**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 bladder3,4. While patients will predominantly (70%) present with non-muscle invasive disease, 30% will have MIUCC5. 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 years3. Lymph node involvement is found in approximately 20%−25% of patients having undergone RC6-8. 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 disease9.

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 CRC10,11. Our lab recently confirmed the tumorigenic effects of LN stromal cells (LNSCs) on UCC and CRCs using patient-derived orthotopic xenograft (PDOX) mouse models12,13.

Developing PDOX models provides an important platform for translational cancer research14,15. 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 research12,15. 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. 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% CO2 humidified incubator.

NOTE: HK cells are normal human follicular dendritic cells and can be grown and expanded for ≤ 15 passages in vitro16.

* 1. To prepare for an experiment, trypsinize the cells.
     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% CO2 humidified incubator at 37 °C for 4 min.
     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.
     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.

1. **Patient specimen collection**
   1. Collect the UCC tumors from consented patient 15 (BlCaPt15, pT3b N1 M0) and 37 (BlCaPt37, pT3b pN0 M0) at resection surgery.
   2. Collect CRC tumors from consented patient 155 (CoCaPt155, T1 N0 M0) and 302 (CoCaPt302, T1 N0 M0) at resection surgery.
2. **Expansion of patient tumor** 
   1. Collect tumors at surgery in cold sterile McCoy’s medium containing penicillin G (500 U/mL) and streptomycin (500 mg/mL).

* 1. Implant tumors directly into the left and right flank of 6−8 week old female NOD/SCID mice.
     1. Mechanically mince tissues into small pieces (~1 mm3) using small surgical scissors.
     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 mm3 divided evenly to both sides of flank.

1. **Tagging and enrichment of luciferase labeled tumors** 
   1. Measure tumor growth bi-weekly using a digital caliper.
   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.

* 1. Monitor tumor weekly by bioluminescent imaging (BLI) in live animals.
     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.
     2. Anesthetize the mouse with 2.5% isoflurane in 100% oxygen, 1 L/min in an induction chamber.
     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.

1. **Select appropriate portion of tumor for enzymatic digestion**
   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.

* 1. Harvest tumor from mouse flank and image.
     1. Euthanize mouse by CO2 inhalation after imaging. Place mouse in the CO2 chamber, turn on gas at 1.4 L/min until respiratory arrest and leave on for 3 min. Follow this with cervical dislocation.
     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.
     3. Remove tumor and place in a sterile petri-dish. Image entire dish in an imaging machine.
  2. Use sterile scissors or scalpel to separate luciferase-negative sections from luciferase positive sections in the tumor and re-image.
  3. Repeat until only most highly positive tumor pieces remain.

1. **Enzymatic digestion of tumor** 
   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.

* 1. 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.
  2. 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.

* 1. 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.

* 1. 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.
  2. 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.
  3. Transfer 1 x 104 to 1 x 106 tumor cells per mouse to a sterile 15 mL conical tube. Add 3 x 105 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.

1. **UCC mouse model**
   1. Preparation of mice for procedure
      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).

* + 1. 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.

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

* + 1. 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.

* + 1. Hold the monopolar pin to the guide wire for 1 s allowing for electrical irritation of the bladder mucosa.
    2. 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.

* + 1. Remove guide wire and angiocatheter from mouse urethra. Insert angiocatheter with syringe of cells attached into urethra.

NOTE: Advancement should be easier than before.

* + 1. 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.

* 1. 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.

1. **CRC mouse model**
   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.

* 1. 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.

* 1. Dilate the anal canal with curved lubricated blunt-tipped forceps to expose the distal anal and rectal mucosa. Remove feces.
  2. 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.
  3. 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.

1. **Bioluminescent imaging**
   1. Monitor the primary tumor, liver, and lung metastatic burden weekly using a bioluminescent imaging system for luciferase activity.
      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.
      2. Anesthetize mouse with 2.5% isoflurane in 100% oxygen, 1 L/min in the induction chamber.
      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.
2. **Harvesting organs and tumor**
   1. When the primary tumor luminescence radiance reaches 1 x 1011 photons or if mice exhibit signs of distress (i.e., weight loss, hunched back, harsh/labored breathing, etc.), euthanize mice by CO2 inhalation (as in step 5.2.1) after luciferin injection and whole body imaging.
   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.

1. **Histological evaluation**
   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 instructions13,17.

**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 bladder13. (**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 104 cells), or BlCaPt37 (5 x 105 cells) with the addition of 3 x 105 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 rectum17. (**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 105 cells), or CoCaPt302 (1 x 104 cells) with the addition of 3 x 105 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 embeddedtumor 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 therapies18. 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 disease19 or subcutaneous implantation of tumor cells or tumor fragments into the flank for localized tumor growth20,21. One laboratory previously reported a bladder cancer murine model by using hydrochloric acid treatments to successfully promote tumor uptake22. 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 patients18,23. 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 involvement24 and the role of the cancer cell/LN stromal interaction in the course of primary tumor progression to metastatic disease10,12,17. 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 results12,13,17. 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 lungs13.

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 metastasis12,17. 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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