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Colonoscopy-based intramucosal transplantation of cancer cells for mouse modeling of colon cancer and lung metastasis

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Abstract

The conventional orthotopic/xenograft models or genetically engineered murine models of colon cancer (CRC) are limited in their scope for a true understanding of tumor growth, progression and eventual metastasis in its natural microenvironment. In the currently used murine models of CRC metastasis, the metastasis occurs primarily in the liver, though lung metastasis accounts for a significant proportion of CRC metastasis. There is an urgent need for a murine model of CRC, which not only allows tumor progression in the colonic mucosa but also metastasis of the lung. The authors describe a minimally invasive murine model of colon cancer progression that may be ideal for a wide range of applications, including evaluating gene function, microenvironment, cancer metastasis and therapeutic translational research.

GRAPHICAl ABSTRACT

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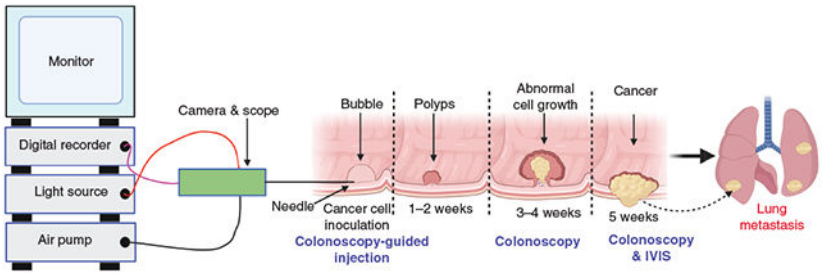
Author contributions

Conceptualization: R Ahmad, W Xu and AB Singh; Data production and acquisition: R Ahmad, B Kumar, W Xu and RL Tamang. GA Talmon performed pathological analyses. R Ahmad and AB Singh wrote the manuscript. AB Singh, AM Mohs and P Dhawan critically reviewed and edited the manuscript.

Ethical conduct of research

All animal experimental procedures were approved (protocol no: 17-126-11FC) by the Institutional Animal Care and Use Committee (IACUC) of the University of Nebraska Medical Center, Omaha, Nebraska.



METHOD SUMMARY

This study describes a procedure for murine modeling of CRC progression and metastasis to the lungs by colonoscopic-guided implantation of colon cancer cells into the host colonic mucosa. This simple and rapid model offers the opportunity for longitudinal monitoring of the cancer progression and lung metastasis with a high success rate (~75% tumor take rate). This technique can be translated to study the gene's function and therapeutics against colon tumorigenesis and its metastasis to the lungs.

Keywords

colon cancer; colonoscopy; lung metastasis; metastasis; xenograft

Among malignancies, colorectal cancer (CRC) is the third-most-common malignancy affecting both men and women. The CRC incidence rate for patients under the age of 50 has dramatically increased such that the median age of younger patients is 44 (75.2%) [1-3]. A leading cause of CRC-related death remains the diagnosis of the disease at advanced stages and metastasis [4,5]. The liver is the most common site for CRC metastasis and the lung is the second-most-common site [4,6,7]. Advanced diagnostic procedures and therapeutic strategies are highly desirable to manage CRC progression and associated patient death. However, exploration of these strategies requires suitable animal modeling of cancer progression and metastasis comparable to its normal niche. Studies aimed at addressing this preclinical need have led to the development of a murine model of colon tumorigenesis by administration of exogenous carcinogens and colitogens as well as genetically engineered mice (GEM) [8,9]. These models, however, rarely show distant metastasis. The widely utilized orthotopic mouse model of CRC metastasis requires injection of the CRC cells into the cecal wall or stitching a dissected tumor from another animal or human patient to the cecal wall of the athymic nude mice [10-12]. Despite the validity of this model for CRC metastasis, it carries several limitations, including little similarity to the histopathological features of natural CRC. Moreover, this model is highly invasive, time-consuming and requires surgical procedures that can not only influence the tumor microenvironment but may also alter the tumor growth and dissemination capacities. Additionally, these models are unable to replicate the initial steps of disease. Metastasis in this model takes place primarily to the liver [13]. Recently, colonoscopy-based syngeneic or CRC cell injections into the colon of C57BL/6 or athymic nude mice were introduced; however, in addition to the inherent variabilities, these models show CRC metastasis primarily to the liver [11]. Murine models that generate colon cancer metastasis to the lung, the second-most dissemination site

during CRC metastasis are needed [14]. To circumvent this limitation, investigators have reported a lung metastasis model of CRC by injecting colon cancer cells into the footpad or the tail vein [15,16]. However, these models lack pathophysiological relevance because they lack the colonic niche and the ability to monitor the tumor progression and metastasis [17].

Rag-1 knock out (*Rag-1*KO) mice are comparable to athymic nude mice in that they are immune-compromised, lack functional B and T cells and have low natural killer (NK) cell activity. These mice also show no leakage of the B and T cells with aging, as can be the case for athymic mice. Most notably, *Rag-1*KO mice are widely used to dissect T cells' role in colonic inflammation and tumor development and progression [18]. The *Rag-1*KO mice are also required in studies where it is necessary to match MHC with MHC restriction of a T-cell receptor [19]. Despite this immune deficiency, the *Rag-1*KO mice are similar to wild-type mice (C57BL/6) in their appearance (with a hairy coat) and thus procedures applicable to *Rag-1*KO mice for imaging can be easily applied to wild-type mice for syngeneic studies if needed.

Based on these advantages, *Rag-1*KO mice were used to develop a reliable, potentially noninvasive model of CRC progression. The authors report a reliable murine model of CRC growth, progression and metastasis to the lung using a high-resolution colonoscopy-guided submucosal injection of human colon cancer cells into *Rag-1*KO mice colon. This minimally invasive procedure may provide a boost to preclinical CRC research.

Materials & methods

Experimental materials

Rag-1KO mice were purchased from Jackson Laboratories and were further expanded by in-house breading. Mice were kept in individually ventilated cages with free access to food and water in compliance with the Animal Welfare Act. Colonoscopic injection procedures (IACUC protocol number: 17-126-11FC) were approved by the Institutional Animal Care and Use Committee (IACUC) of the University of Nebraska Medical Center, Omaha, Nebraska. HCT116 colon cancer cells were obtained from the American Type Culture Collection (ATCC). Anti-HLA ABC (66013-1-Ig) antibody was purchased from Proteintech (IL, USA). A small animal high-resolution colonoscope with an AIDA HD capture system from Karl Storz (Tuttlingen, Germany) and *In Vivo* Imaging System (IVIS Spectrum, Perkin Elmer, Texas) from small imaging core were used in the study. Slide sections (4–5 μM thick) of the paraffin blocks of the samples obtained in the current study were cut using the Leica microtome (IL, USA). Histoclear, hematoxylin and eosin were purchased from Fisher Scientific. The de-locking chamber for unmasking the tissue sections was purchased from Biocare Medical (CA, USA). ABC and DAB kits were purchased from Vector Labs. A Nikon Eclipse microscope (Nikon Instruments, NY, USA) was used to acquire and analyze the images.

Cell culture & infection

HCT116 cells were maintained in RPMI1640 and dynamic growth conditions (5% CO_2 at 37°C). RPMI1640 was supplemented with 10% fetal bovine serum (FBS), 100 U/ml

penicillin and 100 μg/streptomycin. Lentiviral packing plasmid (Lenti-X Packaging Single Shots, catalog no. 631278, Takara) were cotransfected with luciferase-expressing pRRL plasmid in HEK293. Post-transfection (48 h) culture medium was used to infect the HCT116 cells to generate the luciferase-positive cells. Luciferase-positive cells were analyzed by measuring luciferase activity through D-luciferin (Promega #E1500) and then selected for the colonic injection.

Colonoscope-guided injection of CRC cells into the colonic mucosa

A high-resolution mouse colonoscope (COLOVIEW) system with a camera and xenon light source was used. Additionally, an air pump was used to inflate the colon. The entire procedure was viewed on a color monitor and recorded in still and video file formats assisted by an AIDA control platform (Karl Storz).

A colonoscope-guided procedure was used to implant colon cancer cells into the submucosal layer of the lower colon of the *Rag-1*KO mice. Before the injection, mice were fasted (overnight) to render their colon relatively clean of fecal pellets. Moreover, before colonoscope-assisted injection, a colon enema was performed using sterile phosphate-buffered saline (PBS). Thereafter, mice were sedated with isoflurane (4% induction, 1% for maintenance) and restrained on the operating table. The colonoscope-guided mucosal injection of the cancer cell required coordination of two individuals, where one person performed the injection, and the other helped with scope navigation. Submucosal injections were accomplished using flexible stainless steel, 16-inch, 33-gauge, 45-degree bevel hypodermic needles custom made as per specification (cat. no. 7803-05, Hamilton, USA). The needle was inserted through a 30-degree angle port at the right side of the scope with minimal resistance at the junction between the port and the endoscope and placed just before the scope front. The scope was inserted into the mouse colon and the colon was inflated with air pressure. The needle was then placed onto the colon mucosa ~1.5–2 cm away from the anus. Air pressure was released, which led to the insertion of the needle into the submucosa with a flat angle. The formation of an air bubble at the injection site confirmed the successful inoculation of cells into the colonic submucosa [10]. A total of 1×10^6 colon cancer cells in 50 μl of sterile Dulbecco's PBS were injected into the colonic submucosa. The needle was kept in the bubble for 10–12 s to avoid leakage of the colon cancer cells from the injection site.

Longitudinal assessment of tumor progression

Similar to the procedure used prior to the colonoscope-assisted implantation of the CRC cell, mice were fasted and anesthetized prior to the colonoscopic evaluation of the tumorigenesis. Tumor formation, growth and progression were longitudinally monitored by colonoscopy and digitally recorded on tape using high-resolution mice colonoscopy. Colonoscopy was performed at 3 weeks and 5 weeks post-inoculation of colon cancer cells into the colonic submucosa [20,21].

In vivo imaging system imaging

An *in vivo* imaging system (IVIS) was used to access luciferase-expressing HCT116 cells implantation and tumor growth in the *Rag-1*KO mice. The IVIS is routinely used in the

Animal Imaging Core Facility at UNMC. Tumor growth and metastasis were visualized using D-luciferin (catalog number luck-100 [Gold bio]; 15 mg/kg body weight; 100 μl/IP) as substrate before the IVIS imaging. Mice were sedated using isoflurane (4% induction, 1% for maintenance) and then placed into the mouse-imaging platform with the integral anesthetic manifold in IVIS. The platform temperature was maintained at 37°C, and mice were monitored for any distress or discomfort during the entire procedure. Data acquisition and analysis were made according to the manufacturer's recommendations. In brief, at least three images were acquired in the same setting with exposures ranging from 15–45 s.

Histological assessment of tumor development & metastasis to the lung

Organ tissues were collected after the sacrifice of the mice. The tissues were fixed with 10% formalin in PBS overnight (O/N) and then paraffin-embedded. Tissue sections (5 μm) were stained with hematoxylin and eosin (H&E). A gastrointestinal pathologist evaluated the H&E slides from tissues, and representative images were taken by microscope for further evaluation.

Tumor development & metastasis detection by immunohistochemistry

Tumor development and distant metastasis were evaluated by tracking human colon cancer cells by immunohistochemistry (IHC) using an anti-HLA-ABC antibody that is specific for binding to cells of human origin. Briefly, tissue sections were dewaxed and rehydrated using Histoclear and subsequent alcohol gradient in decreasing order. Tris-EDTA buffer (pH 9.0) was used for the antigen unmasking of the tissue sections in the de-locking chamber. Sections were blocked with appropriate blocking serum, then sections were kept in the primary antibody (anti-HLA-ABC) overnight at 4°C. The next day, brown color development was performed using the ABC and DAB kits according to the manufacturer's recommendations.

Statistics analysis

All experiments included more than three mice per group unless stated otherwise. Statistical analyses were performed using Prism 9.0 (GraphPad Software Inc.). All data are plotted as the mean ± standard deviation (SD) and p-values <0.05 were considered statistically significant.

Results & discussion

The current murine models of CRC progression and metastasis are limited in their scope and clinical applicability. Despite concerted efforts to address these limitations, reliable murine models of CRC progression where tumor progresses to cancer in the colonic mucosa and metastasizes to the lungs are lacking. To address the limitations of these models, we used *Rag-1*KO mice, since these mice are similar to athymic/nude mice in the lack of lymphocytes but possess fur similar to C57BL/6 mice. Therefore, the authors anticipate that the outcome of the current study can be readily applied to the syngeneic CRC model. In view of the frequent uses of the *Rag-1*KO mice for studies aimed at understanding the role of the specific immune components in colonic pathobiology, including colon cancer, makes these mice ideal for the desired modeling. A colonoscope-based procedure was adapted

for the inoculation of human colon cancer cells into the colonic submucosa of *Rag-1*KO mice (n#8). HCT116 cells expressing luciferase generated in our laboratory as described in the Materials and Methods were used. Figure 1A & B show the timeline and detailed procedure for the colon cancer cell injection into the submucosa. The bubble formation was used as a positive indicator of the mucosal insertion by the colonoscopy needle. Three weeks post-implantation of the CRC cells into the mouse colon submucosa, mice were subjected to IVIS to determine tumor growth after injecting D-luciferin substrate (intraperitoneal). As shown in Figure 2A, a higher tumor burden was detected in the distal part of the colon than in the mice that did not develop tumors. Following IVIS, mice were subjected to colonoscope-assisted imaging (Figure 2B & C) which validated the IVIS findings and revealed the development of tumors into the colonic wall. The gross anatomical evaluations revealed that six mice (75% success) had well-developed tumor growth in their colon (Figure 3A). All mice were sacrificed 5-weeks post-injection of the CRC cells and the colon was imaged to show the tumor/cancer growth. As shown in Figure 3B & C, significant ($p < 0.05$) tumor mass occupied the entire colonic circumference, potentially depicting the tumor progression by crossing the colon's muscle layer. Histological analysis by H&E showed well-developed tumors underneath the typical architecture of the colon of mice (Figure 3D). Taken together, the data supported the feasibility of our model for preclinical studies of colon tumorigenesis into the vicinity of the colonic mucosa with the potential opportunity for IVIS and/or colonoscopic evaluation of the tumor growth and progression.

To further determine if CRC metastasized to distant organs, mouse lung and liver were dissected and examined histologically under a dissecting microscope. Notably, metastatic foci were found exclusively in the mouse lung and the liver presented no metastatic foci. Gross anatomical examination validated that only mouse lungs had significant ($p < 0.01$) metastatic foci (50% success) while no metastatic foci were found in the liver (Figure 4A-C). While the precise mechanism for why colon cancer metastasizes primarily to the lungs in this model is unclear, the authors speculate that this may be due to the specific homing signal attracting the cancer cells to the lung niche and/or efficient adhesion of the cancer cells to the lung. The mouse tissue sections from the H&E staining were further subjected to evaluation by a gastrointestinal pathologist in a blinded manner. The pathologist used low (10x) and high (20x) objective magnifications to examine tumor/cancer growth and metastatic foci. This analysis helped affirm the initial findings that CRC cell implantation led to tumor growth in the colonic mucosa, which further progressed into adenocarcinoma and ultimately metastasized to the mouse lung. Pathologist evaluation excluded the possibility of any microscopic metastatic lesions into the mouse liver (Figure 4D & E). To further validate that the observed tumor/cancer growth and metastatic foci were indeed originated by the implanted cells, we immune-stained the resultant tissue with anti-HLA-ABC antibody, since this antibody specifically binds to human nucleated cells. Analysis of immunohistochemistry for the HLA-ABC immune-phenotyping confirmed the human origin of the colon tumor/cancer growth (Figure 5A). Immunohistochemical analysis of the lungs and liver tissue sections similarly supported the human origin of the metastatic foci in mouse lung and the lack of such cells in the mouse liver (Figure 5B & C).

The data suggest that the authors have established a noninvasive orthotopic CRC mouse model with metastasis to the lungs that is based on colonoscopic technique using *Rag-1*KO

mice. Importantly, this model offers multiple advantages over the currently used orthotopic model of CRC metastasis: first, the current model does not require surgery, hence mice mortality is significantly lower as compared with the orthotopic model of cecal implantation; second, the current model offers a higher success rate (~75% tumor take rate) and is fast (3–5 weeks for cancer metastasis); third, the current model offers the opportunity for CRC development in its natural site of the colonic mucosal and the opportunity for longitudinal monitoring of the tumor growth and progression; finally, the current model offers an advantage over other murine models of CRC progression in providing exclusive metastatic dissemination to the mouse lung. This new colonoscopy-based model of CRC progression and metastasis has the potential for lung metastasis. This CRC model could be used to study genes of interest and for preclinical testing of new therapeutic agents, especially for lung metastasis, as well as a test model for the development of new diagnostic and surgical imaging agents [22,23].

Future perspective

This study highlights the experimental procedure of CRC development within the natural site of mice colon and its progression to lung metastasis. This model may serve as a suitable platform for the preclinical testing of drugs and imaging techniques to refine cytoreductive surgery. Considering the success of this model with *Rag-1*KO mice, we anticipate that this model can be easily recapitulated to the syngeneic model of CRC, which in turn can offer the possibilities of studying the dynamics of immune deregulations during CRC progression as well as therapies that are aimed at testing checkpoint inhibitors. The described model may also improve preclinical studies aimed at CRC metastasis to the lung and personalized therapeutic and imaging regimens.

Acknowledgments

The authors thank the small animal imaging core of the University of Nebraska Medical Center Omaha, Nebraska for providing access to the small animal colonoscopy facility.

Financial & competing interests disclosure

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No writing assistance was utilized in the production of this manuscript.

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Executive summary

The proposed murine model of colonoscopy-assisted implantation of colorectal cancer into the colonic mucosa for examining cancer progression and metastasis to the lungs offers multiple benefits over the orthotopic model of CRC metastasis:

- The current model offers the ability to examine colon cancer progression in the natural niche of the colonic mucosa and metastatic dissemination primarily to the lung.
- This model is reproducible and minimally invasive, reducing the chance of mortality compared with the orthotopic model of cecal implantation.
- This model is suitable for investigating the therapeutic intervention of novel molecules including specific immune components during CRC progression and lung metastasis.

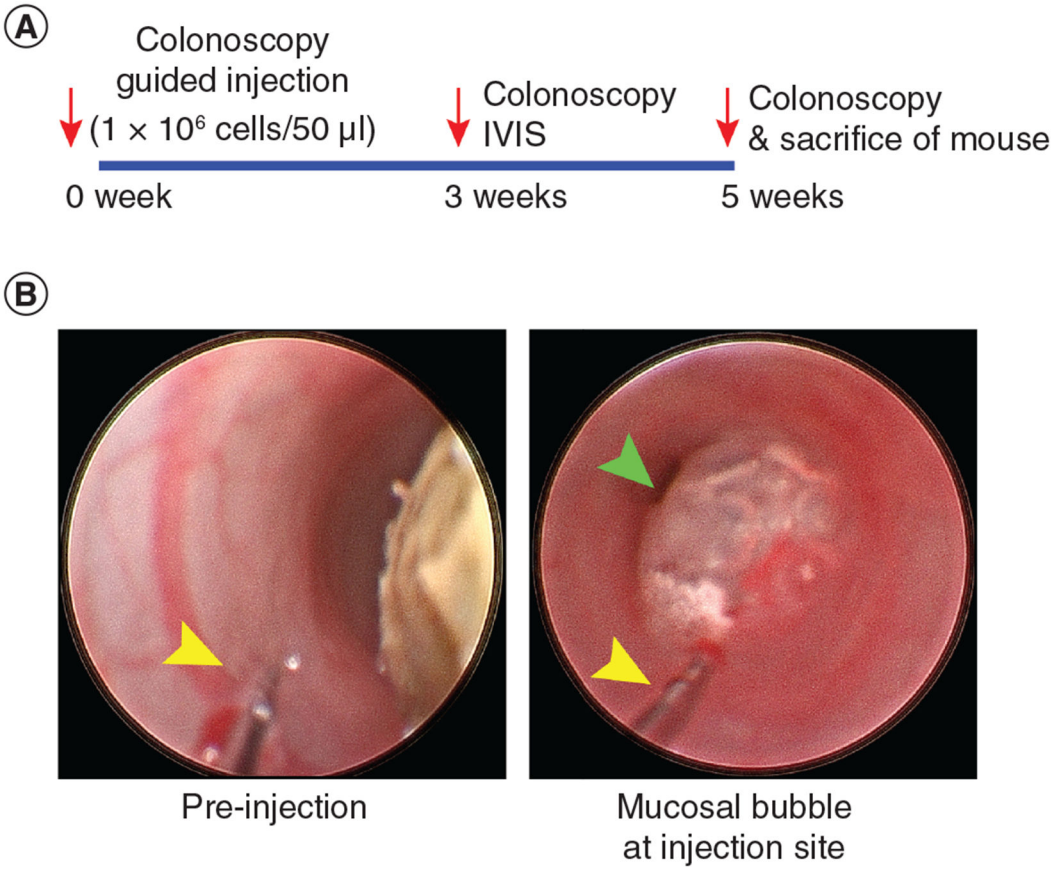

Figure 1. Colonoscopy-guided submucosal injection to implant colon cancer cells in the mucosal wall.

(A) Outline of the experimental procedures and **(B)** the injection needle entering the submucosa, injecting colon cancer cells into a mucosal bubble.

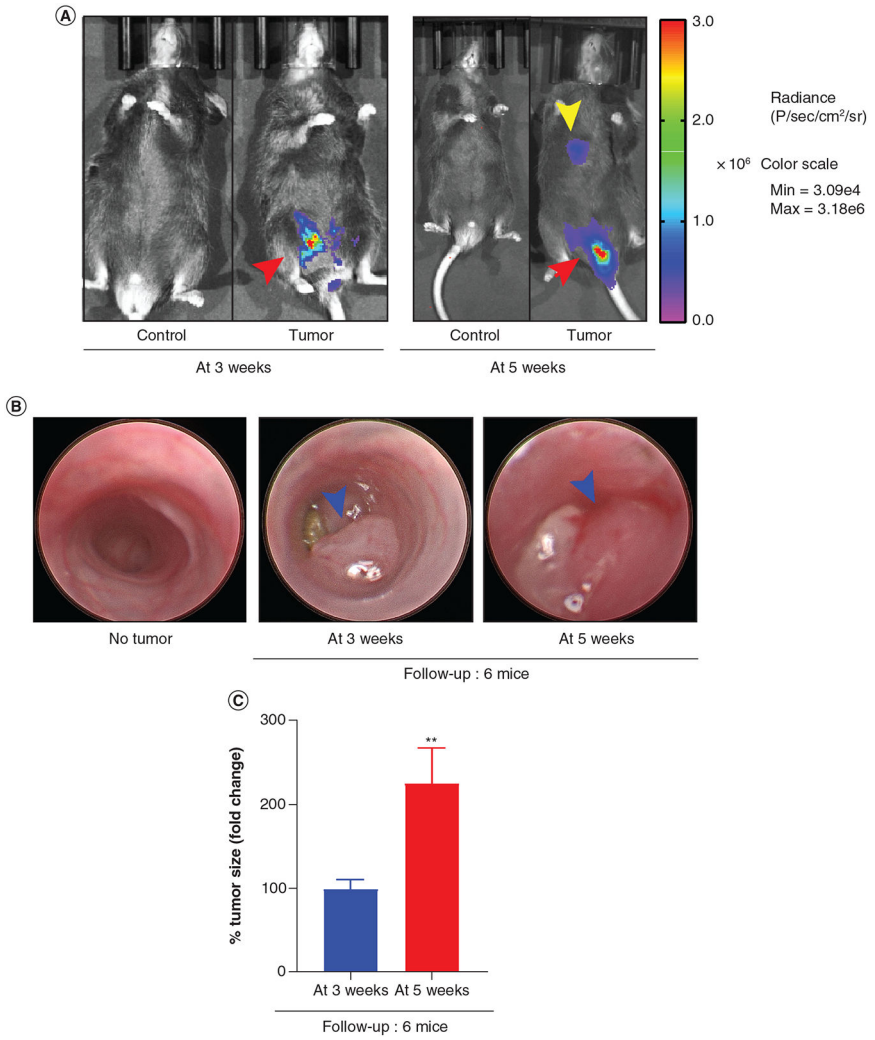

Figure 2. Longitudinal follow-up of tumor growth using IVIS and colonoscopic evaluations.

(A) IVIS images of the colon suggesting tumor development and **(B & C)** colonoscopic images and longitudinal analysis of tumor development.

**p < 0.01.

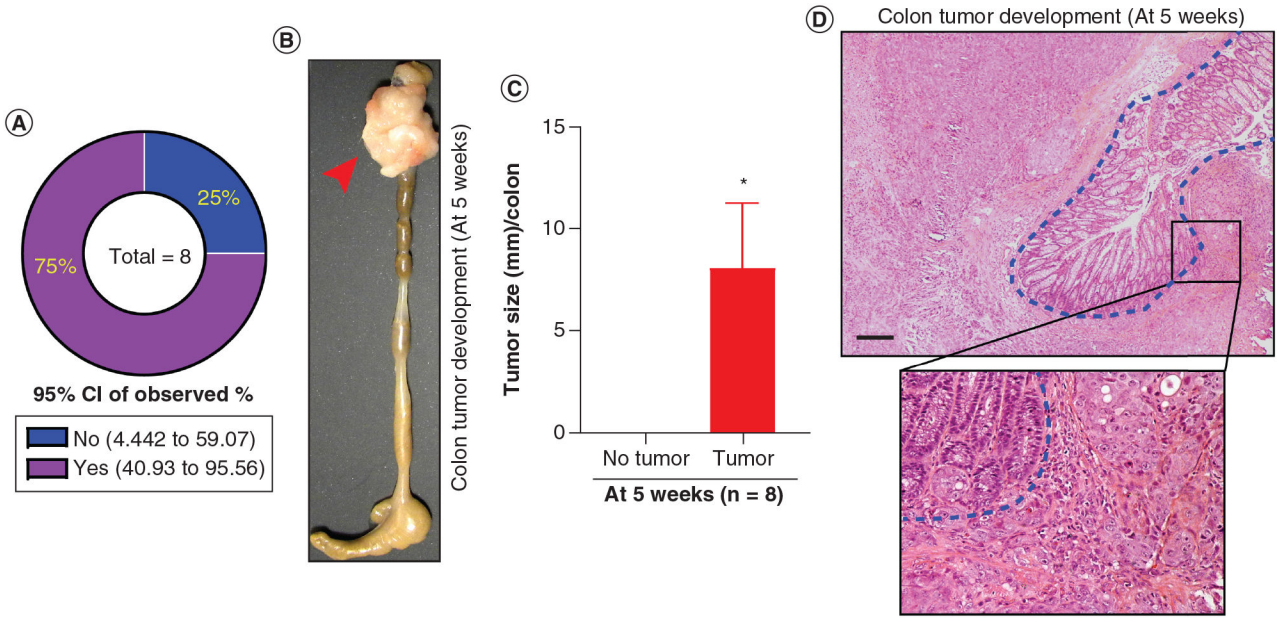

Figure 3. Gross anatomical and histological evaluation of tumor development.

(A) Percentage distribution among mice. **(B & C)** The colon showing tumor growth. **(D)** H&E image of colon showing tumor development and growth. Scale bar = 50 μM.

*$p < 0.05$.

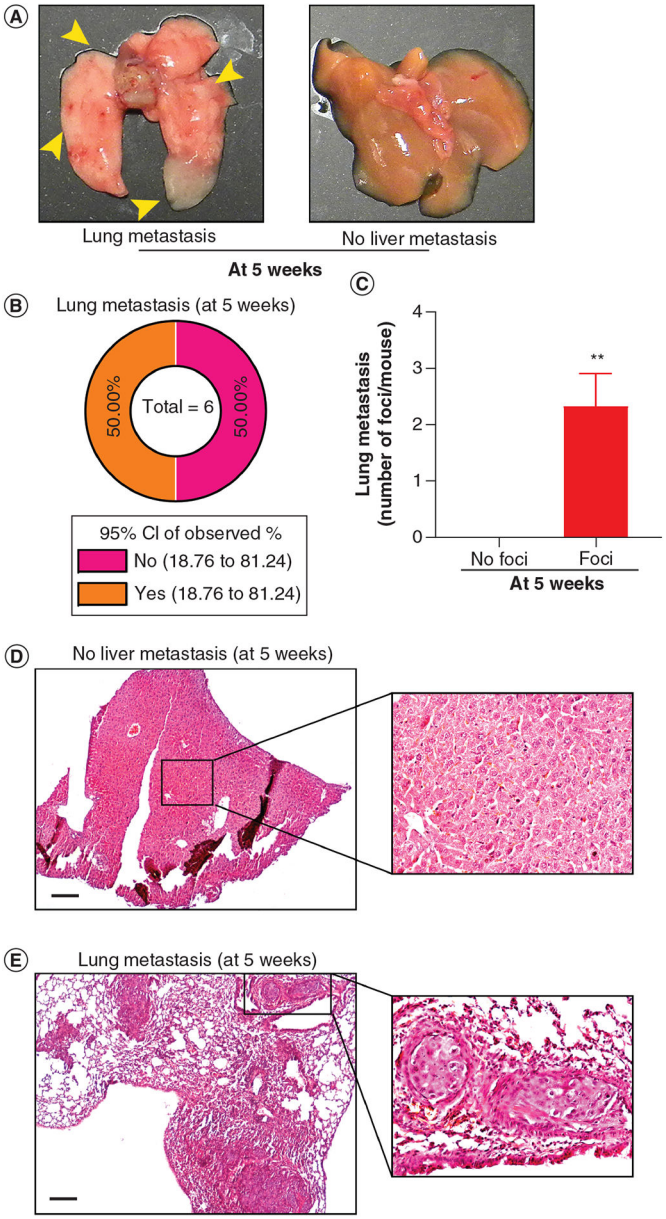

Figure 4. Gross anatomical and histological examination of colon tumor/cancer and its progression to lung metastasis.

(A) The mouse liver and lungs, showing distant colon metastasis. **(B)** Percentage success of lung metastasis. **(C)** Number of metastatic foci/mice. **(D)** Mouse liver showing no tumor metastasis. **(E)** Lungs showing metastasis foci. Scale bar = 50 μM.

**p < 0.01.

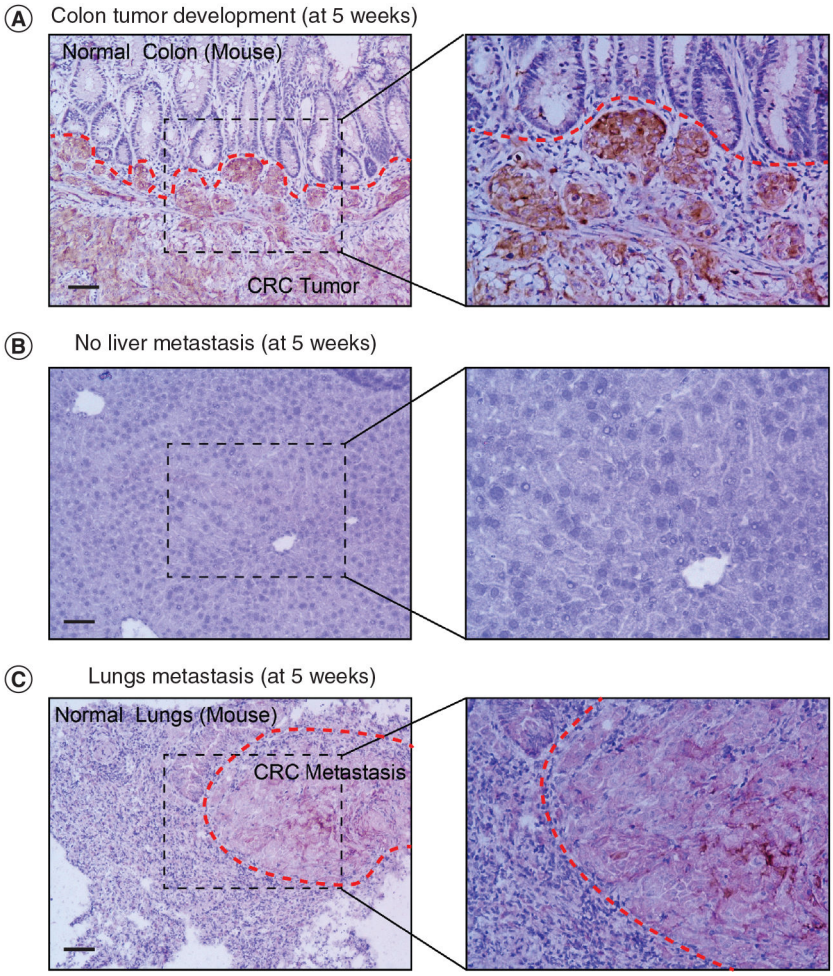

Figure 5. Immunocytochemical analysis using anti-HLA-ABC antibody to validate the human origin of the colon tumors and metastatic foci.

(A) Immunohistochemical analysis showing colon tumor development in submucosa. **(B)** Immunohistochemical analysis showing no metastasis in liver. **(C)** Immunohistochemical analysis showing lung metastasis. Scale bar = 50 μM.