A surgical orthotopic organoid transplantation approach in mice to visualize and study colorectal cancer progression

Arianna Fumagalli[1,2], Saskia J E Suijkerbuijk[1,2], Harry Begthel[1], Evelyne Beerling[1,2], Koen C Oost[3], Hugo J Snippert[3], Jacco van Rheenen[1,2] & Jarno Drost[4]

[1]Hubrecht Institute, Royal Netherlands Academy of Arts and Sciences (KNAW) and UMC Utrecht, Utrecht, The Netherlands. [2]Molecular Pathology, Oncode Institute, The Netherlands Cancer Institute, Amsterdam, The Netherlands, Utrecht, The Netherlands. [3]Molecular Cancer Research, Center for Molecular Medicine, University Medical Center Utrecht, Utrecht, The Netherlands. [4]Princess Máxima Center for Pediatric Oncology, Utrecht, The Netherlands. Correspondence should be addressed to J.D. (J.Drost@prinsesmaximacentrum.nl) or J.v.R. (j.v.rheenen@nki.nl).

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Most currently available colorectal cancer (CRC) mouse models are not suitable for studying progression toward the metastatic stage. Recently, establishment of tumor organoid lines, either from murine CRC models or patients, and the possibility of engineering them with genome-editing technologies, have provided a large collection of tumor material faithfully recapitulating phenotypic and genetic heterogeneity of native tumors. To study tumor progression in the natural *in vivo* environment, we developed an orthotopic approach based on transplantation of CRC organoids into the cecal epithelium. The 20-min procedure is described in detail here and enables growth of transplanted organoids into a single tumor mass within the intestinal tract. Due to long latency, tumor cells are capable of spreading through the blood circulation and forming metastases at distant sites. This method is designed to generate tumors suitable for studying CRC progression, thereby providing the opportunity to visualize tumor cell dynamics *in vivo* in real time by intravital microscopy.

INTRODUCTION

CRC is one of the most common forms of cancer worldwide. Over the past decade, the implementation of preventative screening programs has produced a substantial decrease in mortality rates[1]. Nevertheless, CRC represents the second leading cause of cancer-related deaths in the Western world[2]. Cancer-related mortality is predominantly caused by development of metastases at distant sites. Despite increasing efforts to gain better insight into the mechanisms underlying metastases formation, still little is known.

In vivo studies of CRC are traditionally approached by utilizing murine models that are based on either injection of cancer cell lines[3] or genetically engineered mice (e.g., *Apc*^{min} mice[4]). Cost and pathological relevance vary across models. Although tumor models that are genetically engineered to express oncogenes (e.g., Kras, PI3K) or deplete tumor suppressors (e.g., Apc, Trp53, Smad4) closely resemble human intestinal cancers, due to the high tumor burden through the whole intestinal tract, the mice die before the tumors have progressed to a metastatic stage[4–6]. Moreover, the need to incorporate, for instance, fluorescent reporters is time-consuming, expensive and technically challenging. By contrast, tumor formation upon transplantation of cell lines is in the order of weeks and a whole genetic toolbox already exists, allowing the generation of complicated reporter cell lines. For example, Martin and colleagues have derived cell lines from genetically engineered CRC mice, transplanted them into the cecal wall and showed that *Kras*-mutant CRC depends on oncogenic signaling and on aerobic glycolysis[7]. However, it is often argued that cell lines represent selected subpopulations of the original tumor, due to propagation in tissue culture conditions, which induces selective pressure toward a particular and homogeneous dominant clone[8]. In addition, the developing tumors formed after transplantation of cell lines often do not reflect the histopathological features of human CRC[9].

The recent development of 3D epithelial organoid cultures provides a nearly unlimited *in vitro* source of genetically stable tissue. Organoids can be easily maintained and manipulated *in vitro* and faithfully recapitulate characteristics of *in vivo* tissues during homeostasis and disease such as cancer[10–13]. More recently, the establishment of living tumor organoid biobanks[14,15] containing collections of patient-derived tumor organoid cultures offers a platform for high-throughput drug screens. This potentially allows correlations between tumor genotype and patient prognosis to be defined, as well as allowing the development of patient-specific treatment regimens (personalized medicine). Although the organoid technology represents a powerful resource for finding effective therapeutic strategies directed to specific tumor subtypes[14], it still does not take into account the interplay between tumor cells and the surrounding tissue microenvironment, as this is not recapitulated in a dish. Many studies have pointed to the importance of tumor microenvironment in influencing tumor cell identity and behavior[16–18], leading to the necessity of validating *in vitro* obtained results in animal model systems.

To overcome these limitations, we have recently developed a method to orthotopically transplant murine and human tumor organoids[19]. This transplantation approach allows study of both primary tumor formation and the spontaneous development of metastases. We have recently used this model to demonstrate that transplantation of organoids carrying mutations in the Wnt, EGFR, TP53 and TGFβ/BMP pathways has the ability to form primary tumors that can progress to form distant liver and lung metastases[19]. By transplanting organoids carrying different combinations of mutations, we illustrated that metastasis is the result of niche-independent tumor growth[19].

In this protocol, we present a detailed description of the procedure for orthotopic transplantation of murine and human organoids (**Figs. 1** and **2**; **Supplementary Video 1**). We show that this approach allows the onset of a single intestinal tumor in a native-like environment within the epithelial wall of the intestinal tract (**Fig. 3**). These tumors, due to their long latency, have the chance to spread through the blood circulation and give rise to metastases at distant sites (**Fig. 3**). Moreover, this approach offers the unique possibility of applying intravital imaging technology. By imaging tumor cells, we are able to follow their migration over multiple days and characterize heterogeneous microenvironments within the same tumor mass and among different tumors (**Fig. 4**).

Potential applications

Given the unlimited opportunities that organoids offer in terms of genetic manipulation (e.g., by using shRNA[20] or, more recently, CRISPR/Cas9 technology[21,22]) and expression of fluorescent reporters, the orthotopic transplantation of CRC organoids can be used for a diversity of *in vivo* studies related to CRC biology. Of note, due to the easily accessible tumor location, this technique is specifically designed to induce tumors that can be subjected to *in vivo* imaging applications. Intravital imaging offers the unique power to visualize dynamic processes that cannot be documented with static pictures (e.g., cell migration (**Fig. 4c**,**e**), epithelial-to-mesenchymal transition and dynamic tumor growth[19,23,24]). Moreover, this orthotopic transplantation approach allows the visualization of the complete metastatic cascade (i.e., tumor cell migration, intravasation, arrival at secondary site and metastatic outgrowth) and therefore it is particularly suited for the study of the genetic alterations, and the molecular and cellular mechanisms underlying advanced and terminal stages of CRC[19]. In addition, this method enables *in vitro* organoid technology to be translated to an *in vivo* system and therefore it is suitable for preclinical validation of new targeted-therapies specifically aimed at prevention and/or reduction of metastasis formation.

Comparison with other transplantation methods

The most extensively used CRC mouse models (e.g., *Apc*^{min}-based mouse models[4]) have the disadvantage of generating a multitude of intestinal tumors. Most likely, these mice need to be euthanized, due to intestinal occlusion, before any of these tumors has had the chance to spread and metastasize. Local administration of the Cre recombinase to CRC genetic mouse models can be used to overcome this issue[25,26]. Alternatively, several xenotransplantation-based methods for studying CRC have been developed over the years (see **Table 1**). The implementation of xenotransplantation-based models of CRC, thereby minimizing tumor burden, has its main rationale in the need to study metastatic stages of colorectal carcinogenesis. Moreover, a wide range of tissue material, including human colorectal samples, *in vitro* genetically modified cell lines and, more recently, organoids can be inoculated. Nevertheless, the site chosen for transplantation is crucial in determining the potential of the experimental readout. In fact, s.c. injection[20,21] and subrenal kidney capsule[22] transplantation of *in vitro* engineered mouse and human intestinal tumor organoids allow fine discrimination of tumor-forming capacity and histopathological tumor grading, depending on the specific mutation combination present in each organoid line. However, these tumors grow in ectopic settings, surrounded by stromal characteristics different from those

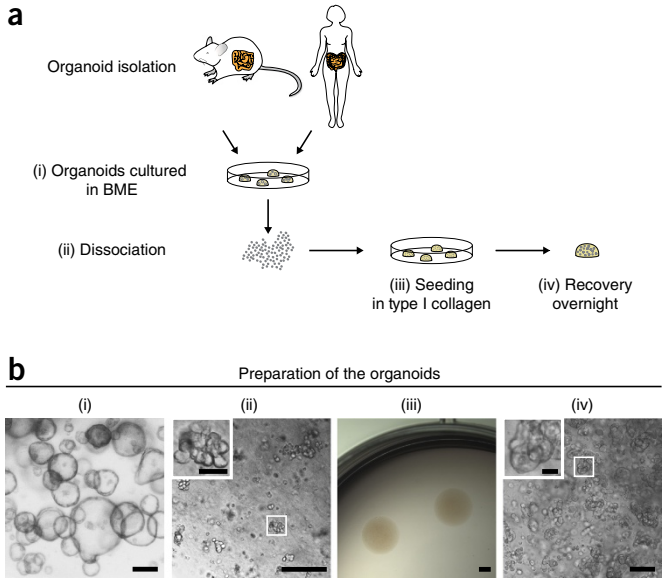

Figure 1 | Isolation and preparation of organoids for orthotopic transplantation. (**a**) Schematic exemplifying the steps required for processing of organoids for orthotopic implantation into mice. (**b**) Representative images of *Apc*$^{fl/fl}$::*Kras*$^{G12D/+}$ ::*Trp53*$^{fl/R172H}$ (AKP) murine intestinal organoid culture[19] (i), organoids after mechanical dissociation (ii), collagen grafts containing dissociated organoids (iii), and organoids recovered overnight, right before implantation (iv). Scale bars, 100 µm (i, ii, iv); 1 mm (iii); 20 µm (insets). BME, basement membrane extract. All experiments were performed in accordance with the Animal Welfare Committee of the Royal Netherlands Academy of Arts and Sciences. Animals were kept at the Hubrecht animal facility in Utrecht, The Netherlands.

of their native environments (i.e., the intestinal epithelial wall), markedly influencing tumor growth and tumor cell behaviors[27]. In this regard, ectopic xenotransplantation approaches cannot inform the aggressiveness of these tumors in terms of tumor-cell spreading and capability of colonizing distant organs. By contrast, transplantations in orthotopic settings have proven to establish intestinal tumors that, surrounded by their natural microenvironment, have the ability to metastasize at clinically relevant distant sites (i.e., liver and lungs). Previously established needle-based cecal injection of CRC cell lines resulted in efficient tumor growth and distant metastasis formation[16,28,29]. However, injection of human and mouse CRC organoids using similar approaches does not lead to tumor growth. Conversely, transplantation of similar CRISPR/Cas9 *in vitro* engineered human tumor colon organoids utilizing the method described here, allowed dissection of the contribution of specific CRC driver mutations to the different steps of the metastatic cascade, including tumor cell dissemination and metastatic outgrowth[19]. Moreover, our transplantation approach is also suitable for implanting 2D cultures, although cecum injection-based techniques might be technically easier alternatives.

Besides our approach, other recent work has reported several alternative orthotopic transplantation methods using organoids. Melo and co-workers[30] adapted a previously developed technique[31], whereby they induced a rectal prolapse and injected organoids into the colonic submucosa. In their elegant work, they defined the Lgr5^{+} tumor cells as the cancer stem cells of colorectal cancer, highlighting the critical role of this cell type in the outgrowth and maintenance of metastatic lesions. Their experiments were performed with relatively fast-growing murine tumor organoids

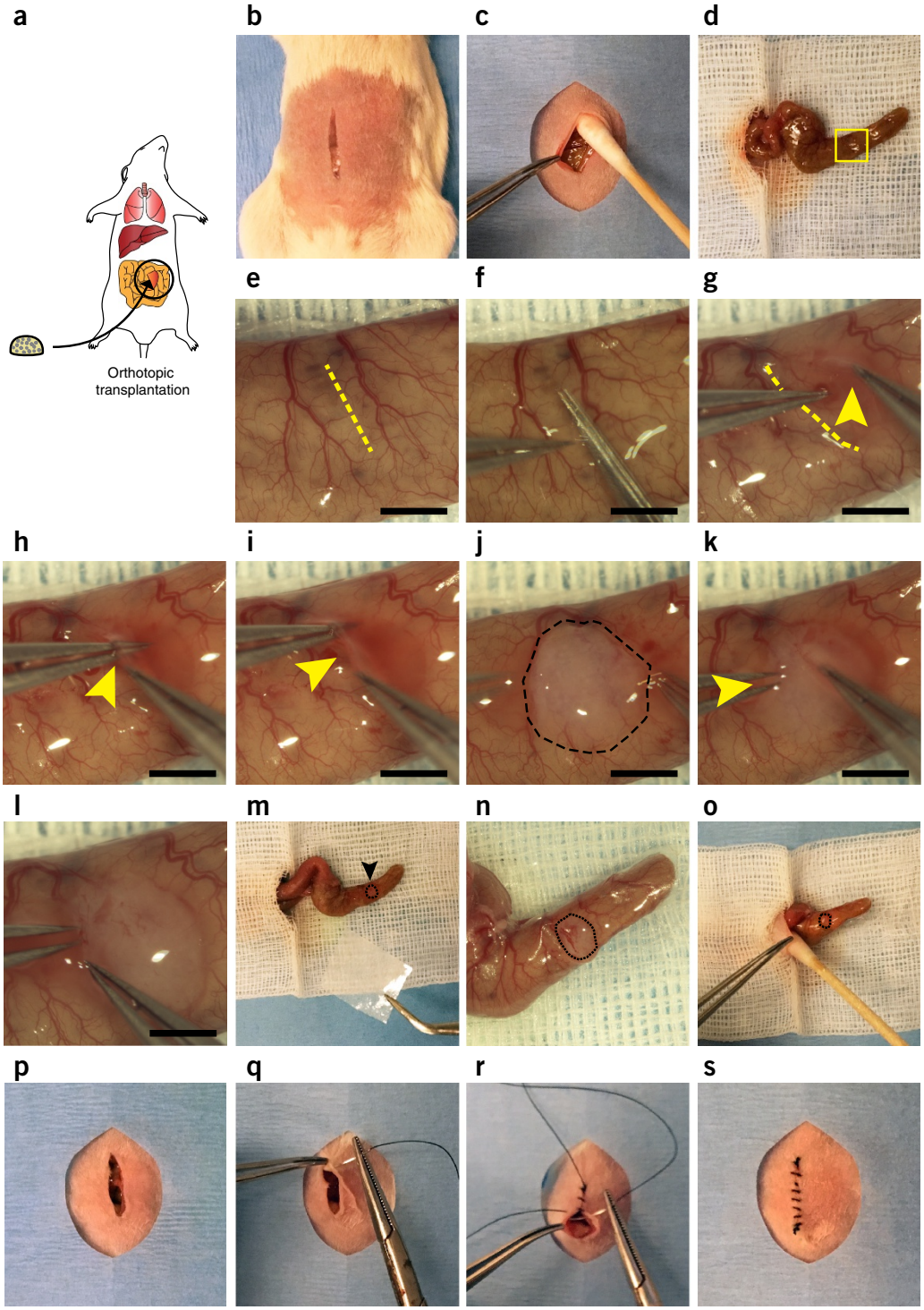

Figure 2 | Orthotopic transplantation of intestinal tumor organoids. (**a**) Schematic representation of the orthotopic transplantation of a collagen graft containing organoids. (**b**–**s**) Step-by-step visual depiction of the orthotopic transplantation procedure (see also **Supplementary Video 1**). (**b**) An incision is made through the skin. (**c**) The peritoneum is opened by an incision, exposing the abdominal organs. (**d**) The cecum is exteriorized. The yellow box indicates the area chosen for transplantation. (**e**) The surface of the cecum is inspected. The yellow dashed line indicates the position chosen to cut the serosa layer. (**f**) The serosa is opened, exposing the muscularis externa. (**g**) The serosa layer of the cecum is held up using mirror-finish forceps. The yellow dashed line indicates the original cut in the serosa. (**h**) Creation of an epithelial pocket by disruption of the muscularis externa. (**i**) The epithelial pocket is enlarged to accommodate the graft. Yellow arrowheads indicate the epithelial pocket. (**j**) The collagen graft containing organoids is placed on the cecum wall in close proximity to the epithelial pocket. The black dashed line highlights the graft. (**k**) The collagen graft is incorporated into the cecal wall inside the epithelial pocket. The yellow arrowhead highlights the action performed by the forceps on the left. (**l**) The edges of the epithelial pocket are rejoined. (**m**) A 1 x 1.5-cm piece of Seprafilm is prepared to cover the transplantation area. Black arrowhead and dashed lines indicate the graft. (**n**) The anti-adhesion barrier is placed on top of the graft. (**o**) The cecum is placed back into the abdominal cavity. (**p**) The edges of the abdominal opening are rejoined. (**q**,**r**) The abdominal opening is sutured (**q**) by stitching skin and abdominal wall (**r**). (**s**) The extremities of the suture are tightened with reef knots . Scale bars, 2 mm. All experiments were performed in accordance with the Animal Welfare Committee of the Royal Netherlands Academy of Arts and Sciences. Animals were kept at the Hubrecht animal facility in Utrecht, The Netherlands.

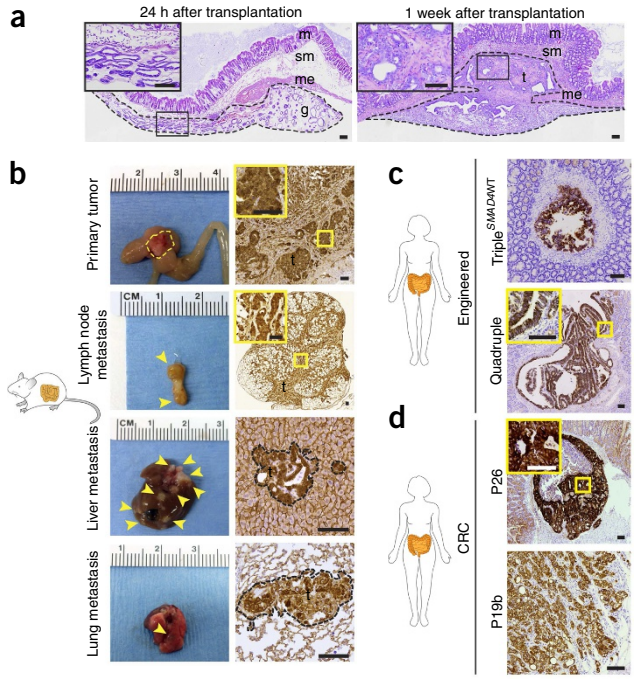

Figure 3 | Analysis of the phenotypic outcome of orthotopically transplanted intestinal tumor organoids. (**a**) Representative merged tile scan image of cross-section of the transplantation area 1 d (left) and 1 week (right) after transplantation. g, graft; m, mucosa; me, muscularis externa; sm, submucosa; t, tumor. Dashed lines highlight the graft (left) and the tumor area (right). (**b**) Orthotopic transplantation of murine AKP intestinal tumor organoids[19]. Representative pictures (left) and β-catenin staining (right, anti-β-catenin clone 14 (BD Bioscience, 1:100) of primary tumor, mesenteric lymph node, and liver and lung metastases. The primary tumor is highlighted by a dashed yellow line. Metastases are indicated with yellow arrowheads. Black dashed lines indicate the borders of tumor tissue (t) and healthy tissue. (**c**) Orthotopic transplantation of Triple*SMAD4*^{WT} (*APC*^{KO}/*KRAS*^{G12D}/*P53*^{KO}) and quadruple (*APC*^{KO}/*KRAS*^{G12D}/*P53*^{KO}/*SMAD4*^{KO}) mutant *in vitro* engineered human intestinal organoids[21]. Representative images of primary orthotopic tumors stained for human-specific cytokeratin (anti-cytokeratin clone Cam5.2 (BD Bioscience, 1:100)). (**d**) Orthotopic transplantation of patient-derived organoid lines P26 and P19b[14]. Representative images of primary orthotopic tumors stained for human-specific cytokeratin. Scale bars, 100 µm.

and a relatively short time window (6–8 weeks). Five weeks after transplantation, the colon appears to be 70–80% occluded, due to tumor growth within the intestinal lumen. However, transplantation of human organoid lines that need a longer latency before developing metastasis at distant sites (on the order of months) may lead to intestinal obstruction[31] and animals dropping out prior to the onset of metastatic lesions. Moreover, the induction of tumor formation in such distal and hardly accessible locations precludes the possibility of performing intravital microscopy (either by surgically implanting an imaging window or via terminal skin-flap experiment) and visualizing tumor cell behavior *in vivo*.

The transplantation method used by O'Rourke and colleagues was adapted from a protocol established for the engraftment of wild-type colon organoids[32] and consists of a rectal enema of tumor organoids in animals previously treated with dextran sulfate sodium (DSS), a colitis-inducing agent[33]. In this case, the mucosal damage induced by DSS promotes a regeneration process facilitating the engraftment of the infused tumor organoids. Although this strategy does not require extensive surgical intervention, the application of DSS, whose mechanisms of action are still not clear, produces

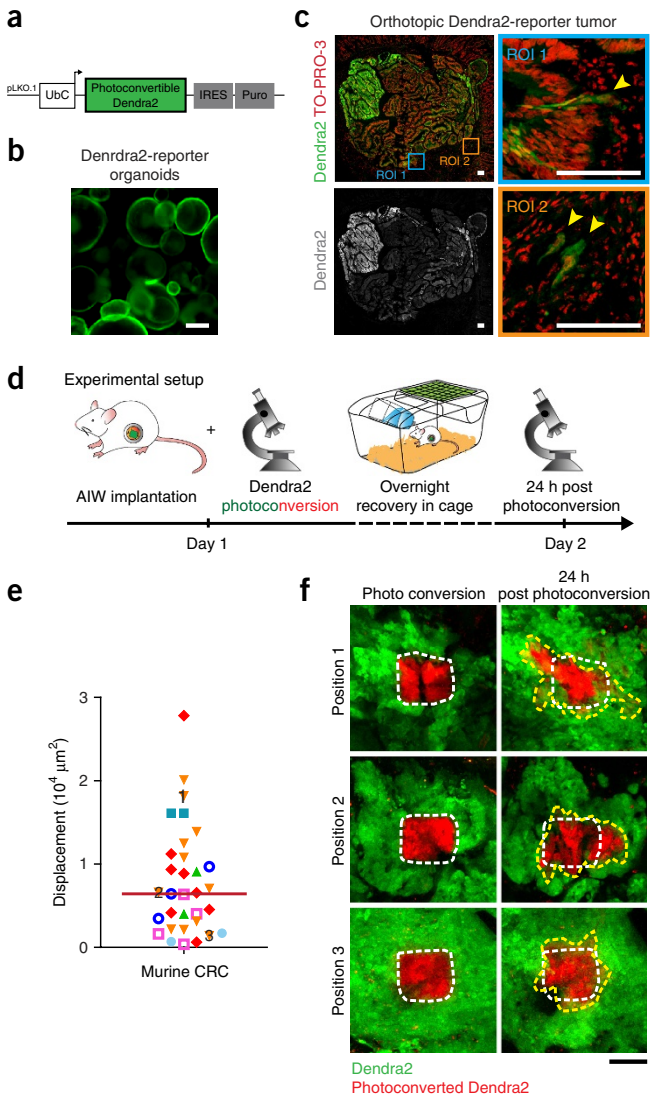

Figure 4 | Intravital imaging of inter- and intra-tumor heterogeneity. (**a**) Schematic representation of the lentiviral vector used for expression of photoconvertible Dendra2. (**b**) Representative image of Dendra2-fluorescently labeled tumor organoids. Scale bar, 100 µm. (**c**) Left, representative picture of Dendra2-fluorescently labeled orthotopic primary tumor. Right, regions of interest (ROIs) zooming on elongated potentially-invasive tumor cells (indicated with yellow arrowheads). Scale bars, 100 µm. (**d**) Cartoon showing the intravital imaging experimental setup. An abdominal imaging window (AIW) is implanted onto the orthotopic primary tumor. Photoconversion is performed in randomly picked regions within the tumor. Mice recover overnight in cages. Twenty-four hours after photoswitching, converted regions are retraced during a second imaging session. (**e**) Analysis of cell migration in orthotopic murine AKP Dendra2-reporter intestinal tumors. The graph shows the displacement of the red Dendra2 areas 24 h after photoconversion. The red line indicates the median. Each symbol represents a microenvironment and different color-coded symbol shapes represent different experimental animals. $N = 8$. (**f**) Representative intravital images of orthotopic murine AKP Dendra2-reporter intestinal tumors. (Left) Photoswitched areas at time point 0 h. (Right) Same imaging microenvironments 24 h after photoconversion. Green represents nonconverted Dendra2, whereas red highlights the photoconverted Dendra2 tumor cells. White dashed lines highlight the photoswitched areas at beginning of the experiment. Yellow dashed lines mark the edges of the red Dendra2 areas 24 h after photoconversion. Scale bar, 100 µm. AIW, abdominal imaging window; IRES, internal ribosome entry site; Puro, puromycin.

TABLE 1 | Comparison of different transplantation methods.

Model	Refs	Advantages	Disadvantages
Subcutaneous injections	21	• Technically easy • No surgical experience required • Injection on both flanks (direct comparison of tumorigenicity between different conditions)	• Ectopic environment • No metastases form
Renal capsule injections	22	• High engraftment rate facilitated by high vascularization of the recipient organ	• Ectopic environment • No metastases form
Cecal needle-based injections	16,28,29	• Tumors progress toward distant metastasis formation	• Injection of organoids does not lead to tumor formation • Implantation site might have a different microenvironment than the rest of the colon • Tumors might generate widespread peritoneal carcinomatosis
Transplantation into the cecal wall	19	• High efficiency of tumor take, for transplanting both organoids and cell lines • Avoids intestinal occlusion upon tumor growth	• Microsurgery experience required • Implantation site might have a different microenvironment than the rest of the colon
Rectal injection via creation of prolapse	30	• Tumors progress toward distant metastasis formation • Suitable for intravital imaging • Tumors progress toward distant metastases formation	• Tumor burden causes colonic occlusion • Unsuitable for intravital imaging
Mucosal damage model + rectal enema	33	• Does not require extensive surgical procedure • Tumors progress toward distant metastasis formation	• Method requires damaging of intestinal mucosa • Low engraftment rate (62%) • Unsuitable for intravital imaging
Colonoscopy-guided submucosal injections	9	• Does not require mucosal damage	• Requirement of a colonoscopy device • Limited metastatic index

general bowel inflammation and severe adverse side effects in mice (loss of body weight, ulcers and intestinal bleeding, diarrhea)[34]. Therefore, preliminary experiments are required to fine-tune the application of the DSS damaging agent in order to minimize animal dropout. Moreover, the organoid engraftment rate for the procedure used by O'Rourke and colleagues appears substantially lower (62%) than that observed upon surgical implantation of comparable organoid lines (*Apc*$^{fl/fl}$::*Kras*$^{G12D/+}$::*Trp53*$^{fl/R172H}$ (AKP), 100%) performed with the method presented here, with an extremely low metastatic rate (1 mouse out of 6 analyzed at 16 weeks post enema, compared to 100% at 10 weeks post transplantation, $N = 22$).

Finally, murine- and patient-derived tumor organoids were successfully engrafted in the colon submucosa by Roper and co-workers[9], using a colonoscopy-guided transplantation technique[35]. A detailed description of this methodology is presented in protocol form by Roper *et al.*[36]. Although this approach does not require injury of the intestinal mucosa for engraftment of tumor organoids, the need for a custom-made colonoscopy device does limit the use of this method to a restricted number of researchers. Moreover, transplantation of AKP murine organoids into immune-compromised mice does lead to formation of only a few metastatic foci that are restricted to the liver[9], whereas the method we describe here leads to tumors that are able to metastasize to both liver and lungs with 100% efficiency (results obtained upon transplantation of five independent lines). Importantly, in addition to the application on the cecal wall, our approach is also suitable for transplantation into the colonic epithelium.

Nevertheless, if researchers intend to perform our method on the colon, the following considerations must be taken into account: (i) the tumor take upon transplantation into the cecal wall (100%) is higher than that in the colon (60%); (ii) tumor growth in the colon can lead to intestinal obstruction and animal dropout before the onset of metastatic lesions. This was never observed upon transplantation of organoids in the cecal mucosa. (iii) Although never carefully analyzed, the presence of potential differences in the microenvironment between colon and cecum might affect tumor growth dynamics. Researchers may want to choose whether to perform the approach in either the colon or cecum depending on their specific experimental needs and interests.

TABLE 2 | Hazard awareness.

Hazard	Precaution
Handling of lentiviruses	• Work in biosafety level 2 (BSL-2) tissue culture hood and under BSL-2 guidelines • Wear suitable protective clothing • Decontaminate culture/spills with 10% bleach
Work with laboratory animals	• Transplantation of human cells into a laboratory animal classifies the animal as a BSL-2 animal, and experiments should be performed under the corresponding guidelines
Isoflurane anesthesia	• Make sure to connect the anesthesia machine to a scavenger unit • Wear suitable protective clothing (e.g., face mask)
Buprenorphine hydrochloride	• Wear suitable protective clothing

Limitations of the method

The orthotopic transplantation approach described in this article is based on surgical implantation of an organoid-containing collagen graft, which is applied to the submucosal layer of the cecal wall. Therefore, tumor growth is not initiated from the luminal side of the intestinal wall. Importantly, we observed that during primary tumor expansion cells invade and colonize the mucosa–submucosa interphase and eventually fuse with the normal intestinal epithelium (**Fig. 3a**,**c**, quadruple).

Moreover, it is important to note that the use of human-derived material implicates the selection of immune-compromised mice as acceptors. The lack of tumor–immune system interactions excludes the possibility of evaluating the role of the immune system in human colorectal cancer progression. Reconstitution of immune-deficient mice with a human immune system could potentially solve this issue[37].

Experimental design

Isolation and preparation of organoids for cecum transplantations (Reagent Setup and Steps 1–20). In this protocol, we present a transplantation technique based on orthotopic implantation of both mouse and human colorectal tumor organoids. We refer to **Table 2** for hazard awareness for our protocol.

Since the establishment of culture systems for mouse and human intestinal organoids[10,11], many excellent protocols have been published describing organoid culture and introduction of viral transgenes in detail[38–41]. Therefore, these procedures we will not be discussed here.

For setting up the system, we have used mouse intestinal carcinoma organoids (AKP) (**Fig 1b** (i)). These organoids have been generated from VillinCreER^{T2}::AKP genetic mice. Administration of tamoxifen to these mice induces specific activation of the Cre enzyme within the epithelial cells of the small and large intestine, with consequential deletion of both *Apc* alleles, expression of an oncogenic form of Kras (Kras^{G12D}) and deletion of one of the two *Trp53* alleles (while the other allele is constitutively mutated i.e., *Trp53*^{R172H}). To obtain organoids, mice were sacrificed 3 d post injection of tamoxifen. The intestine was subsequently processed to obtain organoids as previously described[10]; however, the PROCEDURE should also work with organoids established by other protocols. Mechanical dissociation and or mild trypsinization of organoids the day before transplantation is recommended to stimulate fusion between organoids (**Fig. 1b** (iv)) and therefore ensure optimal tumor growth *in vivo*. AKP organoids showed 100% (N = 22) primary tumor take and metastasis formation.

Organoid transplantation (Steps 21–38). A meticulous step-by-step description of the PROCEDURE is provided below, illustrated in **Supplementary Video 1**. We highly recommend paying particular attention to Steps 31–34 (**Fig. 2f–n**). It is extremely critical to avoid unintentional damage to blood vessels and perforation of the cecal wall during implantation of the organoid-containing collagen graft. With these precautions, we never experienced animal dropout following the transplantation procedure.

Previous attempts to implant CRC patient-derived intact tumor pieces on the surface of the cecum led to formation of widespread peritoneal carcinomatosis[42]. This phenotype is clinically only a secondary effect of colorectal cancer and it could be linked to remaining tumor cells that are left behind within the abdominal cavity upon surgical removal of the intestinal tumor[43]. To address this, we strongly suggest the use of tissue-compatible anti-adhesion barriers (such as Seprafilm), to ensure stability of the graft and proper healing of the epithelium once the transplantation procedure is completed (**Fig. 2m–n**). This prevents inadvertent seeding of tumor cells into the abdominal cavity, avoiding formation of primary abdominal carcinomatosis.

Monitoring tumor formation and disease progression (Anticipated Results). To explore the applicability of the method, we transplanted murine CRC organoids[19], CRISPR/Cas9 *in vitro* engineered human intestinal tumor organoids[21] and patient-derived CRC organoid lines[14] (**Fig. 3b–d**). Tumor growth can be monitored by abdominal palpation of the transplanted mice subjected to mild anesthesia (2% (vol/vol) isoflurane gas for 2 min). Fine detection of tumor formation and metastatic onset can be visualized with bioluminescence or, when available, positron emission tomography and MRI. It is important to note that over time animals can develop a severe metastatic phenotype that can culminate in accumulation of abdominal ascites and multi-organ failure.

Control experiments. To rule out the possibility that the metastatic foci observed upon orthotopic organoid transplantation are not derived from unintentional seeding of tumor cells within the blood or lymphatic circulation while performing the surgery, we suggest including control mice that are sacrificed at specific time points. In our experiments, control mice were inspected at 24 h, 1 week and 4 weeks after transplantation. We never detected the presence of metastases at distant sites (mesenteric lymph nodes, liver and lungs), indicating that the secondary tumors are actually the consequence of the natural progression of the disease.

MATERIALS

REAGENTS

Organoid culture and lentiviral transduction

- TrypLE Express Enzyme (1×), phenol red (Thermo Fisher Scientific, cat. no. 12605-010)
- Dulbecco's modified Eagle medium (DMEM; Thermo Fisher Scientific, cat. no. 31966)
- Advanced DMEM/F12 (adDMEM/F12; Thermo Fisher Scientific, cat. no. 12634-010)
- GlutaMAX, 100× (Thermo Fisher Scientific, cat. no. 35050-061)
- Penicillin–streptomycin (Thermo Fisher Scientific, cat. no. 15140-122)
- HEPES (Thermo Fisher Scientific, cat. no. 15630-056)
- Zeocin Selection Reagent (Thermo Fisher Scientific, cat. no. R250-01)
- Puromycin (InvivoGen, cat. no. ant-pr-1)
- PBS (Sigma-Aldrich, cat. no. P4417)
- Matrigel, growth factor reduced (GFR), phenol red-free (BD, cat. no. 356231)
- B27 supplement, 50×, serum-free (Thermo Fisher Scientific, cat. no. 17504-044)
- Nicotinamide (Sigma-Aldrich, cat. no. N0636)
- *N*-Acetyl-L-cysteine (Sigma-Aldrich, cat. no. A9165)
- A83-01 (Tocris Bioscience, cat. no. 2939)
- Y-27632 dihydrochloride (Abmole Bioscience, cat. no. M1817)
- Recombinant human EGF (PeproTech, cat. no. AF-100-15)
- Recombinant human Noggin (PeproTech, cat. no. 120-10C)
- Recombinant human R-spondin 1 protein (R&D Systems, cat. no. 4645-RS-025) or R-spondin 1-conditioned medium[44] produced by R-spondin1–producing cell line (available from the C. Kuo laboratory (Stanford University) or via commercial sources (e.g., Amsbio, cat. no. 3710-001-01). See also Reagent Setup ▲ **CRITICAL** Low-passage cells, up to 15 passages, must be used and cells must be regularly checked to ensure authenticity and that they are not infected with mycoplasma. ▲ **CRITICAL** We have used only the R-spondin1–producing cell line from the Kuo lab and have no data regarding how similar the various products that are now commercially available are. Pilot studies to check suitability may be necessary.
- Wnt3a-conditioned medium. The L-Wnt3A cell line is used to produce Wnt3a-conditioned medium and is available from the H. Clevers laboratory (Hubrecht Institute) or the American Type Culture Collection (cat. no. CRL-2647). See also Reagent Setup.
- SB 202190 (Sigma-Aldrich, cat. no. S7076)
- FBS (Sigma-Aldrich, cat. no. F7524)
- **Organoids**
- We have used small intestinal organoids derived from VillinCreER^{T2}::*Apc*$^{fl/fl}$::*Kras*$^{G12D/+}$::*Trp53*$^{fl/R172H}$ (AKP) genetic mice[19]. For our experiments, mice of different ages were used, and we have no indications that either organoid establishment or transplantation efficiency are affected by the age of the mice used for derivation of the organoid cultures. To our knowledge, the transplantation efficiencies are not affected by the passage number of the organoids used. We have used human organoids derived from normal intestinal epithelium engineered to harbor mutations in *APC*, *KRAS* and *TP53* (*APC*^{KO}/*KRAS*^{G12D}/*TP53*^{KO}, termed Triple*SMAD4*^{WT}) and *APC*, *KRAS*, *TP53* and *SMAD4* (*APC*^{KO}/*KRAS*^{G12D}/*P53*^{KO}/*SMAD4*^{KO}, termed quadruple) using CRISPR/Cas9 (ref. 21). In addition, patient-derived CRC organoids (P19b and P26)[14] were used. We have used organoids from different passages and we do not have any indications that transplantation efficiencies are affected by organoid passage number ! **CAUTION** Experiments using human tissue must conform to institutional and national regulations and informed consent must be obtained from donors. Approval for human organoid studies was obtained from the ethics committee of the University Medical Center Utrecht. All patients provided informed consent. ! **CAUTION** Experiments using mice must conform to institutional and national regulations. All of our experiments were performed in accordance with the Animal Welfare Committee of the Royal Netherlands Academy of Arts and Sciences.
- Collagen I, high concentration, rat tail (Corning, cat. no. 354249; concentration range: 8–11 mg/ml)
- Minimum essential medium (MEM) alpha, no nucleosides, powder (Thermo Fisher Scientific, cat. no. 12000-014)
- Sodium bicarbonate ($NaHCO_3$)
- Demineralized water

Plasmids

- pLKO.1-UbC-Dendra2-Puro[19]

Immunohistochemistry

- Anti-β-catenin clone 14 (1:100; BD Bioscience, cat. no. 610154)
- Anti-cytokeratin clone Cam5.2 (1:100; BD Bioscience, cat. no. 345779)
- HRP-mouse, ready to use (EnVision+ Single Reagents; DAKO, cat. no. K4001)
- TO-PRO-3 (1:10,000; Thermo Fisher Scientific, cat. no. T3605)

Laboratory animals

- NSG (NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ) mice (The Jackson Laboratory, stock no. 005557) ! **CAUTION** All animal experiments should conform to relevant local guidelines and regulations. All experiments we carried out were performed in accordance with the Animal Welfare Committee of the Royal Netherlands Academy of Arts and Sciences. Animals were kept at the Hubrecht animal facility in Utrecht, The Netherlands ▲ **CRITICAL** For all animal experiments, use age- and gender-matched mice when comparing experimental versus control groups. For example, for transplantation of human organoids carrying different mutation combinations, we always used 8- to 14-week-old male mice.

Animal welfare

- Buprenorphine hydrochloride, 0.3 mg/ml (Buprecare Multidosis; ASTfarma). This compound can be stored at room temperature (our room temperature is always < 25 °C) protected from light and used until 28 d after first opening ! **CAUTION** Buprenorphine hydrochloride may cause prolonged respiratory depression. Wear protective clothing to avoid contact or inhalation. Buprenorphine is a controlled substance and should be handled according to relevant institutional rules.

EQUIPMENT

- Conical 50-ml tubes (Greiner Bio-One, cat. no. 227661)
- Conical 15-ml tubes (Greiner Bio-One, cat. no. 188271)
- Microcentrifuge 1.5-ml tubes (Eppendorf, cat. no. 0030120086)
- 37 °C shaking platform
- Plates, 6-well (Greiner Bio-One, cat. no. 657 160)
- Plates, 12-well (Greiner Bio-One, cat. no. 665 180)
- Plates, 24-well (Greiner Bio-One, cat. no. 662 160)
- Plates, 48-well (Greiner Bio-One, cat. no. 677 180)
- Parafilm (Parafilm, cat. no. PM-996)
- Cell culture dishes, 100 × 20 mm (Greiner Bio-One, cat. no. 664 160)
- Glasstic Slide with hemocytometer counting grid (Kova International, cat. no. 87144E)
- Glass Pasteur pipettes (VWR, cat. no. 612-1701)
- Light microscope (Nikon, model no. Eclipse TS100)
- Dissection microscope (Leica, model no. MZ75)
- Surgical microscope on table-top stand (Leica, model no. M651 MSD)
- Disposable scalpels, no.10 (Swann-Morton, cat. no. 0501)
- Microcentrifuge (Eppendorf, model no. 5424)
- CO_2 incubator (5% (vol/vol) CO_2, 37 °C)
- 0.22-µm sterile syringe filters (Millipore, cat. no. SLGS033SS)
- Level 2 biosafety cabinet
- Seprafilm adhesion barrier (Genzyme Biosurgery, cat. no. 4301-03)
- Histocassette (KP-Megacassette; Klinipath, cat. no. 8064)
- Perma-Hand 5-0 silk sutures (Ethicon, cat. no. 719H)
- Sterile cotton gauze, 8.5 × 5 cm (Klinipress hg compress; Klinion, cat. no. 111 001)
- Cotton swabs (Heinz Herenz, cat. no. 1030118)
- 1 ml Syringe (BD Plastipak, cat. no. 303172)
- Anesthesia machine (Vet Tech Solutions)
- Ventilator (active scavenger unit, Vet Tech Solutions)
- Heating pad (Inventum Holland, cat. no. HNK513)
- Surgical drape (Medline Industries, cat. no. GEM2140)
- Sterile surgical gloves (SemperMed, cat. no. 822751621)
- Surgical facemask (Vet Tech Solutions)
- Medical oxygen (O_2 21.5% (vol/vol), N_2 78.5% (vol/vol); AIRAPY; Linde)
- Isoflurane (100% (wt/wt) solution for inhalation anesthetic; IsoFlo; Abbott, cat. no. B506)
- Hair clipper (Contura; Wella)
- Fine-iris scissors, 24 mm, straight (Fine Science Tools, cat. no.14090-09)
- Fine-iris scissors, 26 mm, straight (Fine Science Tools, cat. no.14090-11)
- Vannas-Tübingen spring scissors (Fine Science Tools, cat. no. 15003-08)
- Curved forceps (Fine Science Tools, cat. no. 11029-14)
- Iris forceps, 0.6-mm tip, curved (Fine Science Tools, cat. no. 11065-07)
- Two Dumont mirror-finish forceps, no. 5 (Fine Science Tools, cat. no. 11252-23)

• Hartman hemostatic forceps, straight (Fine Science Tools, cat. no. 13002-10)

REAGENT SETUP

Basal medium (adDMEM/F12+++) Advanced DMEM/F12 containing 2 mM GlutaMAX, 10 mM HEPES and 100 U/ml penicillin–streptomycin. Store the medium at 4 °C for up to 1 month.

Wnt3a-conditioned medium Production of Wnt3a-conditioned medium using the L-Wnt3a cell line has been previously described in detail in Box 1 of refs. 38,41. Wnt3a-conditioned medium can be stored at 4 °C for up to 6 months. ▲ **CRITICAL** Low-passage cells, up to 15 passages, must be used and cells must be regularly checked to ensure authenticity and that they are not infected with mycoplasma.

R-spondin-1-conditioned medium Production of R-spondin-1-conditioned medium using 293T-HA-Rspo1-Fc cells has been previously described in detail in Box 1 of refs. 38,41,44. R-spondin-1-conditioned medium can be stored at −20 °C for up to 6 months.

N-acetyl-L-cysteine Dissolve 81.5 mg of N-acetyl-L-cysteine per ml of H_2O to prepare a 400× 500 mM stock solution. Store aliquots at −20 °C for up to 1 month.

Nicotinamide Dissolve 1.2 g of nicotinamide in 10 ml of PBS to prepare a 100× 1 M stock solution. Store aliquots at −20 °C until the expiration date.

Recombinant human EGF Dissolve 1 mg of recombinant human EGF in 2 ml of PBS + 0.1% (wt/vol) BSA to prepare a 10,000× 0.5 mg/ml stock solution. Store aliquots at −20 °C for up to 1 month.

Recombinant human Noggin Dissolve 100 µg of recombinant human Noggin in 1 ml of PBS + 0.1% (wt/vol) BSA to prepare a 1,000× stock solution. Store aliquots at −20 °C for up to 1 month.

Y-27632 dihydrochloride Dissolve 50 mg of Y-27632 dihydrochloride in 1.5 ml of H_2O to prepare a 1,000× 100 mM stock solution. Store aliquots at −20 °C for up to 1 month.

SB202190 Dissolve 25 mg of SB202190 in 2.75 ml of DMSO to prepare a 30 mM 10,000× stock solution. Store aliquots at −20 °C for up to 1 month.

A83-01 Dissolve 10 mg of A83-01 in 950 µl of DMSO to get a 25 mM 50,000× stock solution. Store aliquots at −20 °C for up to 1 month.

Mouse colon culture medium To make 50 ml of medium, mix 1.0 ml of B27, 125.0 µl of *N*-acetyl-L-cysteine (500 mM in H_2O), 5.0 µl of EGF (0.5 mg/ml in PBS + 0.1% (wt/vol) BSA), 50.0 µl of Noggin (100 µg/ml in PBS + 0.1% (wt/vol) BSA), and 50.0 µl of R-spondin 1 (500 µg/ml in PBS + 0.1% (wt/vol) BSA) or 5 ml of R-spondin-1-conditioned medium (10%), and top up to 50 ml with adDMEM/F12+++ medium. If organoids stably express Dendra2, puromycin can be added to the culture medium (final concentration = 1 µg/ml) to prevent gene silencing. ▲ **CRITICAL** The prepared medium can be stored at 4 °C for up to 1 week.

Human colon culture medium To make 50 ml of medium, mix 1.0 ml of B27, 500 µl of nicotinamide (1 M in PBS), 125.0 µl of *N*-acetyl-L-cysteine (500 mM in H_2O), 5.0 µl of EGF (0.5 mg/ml in PBS + 0.1% (wt/vol) BSA), 5.0 µl of A83-01 (5 mM in DMSO), 50.0 µl of Noggin (100 µg/ml in PBS + 0.1% (wt/vol) BSA) BSA), 50.0–100.0 µl of R-spondin 1 (500 µg/ml in PBS + 0.1% BSA) or 5 ml of R-spondin-1-conditioned medium (10%), 25 ml of Wnt3A-conditioned medium (50%), and 5.0 µl of SB202190 (30 mM in DMSO), and top up to 50 ml with basal medium. If organoids stably express Dendra2, puromycin can be added to the culture medium (final concentration = 1 µg/ml) to prevent gene silencing. ▲ **CRITICAL** The prepared medium can be stored at 4 °C for up to 1 week.

Matrigel Thaw the original bottle overnight at 4 °C on ice. Mix well by pipetting and divide the Matrigel into 1-ml aliquots in 1-ml cryovials. Aliquots can be stored at −20 °C until the expiration date.

5× Collagen neutralization buffer Dissolve 2.5 g alpha MEM powder (5×) and 2% (wt/vol) $NaHCO_3$ in 45 ml of demineralized water and then add 5 ml of 1 M HEPES, pH 7.5. Filter the solution through a 0.22-µm filter. Store the solution at 4 °C. It can be used for up to 1 year.

Neutralized high-concentration type I collagen Mix high-concentration type I collagen and 5× collagen neutralization buffer in a 4:1 (vol/vol) ratio. The final concentration of type I collagen within the mix ranges between 6.4 and 8.8 mg/ml, depending on the initial concentration of the high-concentration type I collagen stock provided by the manufacturer. Keep the mixture on ice. ▲ **CRITICAL** The mixture should be freshly prepared.

Buprenorphine hydrochloride Dissolve 1 ml of the 0.3 mg/ml buprenorphine hydrochloride stock solution in 9 ml of sterile demineralized water. ▲ **CRITICAL** The solution should be freshly prepared before each surgery session.

EQUIPMENT SETUP

Surgical station The surgery must be performed in an aseptic working environment, with an isoflurane anesthesia machine and ventilator. Position the electric heating pad and disinfect it using 70% (vol/vol) ethanol. Ensure there is sufficient medical oxygen in the tank and isoflurane in the vaporizer to perform surgery.

Surgery preparation Before surgery, the following items must be sterilized: scissors, forceps, Hartman hemostatic forceps. Autoclave the kit together with some surgical drapes and cotton swabs.

At the surgical station, clean the microscope with ethanol before proceeding with surgery. After shaving the mouse, place the sterile drape over the heating pad, and put the ethanol-cleaned facemask on top. Place the surgical tray containing the tools open on the edge of the wrap, and carefully place the sterile needle, sterile cotton gauze and ethanol-cleaned histocassette on it. Open a 15-ml tube and add 10 ml of sterile PBS. Open the Seprafilm cardboard envelope and place the plastic envelope containing the Seprafilm next to the surgical wrap. Make sure to wear sterile surgical gloves during surgery.

Animal housing Animals can be housed under standard conditions. Mice that have undergone abdominal imaging window (AIW) surgery must be housed singly to prevent damage to the implanted imaging window.

PROCEDURE

Orthotopic transplantation of tumor organoids: preparation of the cells ● TIMING 1.5 h + overnight recovery

1| Prewarm tissue culture 6-well plates overnight at 37 °C.

2| Collect organoids (~3–5 d after passaging the culture; **Fig. 1b** (i)) in the culture medium in the well containing the organoids, and transfer them to a 15-ml conical tube.

3| Add 3–5 ml of ice-cold adDMEM/F12+++ medium.

4| Centrifuge at 200*g* for 5 min at 4 °C.

5| Aspirate the supernatant and resuspend the organoids in 500–1,000 µl of prewarmed TrypLE Express plus 10 µM Y-27632.

6| Incubate at 37 °C for 5–10 min and subsequently pipette up and down 5–10 times using a P1000 tip. Check the mixture every 2 min using a light microscope to make sure the organoids are dissociated in clumps of 5–10 cells (**Fig. 1b** (ii)).
▲ **CRITICAL STEP** Keep the dissociation time as short as possible, as trypsinization for too long results in reduced viability.
? TROUBLESHOOTING

7| Add 10 ml of ice-cold adDMEM/F12+++ medium.

8| Centrifuge at 200*g* for 5 min at 4 °C.

9| Aspirate and discard the supernatant.

10| Resuspend the cells in 3–5 ml of ice-cold adDMEM/F12+++ medium.

11| Using a cell counter, count the number of cells within the sample.

12| Dilute the sample in order to collect ~2.5 × 10^5 cells per transplantation procedure (i.e., ~10^6 cells to transplant four mice).

13| Place the collected cells into a 15-ml conical tube.

14| Top up with ice-cold adDMEM/F12+++.

15| Centrifuge the tube at 300*g* for 3 min at 4 °C.

16| While centrifuging, prepare the neutralized highly concentrated collagen mix by gently mixing high-concentration type I collagen and 5× collagen neutralization buffer in a 5:1 (vol/vol) ratio.
▲ **CRITICAL STEP** High-concentration type I collagen is highly viscous. To pipette the right amount to be used, cut the extremity of the pipette tip with 70% (vol/vol) ethanol-sterilized scissors.

17| Aspirate and discard the supernatant from the tube from Step 15.

18| Resuspend the 2.5 ×10^5 cell pellet in 15 µl of neutralized high-concentration collagen mix (i.e. ~10^6 cells to transplant four mice) and place a drop into 1 well of a 6-well plate (**Fig. 1b** (iii)).

19| Place the plate into the CO_2 incubator (5% (vol/vol) CO_2, 37 °C) for 20 min to allow the collagen to solidify.

20| Gently pipette 500 µl of prewarmed (37 °C) culture medium into each well and place the plate into a CO_2 incubator (5% (vol/vol) CO_2, 37 °C) overnight for recovery (**Fig. 1b** (iv).
▲ **CRITICAL STEP** It is necessary to add Y-27632 dihydrochloride to the medium at this stage of the culture. Y-27632 dihydrochloride enhances outgrowth and viability of the cells after plating.

Preparation for the surgery ● TIMING ~10 min
! CAUTION All animal experiments should conform to relevant local guidelines and regulations.

21| Using ~2.0% (vol/vol) isoflurane, anesthetize a mouse in an induction chamber.

22| Use a 1-ml syringe with a 25-gauge needle to inject 100 µl of 0.03 mg/ml buprenorphine subcutaneously.

23| Place the unconscious mouse on the heating pad with its nose in the facemask. Lower the isoflurane to 1.5% (vol/vol).
! CAUTION Do not overheat the mouse because this enhances the depth of the anesthesia and might be lethal due to respiratory failure.
▲ **CRITICAL STEP** Continuously monitor the mouse's breathing and reflexes.

24| Position the mouse on its back and fix the forelegs in a V shape with tape. Shave the abdomen of the mouse. Disinfect the shaved area with 70% (vol/vol) ethanol.
▲ **CRITICAL STEP** It is essential to remove as much hair as possible to prevent contamination of the surgical wound.

25| Remove the medium from the well containing the organoids that are to be transplanted.
▲ **CRITICAL STEP** It is essential to remove the medium a few minutes before starting the surgery to let the grafts settle. This is critical to prevent the grafts from rupturing while being manipulated during implantation into the intestine wall.

Surgical procedure ● TIMING ~20 min
▲ CRITICAL See also **Supplementary Video 1**, which shows this section visually.

26| Using the curved iris forceps, hold the skin and make a 15-mm vertical midline incision through the skin using the 24-mm iris scissors (**Fig. 2b**). Take the small sterile surgical drape and create a rhomboidal incision in the middle of it. Isolate the surgical area by placing the sterile drape around the incision on top of the abdomen. Incise the linea alba to separate the rectus abdominis muscles and open the abdomen (**Fig. 2c**).
! CAUTION Spare the large vessels by performing a midline incision in the abdominal wall.
! CAUTION Make sure to avoid damaging the abdominal organs during the incision. Lift the abdominal wall with the curved forceps and bluntly open the peritoneal cavity. Wait for air to flow into the abdomen and for the release of any organs adhering to the anterior abdominal wall, before enlarging the laparotomy incision.

27| Take the sterile gauze and create a rhomboidal incision in the middle of it. Isolate the surgical opening by placing the gauze on top of the mouse abdomen. Spread some sterile PBS on the gauze to keep the surgical area hydrated.

28| Place the histocassette next to the mouse, on its left side, underneath the sterile gauze. This setup will be used to steadily support the organ during implantation.

29| Drape out the cecum (usually it is located at upper left side of the abdomen) using sterile cotton swabs drenched in PBS.
! CAUTION Try to avoid damaging any organ in the abdomen. Do not use sharp tools to hold the intestine.

30| Place the cecum horizontally on top of the histocassette (**Fig. 2d**).
▲ CRITICAL STEP It is essential to keep the intestine hydrated with generous amounts of PBS while performing extra-abdominal surgery.

31| Using Dumont mirror-finish forceps, gently hold up the serosa layer of the cecum and cut the serosa layer by making a 3- to 4-mm vertical incision with the spring scissors (**Fig. 2f**).
! CAUTION It is critical to cut only the external layer of the intestinal epithelium. Perforating the entire wall can compromise the surgery, leading to premature death of the experimental animal. Perform the cut while looking through a stereomicroscope, using a 25–40× magnification.
! CAUTION Perform the cut of the serosa between the large blood vessels (**Fig. 2e**). This will avoid causing unintentional bleeding.
▲ CRITICAL STEP It is essential to keep the epithelium hydrated with sterile PBS to prevent rupture of the serosa while manipulating it.
? TROUBLESHOOTING

32| Separate the serosa from the underlying submucosa layer by removing the muscularis externa using two pairs of Dumont mirror-finish forceps. Perform this procedure on both sides of the opening to create a pouch with the size of the graft (**Fig. 2g–i**).
! CAUTION Pay attention to avoid perforating the intestinal wall. Perform this operation while looking through a stereomicroscope, using a 25–40× magnification.
? TROUBLESHOOTING

33| Using curved iris forceps, gently pick up a collagen drop containing the organoids (from Step 25), place it on the cecum and push it inside the epithelial pouch (**Fig. 2j–l**).
? TROUBLESHOOTING

34| Cover the transplantation area with a 1 × 1.5-cm piece of Seprafilm (**Fig. 2m,n**).
▲ CRITICAL STEP Use of Seprafilm is critical to prevent post-operative adhesions between the abdominal wall and the injured intestinal area.

35| Place the cecum back into the abdomen using cotton swabs drenched in PBS (**Fig. 2o**).
! CAUTION Try not to touch the graft to prevent it falling out of the pouch.

36| Close the abdominal wall and skin opening, performing continuous stitching. Close the extremities of the suture with reef knots (**Fig. 2p–s**).

Postoperative care ● TIMING 5 min on a regular basis for the duration of the remainder of the experiment

37| Monitor the animal until it is awake and mobile.

? TROUBLESHOOTING

38| The day after surgery, check on the animal to make sure that the sutures are still correctly in place.

? TROUBLESHOOTING

? TROUBLESHOOTING

Troubleshooting advice can be found in **Table 3**.

TABLE 3 | Troubleshooting table.

Step	Problem	Possible reasons	Solution
6	Small organoids/large cell clump pieces remain after trituration	Inefficient trituration	(i) Repeat trituration for a longer time
			(ii) Digest organoids using TrypLE. The duration of the enzymatic digestion is variable and dependent on the organoid line used. Pipette up and down with a fire-polished glass pipette after digestion
31	Bleeding after cutting of the serosa layer	Accidental perforation of the epithelium	Continue the surgical implantation of the collagen graft containing organoids, paying special attention to carefully sealing the implanted area with a Seprafilm adhesion barrier. The animal must be monitored with exceptional care during postsurgical recovery, as peritonitis might occur due to leakage of intestinal contents into the abdominal cavity. In this case, we recommend terminating the experiment at the first sign of discomfort in the animal, according to the local guidelines for animal experiments
		Damaging of the blood vessels underneath	Use a dry sterile cotton swab to stop the bleeding. Do not forget to rehydrate the area afterward using sterile PBS
32	Rupture of the serosa while creating the epithelial pocket	Epithelium was not well hydrated	Create the epithelial pocket only on one side of the serosa opening; it should be large enough to contain the entire collagen blob
33	Collagen blob does not fit into the epithelial pocket	Epithelial pocket is too small	Repeat Step 32, enlarging the epithelial pocket
37	Animal takes a long time to wake up/does not recover properly from anesthesia	Surgical procedure might have taken too long	Administer 200 µl of sterile PBS subcutaneously
38	Animal pulled off the suture	Suture was not correctly executed	Replace the sutures with new ones

● TIMING

Steps 1–20, preparation of the cells: 1.5 h + overnight recovery

Steps 2–15, dissociation of organoids into small cell clumps: ~45 min

Steps 16–19, seeding the cell clumps in neutralized high-concentration collagen mixture: ~45 min

Step 20, recovery of the collagen graft containing organoids: overnight

Steps 21–25, preparation for the surgery: ~10 min

Step 21, mouse sedation: ~5 min

Steps 22–25, preparation of the mouse to surgery (painkiller administration + shaving): ~5 min
Steps 26–36, surgical procedure: ~20 min
Steps 26–30, abdominal incision and exposure of the cecum: ~5 min
Steps 31–32, preparation of the cecal epithelial pocket: ~5 min
Steps 33–35, insertion of the collagen graft containing organoids: ~5 min
Step 36, suturing of the abdominal incision: ~5 min
Steps 37 and 38, postoperative care: ~5 min
Step 37, monitoring of the animal while recovering from anesthesia: ~5 min
Step 38, inspection of the suture integrity (the day after surgery): ~1–5 min

ANTICIPATED RESULTS

With the approach described in this protocol, it is possible to induce the formation of only one tumor mass in a designated location within the intestine of acceptor mice (palpable at 3 weeks after transplantation; **Fig. 3b**). Moreover, as a consequence of the long latency of the orthotopically induced tumors, this approach allows the appearance of tumors at the carcinoma stage capable of forming distant metastatic foci (with an onset ranging between 6 and 10 weeks after transplantation, **Fig. 3b**). Fluorescent labeling of organoids *in vitro* (**Fig. 4a**,**b**) allows easy detection of tumor cells within the intestinal tissue by confocal microscopy and gives a first indication of potential invasive characteristics (**Fig. 4c**, ROIs 1 and 2). Moreover, chimeric labeling (achieved in this case with random lentiviral multi-integration) provides information on the clonal composition of the tumor (**Fig. 4c**) and the relative metastasis[19].

Next, to gain insights into the dynamics of the cells within the tumor, we made use of intravital imaging techniques. By implanting an abdominal imaging window[23] on top of orthotopic intestinal tumors (6–8 weeks after transplantation), we followed in *vivo* migration of tumor cells over a period of 24 h (**Fig. 4c**–**e**). To do this, we exploited the photoconvertible properties of fluorescent Dendra2 (ref. 45). Dendra2 absorption and emission spectra switch to longer wavelengths when the protein is illuminated with blue light. This allows marking and tracing of groups of tumor cells over consecutive days (**Fig. 4e**). **Figure 4d** shows the quantification of the displacement area of different randomly picked photoconverted areas 24 h after photoswitching. The analysis of different areas includes data originating from multiple microenvironments acquired in seven different orthotopic murine AKP Dendra2-reporter primary tumors. Overall, the data highlight the potential of intravital imaging technology in capturing and dissecting tumor cells bearing different migratory properties, both among different tumors and within the same tumor mass (**Fig. 4e**).

Note: Any Supplementary Information and Source Data files are available in the online version of the paper.

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AUTHOR CONTRIBUTIONS A.F. developed the orthotopic transplantation technique; A.F. and J.D. performed the experiments; H.B., E.B. and S.J.E.S. helped with data analysis and preparation of the figures; S.J.E.S edited the video; K.C.O. and H.J.S. provided the patient-derived CRC organoids; J.D. and J.v.R. supervised the study; A.F., J.D. and J.v.R. wrote the manuscript.

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