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Patient-derived Orthotopic Xenograft Models for Human Urothelial Cell Carcinoma and Colorectal Cancer Tumor Growth and Spontaneous Metastasis

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We are submitting a manuscript titled “Patient-derived orthotopic xenograft models that enable human high-grade urothelial cell carcinoma and colorectal cancer tumor implant, growth and spontaneous metastasis”. We would like to submit this manuscript to be considered for publication in *JoVE*. Thanks for your invitation for submission, and we hope our manuscript is in a good stand to be accepted. Looking forward to hearing back from you.

Sincerely,

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TITLE:

Patient-derived Orthotopic Xenograft Models for Human Urothelial Cell Carcinoma and Colorectal Cancer Tumor Growth and Spontaneous Metastasis

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KEYWORDS:

patient-derived orthotopic xenograft, high-grade urothelial cell carcinoma, colorectal cancer, lymph node stromal cells, metastasis, mouse model, tumor microenvironment

SUMMARY:

This protocol describes the generation of patient-derived orthotopic xenograft models by intra-vesically instilling high-grade urothelial cell carcinoma cells or intra-rectally injecting colorectal cancer cells into non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice for primary tumor growth and spontaneous metastases under the influence of lymph node stromal cells, which mimics the progression of human metastatic diseases.

ABSTRACT:

Cancer patients have poor prognoses when lymph node (LN) involvement is present in both high-grade urothelial cell carcinoma (HG-UCC) of the bladder and colorectal cancer (CRC). More than 50% of patients with muscle-invasive UCC, despite curative therapy for clinically-localized disease, will develop metastases and die within 5 years, and metastatic CRC is a leading cause of cancer-related deaths in the US. Xenograft models that consistently mimic UCC and CRC metastasis seen in patients are needed. This study aims to generate patient-derived orthotopic xenograft (PDOX) models of UCC and CRC for primary tumor growth and spontaneous metastases under the influence of LN stromal cells mimicking the progression of human metastatic diseases for drug screening. Fresh UCC and CRC tumors were obtained from consented patients undergoing resection for HG-UCC and colorectal adenocarcinoma, respectively. Co-inoculated with LN stromal cell (LNSC) analog HK cells, luciferase-tagged UCC cells were intra-vesically (IB) instilled into female non-obese diabetic/severe combined immunodeficiency (NOD/SCID) mice, and CRC cells were intra-rectally (IR) injected into male NOD/SCID mice. Tumor growth and metastasis were monitored weekly using bioluminescence imaging (BLI). Upon sacrifice, primary tumors and mouse organs were harvested, weighed, and formalin-fixed for Hematoxylin and Eosin and immunohistochemistry staining. In our unique PDOX models, xenograft tumors resemble patient pre-implantation tumors. In the presence of HK cells, both models have high tumor implantation rates measured by BLI and tumor weights, 83.3% for UCC and 96.9% for CRC, and high distant organ metastasis rates (33.3% detected liver or lung metastasis for UCC and 53.1% for CRC). In addition, both models have zero mortality from the procedure. We have established unique, reproducible PDOX models for human HG-UCC and CRC, which allow for tumor formation, growth, and metastasis studies. With these models, testing of novel therapeutic drugs can be performed efficiently and in a clinically-mimetic manner.

INTRODUCTION:

It has been shown that lymph node (LN) metastasis is a poor prognostic indicator in many solid organ malignancies, including high-grade urothelial cell carcinoma (UCC) of the bladder and colorectal cancer (CRC)[1,2]. Over half of the patients with muscle-invasive UCC (MIUCC), despite curative therapy for clinically-localized disease, will develop metastases and die within 5 years. Metastatic CRC is a leading cause of cancer-related death in the US.

An estimated 81,190 new patients and 17,240 cancer specific deaths are expected to occur in 2018 in the United States due to UCC of the bladder[3,4]. While patients will predominantly (70%) present with non-muscle invasive disease, 30% will have MIUCC[5]. Despite the curative therapy (radical cystectomy [RC] with or without systemic chemotherapy) for clinically localized disease, half of the patients with MIUCC of the bladder will still develop metastases and die within 5 years[3]. Lymph node involvement is found in approximately 20%−25% of patients having undergone RC[6-8]. Five-year survival rate in LN positive patients is less than 35% even after RC, suggesting LN involvement as a crucial negative predictor for the prognosis in UCC patients.

Colorectal cancer is the third most common cancer diagnosed in both men and women in the United States. The patient outcomes largely depend on tumor characteristics and tumor microenvironment, such as depth of invasion, LN involvement, and distant organ metastases. Although the mortality rate in CRC decreased in last decade due to screening and effective

surgeries, it is estimated that almost 50% of CRC patients will develop metastases or recurrent disease[9].

Small animal models provide an expeditious, reproducible, and modifiable platform to study tumor progression and different metastatic patterns. There are currently no described xenograft models that consistently mimic UCC and CRC metastasis seen in patients. The primary route of cancer distant metastasis is via lymphatic spread. New research suggests that the LNs, provide tumor with an unique microenvironment, are not only simply stationary targets where cancer cells transiently pass, but also plays an integral role by interacting with cancer cells in the metastatic process. Indeed, our studies discovered in addition to educate and promote tumor progression and metastases, the LN stromal microenvironment is also responsible for drug resistance in CRC[10,11]. Our lab recently confirmed the tumorigenic effects of LN stromal cells (LNSCs) on UCC and CRCs using patient-derived orthotopic xenograft (PDOX) mouse models[12,13].

Developing PDOX models provides an important platform for translational cancer research[14,15]. By maintaining principal histological and genetic characteristics of their donor tumor, PDOX models make ideal studies and remain stable across passages and make good platforms for translational cancer research[12,15]. PDOX models are being used for preclinical drug evaluation, biomarker identification, and preclinical evaluation of personalized medicine strategies allowing for prediction of clinical outcomes. Currently, there are no described xenograft models that consider the importance of LN involvement and are capable of consistently reproducing primary tumor and distant organ metastasis in UCC and CRC. In this study, we describe the development of PDOX models in NOD/SCID mice with reproduction of metastatic UCC and CRC diseases with LNSC involvement.

PROTOCOL:

All methods described in these animal studies were conducted under the approved guidelines of the animal care and use committee of Ochsner Health System and in accordance with animal research guidelines. All patient tumors for this study were collected from consented patients undergoing cancer resection surgeries in accordance with the Ochsner Health System Investigative Review Board and the ethical standards of the Institutional Committee on Human Experimentation. Board-certified pathologists at Ochsner Health System determined the pathological diagnoses of patient specimens based on the microscopic features of tumor cells, their histological type, and grade level.

NOTE: The following protocol describes the steps for two separate xenograft models, a UCC model via the electrocauterization of the bladder wall to instill UCC cells and an intrarectal injection of CRC cells for study in a CRC model. All steps preparing for and monitoring the experiments are identical for both models, while sections 7 and 8 specifically describe the procedure for UCC instillation and CRC injection, respectively.

1. Culturing cell lines

1.1 Grow HK cells in complete RPMI-1640 medium supplemented with 10% fetal bovine serum, 2 nM glutamine, 100 U/mL penicillin G, and 100 mg/mL streptomycin at 37 °C in a 5% CO_2 humidified incubator.

NOTE: HK cells are normal human follicular dendritic cells and can be grown and expanded for ≤ 15 passages in vitro[16].

1.2 To prepare for an experiment, trypsinize the cells.

1.2.1 Remove media and add 2 mL of 1% trypsin in Hank's balanced salt solution (HBSS) to cells. Place cells back in the 5% CO_2 humidified incubator at 37 °C for 4 min.

1.2.2 Collect cells from dish into a 15 mL tube using a handheld pipet aid with a 10 mL serological pipet attached. Add 8 mL of complete RPMI-1640 medium.

1.2.3 Combine 40 µL of cells and 40 µL of trypan blue in a single well of a 96 well plate. Add 10 µL of mixture to a hemocytometer and count live cells. Add 1 million cells in 25 mL complete RPMI-1640 medium to a 150 mm sterile tissue culture-treated dish.

NOTE: HK cell suspension prepared in this step must be used within an hour to mix with tumor cells for injection.

2. Patient specimen collection

2.1 Collect the UCC tumors from consented patient 15 (BlCaPt15, pT3b N1 M0) and 37 (BlCaPt37, pT3b pN0 M0) at resection surgery.

2.2 Collect CRC tumors from consented patient 155 (CoCaPt155, T1 N0 M0) and 302 (CoCaPt302, T1 N0 M0) at resection surgery.

3. Expansion of patient tumor

3.1 Collect tumors at surgery in cold sterile McCoy's medium containing penicillin G (500 U/mL) and streptomycin (500 mg/mL).

3.2 Implant tumors directly into the left and right flank of 6−8 week old female NOD/SCID mice.

3.2.1 Mechanically mince tissues into small pieces (~1 mm^3) using small surgical scissors.

3.2.2 Implant tissue subcutaneously to the left and right flank using 13 G bone marrow aspiration biopsy needles.

NOTE: Implant a total volume of 8 mm^3 divided evenly to both sides of flank.

4. Tagging and enrichment of luciferase labeled tumors

4.1 Measure tumor growth bi-weekly using a digital caliper.

4.2 At 1 cm in diameter, transduce tumor. Directly inject into tumor with single dose of Luc/red fluorescent protein (RFP)-lentivirus (50 µL/tumor, 1:30 dilution from concentrated high titer lentivirus stock) using a 1 cc syringe with a 27 G needle.

NOTE: The patient tumor typically reaches 1 cm in diameter in 1−2 months. However, the growth rate is extremely variable and based on a number of factors including tumor grade and type.

4.3 Monitor tumor weekly by bioluminescent imaging (BLI) in live animals.

4.3.1 Weigh the mice. Inject conscious mouse with 150 mg/kg luciferin intraperitoneally and wait 5 min for the substrate to circulate in the mouse's body.

4.3.2 Anesthetize the mouse with 2.5% isoflurane in 100% oxygen, 1 L/min in an induction chamber.

4.3.3 Place mouse on stomach in a BLI imaging machine with isoflurane flowing and image. Take sequential images to confirm the presence of Luc/RFP positive tumor regions (false-color bio-luminescent image). Return mouse to cage after imaging is complete.

5. Select appropriate portion of tumor for enzymatic digestion

5.1 On the day of UCC or CRC procedure, image mouse with luciferase tagged tumor as in steps 4.3.1−4.3.3.

NOTE: The length of time for the subcutaneous tumor to grow depends on the speed of tumor growth and the planned number of animals to be injected in the experiment.

5.2 Harvest tumor from mouse flank and image.

5.2.1 Euthanize mouse by CO_2 inhalation after imaging. Place mouse in the CO_2 chamber, turn on gas at 1.4 L/min until respiratory arrest and leave on for 3 min. Follow this with cervical dislocation.

5.2.2 Clean skin with 70% ethanol. Tent skin directly above tumor. With surgical scissors make a small incision in skin. Separate skin from tumor with scissors.

5.2.3 Remove tumor and place in a sterile petri-dish. Image entire dish in an imaging machine.

5.3 Use sterile scissors or scalpel to separate luciferase-negative sections from luciferase positive sections in the tumor and re-image.

5.4 Repeat until only most highly positive tumor pieces remain.

6. Enzymatic digestion of tumor

6.1 Under laminar flow hood, mince luciferase positive tumor pieces (step 5.4) into the smallest possible pieces using sterile surgical scissors and put them into a sterile 50 mL conical tube.

NOTE: Mincing the tumor into the smallest possible pieces will yield more individual cells.

6.2 Prepare digest solution by adding 10 mL of collagenase IV (1.5 mg/mL), 80 µL of hyaluronidase (20 mg/mL), and 160 µL of deoxyribonuclease I (0.1 mg/mL) to 40 mL of HBSS. Mix solution by inverting.

6.3 Add 35−40 mL of the digest solution to minced tumor. Incubate at 37 °C with continuous rotation for 2 h.

NOTE: Vigorously shake tube periodically throughout incubation to prevent tumor tissue from clumping.

6.4 Filter entire digestion through sterile 100 µm cell strainer twice followed by a 40 µm cell strainer to remove debris. Save the flow through and discard debris.

6.5 Wash free cells by adding 20 mL of HBSS and centrifuge at 329 x *g* for 5 min. Discard supernatant and resuspend pellet in 30 mL of HBSS.

6.6 Combine 10 µL of cell solution and 90 µL of trypan blue in a single well of a 96 well plate. Count live cells using a hemacytometer.

6.7 Transfer 1 x 10^4 to 1 x 10^6 tumor cells per mouse to a sterile 15 mL conical tube. Add 3 x 10^5 HK cells from step 1.2.3 per mouse, to the same tube with tumor cells.

NOTE: Use sterile 50 mL conical tube if the total volume exceeds 15 mL. Always calculate more doses for additional animals per study group to account for loss of fluid during syringe use. For example, if a group contains 5 mice, make enough cells for 6 or 7 mice.

6.8 Centrifuge at 329 x *g* for 5 min. Discard supernatant either by decanting or pipetting.

6.9 Resuspend cells in 50 µL per mouse for UCC model or 10 µL per mouse for CRC model in complete RPMI media. Keep cell suspension on ice until ready for use.

7. UCC mouse model

7.1 Preparation of mice for procedure

7.1.1 Obtain six- to eight-week old female NOD/SCID mouse. Shave the lower backs of the mouse using hair removal cream. Anesthetize the mouse in an induction chamber with isoflurane (2.5% in 100% oxygen, 1 L/min).

7.1.2 Once sedated, place mouse in supine position with its snout in an isoflurane nose cone and bare back firmly grounded on a dispersive electrode.

NOTE: Mouse is completely sedated if unresponsive to toe pinch.

7.2 Instill UCC cells prepared in step 6.9 to bladder using an angiocatheter (**Figure 1Aa,Ab**).

7.2.1 Set up a monopolar electrocautery machine and set to a power of 4 W. Lubricate a 24 G angiocatheter with lubricating jelly and insert through urethra of the female mouse.

NOTE: Slight resistance may be felt. Gently push forward or remove angiocatheter and repeat. Do not force.

7.2.2 Insert a 0.025” fixed core straight guide wire 1 mm past the end of the angiocatheter.

NOTE: The wire is marked with tape prior to the procedure to indicate the 1 mm stopping point and insure consistency.

7.2.3 Hold the monopolar pin to the guide wire for 1 s allowing for electrical irritation of the bladder mucosa.

7.2.4 Attach a fresh sterile angiocatheter to 1 cc luer-lok syringe and draw up 200 µL of collected cells from step 6.9.

NOTE: At least 100 µL is lost from the angiocatheter to the syringe. Compensate for loss volume when calculating volume of cell suspension needed.

7.2.5 Remove guide wire and angiocatheter from mouse urethra. Insert angiocatheter with syringe of cells attached into urethra.

NOTE: Advancement should be easier than before.

7.2.6 Instill 50 µL of cells to mouse bladder. Wait a few seconds before removing the angiocatheter to allow for cells to adhere to the bladder wall.

NOTE: Cells remain in the bladder and develop into a primary tumor.

7.3 Remove mouse from isoflurane nose cone and grounding pad. Observe mouse for 1 h following procedure. Look for signs of distress, i.e., hunched back, labored breathing, etc.

8. CRC mouse model

8.1 Anesthetize six-to-eight-week old male NOD/SCID mouse with isoflurane (2.5% in 100% oxygen, 1 L/min) in the induction chamber. Confirm sedation with a toe pinch.

8.2 Place anesthetized mouse in supine position under a dissecting microscope, making sure to secure their snout to an isoflurane nosecone and to secure their front and back limbs with tape for stability.

NOTE: Loupes may be used instead of a dissecting microscope. A small object can be used to improve visibility and angle when placed under the base of the tail, elevating the anus. Typically, small sections of gauze are rolled into a cylinder shape of 1-inch diameter.

8.3 Dilate the anal canal with curved lubricated blunt-tipped forceps to expose the distal anal and rectal mucosa. Remove feces.

8.4 Use a sterile 30 G removable needle on a 50 µL glass syringe to inject 10 µL of tumor and HK cells (from step 6.9) into the distal posterior rectal submucosa 1 to 2 mm above the anal canal. The bevel of the needle should be covered by mucosa. Be careful not to pass into the pelvic cavity.

8.5 Remove mouse from isoflurane nose cone. Observe mouse for 1 h following procedure. Look for signs of distress, i.e., hunched back, labored breathing, etc.

9. Bioluminescent imaging

9.1 Monitor the primary tumor, liver, and lung metastatic burden weekly using a bioluminescent imaging system for luciferase activity.

9.1.1 Obtain a mouse from UCC or CRC experiment and weigh. Inject 150 mg/kg luciferin intraperitoneally and wait 5 min for the substrate to circulate in the mouse's body.

9.1.2 Anesthetize mouse with 2.5% isoflurane in 100% oxygen, 1 L/min in the induction chamber.

9.1.3 Place mouse in BLI Imaging machine with nose fixed in nosecone. When exposing for the image, make sure that the area of interest is facing the camera. For UCC and CRC injection, the ventral side should face the camera for each image. Image mouse in supine position.

10. Harvesting organs and tumor

10.1 When the primary tumor luminescence radiance reaches 1 x 10^{11} photons or if mice exhibit signs of distress (i.e., weight loss, hunched back, harsh/labored breathing, etc.), euthanize mice by CO_2 inhalation (as in step 5.2.1) after luciferin injection and whole body imaging.

10.2 Remove liver and lung, place in a petri dish and image to identify any metastases. Remove tumor, weigh and image. Fix organs and tumor in 10% neutral buffered formalin for 48 h at ambient temperature.

NOTE: Clean/wipe scissors and forceps between each organ to avoid transfer of tissue.

11. Histological evaluation

11.1 Embed formalin fixed tissues in paraffin and slice the tissues at 5 µm thickness on a microtome for hematoxylin and eosin (H&E) and immunohistochemical (IHC) staining.

NOTE: All H&E staining of paraffin slides was done in the pathology laboratory of Ochsner Health System, and all IHC staining used in this paper was performed in research laboratory of Ochsner health System after high-temperature antigen retrieval using Ki67 and cytokeratin 20 antibodies, followed by biotinylated secondary antibody, and avidin-biotin-peroxidase complexes according to manufacturer instructions[13,17].

REPRESENTATIVE RESULTS:

In the UCC PDOX model, UCC patients' BlCaPt15 or BlCaPt37 cells were intra-vesically (IB) instilled in the presence of HK cells into female NOD/SCID mouse bladder (**Figure 1A**). Twenty-five out of thirty (83.3%) animals generated primary tumors and displayed time dependent primary tumor growth based on weekly BLI (**Figure 1B,C** and **Table 1**). Similarly, in the CRC PDOX model, 31 out of 32 (96.9%) mice grew primary tumor when intra-rectally (IR) injected with patients' CoCaPt155 or CoCaPt302 cells plus HK cells (**Figure 1D-F** and **Table 1**). Depending on the patient tumor, mouse tumor growth had a different latency period, which reflects the difference in the patient's clinical characteristics (**Figure 1C,F**).

In both IB and IR models, tumor cell injection not only generated orthotopic primary tumors (**Figure 2A,B**, blue arrows), but many mice tested also developed liver and/or lung metastases. In 10 out of 30 (33.3%) and 17 out of 32 (53.1%) mice instilled with UCC cells and CRC cells with HK cells, respectively, we detected distant organ metastasis via ex vivo BLI (**Figure 2A**,**B** and **Table 1**).

To confirm similar tissue morphology, H&E and IHC staining were performed comparing xenografts and primary patient tumors. Histopathology of patient bladder carcinoma was maintained in xenografts from BlCaPt15 and BlCaPt37 (**Figure 3A**). Results show xenograft tumor corresponding to the muscle invasive growth pattern of the patients' primary tumors. The antibody specific to human cell proliferation marker Ki67 was used in IHC. Ki67 positive nuclear staining indicates highly proliferative, fast-growing human tumor cells. The staining results from xenografts were similar to those of the original surgical biopsies. Similarly, in the IR model, H&E

staining indicates the similarity of architecture between xenografts and patient tumors of both CoCaPt155 and CoCaPt302. IHC using antibody against cytokeratin 20 also showed similar tumor growth pattern in both PDOX models (**Figure 3B**). Thus, our PDOX model recapitulated UCC and CRC patient clinical progression.

FIGURE AND TABLE LEGENDS:

Figure 1: Orthotopic UCC and CRC mouse models. (**A-C**) Intra-vesicle (IB) instillation of UCC cells into mouse bladder[13]. (**Aa**) An angiocatheter was inserted into the bladder of a female NOD/SCID mouse and an electrocautery shock was applied to the bladder wall via a guide wire. (**Ab**) Luciferase tagged UCC tumor cells, BlCaPt15 (2 x 10^4 cells), or BlCaPt37 (5 x 10^5 cells) with the addition of 3 x 10^5 LN stromal HK cells, were instilled into the NOD/SCID mouse bladder through the angiocatheter. (**D-F**) Intra-rectal (IR) injection of CRC cells into the submucosal tissue layer of mouse rectum[17]. (**D**) The anal canal was dilated with lubricated blunt-tipped forceps to allow access to the distal anal and rectal mucosa and a 30 G needle was inserted into the distal posterior rectal submucosa 1−2 mm above the anal canal until the bevel was covered before the injection takes place. Luciferase tagged CRC tumor cells, CoCaPt155 (5 x 10^5 cells), or CoCaPt302 (1 x 10^4 cells) with the addition of 3 x 10^5 HK cells were injected. Tumor burden was monitored and quantified via bioluminescent imaging (BLI; **B** and **E**). Tumor growth of luciferase tagged UCC or CRC cells was monitored kinetically via BLI and analyzed using image analysis software (**C** and **F**).

Figure 2: PDOX models produce spontaneous distant organ metastases. Representative mice (top panels) from the same experiments as in **Figure 1**, e.g., instilled intra-vesically with luciferase tagged UCC tumor cells, BlCaPt15 or BlCaPt37 cells with HK cells (**A**) or intra-rectally with luciferase tagged CRC tumor cells, CoCaPt155 or CoCaPt302 cells with HK cells (**B**) are shown. Yellow arrows indicate mouse bladder (**A**). Photos taken at the time of sacrifice indicate orthotopic tumor formation (blue arrows). Liver, lung, and tumor (middle panels) collected at necropsy and their ex vivo BLI (bottom panels) showed mouse liver and lung metastasis as well as tumor with luciferase activity.

Figure 3: **Xenograft tumors resemble patient pre-implantation tumors.** Paraffin embedded tumor tissue from patient tumor or tumors collected from mice in the same experiments as in **Figure 1** were sectioned and stained by H&E (**A** and **B**) or IHC with antibodies against human Ki67 (**A**) or cytokeratin 20 (CK20; **B**). The brown color indicates positive staining. H&E staining shows tumor nests dissecting into smooth muscle bundles (**A**). Photographs were taken using a digital deconvoluting microscope and analyzed with an image analysis software. Scale bars: 100 µm. All images were taken in original magnification of 200×.

Table 1: Summary of tumor formation, metastasis, and mortality in IB and IR models.

DISCUSSION:

Metastatic disease is responsible for most cancer patient mortalities. In pre-clinical therapeutic tests, it is crucial to establish mouse models that most closely emulate human tumor growth with spontaneous distant organ metastases. Using murine models with implanted patient tumor

derived cancer cells (xenografts) allows for a better understanding of tumor biology and predictive biomarkers as well as testing and prediction of antineoplastic effects of novel therapies[18]. Many models have been used to show UCC and CRC metastases in murine experiments, such as intravenous tail vein injections showing the ability to produce lung disease[19] or subcutaneous implantation of tumor cells or tumor fragments into the flank for localized tumor growth[20,21]. One laboratory previously reported a bladder cancer murine model by using hydrochloric acid treatments to successfully promote tumor uptake[22]. While these methods produce reliable local growth and may demonstrate some metastatic activities, they do not specifically resemble the natural course of cancer developed in humans and do not utilize the metastatic mechanism seen in patients[18,23]. Other murine models were reported to mimic tumor growth by injecting tumor cells directly into organs such as the liver or mesentery, but they carried risks of tumor cell leakage and did not produce significant metastases.

We have previously demonstrated the correlation between cancer cell content in the primary tumor and LN involvement[24] and the role of the cancer cell/LN stromal interaction in the course of primary tumor progression to metastatic disease[10,12,17]. Incorporating our previous work on the influence of the LN stromal microenvironment in metastatic progression, we established orthotopic models (especially the PDOX models) that mimic the natural course of metastatic dissemination, technically reproducible, preserve the heterogeneity of original patient tumors, and generate consistent primary tumor and metastatic results[12,13,17]. Using the tumor-enhancing effects of the LN stromal microenvironment is important because it provides the similar tumor microenvironment in human UCC and CRC, develops all steps in the metastatic cascade, reduced cancer cell number required in mouse model which minimizes number of xenograft passages, and results in reliable and most closest model to tumor growth and metastases in human.

We have established a unique method of IB electro-stimulation using the co-instillation of HK cells that produces a reliable model for developing MIUCC. Our model mimics the natural course of UCC progression by tumor implantation beginning in the mucosa, leading into the muscle, then metastasizing to lungs[13].

Our results also show that the IR model is safe, reproducible, and successful. The orthotopic CRC mouse model features primary tumor growth and spontaneous distant metastasis[12,17]. The IR procedure is quick, easy to learn, technically easy to perform, and not too stressful on the animals. The IB and IR groups had zero mortality (**Table 1**) in the postoperative period before final BLI measurement. However, the technique requires practice. If the intrarectal injection is successful, there should be a visible “bubble” that forms as the fluid is introduced into the rectal submucosa and will result in primary tumor growth that will eventually become palpable as shown in **Figure 1**. If the tumor has been injected too deep into the pelvic cavity, it will be unattached to the colorectal tract and grows very large to fill the pelvis, sometimes causing obstruction. If the injection is too shallow or does not enter the rectal submucosal layer at all, it will leak out resulting in reduced or absent primary tumor burden.

We have established unique, reproducible PDOX models for human HG-UCC and CRC. These models allow for tumor formation and metastasis studies. We can now use these models as the

primary method to continue to study the LN stromal microenvironment and its interaction with patient primary tumors. These models will also allow us to investigate therapies that interfere with the pro-tumorigenic effects of the LNSC on primary tumor. With these models, testing of novel therapeutic drugs can be performed efficiently and in clinically-mimetic manners.

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DISCLOSURES:

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Figure 1

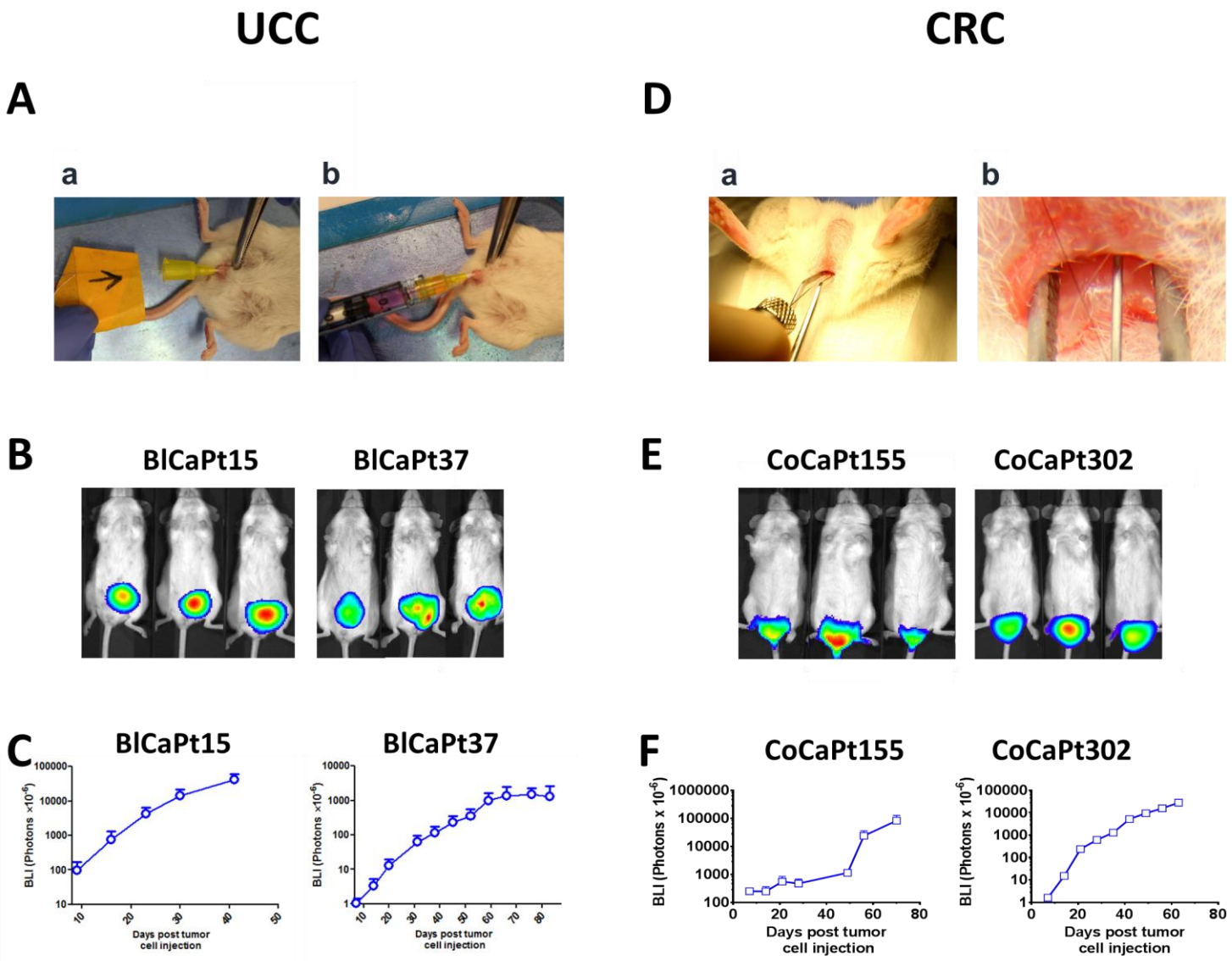
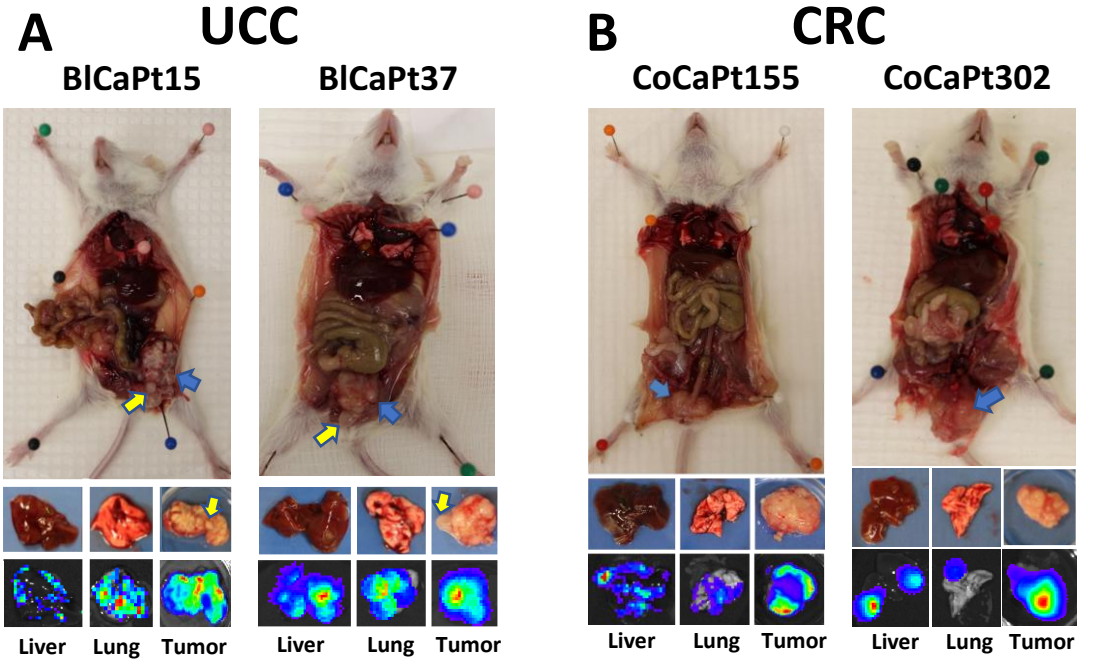


Figure 2


A
UCC
BlCaPt15
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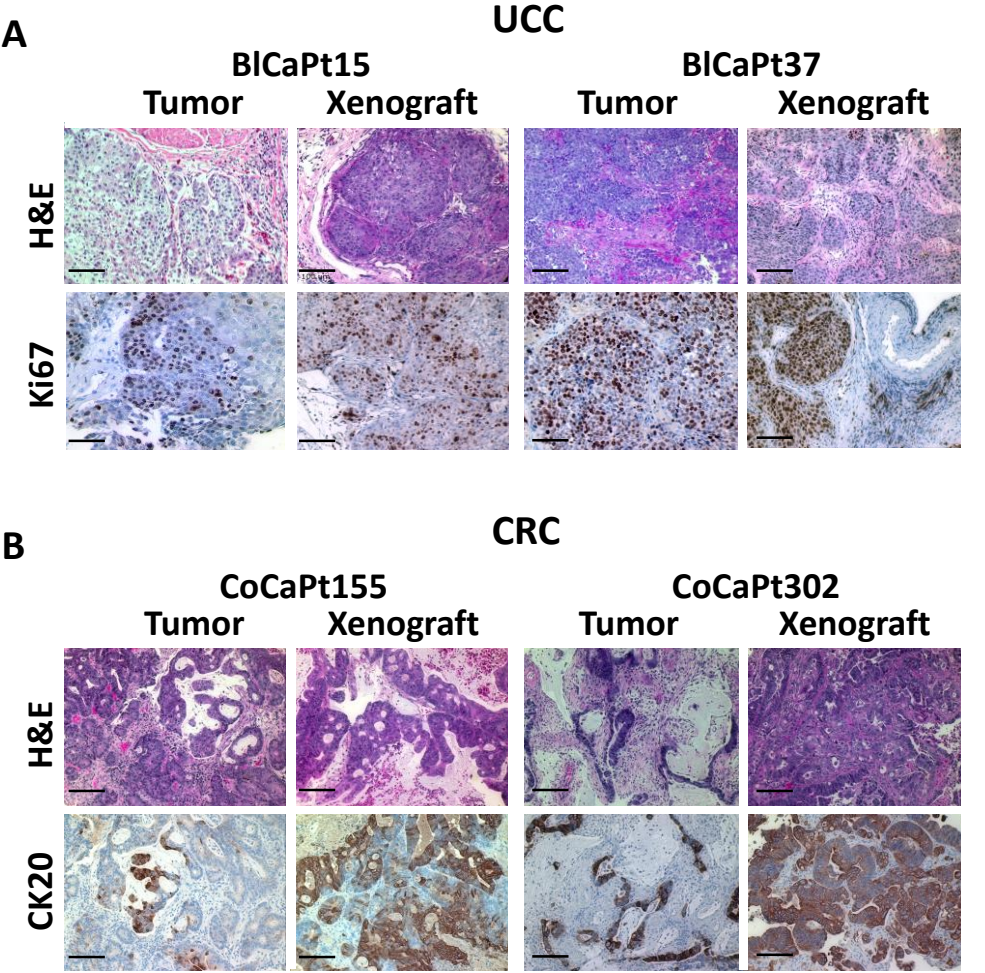
Lung
Tumor

Figure 3


A
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B
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Xenograft
CoCaPt302
Tumor
Xenograft
H&E
CK20

Table 1. Summary of tumor formation, metastasis, and mortality in IB and IR models.

	Tumor implant (%)	Lung/liver metastasis (%)	Mortality (%)
UCC, n=30	83.3	33.3	0
CRC, n=32	96.9	53.1	0

Chemicals/Solutions/Media	**Company**
Avidin-biotin-peroxidase	Vector Labs Inc
Biotinylated secondary antibody	Vector Labs Inc
Collagenase IV (1.5 mg/mL)	Worthington Biochemical Corporation
Deoxyribonuclease I (0.1 mg/mL)	Sigma
D-Luciferin (150 mg/kg)	Perkin Elmer
Formalin (10% neutral buffered)	Leica
glutamine (2 nM)	Fisher Scientific
Hair Removal Cream	Church & Dwight Co., Inc
Hanks Balanced Salt Solution (HBSS)	Fisher Scientific
Hyaluronidase (20 mg/mL)	Sigma
Isoflurane	Henry Schein Animal Health
Luc/RFP-lentivirus	From our collaborators. See reference 13: Gills, J. et al. A patient-derived orthotopic xenograft model enabling human high-grade urothelial cell carcinoma of the bladder tumor implantation, growth, angiogenesis, and metastasis. Oncotarget. 9, 32718-32729, doi:10.18632/oncotarget.26024 (2018)
McCoy's medium	Life Technologies
penicillin/streptomycin 100 mL (100 U/mL)	Fisher Scientific
RPMI-1640 Medium	American Type Culture Collection
Trypan Blue	Sigma
Trypsin/EDTA	Life Technologies
Gas	
100% Oxygen	Airgas Inc
100% CO_2	Airgas Inc
Mice	
6-8 week old NOD/SCID Mice (male)	Jackson Lab
6-8 week old NOD/SCID Mice (female)	Jackson Lab
Immunohistochemistry	
Hematoxylin	Sigma
Ki-67 Rabbit Monoclonal Antibody	Thermo Scientific
Tools	
40 µm cell strainer	Fisher Scientific
100 µm cell strainer	Fisher Scientific
15 mL Conical Tube	Sarstedt
50 mL Conical tube	Sarstedt
150 mm Tissue Culture Dish	USA Scientific Inc
96 Well plate	USA Scientific Inc
Forceps	Symmetry Surgical Inc
Surgical scissors	Symmetry Surgical Inc
Equipment	

5% CO_2 humidified incubator	Thermo Scientific
Bioluminescent (BLI) Imaging Machine	Perkin Elmer
BLI Imaging Machine Software	Perkin Elmer
Centrifuge	Beckman
Deconvoluting Microscope	Intelligent Imaging Innovations
Deconvoluting Microscope Imaging Software	Intelligent Imaging Innovations
Digital caliper	Fowler Tools and Instruments
Dissecting microscope	Precision Instruments LLC
Electrosurgical generator	ValleyLab
Isoflurane Induction Chamber	Perkin Elmer
Microtome	American Optical Corporation
Pipet Aid	Fisher Healthcare
Serological pipet (10 mL)	Sarstedt

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RM-9106-S
08-771-1
08-771-19
11799
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Author(s): Ravan Moret, Linh Hellmers1, Xin Zhang, Jessie Gills, Nathan Hite, Aaron Klinger, Grace A Maresh, Daniel Canter, Stephen Bardot, David A Margolin, and Li Li

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Re: manuscript, JoVE59223 "Patient-derived orthotopic xenograft models enabling human urothelial cell carcinoma and colorectal cancer implant, growth, and spontaneous metastasis"

Editorial Comments:

Changes to be made by the author(s) regarding the manuscript:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Answer: We proofread and made changes were needed.

2. Please revise lines 67-75, 93-99, 253-255, 258-259, 300-302, 322-324 to avoid previously published text.

Answer We revised above listed sentences as instructed.

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Answer: We replaced all published figures with new data.

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Answer: We uploaded each Figure individually as a tiff file as instructed.

5. Figure 3: Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate figure Legend.

Answer: We included a scale bar in images as instructed and added the scale in the corresponding Figure Legend.

6. Please revise the title to be more concise and reflect the content of the protocol.

Answer: We revises the title as instructed.

7. Please provide an email address for each author.

Answer: Ravan Moret (rmoret@ochsner.org), Linh Hellmers (linh.hellmers@ochsner.org), Xin Zhang (xzhang@ochsner.org), Jessie Gills (jgills@ochsner.org), Nathan Hite (nathan.hite@ochsner.org), Aaron Klinger (aaron.klinger@ochsner.org), Grace A Maresh (grace.maresh@ochsner.org), Daniel Canter (daniel.canter@ochsner.org), Stephen Bardot (sbardot@ochsner.org), David A Margolin (damargolin@ochsner.org), Li Li (lli@ochsner.org)

8. Please remove the sub-headings within the Long Abstract.

Answer: All sub-heading in the Long Abstract have been removed.

9. Please define all abbreviations before use.

Answer: Abbreviations have defined prior to use as instructed.

10. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

Answer: Centrifuge speeds are expressed as centrifugal force (x g).

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Answer: All commercial/trademark language has been removed as instructed.

12. Please revise the protocol to contain only action items that direct the reader to do something (e.g., "Do this," "Ensure that," etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as "could be," "should be," and "would be" throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a "Note." Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.

Answer: Protocol has been revised to be in imperative tense throughout all steps.

13. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

Answer: We added more detail and explanation to steps that appeared vague.

14. 2.2: Please specify the reaction conditions (temperature and time) for trypsinization. Please provide the composition of media containing serum.

Answer: Reaction conditions for trypsinization and media composition has been specified in the protocol as instructed.

15. 3.2.1: What is used to mince tissues?

Answer: Surgical Scissors

16. 3.2.2: Where is the tissue implanted? And what volume of tissue is implanted?

Answer: a total volume of 8mm^3 is evenly distributed between the left and right flank.

17. 4.1: How to determine the size of the xenograft?

Answer: Tumors are measured with a digital caliper.

18. 5.1: Please specify the age, gender and species of mouse used.

Answer: The mice used are 6-8 week old female, NOD/SCID mice, describer in step 3.2. This activity is a continuation on the same mouse.

19. 5.3: Please specify the concentration of isoflurane.

Answer: 2.5% isoflurane in 100% oxygen, 1L/min.

20. 5.6: Please describe how to harvest tumor from mouse flank and specify what is imaged and how.

Answer: Tent skin directly above tumor on mouse flank. Cut away skin using surgical scissors. Place tumor in petri dish and image entire dish in imaging machine.

21. 5.7: How to remove non-bright sections?

Answer: Non-bright sections are separated from bright section using sterile surgical scissors or scalpel.

22. 6.1: What is used to mince tumor?

Answer: Surgical Scissors.

23. 7.1.4: How to confirm that mice are sedated?

Answer: To confirm sedation a toe pinch is performed. If mouse is unresponsive to the pinch then it is determined to be completely sedated. (Note added to step)

24. 10.1: Please specify the euthanasia method.

Answer: Mice are euthanized via CO_2 inhalation.

25. 10.4.3: Please specify for how long and at what temperature are the organs and tumor fixed in formalin.

Answer: Tumor and organs are fixed in 10% neutral buffered saline for 48 hours at ambient temperature.

26. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

Answer: Some short steps combined as instructed.

27. Please include single-line spaces between all paragraphs, headings, steps, etc.

Answer: Single line spacing used throughout manuscript as instructed.

28. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

Answer: Essential steps were highlighted (less than 2.75) pages as instructed.

29. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.

Answer: Only complete sentences were highlighted. Highlighted steps describing anesthetization and euthanasia were removed as instructed.

30. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

Answer: All sub-steps and relevant details are highlighted as instructed.

31. Discussion: As we are a methods journal, please also discuss critical steps within the protocol, any modifications and troubleshooting of the technique, and any limitations of the technique.

Answer: We discussed these issues in lines 326-336 of the discussion section.

32. References: Please do not abbreviate journal titles.

Answer: Full names of all journals are listed as instructed.

33. Table of Equipment and Materials: Please sort the items in alphabetical order according to the Name of Materials.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
This manuscript describes methods to generate patient derived tumor xenografts in mice that retain their metastatic ability and are similar to the original tumor.
The methodology is generally clear except for the few points listed below. The model will be very useful for the deeper analysis of tumor metastasis. The authors have generated models with good survival of mice for up to 100 days.

Major Concerns:
None it is a well written manuscript. The manuscript details the development of metastatic orthotopic cancer models.

Minor Concerns:
Line 226, 10.4.2 It is stated "Weigh tumor and image separately.' I am not sure what this means. Are the authors referring to organs perhaps rather than images?

Answer: Step was re-written to be clearer.

Line 190, 7.6.1 It appears that the cells were instilled in the bladder for a few seconds. Do the tumor cells remain in the bladder after the catheter removal? Could the authors clarify this point or clarify the duration that the tumor cells remain in the bladder.

Answer: After installation the catheter remains in the bladder for several seconds allowing for cells to adhere to the bladder wall. Cells remain in the bladder and develop into a primary tumor.

Line 179, How certain are the authors that they are not accidently puncturing the bladder wall with the electro cautery wire?

Answer: The wire has been previously measured to determine the appropriate length. The wire is taped at 0.025" limiting its reach to only 1mm past the edge of the catheter. After removal mice are monitored for signs of bleeding to assure the bladder wall or urethra hasn't be punctured.

Line 120, The source of HK cells are not mentioned anywhere.

Answer: As stated in line 132: "HK cells are normal human follicular dendritic cells and can be grown and expanded for ≤ 15 passages *in vitro*." A reference of Kim et al Journal of Immunology 1995, 155 (3), 1101 was included for more details.

Table 1, The tumor implantation rate of 83% is good but it is lower than the tumor implantation rate via the rectal route. Could this be related to issues with the electro cautery technique or timing of cell instillation in the bladder?

Answer: Implantation of colon cancer tumor cells are using intra-rectal route, while bladder cancer cells are using intra-bladder instillation. No direct comparison was done with same patient tumor cells in both models. In addition, within one model, we have seen different implantation rates with different individual patient tumors. It is difficult to comment on the reason for the difference in tumor implantation rates.

Reviewer #2:

Manuscript Summary:
I reviewed the manuscript titled "Patient-derived orthotopic xenograft models enabling human urothelial cell carcinoma and colorectal cancer implant, growth, and spontaneous metastasis" by Moret and colleagues. The authors reported the development of patient-derived orthotopic xenograft (PDOX) models of urothelial cancer (UCC) and colorectal cancer (CRC). The authors isolated cancer cells from patients' and described technique using dissociated cancer cells from surgically excised patients' tumor samples injected into the bladder and rectum of NOD/SCID mice. Fluorescent/luminescent tumor cells were injected with HK cells (follicular NK cells). Tumor growth and metastasis were monitored by BLI and IR imaging. Tumor metastases were also reported. The authors compared the PDX tissues with the primary tumors. They showed H&E as well as ki67 immunostaining. The manuscript is clearly written. The rational and surgical technical approaches are clearly explained.

Major Concerns:
1. Viral transduction of dissociated cancer cells is not clearly described. The authors should indicate the technique of viral transduction, the source of viruses, multiplicity of infection, method of detection of the transduction efficiency and selection of transduced cells. These details should be described.

Answer: On line 159 it states: "Tumor is directly injected with single dose of Luc/RFP-lentivirus (50ul/tumor, 1:30 dilution from concentrated high tier lentivirus stock)." Revised section 4 and 5 added more details on monitoring and selection of transduced cells.

2. The source of HK cells was not specified. The ratio of the numbers of cancer cells to HK cells was not specified.

Answer: As stated in line 132: "HK cells are normal human follicular dendritic cells and can be grown and expanded for ≤ 15 passages *in vitro*." A reference of Kim et al Journal of Immunology 1995, 155 (3), 1101 was included for more details. 0.3 million HK cells were used for each mouse in both CRC and UCC models, various numbers of cancel cells were used based on disease stage and tumor cell aggressiveness.

3. The authors should show immunostaining of the HK cells (NK cell markers) and inflammatory and immune cell infiltration in the PDX and compare that with the profile of the primary tumors.

Answer: HK cells are a follicular dendritic cell line isolated from human tonsils. They are similar to lymph node stromal cells are reported in our previous publication (*FASEB Journal*. 2015, 29 (8), 3571). HK cells are not NK cells. We have data shown that HK cells will last up to 2 weeks post co-injection with tumor cells using GFP tagged HK cells and detected by live mouse GFP imaging (unpublished). Therefore, at the end point (at least 6 weeks post co-injection of cancer cells and HK cells, HK cells may not be detectable. In addition, NO/SCOD mice are immunodeficient mice that do not have immune cells such as T cells and B cells as well as with reduced NK cells. It will be difficult to compare PDX with patient primary tumor for immune cell infiltration.

4. Since the authors used traumatic cauterization of the bladder, they should show the extent of the inflammatory reactions instigated by the cauterization in the bladders of non-tumor bearing mice.

Answer: As shown in Figure 1, H&E staining of mouse bladders after electro-stimulation with or without tumor cell instillation. Inflammatory cell (small and dense) infiltration is observed between bladder lumen mucosa and tumor growth (left), however, inflammatory reaction is not as obvious in non-tumor bearing mice (right).

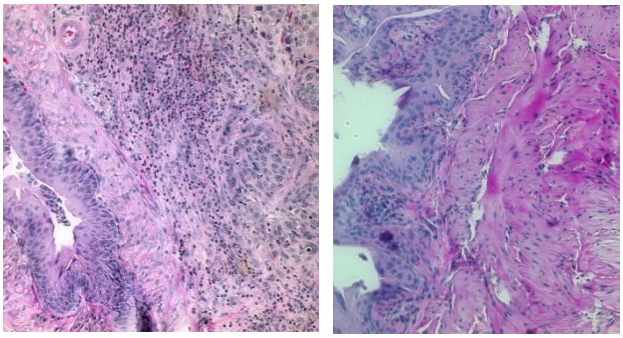

Figure 1. H&E staining of mouse bladder after electro-stimulation. Inflammatory cell (small and dense) infiltration is observed between bladder lumen mucosa and tumor growth (left), however, inflammatory reaction is not as obvious in non-tumor bearing mice (right).