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The impact of surgical extent and sex on the hepatic metastasis of colon cancer

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Abstract

Purpose—Extensive oncological surgeries were previously suggested to increase cancer recurrence rates. We herein studied the impact of different surgical procedures and sex on colorectal cancer liver metastasis, employing several tumor-inoculation approaches in BALB/c mice.

Methods—Experimental hepatic metastases of the syngeneic CT26 colorectal cancer line were induced either by intra-portal inoculation or intrasplenic inoculation, employing different tumor loads. Following intrasplenic inoculation, the entire spleen or an injected hemispleen were removed. Additionally, the magnitude of the surgical trauma accompanying the injection procedure was manipulated.

Results—Increasing the surgical trauma by adding laparotomy or extending the length of the surgery and hypothermia did not significantly affect the number of liver metastases or liver weight for any of the injection methods and tumor loads. The development of metastasis was significantly greater in males than in females under all conditions studied – a difference not explained by the direct effects of sex hormones on *in vitro* CT26 proliferation or vitality.

Conclusion—Concurring with less controlled clinical observations, the surgical extensiveness did not significantly affect CT26 hepatic metastasis, potentially due to a ceiling effect of the surgical trauma on the metastatic process. The sexual dimorphism observed for the CT26 metastasis should be investigated in the context of surgical stress and considering anti-CT26 immunoreactivity.

Keywords

Minimally invasive surgery (MIS); intra-portal injection; intra-splenic injection; hepatic metastasis; sexual dimorphism

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Introduction

Colorectal carcinoma is the second leading cause of cancer-related death in the United States [1], with five-year mortality rates averaging at 65% [2]. Metastatic growth is highly predictive of a poor prognosis [3], is the major cause of death [4] and is primarily localized in the liver. Surgical resection of the primary tumor is a necessary therapeutic approach [5–7], however, metastatic disease following tumor resection occurs in 10% to 60% of patients [8], and is ascribed to pre-existing micrometastases and to the induction of new metastases during the perioperative period [3,9,10].

Importantly, the type of surgical procedure and its physiological consequences were suggested to promote the metastatic process and to facilitate the growth of minimal residual disease in animal studies [9–11]. The potential beneficial effects of minimally invasive procedures on long-term cancer outcomes have recently been studied in colorectal cancer patients, but no definite conclusions have yet emerged [12,13]. Current and ongoing research is focused on understanding the potential mechanism(s) underlying the deleterious impact of the surgical procedure, with the aim of developing prophylactic measures, including the prevention of excess neuroendocrine responses and immune perturbations induced by surgery [10,14,15].

In the common murine models of hepatic colon metastases, colorectal tumor cells are inoculated into the (i) liver parenchyma [16,17], (ii) the hepatic portal vein [18,19] (iii) the spleen [20–22], with or without successive splenectomy, or (iv) into a hemi-spleen that is removed thereafter [23,24]. Unfortunately, as these techniques of tumor inoculation require an *a priori* surgical procedure (e.g. laparotomy), none of them make it possible to assess the unique contribution of surgery to metastatic development, as a no-surgery control group is inherently unobtainable. However, one can manipulate the magnitude of the surgical extent required for tumor inoculation, distinguishing between procedures with minimal and maximal deleterious effects, thus gaining some insight into the impact of surgery on the development of metastases. Potential manipulations of the surgical procedure may include alterations in the extent of tissue damage, the length of the operation, hypothermia and the use of specific anesthetics, all of which were reported to modulate the deleterious effects of surgery in animal models and in patients [10].

The current study evaluated the impact of the surgical extent and the inoculation approach on the development of hepatic experimental metastases, using the syngeneic CT26 colorectal cancer line in male and female BALB/c mice. Such comparisons may yield both practical and theoretical gains with potential clinical ramifications. Because sex differences in the stress responses [25,26], immunity [27] and tumor development [28] are well established, most studies have been conducted in both sexes and included a comparison of the findings in the different sexes.

Materials and methods

Animals and counterbalancing

BALB/c male and female mice were purchased from Harlan laboratories (Jerusalem, Israel) at the age of four weeks. The animals were housed three to four per cage at $22\pm1^{\circ}\text{C}$, on a 12:12 light:dark cycle and were allowed *ad libitum* access to food and water. Animals were used at the age of 8–12 weeks (animals were age-matched within each experiment, across all groups). The order of tumor inoculation into the different animals was counterbalanced across all experimental groups. The Institutional Animal Care and Use Committee of Tel Aviv University approved all studies described herein.

Drugs

Testosterone: testosterone was dissolved in 1 mL of ethanol to produce a concentration of 1.39×10^{-2} M, and was further diluted in complete media (CM – RPMI 1640, supplemented with 10% heat-inactivated fetal calf serum (FCS), 0.05 mg/ml gentamicin, 2 mM L-glutamine, 1 mM sodium pyruvate and 0.1 mM non-essential amino acids (Beit Haemek, Israel)) to the final concentrations (10^{-6}M to 10^{-9}M and 10^{-11}M).

Estradiol: 17- β -estradiol was dissolved in 1 mL of ethanol to produce a concentration of 1.47×10^{-2} M, and was further diluted in CM to the final concentrations (10^{-8}M to 10^{-11}M).

Progesterone: progesterone was also dissolved in 1 mL of ethanol to produce a concentration of 1.39×10^{-2} M, and was further diluted in CM to the final concentrations (10^{-6}M to 10^{-9}M).

The CT26 tumor cell line

The CT26 murine colon carcinoma cell line is a chemically-induced undifferentiated carcinoma, syngeneic to the BALB/c strain [29]. Tumor cells were kindly provided by Prof. Eliezer Flesher (Department of Human Microbiology, Faculty of Medicine, Tel-Aviv University). Cells were grown in monolayer cultures in CM, at 37°C , 100% humidity and 5% CO_2 .

Tumor cell preparation and maintenance during the injection period

Cells were removed from the culture flask with a 0.25% trypsin solution in PBS (phosphate-buffered saline), washed once in PBS containing 0.1 mg/ml BSA (335 g for 10 min), and adjusted to a final concentration of $2\times10^5/\text{ml}$ in PBS-BSA for either spleen or portal vein injection at a volume of 100 μl per animal. The cells were kept on ice during the entire injection procedure in each of the experiments. No effects of the duration of this pre-inoculation *in vitro* tumor cell maintenance on the number of developing liver metastases were evident.

Surgical procedures and tumor inoculation

Hepatic portal vein inoculation—Mice were anesthetized with 6% isoflurane for 20–30 sec, and maintained thereafter with 1.5–2.5% isoflurane. The abdominal skin was shaved and rubbed with 70% ethanol pads, and a 1.5 cm midline abdominal incision was performed.

The hepatic portal vein was exposed, and tumor cells were injected using a 31 Ga needle. To prevent bleeding, pressure was applied to the injection site with a cotton applicator (Q-tip) for four minutes. Finally, the muscle and skin were sutured using 4/0 blue polypropylene monofilament non-absorbable sutures (Johnson & Johnson, Belgium).

Intra-splenic injection—Mice were anesthetized as described above. The skin was shaved and rubbed with ethanol pads, and a 0.5 cm abdominal incision was made adjacent to the spleen (a left flank incision approximately 2 cm left of the abdominal midline) (hereafter, “minimal incision”). For the whole spleen approach, tumor cells were injected into the spleen using a 31 Ga needle, which was maintained in the spleen tissue for two minutes following injection. A 4/0 blue polypropylene monofilament non-absorbable suture was placed across the hilum of the spleen to prevent bleeding, and a splenectomy was then performed. For the hemi-spleen approach [24], the spleen was clamped in the middle of its longitudinal axis using a non-serrated hemostatic clamp, and then was surgically split (using a scalpel) into two hemi-spleens leaving both vascular pedicles intact. Employing a 31 Ga needle, tumor cells were injected into the clamped hemi-spleen. Two minutes after the injection, the needle was removed from the spleen, and the vascular pedicle draining the injected hemi-spleen was ligated. The injected hemi-spleen was then excised using a scalpel, leaving a functional hemi-spleen free of tumor cells. For both of the injection procedures described above, after the excision of the injected spleen/hemi-spleen, the peritoneum and skin were sutured with 4/0 non-absorbable filaments. The animals were allowed to recover in their home cages.

Experimental laparotomy

Mice were maintained anesthetized with 1.5–2.5% isoflurane, and a 1.5 cm midline abdominal incision was made. The small intestine was externalized, rubbed with a PBS-soaked gauze pad and left hydrated with a PBS-soaked gauze pad for 30 minutes. Finally, the small intestine was internalized, and the muscle and skin of the abdomen were sutured.

Assessment of metastatic development

Following both the portal-vein and spleen injection approaches, animals were monitored daily for general well-being after tumor inoculation, and were euthanized with an overdose of isoflurane on the 20th day. Livers were then harvested and weighed, and surface hepatic metastases were counted by an investigator blinded to each animal’s experimental group. Colorectal cancer liver metastases (CRLM) were identified as those larger than 1 mm in diameter, forming a spherical solid and with a distinct formation. As the normal (non-inoculated) livers in both male and female mice weigh about 0.9–0.95 g, the scale shown for the liver weight starts from this value (and not from 0) to better indicate tumor-related increases in liver weight.

Flow cytometry of CT26 cells

A FACS analysis was used to determine the viability of CT26 cells and to assess their numbers (Exp. 4). Cells were stained with 7-amino-actinomycin D (7-AAD), a ready-to-use solution, to identify nonviable cells by a flow cytometric analysis, according to the manufacturer’s protocol [30]. To assess the absolute number of CT26 cells per well, 300 20

µm polystyrene microbeads (Duke Scientific, Palo Alto, CA) were added to each µl of cell sample. Following flow cytometry, the formula used to calculate the absolute numbers of CT26 cells was: $(\#CT26/\#microbeads) \times 300$. The coefficient of variation for this method was found in our laboratory to be less than 6% for identical samples.

Statistical analysis

The two- or four-way analyses of variance (ANOVA) were used to assess the group differences in each of the two dependent variables, the number of metastases and the liver weight. When significant effects were observed, Fisher's protected least significant differences (Fisher PLSD) contrasts were used to test specific pair-wise comparisons with respect to *a priori* hypotheses. A value of $p < 0.05$ was considered to be significant in all analyses.

Results

Exp. 1: Different intra-splenic tumor inoculation approaches, and the impact of adding laparotomy to the minimal incision

In this study, we compared the different intra-splenic inoculation approaches, employing either a whole or hemi-spleen injection procedure, while performing either laparotomy or a minimal incision. Both male and female mice were used, and different loads of CT26 tumor cells were employed. The study was conducted to identify (i) the optimal parameters for quantifying the formation of metastases (by measuring the liver weight and enumerating distinct metastases), and (ii) the least metastasis-promoting procedure to use for tumor inoculation. To accomplish this, a total of 73 mice were used in a $2 \times 2 \times 2 \times 3$ factorial design, studying the following parameters: (i) hemi- versus whole-spleen excision (see the Methods section), (ii) laparotomy + minimal incision versus minimal incision, (iii) males vs. females and (iv) the number of tumor cells administered (1×10^4 , 2×10^4 , 1×10^5). To accumulate sufficient animals, the study was conducted using three replicates on three consecutive days, each including all experimental groups.

No significant differences were evident among the three replications with regard to any of the measures taken, so the data were combined and analyzed collectively. In all animals, no metastatic foci were observed in the peritoneal cavity. A four-way ANOVA indicated a significant main effect for the number of injected CT26 cells, and for sex, on the liver weight ($F(2,65) = 5.768$, $p = 0.049$ and $F(1,65) = 10.341$, $p = 0.002$, respectively). Specifically, the liver weights significantly increased with increasing tumor load, and males presented higher liver weights than females (Fig. 1a).

With respect to the number of CRLM developed, the same pattern of results was evident; there were significant main effects for the number of injected CT26 cells and for sex ($F(2,65) = 16.868$, $p < 0.0001$ and $F(1,65) = 9.328$, $p = 0.0032$, respectively), with the same differences as above (Fig. 1b). Qualitatively, metastases were most distinct and easily enumerated in the animals injected with the two lower doses of injected cells (1×10^4 and 2×10^4), and were difficult to distinguish and enumerate at the highest dose (1×10^5), which most commonly yielded distorted livers.

The intra-splenic injection method (hemi versus whole spleen excision), and the surgery type (laparotomy versus minimal incision), did not significantly affect the liver weights or the numbers of metastases, and had no interaction with any of the other factors. The lack of differences between laparotomy and small incision on the number of metastases is shown in Fig 1c, and the other negative findings are not shown.

A significant correlation between the liver weight and the number of liver metastases was evident ($r^2=0.76$, $p<0.05$) (Fig. 1d). Similar correlations were also evident in Exp. 2 & 3 (not shown, described below).

Exp. 2: Comparing intra-splenic inoculation to intra-portal vein inoculation in both sexes

As several previous studies have used an intra-portal vein inoculation approach, in this study, we also directly compared the CRLM development following the two common approaches for the inoculation of CT26 tumor cells: (i) the hepatic portal vein inoculation approach and (ii) the intrasplenic inoculation approach (that is, followed by splenectomy). The parameters employed in this study were those found most effective in the previous study; those yielding visually distinct liver metastases with seemingly the least trauma.

A total of 40 mice were used in a 2×2 factorial design for this experiment. Male and female mice were inoculated with 1×10^4 CT26 tumor cells, either through the hepatic portal vein or through the spleen, employing the whole spleen inoculation approach. The two-way ANOVA indicated a significant main effect for sex on metastasis ($F(1,36) = 6.699$, $p = 0.0138$), with males presenting higher liver weights than females. There was also a marginally significant main effect for the route of injection ($F(1,36) = 3.716$, $p = 0.061$), with portal vein injection resulting in higher liver weights than spleen injection (Fig. 2a).

Looking at the number of metastases, a similar pattern of effects was evident; the two-way ANOVA indicated a significant main effect for sex ($F(1,36) = 6.834$, $p = 0.01$), with males presenting more than three-fold more metastases than females (Fig. 2b). As would be expected, injection through the portal vein resulted in a greater number of metastases than through the spleen, but this difference did not reach statistical significance ($p = 0.11$) (Fig. 2b). No interaction was evident between sex and the injection approach in either the liver weight or number of metastases.

Exp. 3: The extent of surgery and its impact on CRLM following the intra-portal vein injection approach

This study assessed the impact of the extent of the surgical procedure on CT26 hepatic metastasis, in the context of the portal vein inoculation approach. This study is different from Exp. 1 in that only intra-portal injection was used following laparotomy, and the spleens were left intact in all animals. To manipulate the surgical extent and invasiveness, the length of the procedure and the environmental conditions were manipulated.

Male and female mice (total: 38) were subjected to an extensive surgical procedure or to a minimal surgical procedure (a 2×2 factorial design). The extensive surgery group underwent laparotomy and tumor injection which lasted 45 minutes, and their small intestines were rubbed between two pieces of gauze in four locations. To further increase the

surgical stress, the animals were maintained at room temperature (18°C) during these procedures. The minimal surgery group underwent laparotomy and injection which lasted 20 minutes, in a 23°C environment, without rubbing their small intestines. All mice underwent the injection of 2×10^4 CT26 cells into the hepatic portal vein during laparotomy. To accumulate sufficient animals for the subsequent analyses, the study was conducted with three replicates on three consecutive days, each including all experimental groups.

No differences were evident among the three replications for any of the outcome measures, so the data were combined and analyzed collectively. With respect to the liver weight, a two-way ANOVA revealed a significant main effect for sex ($F(1,31) = 4.678$, $p = 0.03$), with males presenting higher liver weights compared to females (Fig. 3a). No main effect for the surgical severity, and no interaction with the liver weight was evident. With respect to the number of liver metastases, although the same pattern of differences was found, none of the differences were significant (Fig. 3b).

Experiment 4: The *in vitro* effects of sex hormones on CT26 proliferation and viability

As the previous three experiments indicated significant sex differences in the susceptibility to CT26 CRLM, we started to explore the potential mechanisms mediating this sexual dimorphism. Specifically, we examined whether the evident *in vivo* sex differences could be attributed to the direct effects of sex hormones on CT26 tumor cell viability or proliferation, as assessed *in vitro*.

CT26 cells were cultured in fresh CM and incubated in a 96-well flat-bottom plate (30,000 cells in 200 μ l CM per well) with: (i) testosterone (10^{-6} M/ 10^{-7} M/ 10^{-8} M/ 10^{-9} M/ 10^{-11} M); (ii) estradiol (10^{-8} M/ 10^{-9} M/ 10^{-10} M/ 10^{-11} M); (iii) progesterone (10^{-6} M/ 10^{-7} M/ 10^{-8} M/ 10^{-9} M) (all dissolved in ethanol and further diluted in CM); (iv) ethanol as a vehicle control (10^{-6} M/ 10^{-7} M/ 10^{-8} M/ 10^{-9} M/ 10^{-10} M/ 10^{-11} M) or (v) CM as another control. Each condition was repeated in six different wells. After 24 or 48 hours of incubation, the cells were trypsinized and collected from each well. Plates were inspected under a microscope to verify that no adherent cells remained. Cells were counted, and inspected for viability by a flow cytometric analysis, as detailed in the Methods section.

Under control conditions, the CT26 cells proliferated and increased their numbers by approximately five-fold after 24 hr, and 15-fold after 48 hr. No differences were noted between the different control groups (CM with or without different concentrations of ethanol), and therefore, these groups were combined (under the label “vehicle”). The highest and lowest concentrations of each hormone were separately combined to provide greater statistical power, as their influences seemed similar. No effect of any of the sex hormones was evident after 24 hr. After 48 hours, estradiol and progesterone had increased the cell proliferation in a dose-dependent manner by up to approximately 20–25% (Fig. 4; $p=0.0001$ and $p=0.0006$ at 48 hr, respectively). Notably, these findings were in the opposite direction than that predicted based on the *in vivo* sex differences. The cell viability values ranged between 92% to 94% after both 24 and 48 hours, and did not differ significantly among the groups (not shown).

Discussion

Understanding the impact of the extent of surgery on CRLM is of clinical significance. In the current study, we employed the syngeneic CT26 colon carcinoma cell line in BALB/c mice and used several approaches for inducing experimental hepatic metastasis, and then manipulated the extensiveness of the surgical procedure used.

In comparison to major open surgeries (e.g. conventional surgery for colorectal cancer), it is well established that minimally invasive surgeries (MIS) are characterized by less immunosuppression [31–33], as well as lower short-term morbidity and mortality rates [34]. Given the aforementioned short-term benefits of MIS in both human and animal studies, one would expect lower recurrence rates and higher survival rates in patients undergoing MIS for cancer. However, both animal and human studies addressing this issue have been inconclusive, reporting contradictory results [32,35,36]. For example, a randomized trial comparing laparoscopic and traditional open surgery in patients with stage II or III colon cancer (undergoing left hemicolectomy) revealed that, although the postoperative proinflammatory and immunosuppressive responses were significantly lower in the laparoscopic group, the estimated cumulative recurrence rates were similar for the two approaches [36].

Numerous studies have indicated that more extensive surgical procedures induce greater physiological perturbations [31–33], and some have also shown increased cancer recurrence rates [37–40]. A major finding of the present study is that there was a similar course of metastatic development when the animals were subjected to mild or more severe surgical trauma. In one experiment we added laparotomy to a minimal incision, and in a second experiment, we extended the duration of surgery, added physical manipulations to the colon, and maintained a lower room temperature during the operation, all of which have been reported to modulate the deleterious effects of surgery [10]. Admittedly, we did not assess the neuroendocrine responses in these same animals, in order not to interfere with the studied oncological outcomes. However, it is reasonable to assume that at least some physiological indices would show greater perturbations due to the manipulation of these specific aspects of the surgical procedures, and with reference to known clinical variations in surgical procedures [31–33].

The importance of our findings, which suggest that there were no significant differences in the CRLM, stems from their well-controlled nature, and from their occurrence in both the portal injection and the splenic injection approaches, employing different tumor loads that induced markedly different metastatic outcomes. A plausible explanation for this lack of a difference may be a ceiling effect of surgical stress responses caused by the injection procedures, as the inoculation techniques themselves cause bleeding and/or involve a massive manipulation of the internal organs. Beyond these threshold perturbations, additional skin and muscle injuries, or the extension of the procedural duration or hypothermia, may be secondary, and not significantly increase the risk of CRLM.

Using a different tumor model and outcome (the long-term survival rates following excision of a primary melanoma (B16F10 in C57BL mice)), we similarly found no significant

deleterious effects of adding laparotomy to tumor excision [14]. Additionally, we were able to reduce mortality rates with similar success following both mild and more severe surgical trauma by targeting the excessive release of catecholamines and prostaglandins during the immediate perioperative period [14]. Thus, the potential deleterious effects of surgery, and the potential use of prophylactic measures, should also be considered when employing MIS in clinical practice.

Several approaches for inducing CRLM are commonly used. The intra-portal vein injection of colon carcinoma cells simulates the natural tumor-cell spread through the blood system, and the consequent establishment of metastatic foci in the liver. However, this approach is technically difficult in mice, and often results in bleeding, tumor spillage and peritoneal carcinomatosis (in addition to hepatic metastases) [23], as was also evident in our pilot studies (not presented in this report). The intra-splenic inoculation approach, which also includes splenectomy, was reported to reliably induce metastases that are restricted to the liver [41,42], and its potential advantages include (i) a small subcostal incision, minimizing tissue damage and potentially reducing the surgical stress responses, and (ii) lower procedure-related mortality rates compared to the intra-portal approach. In the current study, both approaches reliably induced metastases that were mostly restricted to the liver. When the spleen was removed following inoculation of this organ, no peritoneal carcinomatosis occurred. Overall, the two approaches differ with respect to removing the spleen, the overall tissue damage and the potential tumor leakage and bleeding following inoculation.

Based on our overall experience with this model, we found the splenic approach to be advantageous in terms of the animal recovery and survival following the injection procedure. Based on the literature, if the spleens are not excised, large splenic malignancies develop. However, excision of the entire spleen may also be deleterious, as the spleen constitutes an important site of antigen presentation. Thus, we also used the “hemi-spleen” approach [24], in which half the spleen, uncontaminated with tumor cells, is retained in the animal, providing the host with an putative immunological advantage compared to a complete splenectomy. To the best of our knowledge, no previous direct comparison of the hemi- versus complete splenectomy approaches has been conducted. We found no significant differences between the two approaches regarding metastases. It is possible that retaining a wounded organ (along with the development of metastases) may induce an inflammatory environment associated with immune suppression and stress responses, which could accelerate the metastatic process [43] and counteract the potential benefits of retaining an un-contaminated hemi-spleen.

Another major finding of the current study is that there was sexual dimorphism with regard to the development of metastases of the CT26 tumors; males were more susceptible than females following all of the injection approaches and surgical procedures used. Epidemiological findings have shown that the incidence of colon cancer is higher in males than in females (59 vs. 43.6 in 100,000) [1], but the recurrence rates following tumor excision were not reported to be different. However, many clinical studies have shown that either estrogen replacement therapy (ERT) or hormone replacement therapy (HRT, estrogen and progesterone) can significantly reduce the risk of colon cancer in postmenopausal women [44–46]. Similarly, in ovariectomized rats, 17- β -estradiol (E2) treatment reduced the

number of dimethylhydrazine-induced tumor lesions in the colon by 71% [47]. These findings propose a potential *in vivo* protective role for estradiol in females, at least with regard to the development of the primary tumor.

Unlike some of the aforementioned studies, which addressed the development of primary tumors, the current study examined an *in vivo* metastatic process in the context of surgical procedures, and showed a remarkable relative resistance in female mice to the CT26 carcinoma. In an effort to start identifying the mechanism(s) underlying this difference, we examined the influence of estradiol, testosterone and progesterone under a wide range of concentrations (10^{-11} - 10^{-6} M) on the *in vitro* tumor cell proliferation and viability. In contrast to our *in vivo* effects, we found that both estradiol and progesterone had a stimulatory (rather than the expected inhibitory) effect on CT26 cancer cells. These effects were evident only at the higher concentrations (between 10^{-6} and 10^{-9} M) and after 48 hours of incubation, and did not exceed a 25% increase. Testosterone had no significant effect on the tumor cells at any of the concentrations or time points studied. Thus, the direct effects of sex hormones on CT26 cell proliferation and viability do not seem to underlie the *in vivo* sexually-dimorphic metastatic capacity of CT26 cells.

In general, both the animal and human literature is inconsistent regarding such *in vitro* effects of estradiol on colon cancer cells, reporting both enhanced [48–51] and suppressed [51,52] proliferation. A plausible explanation for this inconsistency may stem from the different types of estrogen receptor (ER) expressed by different colon tumor lines. Specifically, those expressing ER-beta were shown to be inhibited by estradiol [50,52–54], whereas those expressing ER-alpha [48,49] were shown to have accelerated growth in response to estradiol, similar to the CT26 line used in the present study. As the MC-26 cell line, which expresses the stimulatory ER-alpha [55] receptor, was derived from the CT26 cell line used in the present study, it is reasonable to assume that the CT26 cells also express ER-alpha.

Finally, we and others have reported that male rats are markedly more susceptible to the immunosuppressive and tumor-promoting effects of stress hormones, specifically catecholamines and corticosterone [26,56]. Human studies have reported similar dimorphism with respect to other physiological effects of these hormones. As the context of CT26 inoculation in the current study involves surgical stress responses, these differences may also have played a role in the sexual dimorphism observed in the development of metastases in the present study.

This study has several limitations, including the lack of mechanistic understanding of the observed sex differences, which is a current focus of our ongoing studies. Additionally, although the “more severe” surgical approaches are characterized by the addition of laparotomy, a prolonged surgery and/or colder ambient conditions, we did not assess the actual surgery-related physiological perturbations in these animals to verify that greater impacts of these factors. The strengths of the study include the assessment of the clinically relevant index of actual metastatic lesions, and the various conditions in which sex differences and the lack of effect of the magnitude of surgical trauma were shown, including various inoculation approaches and tumor loads.

In summary, manipulating the extent of the surgical procedure while administrating CT26 cells did not significantly affect CRLM development, even when tested for various approaches and tumor loads. The male-female differences regarding CT26 metastatic development were robust and consistent across all conditions. These differences should be further investigated in the context of surgical stress, focusing on the potential mechanism(s) underlying these findings, including host immune resistance and the tumor cell capacity to extravasate, seed and develop new tumors.

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Figure 1. The effects of sex and the number of injected tumor cells on the liver weight and number of surface hepatic metastases

Female and male mice were intra-splenically injected with increasing doses of CT26 tumor cells. Both the liver weight (**a**) and number of surface hepatic metastases (**b**) significantly increased as a function of the tumor load. Males presented significantly higher liver weights and more metastases than females, as indicated by an asterisk (*) at each tumor load (**a** & **b**). No significant differences between laparotomy and small incision with regard to the metastatic development were evident (**c**). The data are presented as the means + SEM (**a–c**). A significant correlation between the liver weight and the number of liver metastases was evident (**d**). The scales used to show the liver weights start at 0.9 g, as this is the baseline weight in tumor-free animals of both sexes.

Figure 2. The effect of the tumor cell inoculation approach and sex on the liver weight and number of surface hepatic metastases

Male and female mice were injected either intra-splenically or via portal vein inoculation with 1×10^4 CT26 tumor cells. Both injection approaches yielded similar loads of metastases (**a & b**), with a marginally significantly greater metastatic load following portal vein inoculation. Males presented significantly higher liver weights (**a**) and developed more metastases than females (**b**) as indicated by an asterisk (*) for each injection approach (**a & b**). Data are presented as the means + SEM.

Figure 3. The effects of surgical trauma and sex on the liver weight and the number of surface hepatic metastases

Male and female mice were injected via portal vein inoculation with 2×10^4 CT26 tumor cells. Males presented significantly higher liver weights (a) and developed more metastases than females (b). The magnitude of the surgical trauma (surgical severity) had no effect on either the liver weights or the number of metastases, and no interactions were evident. The data are presented as the means + SEM.

Figure 4. The *in vitro* effects of sex hormones on CT26 proliferation

CT26 cells were incubated in complete medium (CM) with various concentrations of each of the major three sex hormones: testosterone (Testo), estradiol (Est.) or progesterone (Prog.), or with ethanol as a vehicle control or CM as another control. No significant *in vitro* effect of any of the sex hormones on CT26 proliferation or viability was evident after 24 hours. After 48 hours, estradiol and progesterone increased the cell proliferation in a dose-dependent manner, as indicated by an asterisk (*), compared to the vehicle levels. The data are presented as the means + SEM.