Video Article

A Genetically Engineered Mouse Model of Sporadic Colorectal Cancer

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URL: https://www.jove.com/video/55952
DOI: doi:10.3791/55952

Keywords: Cancer Research, Issue 125, Genetically engineered mouse model, GEMM, colorectal cancer, colon cancer, segmental infection, adeno-cre, colonoscopy

Date Published: 7/6/2017

Citation: Betzler, A.M., Kochall, S., Blickensdörfer, L., Garcia, S.A., Thepkaysone, M.L., Nanduri, L.K., Muders, M.H., Weitz, J., Reissfelder, C., Schölch, S. A Genetically Engineered Mouse Model of Sporadic Colorectal Cancer. *J. Vis. Exp.* (125), e55952, doi:10.3791/55952 (2017).

Abstract

Despite the advantages of easy applicability and cost-effectiveness, colorectal cancer mouse models based on tumor cell injection have severe limitations and do not accurately simulate tumor biology and tumor cell dissemination. Genetically engineered mouse models have been introduced to overcome these limitations; however, such models are technically demanding, especially in large organs such as the colon in which only a single tumor is desired.

As a result, an immunocompetent, genetically engineered mouse model of colorectal cancer was developed which develops highly uniform tumors and can be used for tumor biology studies as well as therapeutic trials. Tumor development is initiated by surgical, segmental infection of the distal colon with adeno-cre virus in compound conditionally mutant mice. The tumors can be easily detected and monitored via colonoscopy. We here describe the surgical technique of segmental adeno-cre infection of the colon, the surveillance of the tumor *via* high-resolution colonoscopy and present the resulting colorectal tumors.

Video Link

The video component of this article can be found at https://www.jove.com/video/55952/

Introduction

Colorectal cancer (CRC) continues to be one of the leading causes of cancer-related death in western countries.[1] While the prognosis of patients with early stage disease is good, many tumors are diagnosed at later stages in which, despite numerous treatment options, the prognosis is limited.[2,3,4,5]

The majority of current mouse models of CRC are based on the implantation of tumor cells derived from cell lines or patient tumors into immunodeficient mice.[6,7,8] This leads to local and, depending on the injection site and the tumor cells used for injection, sometimes metastatic tumors.[9,10] However, the resulting xenograft models have major limitations. They must be established in immunodeficient mice, thus eliminating the complex interaction between the tumor and the host immune system. In addition, as the tumor stroma is derived from host cells, the interaction between human tumor parenchyma and murine stroma is defective and therefore not representative of the disease. These deficiencies can be avoided by the use of murine cell lines for injection. However, only few murine CRC cell lines are available and, similar to most available human CRC cell lines, are monoclonal and highly anaplastic.[11] In summary, most currently available CRC mouse models are highly artificial and not fully representative of the human disease.

Genetically engineered mouse models (GEMMs) of CRC can avoid these drawbacks as they feature genuine mouse tumors which are created via induction of key mutations of CRC in the colon.[12,13,14] This can be achieved by the activation of conditional (floxed) germline mutations by cre recombinase within the colorectal mucosa. While in GEMMs of many other tumor entities germline (inducible) cre expression driven by tissue-specific promoters is used, germline cre cannot be used in the colon as this leads to a great number of adenomas throughout the colon causing death by benign tumor load at a very young age. Therefore, in the here described model an adenoviral vector expressing cre is used to infect a short colon segment. This leads to the induction of tumorigenesis within this segment of the mucosa at a time point defined by the investigator, resulting in adenomas ultimately progressing to invasive and metastatic carcinoma. The tumors are genuine mouse tumors, grow in an intact microenvironment and are therefore able to simulate the entirety of colorectal oncogenesis including tumor–host interaction and the metastatic cascade. This model is therefore an attractive platform for studies of cancer biology and preclinical therapeutic trials.

A major disadvantage of genetically engineered mouse models of CRC is their technical complexity. Local cre delivery using rectal adeno-cre enemas in mice carrying floxed Apc alleles has been described before; however, the incidence, multiplicity and location of the intestinal tumors can be highly variable with this technique.[15] Therefore, the technique of confining the adeno-cre infection by surgical clamping of the segment to be induced has been developed.[13] We have modified this procedure in order to improve animal welfare, as well as reduce mortality and the number of resulting tumors. With this protocol, all labs with experience in small rodent surgery should be able to reproduce the model and to produce tumors which are highly reproducible and easily accessible to colonoscopy. Depending on the conditional mutations used for tumorigenesis, the full spectrum of adenoma, invasive carcinoma and metastases can be observed. As the tumors are located in the distal colon, serial endoscopic assessment is easily possible in this model.

Protocol

The animal experiments presented here were independently reviewed and approved by an institutional and a governmental Animal Care and Use Committee and were conducted according to Federation of Laboratory Animal Science Associations (FELASA) guidelines. All possible measures were taken to minimize suffering including anesthesia and analgesia or, if necessary, premature euthanasia.

1. Local Tumor Induction *via* Surgical Adeno-cre Infection

1. **Preparation of animals for surgery**
   NOTE: Virtually any conditional ("floxed") mutation can be induced via the here described method. The use of mutations in genes relevant in colorectal cancer such as Apc, Kras or Tp53 is recommended. The efficiency of cre recombination is dependent on the size of the construct to be excised. Large floxed sequences are excised less efficiently. The recombination of all alleles should be confirmed in the tumors by PCR.
   1. For the development of colorectal tumors, use a cross of the following conditional alleles for a basic model of CRC (MGI database number in brackets):
      Apc^{tm2Rak} (MGI: 3688435)[16]
      $Kras^{tm4Tyj}$ (MGI: 2429948)[17]
      $Tp53^{tm2Tyj}$ (MGI: 3039263)[18]
   2. If a fluorescence reporter allele is required (*e.g.*, to detect micrometastases), use the following allele:
      $Gt(ROSA)26Sor^{tm6(CAG-ZsGreen1)Hze}$ (MGI: 3809522)[19]
      NOTE: All above strains are available via the NCI Mouse Repository or the Jackson Laboratory. No fasting is required as all remaining fecal matter can be flushed out prior to the adenoviral infection. Preoperative fasting leads to higher perioperative mortality and constitutes tremendous stress for small rodents.
   3. Use sevoflurane at 3 - 3.5 vol% for general anesthesia. A loss of the toe pinch reflex indicates sufficient anesthesia.
   4. Prior to the first incision, inject 0.05 mg/kg of buprenorphine subcutaneously.
   5. Cover the eyes of the anesthetized mouse with ophthalmic ointment to avoid desiccation of the cornea.
   6. Place the mouse in a supine position on a small table. Use non-traumatic adhesive tapes to restrain the mouse.
   7. Shave the abdomen with an electric shaver (depilatory cream can be used alternatively) and disinfect with alcohol swabs or iodine. Use the contact time recommended by the manufacturer.
   8. Cover the surgical field with sterile drapes.
      NOTE: The use of perioperative antibiotics is optional and subject to institutional guidelines.
   9. Use sterile single use or sterilized instruments for all surgical procedures.

2. **Midline laparotomy and exposure of the colon**
   1. Use scissors (scalpels can be used alternatively) to make a midline incision (~ 15 mm) of the skin on the lower abdomen.
   2. Pick up the abdominal wall musculature with forceps and carefully incise it with scissors, thus opening the abdominal cavity.
   3. Identify the distal colon, only touch it with atraumatic forceps. Clamp the colon with a delicate clamp (*e.g.*, a Micro Serrefine vascular clamp) approximately 15 mm proximally of the anus.
      NOTE: Give special attention to the vulnerability of the colon at all times. Perforation inevitably leads to peritonitis and sepsis and requires euthanasia of the animal.

3. **Segmental colon infection with Adeno-cre virus**
   1. Insert a flexible Teflon tube transanally and carefully advance it until it reaches the lumen occlusion achieved by the clamp previously placed at 15 mm from the anal verge. Do not use excess force as this may lead to perforation.
   2. Cannulate the tube with a 30G cannula, connect a standard 1 mL syringe and flush the colon with normal saline in order to evacuate remaining fecal matter. This may require several mL of saline.
   3. Once the distal colon is empty remove the tube and replace it with a fresh Teflon tube and again position it directly distal to the clamp as described above.
   4. Occlude the colon with a second clamp ~ 3 mm distal to the proximal clamp (*i.e.*, over the inserted tube, ~ 12 mm from the anal verge), resulting in a 3 mm isolated segment to be infected.
      NOTE: For distal occlusion of the segment Fogarty coronary artery clips have proven most suitable as they are rubberized, leading to tight occlusion of the colon despite the intraluminal tube between the clamp's branches.
   5. Use a second syringe (standard 1 mL with a 30G cannula) to carefully inject 50 - 80 µL of 0.25% Trypsin-EDTA into the clamped colon segment and incubate for 10 min. Leave the cannula and the syringe attached to the Teflon tube to prevent the fluid from leaking back.
      NOTE: The colon must be inflated to break up the mucosal barrier and to reach the crypt stem cells, yet not too much inflated to avoid perforation of the clamped segment.
   6. First remove the distal clamp, and then the trypsin tube.
   7. Flush the distal colon with ~ 500 µL of normal saline to remove remaining trypsin.
   8. Insert a new Teflon tube, put the distal clamp back in place and inflate the colon segment with 50 - 80 µL of adenoviral solution (10^{11} plaque-forming units (PFU)/mL in phosphate-buffered saline) and incubate for 30 min (**Figure 2A**).
      NOTE: Do not spill viral solution as contact with adeno-cre may lead to tumor development in any tissue of conditionally mutant mice.

9. Remove the clamps and the tube.

4. **Closure of the abdomen and postoperative recovery**
    1. Close the abdominal wall with 6-0 rapidly absorbable running sutures (*e.g.*, polydioxanone (PDS)).
    2. Close the skin with surgical wound clips.
    3. Place the mouse on heating pad set to 38 °C until it has fully recovered from the anesthesia.
    4. Administer another bolus of 0.05 mg/kg buprenorphine i.p. 12 h after surgery, followed by additional buprenorphine boluses every 12 h if needed.
    5. Monitor the mice at least once daily for signs of distress due to tumor growth.

2. Colonoscopy

NOTE: Depending on the conditional mutations used, adenoviral infection leads to endoscopically visible tumors within 2 - 4 weeks. Therefore, perform the first postoperative colonoscopy 2 weeks after the adenoviral induction and repeat every 2 weeks. A commercially available system is recommended for murine colonoscopy.[20]

1. **Preparation of animals for colonoscopy**
   NOTE: No fasting is required. Remaining fecal matter is usually well-formed in the distal colon and can be pushed beyond the tumor during colonoscopy, thus making the stressful process of repeated fasting unnecessary.
    1. Use sevoflurane at 3 - 3.5 vol% for general anesthesia. A loss of the toe pinch reflex indicates sufficient anesthesia.
    2. Cover the eyes of the anesthetized mice with ophthalmic ointment to avoid desiccation of the cornea.
    3. Restrain the mice in a supine position on a small table.

2. **Colonoscopy**
    1. Insert the scope (diameter 1.9 mm; length 10 cm) into the intestinal tract through the anus and carefully insufflate air under visual control to distend the colon. Do not insufflate more air than required for the examination.
       NOTE: For air insufflation, the anti-fog air pump of the colonoscopy system may be used. If no anti-fog air pump is available, any other air pump with very low pressure settings can be used, or pressurized air with a delicate pressure-reducing valve. Carbon dioxide (CO_2) easily leads to acidosis in small rodents and must therefore be avoided.
    2. Carefully push the scope forward until a mucosal lesion in the distal colon can be identified (**Figure 1A - 1D**).
    3. Save endoscopic images for later evaluation. An endoscopic scoring system for intraluminal tumors has been described before.[20]
    4. Carefully remove the scope and place the mouse on heating pad set to 38 °C until it has fully recovered from the anesthesia.

Representative Results

If performed adequately, > 85% of the animals develop tumors. The mortality of the here presented surgical procedure is < 5%, mortality of the colonoscopy is virtually non-existent. In the majority of mice, a single lesion is detected; in about 30% 2 - 3 small adenomas can be detected which usually fuse to a single tumor within 2 - 3 weeks after tumor induction.

The phenotype and biological behavior of the resulting tumors is highly dependent on the conditional mutations of the animals. The local cre-mediated knockout of Apc is sufficient to induce tumorigenesis and initially leads to adenoma, which can be detected 2 - 4 weeks after adenoviral infection and which progresses to invasive adenocarcinoma within 12 - 16 weeks. This process can be accelerated by adding a conditional oncogenic Kras mutation (Kras G12D), the resulting tumors quickly progress to invasive adenocarcinoma. Upon the addition of oncogenic Tp53 R172H the tumors rapidly progress to invasive and metastatic carcinoma. Survival strongly depends on the genotype of the tumors; median survival is usually about 80 - 200 days after tumor induction. The cause of death in the great majority of cases is large bowel obstruction secondary to tumor growth.

The tumors can be easily and repeatedly monitored via colonoscopy (**Figure 1**) without major stress for the animals.

The disease manifestations are very similar to human CRC. The animals develop adenomas and ultimately adenocarcinomas of the distal colon (**Figures 2B, 2C, 2F**). Depending on the conditional genotype of the mice, the tumors also metastasize to the peritoneum (**Figure 2D**) the livers (**Figure 2E**) and rarely the lungs (not shown). If a cre reporter allele (*e.g.*, ZSGreen[19]) is used, all tumor manifestations can be easily identified. The ZSGreen reporter allele features a fluorescent protein expression bright enough to be visible at daylight (**Figure 1B**, **Figure 2C**).

Histologically, 95% of the developing tumors are adenocarcinomas. About 5% of tumors are of mesenchymal origin, *e.g.*, fibrosarcoma or leiomyosarcoma, presumably developing from stromal cells which have been accidentally infected with adeno-cre. The histomorphology of the adenocarcinomas closely resembles human CRC, featuring the entire spectrum from non-invasive adenoma to adenocarcinoma invading surrounding structures (**Figure 3A - 3C**).

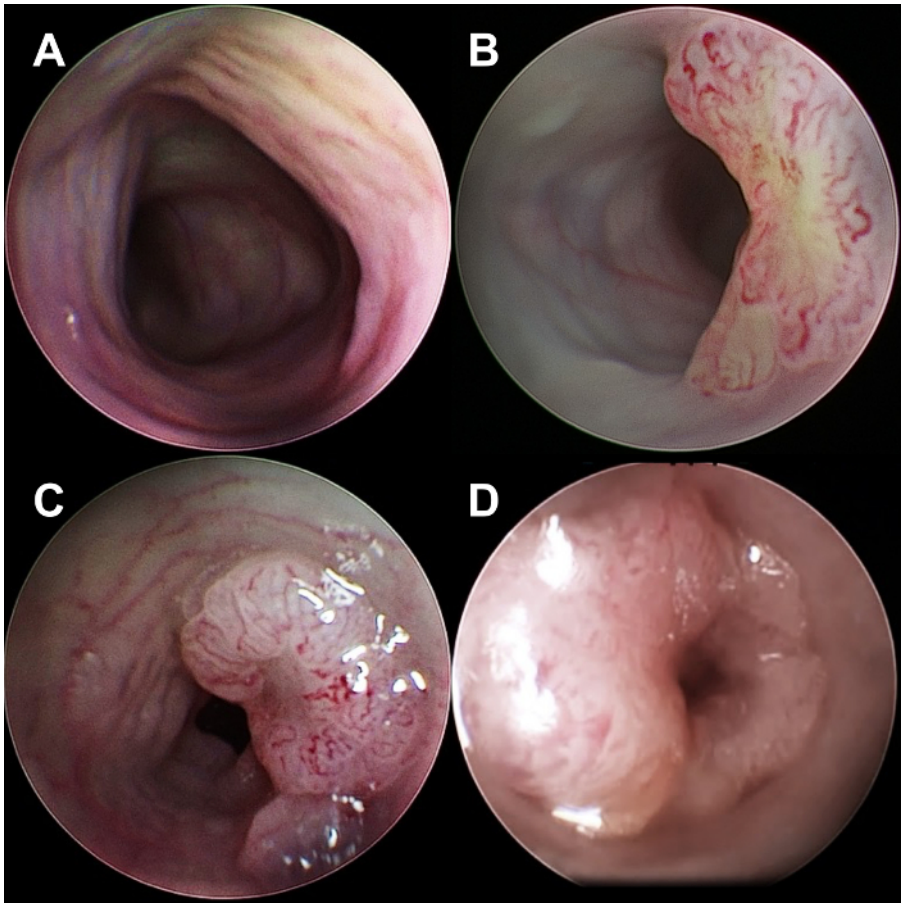

Figure 1. Colonoscopic Images of Colorectal Tumors (Conditional Alleles of the Given Animal in Brackets).
A. Normal distal colon. **B.** Early adenoma 2 weeks after adeno-cre infection. Note the green color due to a GFP reporter allele in this mouse. (Apc^{tm2Rak}, $Kras^{tm4Tyj}$, $Gt(ROSA)26Sor^{tm6(CAG-ZsGreen1)Hze}$). **C.** Late adenoma ($Apc^{tm2Rak}$, $Kras^{tm4Tyj}$). **D.** Colorectal adenocarcinoma (as diagnosed by pathology after the colonoscopy images were obtained; Apc^{tm2Rak}, $Kras^{tm4Tyj}$, $Tp53^{tm2Tyj}$). Please click here to view a larger version of this figure.

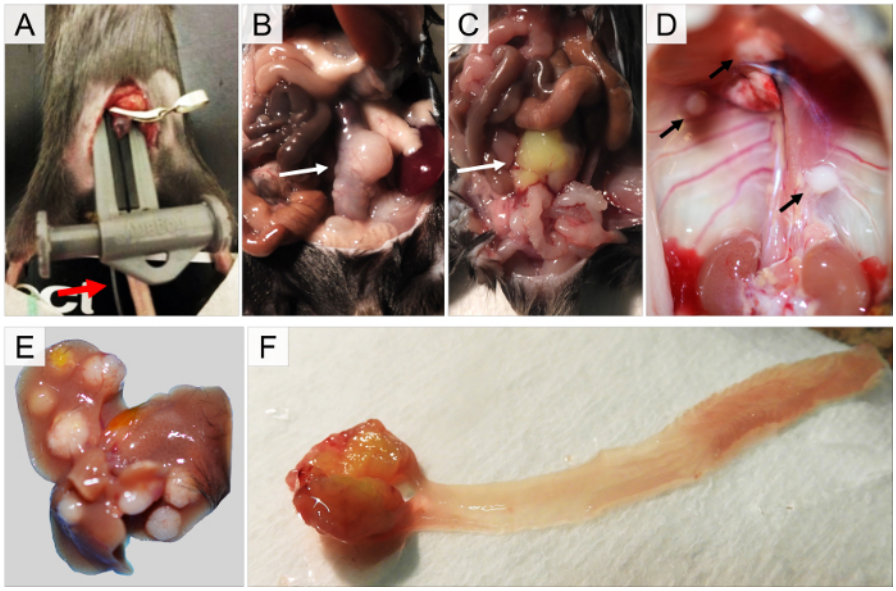

Figure 2. A. Intraoperative Situs**.** Note the large, rubberized Fogarty clip at the bottom and the transanally inserted tube for adeno-cre injection (red arrow). **B.** Colorectal tumor (white arrow) with consecutive large bowel obstruction 8 weeks after adeno-cre infection (Apc^{tm2Rak}, $Kras^{tm4Tyj}$, $Tp53^{tm2Tyj}$). **C.** Colorectal tumor (white arrow) 10 weeks after adeno-cre infection (Apc^{tm2Rak}, $Kras^{tm4Tyj}$, $Gt(ROSA)26Sor^{tm6(CAG-ZsGreen1)Hze}$). Note the greenish color due to a green fluorescent protein (GFP) reporter allele in this mouse. **D.** Peritoneal carcinosis (black arrows) in the GEMM (Apc^{tm2Rak}, $Kras^{tm4Tyj}$, $Tp53^{tm2Tyj}$). Liver and intestine have been removed to expose the kidneys and the diaphragm. **E.** Gross hepatic metastases in the GEMM 12 weeks after adeno-cre infection (Apc^{tm2Rak}, $Kras^{tm4Tyj}$). **F.** Colon with tumor after removal from the animal depicted in **Figure 2C**. Please click here to view a larger version of this figure.

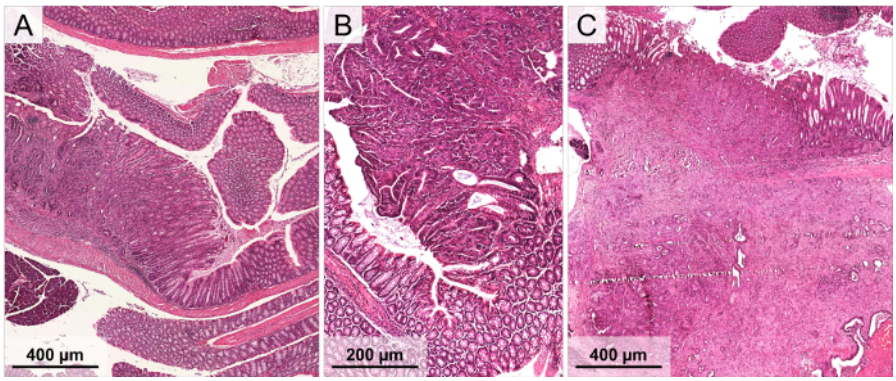

Figure 3. Hematoxylin /Eosin (H/E) Stained Sections of Colorectal Tumors from the GEMM.
A. Transitional zone from normal mucosa to adenocarcinoma with infiltration of mucosa, submucosa and muscularis mucosae (Apc^{tm2Rak}, $Kras^{tm4Tyj}$). **B.** Transitional zone from normal colonic mucosa to invasive adenocarcinoma (Apc^{tm2Rak}, $Kras^{tm4Tyj}$). **C.** High grade adenocarcinoma with infiltration of surrounding tissue (Apc^{tm2Rak}, $Kras^{tm4Tyj}$, $Tp53^{tm2Tyj}$). Please click here to view a larger version of this figure.

Discussion

While they are generally easy to generate and maintain, classical CRC mouse models based on cell line injection are artificial and are not able to fully recapitulate the human disease. As a consequence, GEMMs have been developed. The first CRC GEMM was the Apc^{Min} mouse, which harbors a heterozygous null mutation in the Apc gene, therefore mimicking the human hereditary disease familial adenomatous polyposis (FAP).[21] However, Apc^{Min} mice invariably develop multiple intestinal adenomas not limited to the colon; also, the time and exact location of adenoma formation is random and the tumors rarely develop into malignant lesions as the mouse dies of benign tumor load before the adenomas can progress. Therefore, the Apc^{Min} mouse is a model of FAP, not sporadic CRC. Other models present mutations in DNA mismatch repair genes.[22,23] Although some of these mice are excellent models for hereditary gastrointestinal cancer, they do not represent sporadic colorectal cancer.

Aside from the underlying genetic alteration it is the location of the genetic lesion which sets apart models of sporadic and syndromal colorectal cancer. All of the above-mentioned models feature mutations which are constitutively active or induced throughout the colon or even the entire gastrointestinal tract. This results in the formation of multiple adenomas, which is not representative of human sporadic CRC, is hardly assessable by colonoscopy and, in addition, usually does not leave the tumors enough time to develop before the animal succumbs to the extensive tumor load. Therefore, a mouse model for sporadic colorectal cancer must incorporate local activation of colorectal tumorigenesis.

The tumors arising in the here presented GEMM are strictly confined to the surgically clamped and adeno-cre-infected segment of the colon. This results in the formation of a single tumor which is easily accessible to detection and surveillance by colonoscopy. In addition, the distal location makes bowel obstruction a late complication of tumor growth, allowing the tumors to develop into invasive and, depending on the genotype, metastatic carcinoma before the animal requires euthanasia. Another advantage of segmental cre infection is the unaffected environment. While in Apc^{Min} and other hereditary mouse models, the surrounding mucosa and even the stromal cells suffer the same genetic aberrations as the tumors, the tumors in the here proposed model develop within normal colonic epithelium and stroma. This enables more realistic tumor-host interaction and molecular studies without restrictions. Furthermore, the time point of tumor induction is well-defined in this model. While in the Apc^{Min} mouse, tumors develop after the "second hit", during which the second Apc allele is stochastically lost at a random time point, all desired mutations are activated simultaneously in the here described model, thus allowing studies of very early lesions or consecutive biopsies in a very defined manner.

However, this model also comes with limitations. The crossing of multiple alleles into one model requires tremendous time and resources; in addition, often not all offspring can be used for the experiments due to an unsuitable genotype. The procedure itself requires practice and is time-consuming. The production of adeno-cre virus suspension with sufficiently high titers and in large amounts is cost-intensive. Therefore, this model is not suitable to replace all other CRC mouse models and will be limited to laboratories with a distinct focus on GEM models.

The protocol itself is technically demanding, yet with some training non-surgical personnel can perform the procedures adequately. The critical step during the adeno-cre infection is the inflation of the clamped bowel segment – excess intraluminal pressure leads to perforation, insufficient pressure reduces the rate of successful infection. This step requires training.

A protocol for adenoviral infection of the distal colon has been published by Hung et al. before.[13] The here presented protocol differs from the aforementioned protocol in several key aspects. As it frequently causes bowel perforation in unexperienced hands, mechanical abrasion of the mucosa before adeno-cre incubation is skipped in the present protocol. This results in a reduced rate of tumor formation, which however can be counteracted by increased viral titers. This way, the rate of mesenchymal tumors can also be reduced as without mucosal abrasion less mesenchymal cells within the bowel wall are exposed to adeno-cre.

Also, in contrast to the above mentioned protocol[13] the here described protocol does not recommend overnight fasting prior to surgery or colonoscopy. Instead, flushing of the bowel or, in case of colonoscopy, simple mechanical manipulation is used to remove remaining fecal matter. This refinement of the protocol dramatically improves animal welfare. Overnight fasting is a highly stressful procedure for small rodents and known to strongly influence the murine metabolism, therefore also influencing experimental results.[24,25] These unwanted effects of overnight fasting can be avoided in the here presented manuscript.

Another important difference between the here presented protocol and the protocol by Hung et al. is the length of the clamped (and thus infected) segment. While the here described protocol recommends a short length (~3 mm), Hung et al. recommend 20 mm of the distal colon to be infected. The shorter segment length has been chosen to reduce the number of recombined crypts and thus resulting tumors. As the majority of patients with sporadic CRC develop a single tumor rather than multiple tumors, this measure increases the clinical relevance of the model. The rate of tumor formation does not seem to be affected by the reduced surface of infected colon.

Future applications of this technique include all protocols requiring local cre-recombination within the colon mucosa. Most applications in cancer research will include conditional oncogenes, leading to the formation of colorectal tumors; however, all other applications such as inducing reporter constructs within the colon mucosa can be achieved with the here presented protocol as well.

In conclusion, the here presented combination of a highly sophisticated genetically engineered mouse model of colorectal cancer along with the option of high-resolution colonoscopy for the detection and surveillance of the developing tumors provides an excellent setting to study the biology and treatment of CRC.

Disclosures

The authors have nothing to disclose.

Acknowledgements

This work is dedicated to the memory of Professor Moritz Koch.

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