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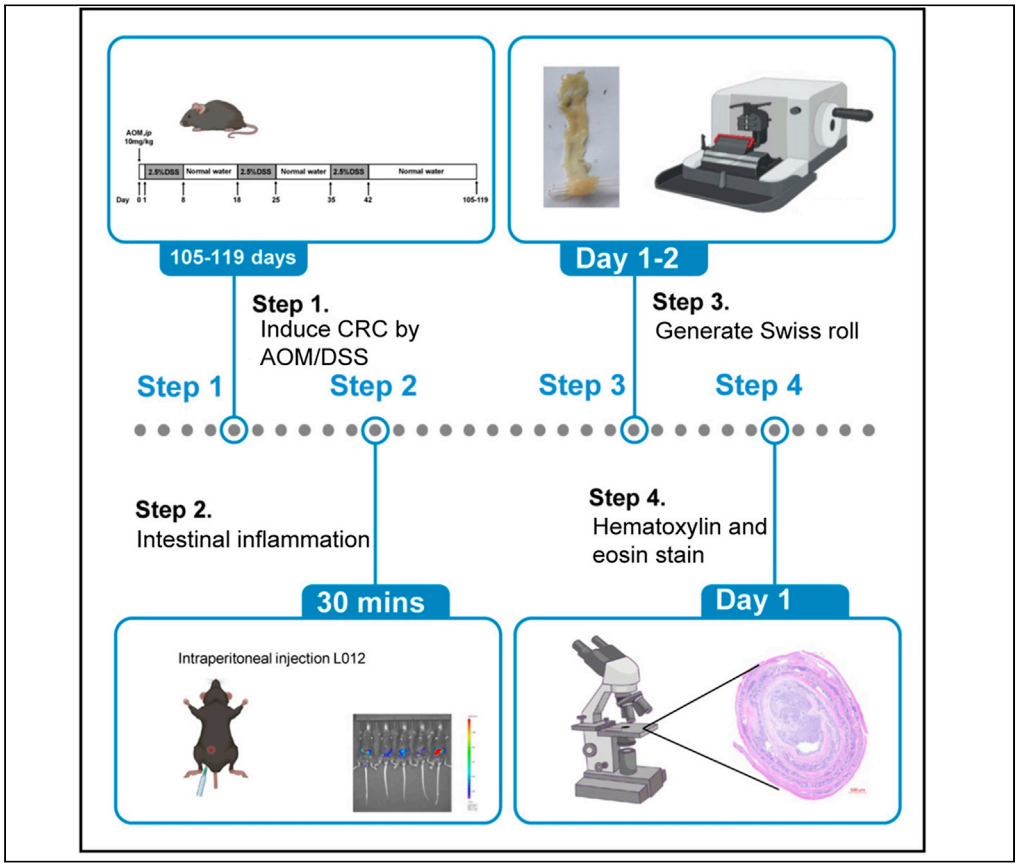
Protocol for colitis-associated colorectal cancer murine model induced by AOM and DSS



Inflammatory bowel diseases (IBDs) contribute to the tumorigenesis of colorectal cancer (CRC). Here, we describe a step-by-step protocol for the construction of colitis-associated CRC murine model by sequential utilization of azoxymethane and dextran sulfate sodium. We also detail steps to determine the degree of murine intestinal inflammation and generate colorectum Swiss roll for further histopathological analyses. This is a convenient and reproducible protocol for colitis-associated CRC murine model by the induction of general chemical reagents.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

A detailed protocol for colitis-associated CRC murine model induced by AOM and DSS

Processing and examination of colorectum-based Swiss roll

In vivo imaging for the quantification of intestinal inflammation by using L-012

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Protocol for colitis-associated colorectal cancer murine model induced by AOM and DSS

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SUMMARY

Inflammatory bowel diseases (IBDs) contribute to the tumorigenesis of colorectal cancer (CRC). Here, we describe a step-by-step protocol for the construction of colitis-associated CRC murine model by sequential utilization of azoxymethane and dextran sulfate sodium. We also detail steps to determine the degree of murine intestinal inflammation and to generate colorectum Swiss roll for further histopathological analyses. This is a convenient and reproducible protocol for colitis-associated CRC murine model by the induction of general chemical reagents. For complete details on the use and execution of this protocol, please refer to Yang et al. (2022).[1]

BEFORE YOU BEGIN

IBD can contribute to the tumorigenesis of CRC, which is known to be correlated with genetic predispositions and the dysregulated function of immune cell, epithelial cells and commensal microbiota.[2] A colitis-associated murine model is useful for studying the pathophysiology and therapy of inflammation-dependent CRC. Here, we describe a step-by-step protocol for the construction of the colitis-associated CRC murine model by a sequential utilization of AMO and DSS.[3] This murine model exhibits severe colitis with loss of body weight and bloody diarrhea, followed by the development of multiple colon tumors. The cumulative symptoms of this AOM- and DSS-induced model include the aberrant crypts with crypt fission, the emergence of microadenomas, the dysregulated diversity of the microbiome, the deleterious inflammatory immune responses, the high frequency of genetic mutations, et al.[2,4,5]

Compared to the gene-manipulation[6] or T cell-transfer based colitis models,[7] this chemical induction method is convenient and reproducible in immuno-competent mice with wild-type background. Moreover, this AOM- and DSS-induced murine CRC model exhibits similarity and relevance with histological, pathological, and molecular features of human IBD-associated CRC, rendering it a suitable model for the investigation of CRC diseases.

Institutional permissions

All experimental mice shall be maintained under specific-pathogen-free conditions in qualified animal facility. All mice experiments were performed according to the governmental and institutional guidelines to guarantee animal welfare, and were also approved by the Institutional Animal Care and

Use Committee (IACUC) of Tsinghua University. Usage or reproduction of this protocol for research purpose will not require the acquisition of permissions from the relevant institution.

Preparation of induction reagents

Timing: 1 h

1. Dissolve 25 mg AOM powder in 2.5 mL sterile water to make 10 mg/mL stock solution. Rigorous Vortex is required for a complete homogenized solution. Prepare aliquots and store them at −20°C for up to 1 year.

   Note: AOM may cause heritable genetic damage and cancer. Protective clothing, gloves, and face/eye protection are highly recommended.

2. Dissolve 25 g of DSS powder (molecular weight 35–50 kDa) in 1,000 mL of sterile water to make a 2.5% DSS solution. Rigorous Vortex is highly recommended for a complete homogenized solution. This 2.5% DSS solution can be stored at 4°C for up to 4 weeks, although fresh DSS solution is preferred.
3. Dissolve 5 mg L-012 powder in 5 mL sterile water to make a 1 mg/mL stock solution. Prepare aliquots and store them at −20°C for up to 1 month.

Preparation of mice

8 to 10-week-old male C57BL/6J mice shall be used in this AOM- and DSS-induced murine CRC model.

⚠ CRITICAL: Female mice are not recommended in this protocol, because female mice are resistant to AOM and DSS-induced tumorigenesis.[8]

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Azoxymethane (AOM)	Sigma-Aldrich	A5486
Dextran sulfate sodium salt (DSS, molecular weight 35–50 kDa)	MP Biomedicals	160110
PFA	Sigma-Aldrich	30525-89-4
L-012	Sigma-Aldrich	143556-24-5
NaCl	Solarbio	S8210
KCl	MACKLIN	P816347
Na_2HPO_4	MACKLIN	USP S818102
KH_2PO4	MACKLIN	P815660
Weigert's Hematoxylin Stain Kit	Solarbio	G1142
Eosin Y Stain Solution	Solarbio	G1100
Xylene	Merck	534056
Acidified water	Coolaber	SL2240-100ml
Paraffin	Sangon	B500301-0100
Ethanol	MACKLIN	E809065
Molds	CITOTEST	155967
Isoflurane	RWD Life Science	R510-22-10
Experimental models: Organisms/strains		
Mouse: 8 to 10-week-old male C57BL/6J	The Jackson Laboratory	JAX:000664

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Software and algorithms		
Living Image Software	PerkinElmer Inc.	https://www.perkinelmer.com/product/spectrum-200-living-image-v4series-1-128113
Illustration	BioRender	N/A
Adobe Illustrator CC 2018	Adobe	N/A
Other		
Scissors	Sanyou	042000
Forceps	Sanyou	044570
1 mL syringe	Jiangsu Zhiyu Medical Instrument Co., Ltd	N/A
Coverslip	CITOTEST	10212450C
Glass slide	ZSGB-BIO	ZLI-9506
Capillary tube	Skills model	S7-MXG
Labquake rotator/shaker	Barnstead Thermolyne	Model 40011
Digital pathology biopsy scanner	KFbio	KF-PRO-120
Microscopy	Nikon	Eclipse 90i
IVIS Spectrum system	PerkinElmer Inc.	IVIS Spectrum 3D
Electronic balance (for weighting mice)	Beyotime	E0266
Analytical balance (for weighting chemicals)	Mettler Toledo	ME104E
Portable Laboratory Anesthesia Machine	RWD Life Science	R520
35 mm dish	Eppendorf	30700112
Embedding workstation	Leica	HistoCore Arcadia H+C
Microtome	Leica	RM2255
Staining jar	Easybio	BE6090
Water bath	SHANGHAI BLUEPARD INSTRUMENTS	DK-S12

MATERIALS AND EQUIPMENT

Phosphate-Buffered Saline (PBS)

Reagent	Final concentration (mM)	Amount
NaCl	137.00	8.01 g
KCl	2.70	0.20 g
Na_2HPO_4	10.00	1.42 g
KH_2PO_4	1.47	0.20 g
ddH_2O	N/A	1,000 mL
Total	**N/A**	**1,000 mL**

Note: Adjust pH to 7.4 $\pm$ 0.1 at 25°C. Store at 25°C for up to 1 month. Store at 4°C for up to 6 months.

Alternatives: Commercial PBS is available from vendor such as Solarbio (P1020).

AOM solution

Reagent	Final concentration	Amount
AOM	10 mg/mL	25 mg
ddH_2O	N/A	2.5 mL
Total	**N/A**	**2.5 mL**

⚠ CRITICAL: Store at −20°C for up to 1 year. Avoid repeated freeze-thaw cycles. Aliquots are highly recommended.

Note: AOM may cause heritable genetic damage and cancer. Protective clothing, gloves, fume hood, and face/eye protection are highly recommended.

DSS solution		
Reagent	Final concentration	Amount
DSS	25 mg/mL (2.5%)	25 g
Sterilized water	N/A	1,000 mL
Total	**N/A**	**1,000 mL**

⚠ CRITICAL: Preparing fresh 2.5% DSS solution right before the initial usage and changing fresh 2.5% DSS solution twice a week are recommended in the experiment since DSS solution is not very stable at room temperature. Molecular weight of DSS should be between 36–50 kDa because DSS with less or more molecular weight may cause less colitogenesis or substantial mortality.

4% PFA		
Reagent	Final concentration	Amount
PFA	40 mg/mL	2 g
PBS	N/A	50 mL
Total	**N/A**	**50 mL**

Note: The mixture of PFA powder and PBS can be incubated at 56°C overnight to accelerate PFA dissolving in PBS. Store at 4°C for up to 1 year.

Note: PFA irritates the respiratory tract and is carcinogenic. PFA powder should keep away from fire. Therefore, protective clothing, gloves, fume hood, and face/eye protection are highly recommended.

Alternatives: Commercial 4% PFA is available from vendor such as Solarbio (P1110).

L-012 solution		
Reagent	Final concentration	Amount
L-012	1 mg/mL	5 mg
ddH_2O	N/A	5 mL
Total	**N/A**	**5 mL**

⚠ CRITICAL: Store at −20°C for up to 1 month. Avoid repeated freezing and thawing. Aliquots and storage in a dark place are highly recommended.

STEP-BY-STEP METHOD DETAILS

The step-by-step method details are divided into four main parts.

Induction of colitis-associated CRC

Timing: 15–17 weeks

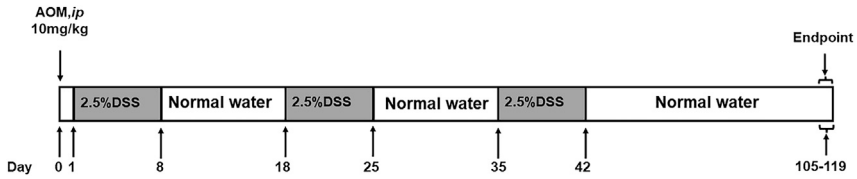

Figure 1. A schematic protocol of the AOM- and DSS-induced murine CRC model

On day 0, each mice was injected intraperitoneally with 10 mg/kg (body weight) of AOM. Then each mice was providedwith 2.5% DSS-containing drinking water for 7 days, followed with normal water for 10 days. This cycle of DSS –containing water and normal water shall be repeated three times, and then the normal water treatment stays last to the experimental endpoint.

Here we describe the details of the colitis-associated CRC model in mice by using AOM and DSS. It takes about 15–16 weeks, but the exact time differs depending on the treatments and conditions. This protocol includes one dose of AOM injection, three cycles of DSS administration, and three cycles of regular drinking water (Figure 1). Mice were monitored for weight loss twice a week and were detected for intestinal inflammation at the end of each DSS treatment cycle.

1. On day 0, mark and weight each mice.
2. Prepare 1 mg/mL (1 μg/μL) AOM working solution in PBS, and inject each mouse intraperitoneally with 10 mg/kg (body weight) of AOM (For example, a 20 g mouse shall be injected with 200 μL AOM solution).
3. On day 1, replace the drinking water with 2.5% DSS solution for 7 days (Always prepare at least 5 mL of DSS solution per mouse per day, changing fresh 2.5% DSS solution twice a week is recommended since DSS solution is not very stable at room temperature). This is cycle 1.
4. Monitor the weight loss of the mice twice a week until they are euthanized.
5. On day 8, change the 2.5% DSS solution to normal water for another 10 days.
6. On day 18, replace the drinking water with 2.5% DSS solution for 7 days. This is cycle 2.
7. On day 25, change the 2.5% DSS solution to normal water for another 10 days.
8. On day 35, replace the drinking water with 2.5% DSS solution for 7 days. This is cycle 3.
9. On day 42, change the 2.5% DSS solution to normal water until the mice are euthanized.
10. Day 105–119 is a window phase to euthanize mice, but the exact endpoint of the experiment depends on the situation (recommended criteria can include weight loss of 10%–20%, occult blood in stool and energies).

In vivo imaging of intestinal inflammation

Timing: 30 min

It has been shown that inflammatory microenvironment of the intestine promotes tumor establishment. Therefore, we also provide a noninvasively proposal by the usage of *in vivo* imaging technique to determine the degree of intestinal inflammation. Briefly, noninvasive imaging is achieved by using a luminol-based chemiluminescent probe, L-012, for the detection of reactive oxygen species (ROS) and reactive nitrogen species (RNS). Both ROS and RNS are closely associated with inflammation.

11. Initialize the IVIS Spectrum system and start Living Image software according to the manufacturer's instructions.
12. Anesthetize mice in a chamber with the concentration of 2.0%–3.0% isoflurane.
13. During anesthesia, intraperitoneally inject the mice with 25 mg/kg L-012 solution (For example, a 20 g mouse shall be injected with 500 μL L-012 solution).[9]
14. Place processed mice into the anesthesia manifold of the imaging chamber of the IVIS Spectrum system in a supine position.

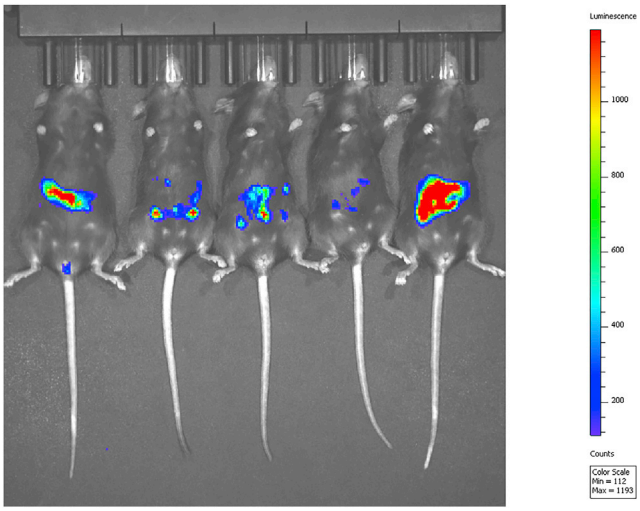

Figure 2. *In vivo* imaging of murine intestinal inflammation at week 16 after AOM- and DSS-treatment following intraperitoneal injection of L-012 solution

Bioluminescent images were obtained under isoflurane anesthesia using an IVIS Spectrum CT system.

15. 2 min after the injection of L-012, adjust the acquisition parameters and acquire bioluminescent images.
16. Place the mice back to the cages, and keep them warm for recovery.
17. For quantitative analyses, use Living Image software to calculate the intensity of bioluminescent signals in the standardized regions of interest (ROI) for each mouse (Figure 2).
18. It is recommended to use L-012 to image the intestinal inflammation at the end of DSS cycle and in normal water, triweekly after DSS-normal water cycles.

Generation and fixation of a Swiss roll

Timing: 2 days

19. Day 1. At a selected time point, sacrifice the mice using an approved method.
20. Position the mouse supine on a surgical pad, perform a ventral midline incision with sterile surgical forceps and scissors, and remove the entire colorectum (Figure 3).
21. Place the entire colorectum in a sterile dish (if further *in vitro* culture is required) or on any suitable surface if further culture is not required. Use a steel gavage needle attached to a syringe to flush the stools out with sterile PBS (Figure 4A).
22. The length of the colon can be measured now (Figure 4B).
23. Cut open the colon longitudinally (avoid mechanical injury) and roll it into a Swiss roll using two capillary tubes (Figures 4C–4I).
    a. Clamp the opened colon with two capillary tubes
    b. Roll them (capillary tubes-colon-capillary tubes) into a Swiss roll.
24. Fix the fresh Swiss roll by inserting a syringe needle in the space between two capillary tubes and place it in a 30 mm dish containing 4 mL 4% PFA for 24 h at 4°C (Figures 4J–4L).
25. Day 2. Transfer the fixed Swiss roll samples to the 50 mL tube tissue processor for dehydration by a sequential incubation order as below:
    a. In 50% ethanol for 25 min,
    b. In 70% ethanol for 25 min,
    c. In 80% ethanol for 25 min,
    d. In 95% ethanol for 15 min, repeat this step one more time,
    e. In 100% ethanol for 30 min, repeat this step one more time,

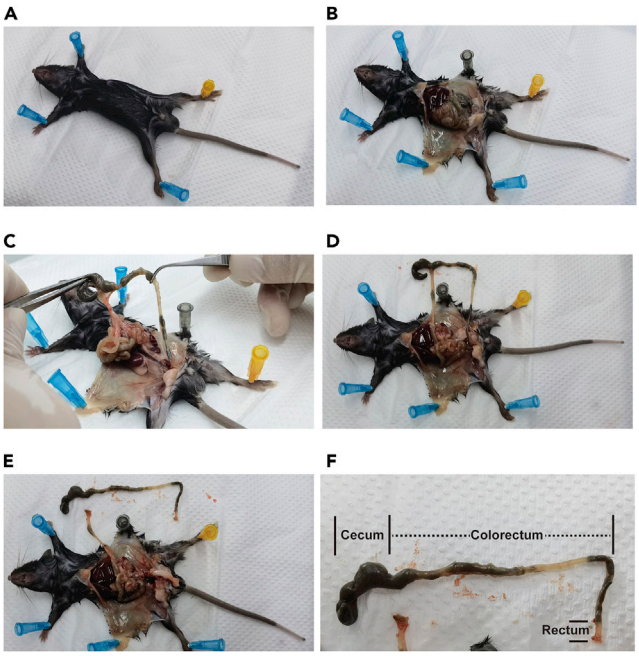

Figure 3. Separation of murine colorectum

(A) Position the murine supine on a surgical pad.

(B) Open the enterocoelia.

(C and D) Expose the entire colon.

(E and F) Remove the entire colon including cecum, colorectum and rectum.

f. In ethanol and xylene mixture buffer (1:1 v/v) for 30 min,
g. In 100% xylene for 20 min, repeat this step one more time,.
h. Finally, incubate them in paraffin tissue embedding medium at 60°C for 1.5 h, repeat this step one more time.

26. Transfer the infiltrated tissues into the embedding workstation (working temperature: 60°C) and embed them with an appropriate embedding mold.
27. Generate 5 μm cross-sections using a microtome.
28. Float the tissue sections in a PBS bath and use glass slides to pick the tissue sections from the PBS bath.
29. Dry the slides on a 42°C slide dryer and then in a 56°C oven for 1 h.

⚠ CRITICAL: Fully processed slides can be stored at 4°C for up to 3 months.

Deparaffinization and H&E staining of a Swiss roll

Timing: 1 day

30. Deparaffinization:
    a. Incubate the slides with 30 mL 100% xylene in a staining jar for 10 min,
    b. Repeat twice this incubation step in 30 mL xylene,
    c. Incubate the slides with 30 mL 100% ethanol for 10 min,
    d. Repeat this incubation step,
    e. Then proceed to 90% ethanol in water (v/v) for 5 min,
    f. 80% ethanol in water (v/v) for 5 min,
    g. 70% ethanol in water (v/v) for 5 min,
    h. And wash the slides in ddH_2O for 5 min three times.

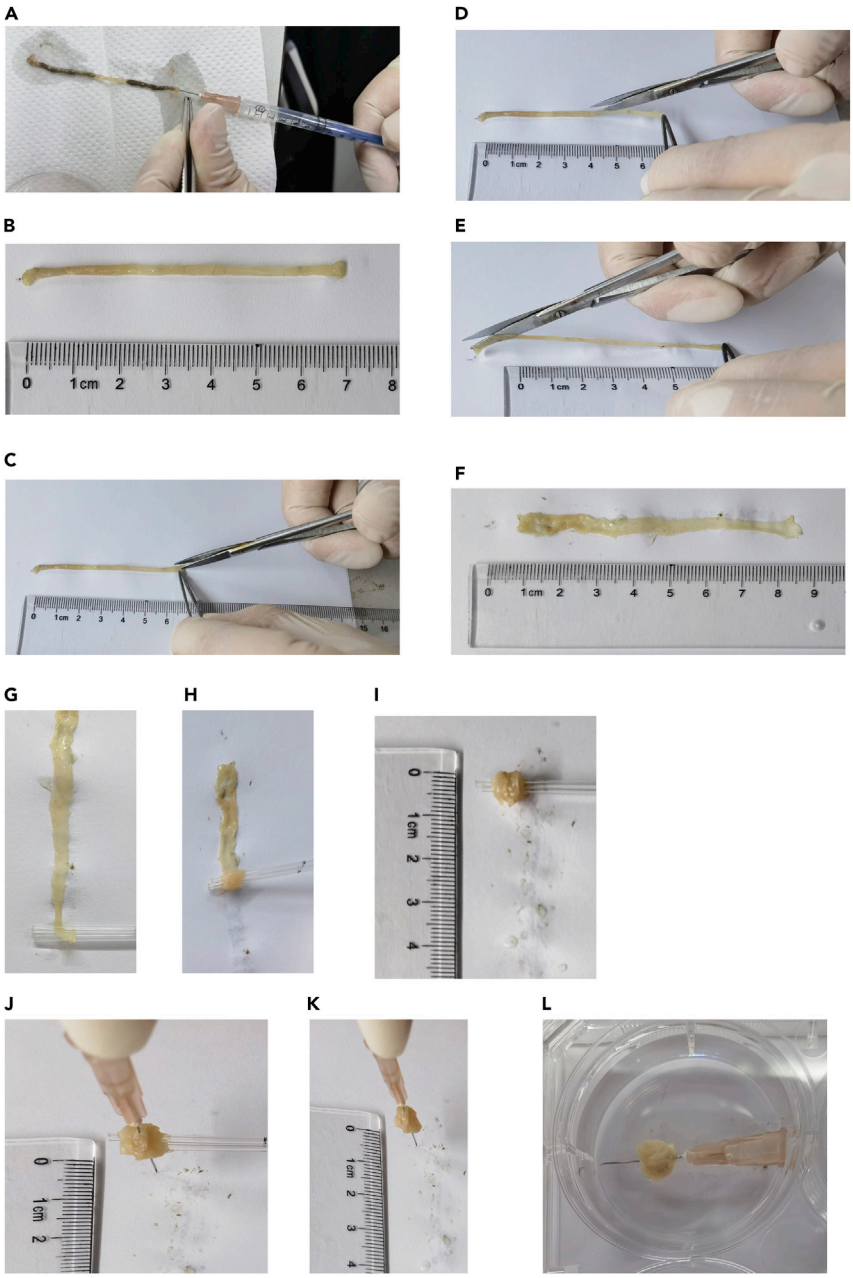

Figure 4. Process of Swiss-rolling for murine colon

(A and B) Using a steel gavage needle attached to a syringe to flush the stools out with sterile PBS.

(C–F) Open the colon longitudinally.

(G–I) Roll the opened colon into a Swiss rolls using two capillary tubes.

(J–L) Fix a fresh Swiss roll with a syringe needle and place it in 4% PFA for 24 h.

31. Stain the sections with hematoxylin and eosin (H&E) using standard H&E procedures.
    a. Incubate the slides for 5 min with hematoxylin solution to stain the nuclei using Weigert's Hematoxylin Stain Kit,
    b. Wash the slides for 10 min in running ddH_2O.
    c. Incubate the slides for 30 s in Eosin Y staining solution.
    d. Wash the slides three times in acidified water,
    e. Add a coverslip.

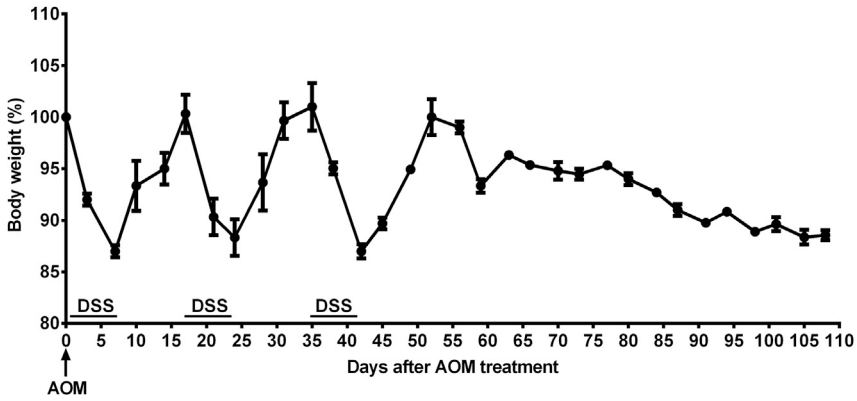

Figure 5. Body weight dynamics of AOM- and DSS-induced murine colorectal cancer model

8 to 10-week-old male C57BL/6J mice were injected intraperitoneally with a single dose of the organ-tropic carcinogen AOM solution on day 0. Mice were treated with 2.5% DSS solution for 7 days in each cycle, followed by a recovery phase for 10 days with normal drinking water. Each mice were then monitored for weight loss twice a week.

32. Score histological alterations using a digital pathology biopsy scanner (KFbio, KF-PRO-120) or Microscopy (Nikon, Eclipse 90i).

EXPECTED OUTCOMES

Here we describe the details of the inflammation-dependent colitis-associated CRC model through a combined induction of AOM and DSS, in which genotoxic agent AOM induces O^6-methylguanine adducts resulting in G → A transitions in DNA, while discontinued DSS administration triggers colitis-dependent neoplasia leading to inflammatory microenvironment of the intestine. All these promote intestinal tumorigenesis. Therefore, it is crucial to monitor inflammatory responses in intestine. In this protocol, we provide a noninvasive method to determine the degree of intestinal inflammation by using the luminol-based chemiluminescent probe L-012, which is a sensor to monitor reactive oxygen species (ROS) and reactive nitrogen species (RNS). Both ROS and RNS are closely associated with inflammation (Figure 2), which would be reinforced by DSS. Since DSS administration could cause significant weight loss (Figure 5), detection of intestinal inflammation is highly recommended at each end of DSS treatment cycle, the end of normal water cycle, and triweekly after DSS-normal water cycle. The disease activity index should also be recorded during the induction cycle. Here, we also provide a scoring system to approach this aim (Table 1).[10] As for the determination of the experimental endpoint, we suggest that day 105–119 is a window phase to euthanize mice when the mice lose 10%–20% of body weight, develop fecal occult blood, and are lethargic. It is worth mentioning that the exact endpoint of the experiment depends on the situation of each mice. Histological evaluation of inflammation and tumorigenesis shall be performed by H&E staining. Here we provide a scoring system to approach this aim (Figure 6 and Table 2).

LIMITATIONS

As with any other model, the AOM and DSS induced murine model of inflammation-dependent colorectal cancer has its limitations. For example, Kras or p53 mutations are typical in humans, but they have not been detected in this mouse model. Another factor is diet, which significantly affects the outcome of AOM and DSS-induced colorectal cancer. These alterations are partially due to microbiota and many other host genetic factors. In summary, this AOM and DSS murine model exhibits close similarity and relevance with human IBD associated colorectal cancers including histological, pathological, and molecular features. Thus, it is a suitable model for the investigation of human CRC.

TROUBLESHOOTING

Problem 1

Animal death (steps 3–8).

Table 1. Scoring system for disease activity index based on weight loss, feces consistency, and the degree of intestinal bleeding

Score[a]	Weight loss	Feces consistency	Intestinal bleeding
0	None	Normal	Normal
1	0%–5%	Soft but formed	Blood traces in stool visible
2	5%–10%	Soft and unformed	Blood traces in stool
3	10%–18%	Very soft and wet	Archorrhagia
4	>18%	Diarrhea	Rectocele

[a]The sum of the three subscores results in a combined score ranging from 0 (no changes) to 12 (severe disease activity).

Potential solution

Some mouse strains may have a high predisposition for induction of colitis due to unique genetic background. Reduce the dose of DSS to 2% or reduce the duration of DSS treatment in each cycle (7 days–5 days). Prolonging normal water cycle to two weeks is another alternative.

Problem 2

No or weak colitis/tumor (steps 3–8).

Potential solution

Some mouse strains may have a low predisposition for the induction of colitis due to unique genetic background. Increase the dose of DSS to 3% or increase the number of treatment cycles from 3 to 4.

In addition, female mice drink less water and have a relatively low predisposition for induction of colitis. Thus, use male mice instead of female mice in this protocol.

RESOURCE AVAILABILITY

This study did not generate new materials.

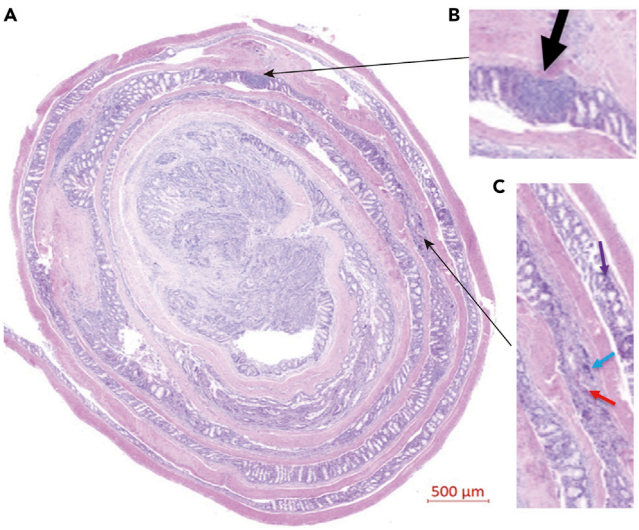

Figure 6. H&E staining of AOM- and DSS-induced murine colorectal cancer

(A) Swiss roll of colorectal sample from the AOM- and DSS-induced murine model. Standard process of H&E staining was applied to represent the state of the colorectum.

(B) The black arrow indicates the tumors in the colon.

(C) The red arrow indicates the area of strong transmural inflammation with loss of crypt structure and depletion of goblet cells. The blue arrow indicates the neutrophilic infiltrates. The purple arrow indicates the crypt abscess.

Table 2. Scoring system for inflammation-associated histological changes in the colon based on tissue damage, lamina propria inflammatory cell infiltration, and the number of tumors

Score[a]	Tissue damage	Lamina propria inflammatory cell infiltration	The number of tumor
0	None	Normal	0
1	Isolated focal epithelial damage	Some neutrophils	1–2
2	Mucosal erosions and ulcerations	Submuscosal presence of inflammatory cell clusters	3–5
3	Extensive damage deep into the bowel wal	Transmural cell infiltrations	>5

[a]The sum of the three subscores results in a combined score ranging from 0 (no changes) to 9 (severe disease activity).

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Wanli Liu (liulab@tsinghua.edu.cn).

Materials availability

This study did not generate new materials.

Data and code availability

This study did not generate new materials.

ACKNOWLEDGMENTS

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AUTHOR CONTRIBUTIONS

All authors contributed to the writing of the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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