert the injection needle (with the screw cap attached) into the working channel of the colonoscope (Video 1).
35. Assistant: Draw up 50–70 μ l of sample using the transfer needle. Lower volumes can be injected, if desired.
36. Assistant: Unscrew the transfer needle from the syringe (Video 1).
37. Assistant: Attach the syringe onto the screw cap that is on the injection needle (Video 1).

CRITICAL: The syringe must be secured tightly to the injection needle to avoid leaking of the payload. The injection needle should be held tightly by the operator while the syringe is being secured to the needle. The injection needle tip should always be visible. These steps will help avoid perforation of the colon.

38. Operator: Insert the tip of the injection needle into the mucosa of the colon (Figure 2a, Figure 2b, Video 2). The correct position of the injection needle is based on sight alone. Since the needle exits the colonoscope anteriorly, the injection should be performed into the anterior surface of the colon. The ideal colon site is parallel to the colonoscope, not sloping down or up, with a 30-degree angle relative to the colon wall. Keep the endoscope close to the colon wall so that you can clearly see the needle entering the mucosa. Abort the injection procedure if the view is obstructed by bubbles, stool, water, or debris. Begin proximally (approximately 3 cm from the anal verge) to allow space for additional injections, then move distally for additional injections (if desired). Space injections by at least 0.5 cm to avoid leakage of the sample to the adjacent injection site. You can flip the mouse over into the prone position to inject into the posterior surface of the colon.

CRITICAL: If you feel a “pop” then you passed the needle through the muscularis propria and serosa. Overt pneumoperitoneum is rare due to the relatively small caliber of the needle. Overall morality from mucosal injection is less than 1%.

39. Operator: Ask the Assistant to inject a “test” volume of approximately 10 μ l (Figure 2a, Figure 2b, Video 2). The purpose of the “test” injection is to identify if a mucosal bubble is forming or if the sample is leaking into the lumen. If no bubble and no fluid is seen, then the needle perforated the colon wall and the sample was injected into the peritoneum.

40. Operator: If you see a small bubble form, ask the Assistant to quickly inject the remaining volume. If successful, you will see a bubble form with overlying mucosa that fills most of the colon lumen (Figure 2a, Figure 2b, Video 2). and the Assistant will feel resistance to the injection. Keep the needle in the bubble for approximately 10 seconds to prevent leakage of the sample from the injection site. If unsuccessful, you will either see the sample leak into the colon lumen (if the needle was not placed into the mucosa) or not see the sample at all (if the needle passed through the colon wall). In this case, either readjust the needle (for example, insert it slightly deeper) or identify a new location for injection at least 0.5 cm away from the prior injection site. Up to 3 bubbles can be placed in a typical adult mouse. Smaller volumes can also be delivered if desired.

CRITICAL: The injection must overcome the inherent resistance of the colon mucosa to form a bubble. Therefore, the Assistant must inject the entire payload forcefully and quickly (i.e., in less than 1 second).

41. Following the procedure, place mice in their cages with a heating lamp for warmth to recover.

Tumor monitoring with colonoscopy: (TIMING: 5 minutes or less per mouse)

42. For most CRC models, tumors can be visualized with colonoscopy 4–6 weeks after initiation. Perform tumor monitoring using the Karl Storz colonoscopy

system with white light, SPIES (Storz Professional Image Enhancement System), GFP fluorescence, and RFP (or tdTomato) fluorescence. SPIES, a version of narrow band imaging (NBI), facilitates detection of small tumors by reducing conventional white light to two 30-nm-wide spectra corresponding to blue and green light, which enhances the contrast for blood vessels²⁹. Fluorescence is useful for detecting tumors driven by GFP or tdTomato-expressing lentiviral vectors or in mice with GFP or tdTomato expression (Figure 3a-3d, Videos 3–5).

Troubleshooting: see Table 2 for troubleshooting guidance.

Timing

Step 1-11, Lentivirus preparation: 5 days

Step 12-14, Lentivirus titer measurement: 3 days

Step 15A, Isolation and culture of murine intestinal crypts: 2 hours

Step 15B, Human colorectal cancer organoid culture: 2 hours

Steps 16-23, Infection of mouse intestinal organoids: 6 hours

Steps 24–26, Selection of infected organoids: 5 days

Step 27A, Colonoscopy-guided mucosal injection, preparation of mouse tumor organoids: 30 minutes

Step 27B, Colonoscopy-guided mucosal injection, preparation of human CRC organoids: 30 minutes

Steps 28-29, Colonoscopy-guided mucosal injection, Preparation of mice: 10 minutes

Steps 30-33, Colonoscopy-guided mucosal injection, Colonoscopy and preparation of equipment: 10 minutes

Steps 34-41, Colonoscopy and colonoscopy-guided mucosal injection: 5 minutes per mouse

Step 42, Tumor monitoring with colonoscopy: 5 minutes per mouse at appropriate time points post injection.

Anticipated Results

Tumor assessment.

We evaluate tumors generated with mucosal injections by three criteria, all determined by colonoscopy: 1) tumor initiation rate, or number of tumors / number of successful injections; 2) tumor number per mouse, or number of tumors / number of mice receiving two or more successful injections; and 3) tumor size index, or tumor area / lumen area, as previously

described⁵¹. Examples of the typical results we see are given below. Following the first identification by colonoscopy, tumors are subsequently evaluated by histology.

4-hydroxytamoxifen into *Apc^{fl/fl}; Villin^{CreER}* mice:

Mucosal injection of 100 μ M 4-hydroxytamoxifen into *Apc^{fl/fl}; Villin^{CreER}* mice is extremely efficient and results in rapid tumorigenesis (within 1–2 weeks) with every successful injection (Figure 3a).

Viral injection:

The success of viral injection (either adenovirus or lentivirus) is dependent on the viral titer because the colon epithelial cell population (including stem cells) is relatively resistant to viral infection in comparison to the stromal cell population. Thus, injection of lentivirus with titers of less than 1,000 TU/ μ l will infect predominantly stromal cells and not epithelial cells. We typically use lentiviral titers of at least 10,000 TU/ μ l. For adenoviral experiments, we use titers of 100,000–300,000 TU/ μ l, but we hypothesize that 10,000 TU/ μ l would also be effective. For Cre-mediated *in situ Apc* excision with viral vectors or for CRISPR-Cas9-mediated *in situ Apc* editing, titers of 10,000 TU/ μ l or greater (300,000 TU/ μ l for adenoviral vectors) will produce approximately 1 tumor per mouse in 90% of mice ($n = 117$) within six weeks, if at least two successful injections are performed per mouse (Figure 3b, Videos 3–5). Therefore, it is essential to measure viral titer for custom-made lentivirus to ensure that the concentration is high enough to generate colon tumors.

Mouse organoid transplantation:

Syngeneic transplantation requires immune compatibility between donor organoids and recipient mice. Syngeneic engraftment is more successful with organoids harboring advanced mutations (i.e., *Apc* / ; *Kras^{G12D/+}; Trp53* /) than with *Apc* deficiency alone, most likely due to an immune response to the injection. For most mouse organoid transplantation studies, we use NSG mice as recipients. In these experiments, *Apc* / organoids will form primary colon tumors in all mice within six weeks but will not invade the muscularis propria or metastasize to the liver (Figure 3c). With *Apc* / ; *Kras^{G12D/+}; Trp53* / organoids, liver metastasis occurs in approximately 33% of mice 3 months after engraftment³⁴.

Human CRC organoid transplantation:

Human CRC organoid transplantation is performed in immunodeficient (e.g., NSG) mice. Primary tumors form in 92% of recipient mice within six weeks (Figure 3d). Primary tumors then invade the muscularis propria and metastasize to the liver in approximately 33% of mice 3 months after engraftment.³⁴

Inflammatory benign polyps:

Despite successful “bubble” formation, tumors may fail to form for a number of reasons (Table 2). In these cases, inflammatory benign polyps often form at the injection site in immunocompetent mice secondary to an immune response to the injection. These polyps are thin, soft, small, and contain inflammatory cells on histology. The polyps are not

adenomatous by histology and do not grow. Inflammatory benign polyps are present for at least six months following mucosal injection and are unchanged in size (Figure 3e, Video 6).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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a **b** **Figure 1. Colonoscopy equipment.**

(a) The colonoscopy system is composed of an anesthesia set up (including isoflurane, anesthesia chambers, oxygen, and tubing), LCD monitor, image processor, image capture device, and light source. (b) The colonoscope we use is a repurposed 1.9 mm / 9.5 French, 11 cm long Richard Wolf pediatric uteroscope. The colonoscope has an integrated working channel for insertion of the mucosal injection needle. The needle then exits at the anterior aspect of the colonoscope tip. The light connection with compatible with the Storz light source and cable.

Figure 2. Colonoscopy-guided mucosal injection technique.

(a) Cartoon of the injection needle entering the lamina propria and injecting virus or organoids into a mucosal “bubble”. **(b)** Colonoscopy images of the injection needle entering the lamina propria and forming a mucosal bubble”. Appropriate institutional regulatory board permission was obtained for these experiments.

Figure 3. Colorectal cancer modeling with colonoscopy-guided mucosal injection.

(a) Tumors in the distal colons of *Apc^{fl/fl}; Villin^{CreER}* mice following mucosal injection of 100 μ M 4-hydroxytamoxifen. Tumors were imaged with white light colonoscopy, SPIES colonoscopy, white light + SPIES colonoscopy, and H&E staining. (b) Tumorigenesis in *Rosa26^{LSL-Cas9-eGFP/+}; Villin^{CreER}* mice treated with tamoxifen and then injected with lentiviruses encoding an sgRNA against *Apc* and turboRFP (U6::sgApc-EFS::turboRFP, 10,000 TU/ μ l) into the colon mucosa. Tumors are imaged with white light colonoscopy, GFP fluorescence colonoscopy, and tRFP fluorescence colonoscopy, and GFP/tRFP/DAPI

immunofluorescence. **(c)** Tumors in the distal colons of NSG mice following orthotopic transplantation of wild-type colon organoids infected with U6::sgApc-EFS::Cas9-P2A-GFP lentivirus. Tumors are visualized with white light/GFP fluorescence colonoscopy and GFP immunofluorescence. **(d)** Tumors in NSG mice following orthotopic transplantation of patient-derived CRC organoids. Tumors are demonstrated with colonoscopy H&E staining, and compared to the histology of the patient cancer. **(e)** Inflammatory polyps in mice six weeks following successful colonoscopy-guided mucosal injection that did not result in tumor formation. Unlike adenomas, these polyps are thin-walled and exhibit large blood vessels in a pattern similar to normal colon mucosa (arrows). On H&E staining, the polyps feature nondysplastic crypts and a diffuse lymphocytic infiltration (Scale bar: 200 μ m). H&E: Hematoxylin and eosin; R26: Rosa26; SPIES: Storz Professional Image Enhancement System; tRFP: Turbo RFP; NSG: NOD-SCID-gamma. DAPI antibody: VectorLabs, catalog # H1200; EPCAM antibody: rat EpCAM-APC, 1:500, Biolegend, catalog # 17-5791-82. Appropriate institutional regulatory board permission was obtained for these experiments.

Table 1.

Mouse models of colorectal cancer. Models in bold are described in this paper.

Model	Description	Mouse genotype or background	Tumor location	Advantages	Disadvantages	Reference
Chemical Injury	DMH, AOM, or DSS + AOM	Wild-type	Distal colon	-Can be used in mice of any genetic background -inexpensive	-Requires chemical injury to the colon -Mutations are not genetically defined	52–54
GEMM	Rectal enema of Ad5CMV::Cre under surgical guidance	<i>Apc^{fl/fl}</i> <i>Apc^{fl/fl};LSL-Kras^{G12D/+}</i>	Distal colon and liver metastasis	-Produces 1-2 spatially defined tumors in the distal colon -Liver metastasis reported	-Requires genetically engineered mice -Requires surgery	6
GEMM	Rectal enema of Ad5CMV::Cre via GelFoam	<i>Apc^{fl/fl}</i>	Distal colon	-Produces genetically defined tumors in the distal colon	-Requires genetically engineered mice -Requires specialized GelFoam delivery agent	7
GEMM	Rectal enema of 4-hydroxytamoxifen	<i>Apc^{fl/fl};Trp53^{fl/fl};Villin^{creER}</i>	Distal colon and liver metastasis	-Inexpensive, simple procedure -tumor limited to distal colon -liver metastasis reported	-Requires genetically engineered mice	55
GEMM	Deletion of <i>Apc</i> in carbonic anhydrase 1 (<i>Car1</i>)-expressing cells	<i>Apc^{fl/+};Car1-cre (CAC)</i> with and without DSS colitis	Cecum to distal colon	- Tumorigenesis is limited to the colon	-Requires genetically engineered mice	56
GEMM	Tamoxifen-inducible deletion of <i>Apc</i> in carbonic anhydrase 1 (<i>Car1</i>)-expressing cells	<i>Apc^{fl/fl};Car1^{CreER}</i>	Cecum	- Tumorigenesis is limited to the colon	-Requires genetically engineered mice	57
GEMM	Tamoxifen-inducible or constitutive deletion of <i>Apc</i> in CDX2-expressing cells	<i>Apc^{fl/+};CDX2P-NLS Cre</i>	distal small intestine, cecum, and distal colon	- Tumorigenesis is predominantly in the distal colon	-Requires genetically engineered mice	58
GEMM	Doxycycline-inducible short hairpin suppression of <i>Apc</i>	<i>shApc;LSL-rtTA3;Lgr5^{creER}</i>	colon and duodenum/proximal jejunum	- <i>Apc</i> expression is turned on and off as desired	- Requires genetically engineered mice	59
GEMM	Adeno-cre, Lenti-cre, 4-hydroxytamoxifen delivery by colonoscopy-guided mucosal injection	<i>Apc^{fl/fl}</i> <i>Apc^{fl/fl}; Villin^{creER}</i>	Distal colon	-Tumors form in selected sites in distal colon -Tumors are monitored with colonoscopy	- Requires genetically engineered mice -Requires colonoscopy system and special equipment	34
GEMM	Epithelial genome editing with colonoscopy-guided injection of lentiviral	<i>Wild-type</i> <i>Rosa26^{LSL-Cas9-eGFP/+}</i> <i>Rosa26^{LSL-Cas9-eGFP/+}; Villin^{creER}</i> <i>Apc^{fl/fl}; Rosa26^{LSL-Cas9-eGFP/+}</i>	Distal colon	-Tumor induction in mice without germline mutations	-Requires expertise with molecular cloning and lentiviral production -Requires colonoscopy system and special equipment	34

Model	Description	Mouse genotype or background	Tumor location	Advantages	Disadvantages	Reference
	CRISPR-Cas9 components			-Tumors form in selected sites in distal colon -Tumors are monitored with colonoscopy		
Transplantation	Subcutaneous flank injection of tumor organoids	Human CRISPR-engineered tumor organoids into NSG mice	Flank	-Ease of use -Rapid tumor formation	-Tumors are not present in the correct microenvironment -Does not permit study of metastasis	11
Transplantation	Kidney capsule injection of tumor organoids	Human CRISPR-engineered tumor organoids into <i>Nod-scid/IL2Rγ</i> null (NOG) mice	Kidney capsule	- The kidney capsule is a permissive environment for organoid engraftment	-Requires surgery -Tumors are not present in the correct microenvironment -Does not permit study of metastasis	12
Transplantation	Orthotopic colonoscopy-guided cancer cell transplantation into the colon mucosa	Mouse and human cell lines into NSG mice	Distal colon and liver metastasis	-Tumors are located in the distal colon -Liver metastasis	-Tumors from cell lines do not reflect the histology of human colorectal cancer	32,33
Transplantation	Orthotopic cancer cell transplantation into prolapsed rectal mucosa	Human CRC cell lines and mouse tumor organoids into NSG mice	Distal colon and liver metastasis	-Tumors are located in the distal colon -Genetically engineered recipient mice are not required -Liver metastasis	-Primary tumors form only in the distal 1-1.5 cm of the rectum and	9,16
Transplantation	Orthotopic organoid transplantation into the cecal serosa	CRISPR-Cas9-engineered human cancer organoids into NSG mice	Cecum	-Genetically engineered recipient mice are not required -Liver metastasis	-Requires surgery -Tumors are located in the serosal surface of the cecum, which is not where human cancers typically form	13,14
Transplantation	Rectal enema of mouse cancer cells	C57BL/6 AKP mouse cancer cells into syngeneic recipient mice	Distal colon	-Genetically engineered recipient mice are not required -No specialized procedure or equipment are required	-Engraftment is inefficient	60
Transplantation	Rectal enema of tumor organoids into DSS colitis mice	Mouse and human CRC organoids into DSS colitis mice	Distal colon and liver metastasis	-Genetically engineered recipient mice not required -No specialized procedure or equipment are required -Liver metastasis	-Requires induction of colitis -Difficult to control the number of engrafted organoids	10
Transplantation	Colonoscopy-guided orthotopic organoid transplantation	Mouse and human CRC organoids into NSG or syngeneic mice	Distal colon and liver metastasis	-Genetically engineered recipient	-Requires colonoscopy system and special equipment	34

Model	Description	Mouse genotype or background	Tumor location	Advantages	Disadvantages	Reference
	into the colon mucosa			mice not required -Tumors form in selected sites in distal colon -Liver metastasis		

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Table 2:

Troubleshooting problems with colonoscopy-guided mucosal injection models of colorectal cancer.

Step Number	Problem	Possible Reasons	Solutions
16	Mouse is not anesthetized	-Oxygen or isoflurane supply is low	-Replace oxygen or isoflurane
16	Mouse is over anesthetized or breathing very slowly	-Isoflurane setting is high -Mouse is cold	-Reduce setting for isoflurane -Warm mouse with heating pad, warm glove, or your hands
17	Cannot remove stool from colon	-Did not flush with enough water -Did not adequately massage the abdomen	-Flush the colon again with more water -Massage the abdomen until stool is removed
17	Injection syringe is leaking	-Syringe cap is loose -Defect in injection syringe	-Tighten screw cap -Replace injection syringe
18	Injection needle is bent	The needle is small caliber and can easily bend	Replace injection needle
21	Cannot fit colonoscope into mouse colon	-Colon is dry -Mouse is too small	-Lubricate the mouse colon with a 1-3 ml tap water enema -Wait until mouse is older or use an older mouse -Switch to the Karl Storz endoscope, which has a smaller diameter than the Richard Wolf endoscop.
21	Mouse abdomen is overdistended during colonoscopy	-Air pump setting is too high	-Reduce air flow with either a nozzle on the air pump tube or by releasing air from the endoscope
21	Too many bubbles to see the colon properly	-Too much water in the colon	-Invert the mouse to allow water to pass, then start again
28	The sample disappeared after injection	-Sample leaked around the syringe -Needle is in the peritoneum, not the lamina propria	-Tighten screw cap -Be careful to insert only the tip of the needle, and then inject the sample
28	The sample leaked into the colon	-Needle is not in the lamina propria -Assistant is injecting too slowly	-During the same injection attempt, insert the needle slightly deeper, then inject another 10 µl of sample to see if you get a bubble -Inject rapidly (entire volume over less than 1 second) to overcome the resistance of the mucosa
28	The needle is slipping off the mucosa	-Needle is dull -Colon is wide or too distensible	-Replace needle -Choose a younger (i.e., 6-8 weeks old) mouse, or a female mouse
28	I felt a “pop” as the needle went completely through the colon wall	-Deep insertion of the needle	-Do not inject virus or organoids into peritoneum -Remove the needle and attempt injection at another site -Frank perforation is not a concern due to the small diameter of the needle
28	The mouse is bleeding from an injection attempt	-Self-limited bleeding is an expected complication	-Wait a few minutes, then try again -Flush the colon with water to wash out the blood
28	The bubble is small or deflated even though I injected 50 – 70 µl of sample	-The sample leaked from the injection site or from a previous injection site	-Space the injections at least 0.5 cm apart -Flip the mouse over to perform injection on the opposite (posterior) side of the colon
28	Tumors did not form despite multiple successful injections in each mouse	-Low viral titer -Low concentration of organoids -Organoids were not dissociated enough and got caught in the needle -Immune rejection of organoids -Technical difficulties in placing the needle correctly	-Increase viral titer to at least 10,000 TU/µl. -Increase organoid concentration -Dissociate organoids well, either mechanically or enzymatically -Use immunodeficient recipient mice -Use young (6-8 week old) female recipient mice -Keep the needle close to the mucosa -Ensure a good view of the mucosa, free of water, bubbles, stool, debris -Needle should enter the mucosa at a 30 degree angle -Reduce air flow rate to reduce tension on the colon