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Development of a metastatic murine colon cancer model

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ABSTRACT

Background: It has now become clear that the complex interplay of cancer and the immune responses against it plays a critical role in the tumor microenvironment during cancer progression. As new targets for cancer treatment are being discovered and investigated, murine models used for preclinical studies need to include intact immune responses to provide a closer correlation with human cancer. We have recently developed a modified syngeneic orthotopic murine colon cancer model that mimics human colon cancer progression with consistent results.

Materials and methods: Tumors were created using the murine colon adenocarcinoma cell line, CT26, modified to overexpress the firefly luciferase gene (CT26-luc1), which allowed real-time *in vivo* monitoring of tumor burden when the substrate, D-luciferin, was injected intraperitoneally using the *In Vivo* Imaging System. Mice are Balb/c (Harlan), syngeneic with the CT26-luc1 cells. Cells are injected submucosally, suspended in Matrigel, into the cecum wall under direct visualization.

Results: The model has demonstrated consistent implantation in the cecum. *In vivo* bioluminescence allowed real-time monitoring of total tumor burden. Perioperative preparation had a significant impact on reproducibility of the model. Finally, total tumor burden quantified with bioluminescence enabled estimation of lymph node metastasis *ex vivo*.

Conclusions: This method maintains an intact immune response and closely approximates the clinical tumor microenvironment. It is expected to provide an invaluable murine metastatic colon cancer model particularly in preclinical studies for drug development targeting those mechanisms.

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1. Introduction

Colorectal cancer (CRC) is the third most common cause of death by cancer in both sexes in the United States, and over 50,000 are expected to succumb to CRC in 2013 [1]. With best care, the 5-y survival rate of all cases of CRC is 65%. It is clear that new therapies are still needed to improve survival in advanced disease. Although some promising therapies have been developed in recent years, the vast majority of compounds validated by animal models fail to show benefit in clinical trials. For drugs tested from 1993–2002, only 26% of oncology drugs tested in phase I trials resulted in Food and Drug Administration approval for treatment [2]. In recent years, a number of voices have brought awareness to the growing concern that our preclinical animal models are inappropriate for assessing the utility of novel therapies in actual human patients [3–5]. Criticisms point out the poor predictive value of many preclinical trials, the lack of relevance of *in vitro* data, and the differences between mouse models and human immunology and cancer biology.

Despite these criticisms, murine models for human cancer are still the mainstay of preclinical evaluation in drug discovery and emerging technologies. Thus, models that more closely replicate the biology and progression of human cancer are in urgent need. Xenograft models that implant human colon cancer cells into immune-deficient nude mice have been used since the 1960s [6] and remain the most commonly used model for drug development. Xenograft models are useful for assessing drug effectiveness against human cancer cells in an animal setting; however, as targeted therapies emerge, the immune-deficient nude mouse model is no longer an ideal model for novel therapies that take advantage of the immunologic characteristics of cancer. Even the latest patient-derived xenograft models that maintain the heterogeneity of the tumor by transplanting a part of a tumor from a patient, argued by many to be the best mimic of a human tumor, are not free from this limitation because the tumor can only be implanted onto immune-deficient nude mice [7].

Although syngeneic models that implant murine cells into immune intact normal mice are limited to using relatively less studied murine colon adenocarcinoma cell lines, these models are capable of demonstrating the complex interaction between the immune system and the tumor microenvironment in cancer progression. Subcutaneous tumors were for many years the mainstay of murine tumor models, with direct measurement of tumor size used to study effects of drugs and targeted therapies. We have recently reported that even these genetically homogenous cell lines have markedly different gene expression depending on the location of the tumor in the mouse, with subcutaneous tumors having a different gene expression profile from orthotopically implanted tumors, and models of metastasis, demonstrating the importance of orthotopic models in studying the effects of the tumor microenvironment on cancer growth and progression [8–10].

Real-time monitoring of cancer progression *in vivo* is now possible through genetic overexpression of reporter genes, such as green fluorescent protein (GFP) or luciferase, within the implanted cell lines. Methods using GFP were the first to be developed [11]; however, the strength of the signal is known to dissipate when it travels through the body wall. Syngeneic cancer cell lines engineered to express firefly luciferase have been used successfully in our laboratory to track breast cancer progression and metastasis to the lymph nodes and lungs [10,12] and are optimally suited for following intra-abdominal tumors and subsequent metastasis. Here, we demonstrate our newly established syngeneic colon cancer cell implantation method and the utility of our luciferase positive model for monitoring cancer progression.

2. Material and methods

2.1. Cell culture

CT26-luc1 cells generated from CT26 (American Type Culture Collection, Rockville, MD), a murine colon adenocarcinoma cell line derived from BALB/c colon, have been engineered to express firefly luciferase through a retrovirus vector-mediated process by our collaborators at the National Cancer Research Institute in Tokyo, Japan [13]. CT26-luc1 cells were cultured in RPMI Medium 1640 (Life technologies, Carlsbad, CA) with 10% fetal bovine serum. Before implantation, CT26-luc1 cells were cultured in a 37°C humidified incubator with 5% CO_2 and grown to 80% confluence using RPMI with 10% fetal bovine serum. Cells were resuspended in phosphate-buffered saline (PBS) and mixed with Matrigel Basement Membrane Matrix (BD Bioscience, San Jose, CA) at a ratio of 1:9 for a final concentration of 50,000/10 μL before implantation.

2.2. Animals

All animal studies were conducted in the Animal Research Core Facility at Virginia Commonwealth University School of Medicine in accordance with institutional guidelines. Surgical procedures were approved by the Virginia Commonwealth University Institutional Animal Care and Use Committee, accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Female BALB/c mice (8–10 wk, weight 20–25 g; Harlan, Indianapolis, IN) were anesthetized with continuous vaporized isoflurane for general anesthesia, and mice were given analgesia (Buprenorphine SR, Zoopharm, Windsor, CO) for at least 72 h postoperatively and closely monitored throughout the perioperative period. Any animals appearing to be in significant distress or showing physical signs indicating unlikely survival for an additional 24 h were euthanized as a humane end point.

2.3. In vivo bioluminescence

D-Luciferin (0.2 mL of 15 mg/mL stock; PerkinElmer, Waltham, MA) was injected intraperitoneally into mice previously implanted with CT26-luc1 cells at indicated times. Bioluminescence was detected and measured using the *In Vivo* Imaging System (IVIS; Caliper–PerkinElmer). Living Image Software (Xenogen–PerkinElmer) was used to quantify the photons per second emitted by the cells. Bioluminescence was measured and quantified at 5-min intervals over 30 min using a subject

height of 1.5 cm, medium binning, and an exposure time of 0.5 s–1 min. The peak number of photons per second calculated over this time frame then determined bioluminescence.

3. Results

3.1. *Development of our metastatic syngeneic orthotopic colon cancer model*

The surgical technique to generate a metastatic syngeneic orthotopic colon cancer model was established (Fig. 1). CT26-luc1 cells were suspended in a Matrigel Basement Membrane Matrix. Matrigel suspension enables an even distribution of cells throughout the inoculum, and its gel-like consistency when warmed by body temperature prevents the cells from leaking out into the peritoneal cavity and causing carcinomatosis. It also provides an initial scaffold for tumor growth. The abdomen is prepped with povidone iodine after fur removal and draped using aseptic technique. Laparotomy is obtained through midline incision. The cecum is eviscerated, then draped in separate sterile gauze, and moistened with PBS. After cecotomy, Matrigel-suspended syngeneic CT26-luc1 colon adenocarcinoma cells are injected into the submucosal layer of the cecal wall using a 1-mL syringe with a 28-gauge needle (50,000 cells in 10 μL). We found that cecotomy allows identification and inoculation of the cells to the appropriate layer of the thin murine cecal wall more easily than surface injection. The cecotomy was closed with 5-0 silk sutures in interrupted Lembert fashion. The cecum is then again moistened with PBS and gently returned to the left upper quadrant, taking care to avoid malrotation of the viscera. The fascial edges are approximated with 5-0 silk sutures and the skin closed primarily with continuous sutures.

3.2. *Bioluminescence allows monitoring of chronological tumor burden growth*

We have generated a CT26 murine colon adenocarcinoma cell line with stable overexpression of firefly luciferase (CT26-luc1

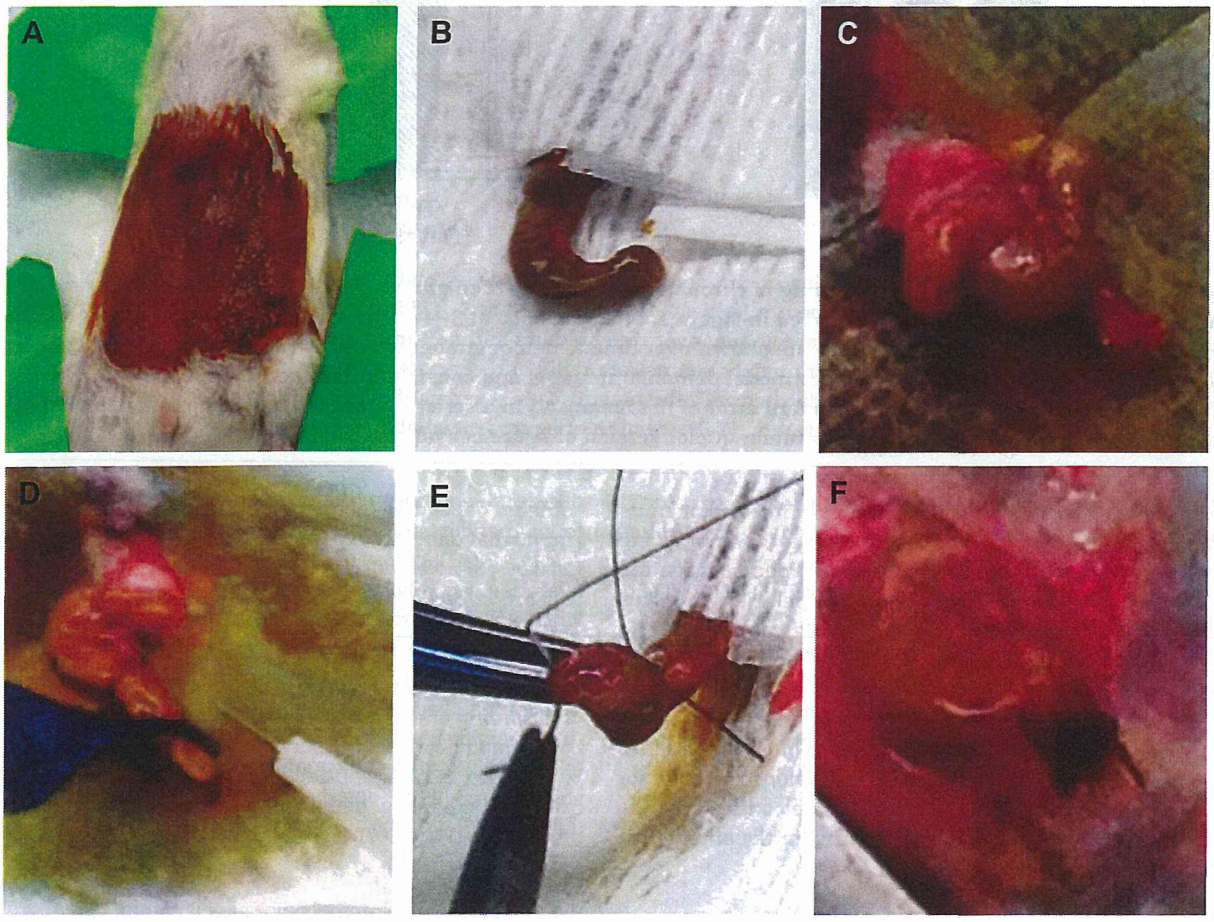


Fig. 1 – Our metastatic murine colon cancer model has a simple, reproducible surgical technique. (A) Fur was clipped on syngeneic Balb/c mice, animal was anesthetized with isoflurane and sterilely prepped and draped. (B) A laparotomy incision was made, and the cecum was grasped and eviscerated. (C) The tip of the cecum was sharply removed, making a cecotomy. (D) CT26-luc1 (5×10^5) cells in Matrigel suspension were injected through the lumen submucosally. (E) The cecotomy was then closed, followed by (F) two-layer closure of the abdominal wall. Animals were monitored closely in the perioperative period, with Buprenorphine SR used for analgesia. (Color version of figure is available online.)

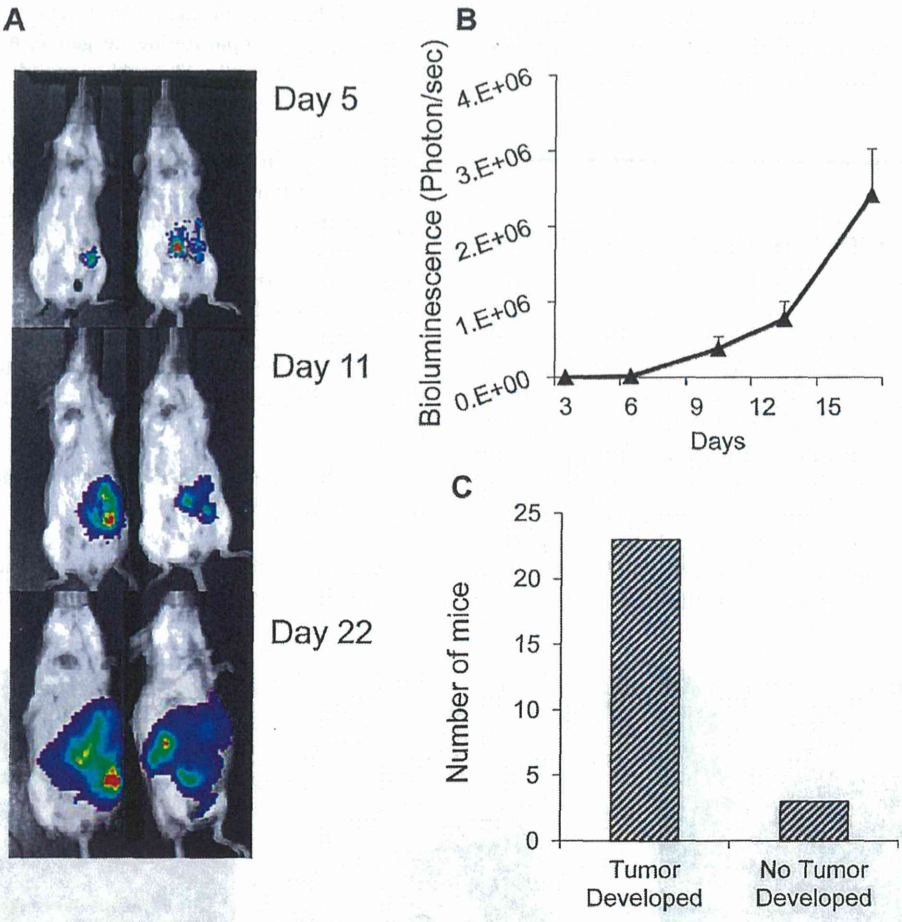


Fig. 2 – Bioluminescence allows monitoring of chronological tumor burden growth. (A) Balb/c mice underwent surgical implantation of CT26-luc1 cells as described in Figure 1. IVIS was used at 3–4-d intervals to follow cancer progression. Representative photographs of two mice are shown over time to demonstrate tumor growth. (B) Photon counts allow quantification of tumor burden and in our model demonstrate rapid and consistent rise over time (representative experiment shown, $n = 10$, mean ± standard error of the mean). (C) In a series of experiments comprising 26 mice, the majority (23) animals proceed to develop tumor. (Color version of figure is available online.)

cells). When D-Luciferin is intraperitoneally injected, it emits a photon signal that can be detected using IVIS and Living Image Software. Note the similar tumor burden between the two animals that were followed chronologically (Fig. 2A). This technology allows quantification of the amount of live CT26-luc1 cells (total tumor burden), which allows development of tumor to be assessed *ex vivo*. Figure 2B demonstrates the growth curve of the CT26-luc1 tumor burden after implantation using our method in the same animals ($n = 10$). In a series of experiments, most animals, 23 of 26, developed cecal tumor using this technique (Fig. 2C). Failure to develop tumor may be because the inoculation was not localized in the appropriate layer of the cecal wall.

3.3. *Consistent and reproducible results depend on meticulous technique and preoperative preparation*

Even among the animals that underwent the same new method, injection of CT26-luc1 cells suspended in Matrigel into the cecum, postoperative morbidity and mortality differed depending on the preoperative preparation used. We found that our early experiments had low survival with 63% at 20 d ($n = 37$). Necropsy of these animals revealed severe bowel dilatation secondary to extensive adhesions and obstruction with ischemia. After these initial experiments, the perioperative preparation was re-examined and modified to include clipping of the fur on the abdominal wall and frequent moistening of the exposed bowel during the operation. After these modifications, the survival at 20 d was improved to 89% ($n = 47$; Fig. 3C). Log-rank analysis of the overall Kaplan–Meier survival modeling demonstrates a statistically significant difference ($P = 0.007$).

3.4. *In vivo bioluminescence allows noninvasive estimation of metastasis*

To investigate whether IVIS measurements can assess metastasis in the model, mice were implanted with 5×10^5 CT26-luc1 cells using our newly established method. Of the 13 animals that survived until approximately 1 mo after the

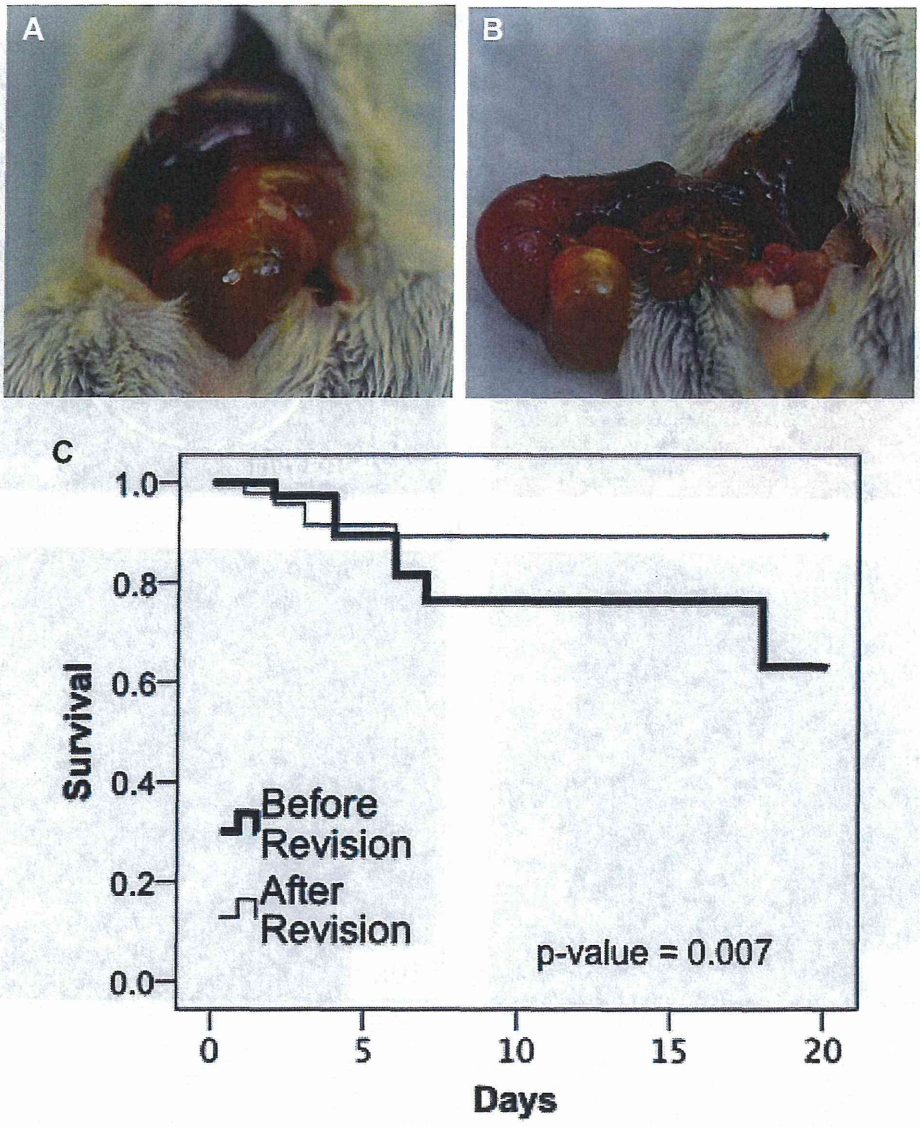
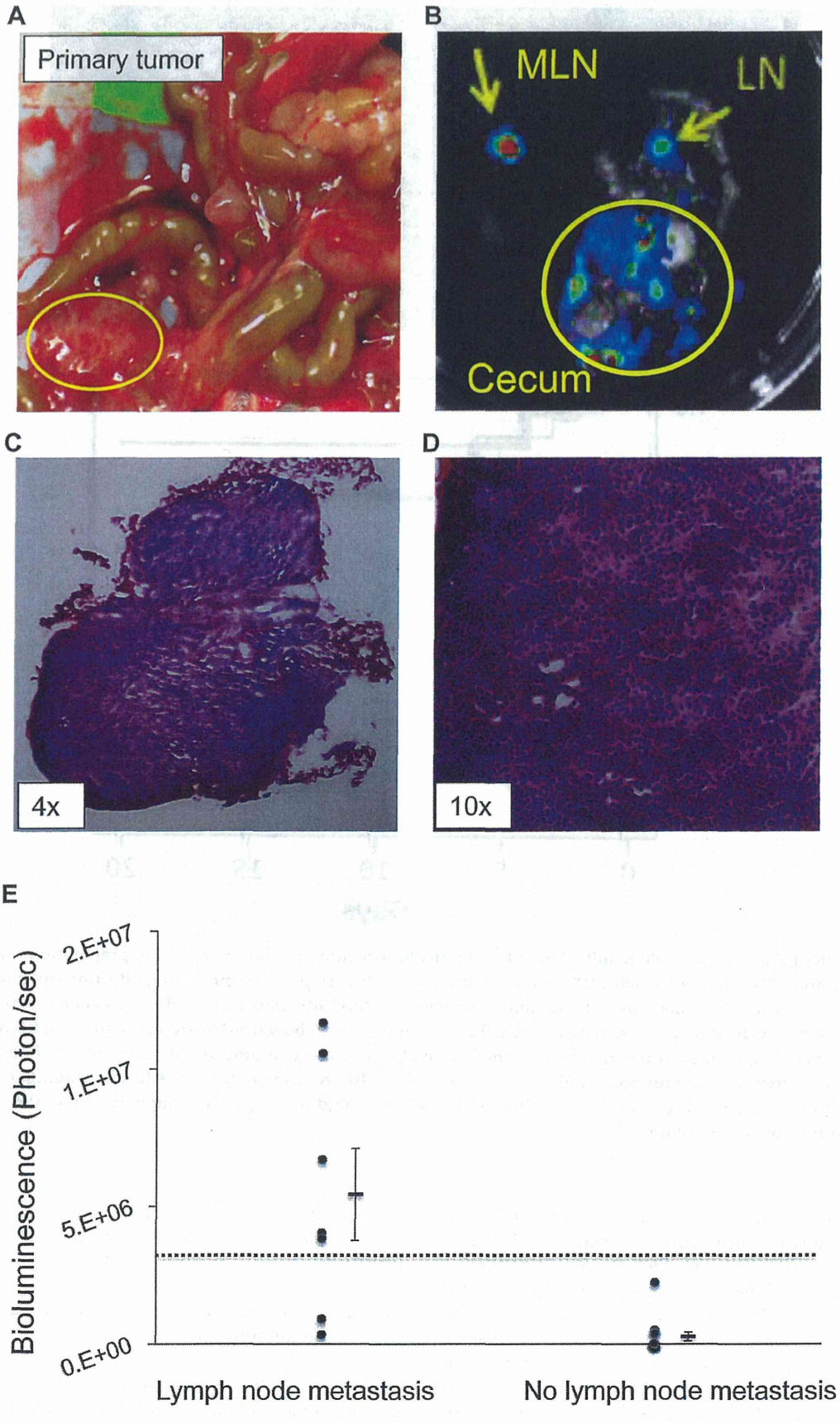


Fig. 3 – Consistent and reproducible results depend on meticulous technique and preoperative preparation. (A) Early attempts at orthotopic implantation with CT26 were plagued by high perioperative morbidity, affecting the reproducibility of the model. A representative necropsy photograph is shown. (B) Morbidity was attributed to bowel obstruction, as is evident by the severely distended bowel with dense adhesions and necrotic bowel associated with internal hernia, noted in this representative image taken at necropsy (C) Animal survival significantly improved after refinement of method. The thick line depicts survival before revision, and the thin line depicts after revision of the procedure, including thorough hair clipping and frequent moistening of the bowel with sterile PBS solution during operative implantation of the cells. (Color version of figure is available online.)

injection, 10 had enlarged tumors in the cecum (Fig. 4A). Immediately before sacrifice, animals were injected intraperitoneally with D-luciferin to enable *ex vivo* assessment of bioluminescence. As shown in Figure 4B and C, some removed mesenteric lymph nodes demonstrated metastasis detected by bioluminescence. IVIS had been performed on the animals within 5 d before sacrifice to investigate whether the total IVIS measurement can predict metastasis. Significant difference was noted in the last whole-body IVIS photon counts in those animals with grossly positive nodes and nodes positive on *ex vivo* IVIS (7 of 13) compared with animals without positive nodes (6 of 13; Fig. 4C). All animals with a count >3,000,000 photon/s had positive mesenteric lymph nodes at the time of sacrifice.

4. Discussion

The past decade of cancer biology research has resulted in a growing understanding of the importance of the tumor microenvironment in cancer progression. A PubMed search for the keyword "tumor microenvironment" reveals a 15-fold


A
Primary tumor
B
MLN
LN
Cecum
C
4x
D
10x
E
Bioluminescence (Photon/sec)
2.E+07
1.E+07
5.E+06
0.E+00
Lymph node metastasis
No lymph node metastasis

increase in publication counts since 2004, with 94% of studies published within the past 10 y. It is increasingly recognized that cancer cells do not manifest disease independently but rather form collaborative interactions with conscripted and corrupted resident and recruited normal inflammatory cells that allow the cancer to progress through tumor growth, invasion, and metastasis. The contributions of T-cells in particular to cancer progression have been well established. Despite the known importance of recruited immune cells and the role these cells play in the tumor microenvironment, xenograft models in immunologically deficient nude mice, where T-cell functions are eliminated, remain commonly used animal models for cancer drug development.

Xenograft models that implant human colon cancer cells into immune-deficient nude mice take one of several typical approaches: injection of human colon cancer cells into the cecum or colon subserosally or surgical excision of a tumor grown subcutaneously in another animal with replacement onto the cecum of the study animal. Tumors can also be grown subcutaneously, or hepatic metastatic models can be created directly through injection into the ileocecal vein or the portal vein [14,15]. In addition, tumors have been taken directly from human patients and assayed for metastatic potential using these models [16].

A popular approach pioneered by Hoffman [11] at the University of California, San Diego involves the patented MetaMouse models. For colon cancer, this involves subcutaneous growth of the cancer cells overexpressing GFP or red fluorescent protein and suture fixation of a small piece of the tumor to the colon wall in a separate animal that can be assessed using whole-body imaging for fluorescence. The published literature using this model is predominantly in nude mice with human CRC-established cell lines or histologically intact tumor [17,18].

Xenograft models such as those described are useful for assessing effectiveness against human tumors in an animal setting; however, as targeted therapies emerge, the immunologically deficient nude mouse model is no longer an ideal model for therapies that take advantage of the immunologic characteristics of cancer. Syngeneic models have an advantage over xenograft models in that they are immunologically intact and can demonstrate the interdependence of cancer and the immune system [19]. Techniques developed by Hoffman *et al.*, while allowing the metastasis of a primary tumor, have a tumor that is located extraluminally, making their spread markedly different from that seen in the clinical setting where the tumor originates intraluminally then invades extraluminally while undergoing lymphatic and hematologic spread. Although orthotopically transplanted colon tumors do have hepatic metastasis, they do not regularly undergo lymphatic metastasis; therefore, it is not an optimal setting to enable the study of drugs aimed to affect or prevent early metastasis. Furthermore, because of the extraluminal location of the tumor, these models are reported to have a high rate of carcinomatosis [20]. Although our model is not without carcinomatosis, it occurs uncommonly around 10% of the time. It is clear that each existent model is likely optimal for a particular set of clinically relevant research questions and that comparison of effects in multiple *in vitro* and *in vivo* models is likely ideal for predicting the utility of an emerging compound. There is no perfect model that can ultimately equal the value of actual clinical results; however, it is best to realize the relative weaknesses of the various models when choosing one to use.

Luciferase transgenes have emerged over the past decade as a useful method allowing *in vivo* imaging of tumors and metastasis through bioluminescence. This allows for noninvasive serial monitoring of tumor growth and responses to treatments or experimental variables in real time in living animals [8–10,12]. Although use of syngeneic or xenograft transgenic tumor cells for subcutaneous or orthotopic implantation is well established; recently, there have been concerns of potential activation of cellular adaptive immunity and sensitization against the viral agents used to transduce the luciferase transgene or to the luciferase enzyme itself. Although some authors have shown that high levels of expression of firefly luciferase result in an immune response [21,22], there is evidence from multiple studies that if luciferase expression starts low and increases gradually over time, as in scenarios resulting from implantation of luciferase expressing cancer cells, the immune response is minimal [22–24]. It is thought that there is immune tolerance to firefly luciferase at smaller levels of expression, and that it is only at sustained expression levels equivalent to 10^9 photon/s/cm^2 that an immune response is elicited, with other studies directly demonstrating that there is no effective alteration in the immune response from luciferase used in the context of tumor imaging [25,26], making it an ideal reporter to use in our model. In our model, luciferase expression as quantified using IVIS directly correlated with tumor burden and was able to be used to set a predictive threshold for lymph node metastasis before euthanasia. In addition to the possible effect of luciferase transgene in inciting an immune response, there is a known phenomenon of "quenching" of the luciferase signal, which is an artifact due to less delivery of luciferin to the tumor as the tumor grows and vascularity to the interior part

Fig. 4 – *In vivo* bioluminescence allows noninvasive estimation of metastasis. (A) Representative necropsy picture demonstrates primary tumor in the cecum (circled yellow) without significant bowel dilatation to suggest bowel obstruction. No carcinomatosis was observed in this case. (B) *Ex vivo* IVIS, where luciferin was administered *in vivo* and the organs were imaged at fixed timing extracorporeally, was performed to verify metastasis 23 d after implantation. Primary tumor in the cecum and metastases to the regional (LN) as well as to the mesenteric lymph nodes (MLN) are indicated with yellow arrows. (C and D) hematoxylin and eosin staining of the mesenteric lymph nodes confirmed metastasis by CT26-luc1 cancer cells in both low (C) and high (D) magnifications. (E) *In vivo* bioluminescence of the live animal before euthanasia was found to correlate with the presence of mesenteric lymph node metastases. Photon counts in animals with lymph node metastasis were notably higher, reflecting increasing tumor burden, with a threshold of 3,000,000 photons/s for 100% metastasis. (Color version of figure is available online.)

of the tumor becomes compromised. This phenomenon is frequently observed in subcutaneously or intramammary implanted models where the size of the tumor and bioluminescence signal become nonlinear; however, this was not observed in this model most likely because the tumor metastasized to the lymph nodes before quenching of the signal became an issue. In addition, the extensive tumor size that causes this phenomenon is likely anatomically prohibited by the consequence of bowel obstruction.

In our laboratory, we are committed to using and developing advanced cancer model systems with a high degree of verisimilitude to the pathology of disease progression seen in human cancer patients. To that end, we have worked on refining our methodology in murine colon cancer modeling to achieve a realistic approach to metastatic CRC modeling. During development of this model, we used several different surgical techniques in an attempt to make a reproducible metastasis model. Initial attempts simply used injection of cells through the serosa of the colon; however, in our experience, this led to early carcinomatosis before lymphatic metastasis as the inoculum leaked through the needle tract and seeded the peritoneum. We used transplantation of subcutaneously grown tumors to the surface of the cecum but again experienced a higher likelihood of carcinomatosis without lymphatic spread. A third method used a novel technique in which the cecum was invaginated, creating a pouch into which Matrigel cell suspensions were injected. This method yielded large tumors, but many of the animals became moribund before development of metastasis due to colonic obstruction.

Our current approach entails the use of a mildly immunogenic syngeneic murine colon cancer cell line CT26, originally developed in 1975, with a high metastatic potential [27], which has been altered to express luciferase. Tumors are created through orthotopic injection of the cell line prepared in a Matrigel suspension into the wall of the cecum, allowing invasion through the colon wall and lymphatic spread. Use of Matrigel is imperative in this method. Attempts without Matrigel, with cells in a PBS suspension, in our experience led to excessive leakage with low take and/or carcinomatosis. Implantation in the long, easily mobilized murine cecum is also critical in our approach. The cecum of a healthy adult mouse is approximately 2.5–3.0 cm in length. Implantation in the cecum allows the tumor to grow large enough for metastasis to occur before colonic obstruction from the tumor. In studies using implantation in other colorectal locations, with early obstruction has been a barrier to studying metastatsis. A recent rectal cancer model using CT26 was able to demonstrate an excellent take rate of tumor and frequent spontaneous metastasis; however, because the lower rectum was used, the pattern of spread is through the systemic venous circulation, limiting the model to exemplifying low-lying rectal cancer without broader applicability to colon cancer [28].

Recent advances in cancer biology have resulted in a new and growing emphasis on the role of the immune system in forming a tumor microenvironment that nurtures and promotes cancer progression. Potential applications for the model include experiments with targeted therapies and conventional drugs that affect the tumor microenvironment in a neoadjuvant setting, as well as studies into the role of the tumor microenvironment and the immune system in facilitating or potentially thwarting colon cancer progression. The tumor microenvironment is especially important in the context of metastatic disease, where significant need still exists for new treatments. As drugs that attempt to modulate these factors of cancer progression are developed and evaluated preclinically, it is clear that advanced model systems such as that presented herein will have a large role to play.

5. Conclusions

We report a new murine model for metastatic colon cancer. The important combination of luciferase imaging allowing prediction of metastasis before euthanasia, Matrigel implantation to reduce peritoneal dissemination, and cecum orthotopic implantation to decrease likelihood of bowel obstruction before metastasis is key to our approach, and through relatively simple refinements in technique, the mortality associated with the implantation procedure is minimized. This model replicates the invasion and metastasis of colon cancer closely to that of the human patient and has a role to play in the development of new cancer therapeutics and new understandings of colon cancer metastasis.

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Disclosure

There are no potential conflicts of interest to disclose.

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