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Orthotopic endometrial cancer model with retroperitoneal lymphadenopathy from in vivo propagated and cultured VX2 cells.

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TITLE:

An Orthotopic Endometrial Cancer Model with Retroperitoneal Lymphadenopathy Made From In Vivo Propagated and Cultured VX2 Cells

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KEYWORDS:

endometrial Cancer, orthotopic cancer model, VX2 cell line, lymphadenopathy, rabbit model, surgical model

SUMMARY:

This protocol presents a standardized method to grow VX2 cells in culture and to create an orthotopic VX2 model of endometrial cancer with retroperitoneal lymph node metastases in

rabbits. Orthotopic endometrial cancer models are important for the pre-clinical study of novel imaging modalities for the diagnosis of lymph node metastases.

ABSTRACT:

Endometrial cancer is the most common gynecologic malignancy in North America and the incidence is rising worldwide. Treatment consists of surgery with or without adjuvant therapy depending on lymph node involvement as determined by lymphadenectomy. Lymphadenectomy is a morbid procedure, which has not been shown to have a therapeutic benefit in many patients, and thus a new method to diagnose lymph node metastases is required. To test novel imaging agents, a reliable model of endometrial cancer with retroperitoneal lymph node metastases is needed. The VX2 endometrial cancer model has been described frequently in the literature; however, significant variation exists with respect to the method of model establishment. Furthermore, no studies have reported on the use of cultured VX2 cells to create this model as only cells propagated in vivo have been previously used. Herein, we present a standardized surgical method and post-operative monitoring method for the establishment of the VX2 endometrial cancer model and report on the first use of cultured VX2 cells to create this model.

INTRODUCTION:

Endometrial cancer, or cancer of the lining of the uterus, is the second most common gynecologic malignancy worldwide and the most common malignancy in developed nations[1]. The incidence of endometrial cancer has steadily increased, rising by 2.3% per year between 2005-2013 with a corresponding 2.2% increase in mortality[1–3]. The diagnosis of lymph node metastases is paramount as the presence of positive lymph nodes is a strong negative predictor of survival[4–7] and can guide the administration of adjuvant therapy[8–13]. Lymph node metastases are currently diagnosed by surgically removing the lymphatic tissue overlying the major blood vessels in the pelvis and abdomen. This procedure, known as a lymphadenectomy, is controversial due to conflicting survival data from two large trials[14–18] and the known risk of intra-operative[15,19,20] and post-operative morbidity[21–23]. As current non-invasive imaging modalities do not have the required sensitivity and specificity to replace lymph node dissection[24], there has been a push to develop new diagnostic imaging techniques. To test these novel techniques in a pre-clinical setting, a reliable model of endometrial cancer with retroperitoneal lymph node metastases is required.

The rabbit VX2 tumor model is a well-established model which has been used extensively to study multiple human solid organ tumors[25] including lung[26], head and neck[27,28], liver[29], kidney[30], bone[31,32], brain[33], pancreas[34] and uterus[35–37]. The VX2 model was originally developed in 1940 by Kidd and Rous[38] by successfully transplanting a cottontail rabbit papilloma virus discovered by Shope in 1933[39]. Since that time, the VX2 model has been maintained in vivo, requiring serial passage in the quadriceps muscle of White New Zealand rabbits[40]. More recently however, multiple groups have successfully grown VX2 cells in vitro[40–42] and demonstrated the retained tumorigenecity of the cultured cell line[31,42,43]. VX2 tumors are histologically defined as anaplastic squamous cell carcinomas[44] and contain glandular features which resemble adenocarcinoma[26]. Tumors are characterized by ease of implantation, rapid growth and hyper-vascularity[44,45] and reliably metastasize, most commonly to regional and distant lymph nodes[45]. Similarities in

uterine vascular and lymphatic anatomy[46] as well as the orthotopic growth site ensure that the metastatic pattern of rabbit VX2 carcinoma mimics that of human endometrial cancer, making the VX2 model a reliable model for studying human metastatic disease. Furthermore, histologic features such as abnormal microvascular proliferation[47] , as well as immunological[48] and genetic similarities[49,50] between humans and rabbits suggest that the tumor microenvironment may reflect that of human endometrial cancer.

Multiple groups have reported on the use of VX2 to create a model of endometrial cancer with retroperitoneal metastases with a high reported rate of success[36,51,52]; however, significant variation exists within the current literature with respect the method of model creation. Cell suspension doses as low as 4 x 10^5 cells/uterine horn[51] and as high as 5 x 10^9 cells/uterine horn[37,53] have been reported with no standard consensus on the required VX2 cellular dose. As well, a variety of inoculation methods have been reported including micro-surgical implantation of tumor into the uterine myometrium[36], injection of VX2 cell suspension[37,44,52,53] and in some cases, the addition of uterine horn suturing prior to innoculation[52]. Finally, no groups have reported the use of cultured VX2 cells to create this model. Thus, the purpose of this study is to demonstrate a successful standardized method of VX2 model creation and to report the first use of cultured VX2 cells to create a model of endometrial cancer with retroperitoneal metastases in a rabbit.

PROTOCOL:

All animal studies were conducted in Animal Resource Center (ARC) approved facilities of the University Health Network and in accordance with approved animal use and care protocols (AUP #3994/#4299). VX2 cell line was obtained from Dr. Aken's Lab at the University Health Network.

1. Creation of in vitro VX2 cell line

1.1. Harvest the VX2 tumor from rabbit quadricep muscle and growth in mouse flank.

1.1.1. Thaw frozen VX2 tumor blocks (1 cm x 1 cm), mince on a culture plate using a scalpel blade and strain through a 70 µm filter by adding small amounts of Hanks Balanced Salt Solution (HBSS) sequentially to ensure all cells are strained for a final volume of 1 mL.

1.1.2. Count cells and dilute with 0.9% phosphate buffered saline solution to a concentration of 1 x 10^7/mL and place on ice in a sterile tube.

NOTE: Tumor blocks were obtained from a lab collaborator from previous propagation of VX2 tumors in rabbit quadriceps muscles.

1.1.3. Anesthetize a female white New Zealand rabbit (weight 2.5-3.5 kg) with 5% inhaled isoflurane, remove the fur overlying the injection site and cleanse the injection site with povidone-iodine solution. Inject 500 µL of the prepared VX2 cell suspension (5 x 10^6 cells/mL) into the quadriceps muscle of the rabbit. Monitor the rabbit clinically for tumor growth starting on day 10.

1.1.4. Euthanize the rabbits after tumors reach 1-1.5 cm in diameter (measured externally with calipers) using an intravenous pentobarbital injection (100 mg/kg). Confirm euthanasia by checking vital signs including heart rate and respiratory rate. Excise the tumor using sterile instruments after cleaning the area with betadine solution.

1.1.5. In a biological safety cabinet using sterile instruments, mince the tumor in to small pieces (0.2 cm) on a 10 cm tissue culture dish using a scalpel blade. Pass the tumor fragments through a 70 µm cell strainer using 1 mL of HBSS as described in step 1.1.1. Count cells and dilute using 0.9% saline solution to obtain 7.5 x 10^5 cells in 200 µL.

1.1.6. Anesthetize male NOD scid gamma mice (weight 25 g) using 4% isoflurane at 1 L/min and maintain anesthesia using 2% isoflurane. Once mice are anesthetized, inject 200 µL of the solution from step 1.1.5 into the subcutaneous tissue of the mouse flank using a 27 gauge needle.

1.2. Harvesting and passaging of VX2 cells in vitro

1.2.1. Monitor xenograft growth clinically twice weekly until tumors reach 1 cm in diameter using calipers.

1.2.2. Euthanize mice by placing in a CO_2 chamber or performing cervical dislocation after anesthetizing with 4% isoflurane gas. Excise tumors under sterile conditions in a biological safety cabinet.

1.2.3. Mince tumors with a scalpel into 1-2 mm pieces in a 6 cm tissue culture dish containing 3 mL of Dulbecco's Modified Eagle Medium (DMEM)/Ham's F12+ 10% fetal bovine serum (FBS) media.

1.2.4. Leave tumor fragments undisturbed in a 37 °C incubator with 5% CO_2 for two days until cells begin to grow out from the tumor pieces. Subsequently, check cultured plates daily by light microscopy for cell confluency. Upon reaching 70% confluency, trypsinize cells by adding 1 mL of 0.05% trypsin to the flask and placing back in the 37 °C incubator for 5 min.

1.2.5. Neutralize trypsin with 6 mL of DMEM/Ham's F12 + 10% FBS medium, collect cells and centrifuge at 4 °C for 5 min at 300 x *g*. Remove supernatant and re-suspend pellet in 3.5 mL of new DMEM/Ham's F12 + 10% FBS media. Seed fresh 6 cm collagen I coated plates with 1.6 x 10^6 cells per plate to achieve approximately 50% cell seeding density.

NOTE: 50% cell density refers to the cells taking up 50% of the surface area of the plate when attached.

1.2.6. **Early passaging:** Repeat the above process (trypsinizing, seeding) until cells have been passaged 5 times and then assess cell line purity with PCR using a quantitative PCR assay to distinguish rabbit genomic DNA from mouse genomic DNA (see step 1.3.1-1.3.4).

NOTE: After 8 passages, cells can be propagated on non-collagen coated regular adherent tissue culture plates.

1.2.7. Passage, trypsinize and freeze aliquots of cells in DMEM/HAM F12+30% FBS+10%DMSO at a concentration of 2 x 10^6 per vial. Store cells in liquid nitrogen until required for experiments.

1.3. Confirmation of VX2 origin of surviving cells:

1.3.1. Create a standard cure from purified genomic mouse and rabbit DNA (Step 1.3.2 and Step 1.3.3). Use primers which target species-specific sequences within the second open reading frame of the LINE-1 retrotransposon element.

NOTE: Primer sequences for CRPV E6 are: 5'- GATCCTGGACCCAACCAGTGand 5'-CCTGCCGGTCCCTGATTTAT. The LINE-1 retrotransposon elements were used. Primer sequences for rabbit LINE-1 are: 5'-TCAGGAAACCCCAGAAAGTATGC and 5'-TTTGATTTCTTGAATGACCCAGTGT Primer sequences for mouse LINE-1 are: 5'-AATGGAAAGCCAACATTCACGTG and 5'-CCTTCCTTGACCAAGGTATCATTG

1.3.2. To create a standard curve for CRPV E6, purify VX2 tumor cell lysate (Step 1.1.1) using a commercial kit following manufacturer's protocol. Quantify this DNA using a commercial assay. Perform 6 serial dilutions from 225 pg/µL to 0.925 pg/µL in low EDTA-TE buffer. To create a standard curve for mouse genomic DNA, dilute 100 µg of commercial mouse genomic DNA in low EDTA-TE buffer in 5 dilutions from 125 pg/µL down to 0.0125 pg/µL.

1.3.3. Place 4 µL from each diluted solution from the samples from step 1.3.2 in wells and run samples in a PCR thermocycler. Use the Cq values from each run along with the DNA amounts per well to calculate a standard curve using the thermocycler software auto-threshold settings.

1.3.4. Use standard qPCR procedures to perform PCR with 40 cycles of 2-step cycling (98 °C and 60 °C) on a thermocycler. Establish threshold values, and set delta Ct values at >35 to ensure there is minimal mouse contamination in the rabbit VX2 cell line. The total volume of each reaction was 10 µL, consisting of 5 µL of 2x Mastermix, 1 µL of forward + reverse primers with final primer concentration in reaction at 250 nM, and 4 µL of DNA sample.

NOTE: Please see references[54,55] for details on performing PCR. Threshold values are established, and delta Ct values are set at >35 to ensure there is minimal mouse contamination in the rabbit VX2 cell line. The recommended level of VX2 DNA for reliable detection by qPCR is 1-10 pg.

2. VX2 cell culture and creation of cell suspension

2.1. Thaw vials containing VX2 cells (frozen in step 1.2.7) in a water bath at 37 °C for 1 minute and transfer cells to a conical centrifuge tube with 10 mL of culture medium (1:1 DMEM/F-12 + 10% FBS and 1% Penicillin-streptomycin). Centrifuge for 8 min at 107 x *g*, discard the supernatant

and re-suspend the cell pellet in 9 mL of media.

2.2. Transfer re-suspended cells to a large culture flask. Incubate cells without shaking at 37 °C. Check cells daily for confluence using light microscopy and change culture media every three days.

2.3. Creation of cultured VX2 cell suspension for injection

2.3.1. Trypsinize cells by adding 3 mL of 0.25% trypsin to flask once cells have reached 80% confluence. Place flask in incubator (37 °C) for 5 min. Transfer solution to a conical centrifuge tube and centrifuge for 8 min at 107 x *g* and then remove supernatant.

2.3.2. Wash the cell pellets 3 times with 9 mL of phosphate buffered saline, centrifuge and remove supernatant as above. Count the cells and dilute with 0.9% phosphate buffered saline solution to a concentration of 4 x 10^7 cells/mL and place in a sterile conical centrifuge tube on ice.

2.4. Creation of in vivo propagated VX2 cell suspension for injection

2.4.1. Thaw frozen VX2 tumor blocks (1 cm x 1 cm), mince on a culture plate using a scalpel blade and strain through a 70 µm filter as in step 1.1.1. Add small amounts of HBSS sequentially to ensure all cells are strained for a final volume of 1 mL. Count cells and dilute with 0.9% phosphate buffered saline to a concentration of 1 x10^7/mL and place on ice in a sterile tube.

NOTE: Tumor blocks were obtained from a lab collaborator from previous propagation of VX2 tumors in rabbit quadriceps muscles.

3. Surgical Model Establishment

3.1. Establishment of surgical anesthesia and pre-operative preparation

3.1.1. Pre-medicate female white New Zealand rabbits (2.5-3.5 kg) 1 hour prior to the planned surgical procedure using injections of acepromazine (1 mg/kg IM) and meloxicam (0.2 mg/kg SQ). Anesthetize animals using inhaled isoflurane (4%) and an anesthetic machine with a Bain circuit. Ensure anesthesia is adequate by checking there is no response to stimuli (brush eyelashes with fingers, put pressure on toe webbing).

3.1.2. Intubate anesthetized rabbits using a laryngeal mask airway (LMA) and secure in place with tape, maintaining deep anesthesia by titrating the inhaled isoflurane dose between 2.5-3%. Monitor surgical anesthesia regularly throughout the procedure by checking vital signs (respiratory rate, capillary refill, oxygen saturation if available) and monitoring for signs suggestive of pain (movement, withdrawal from stimuli, noises) or light anesthesia (movement, chewing on LMA tube).

NOTE: This should be done by someone with experience monitoring surgical anesthesia.

3.1.3. Place a 22-gauge ear-vein catheter in the marginal dorsal vein. Administer cefazolin (20 mg/kg) intravenously 10 min prior to surgical skin incision.

3.1.4. Place rabbits in a dorsal position on the operating table and clip hair over the pelvis and abdomen. Clean the surgical field using a 3-step surgical skin prep (betadine soap, chlorhexidine solution, betadine solution). Drape the surgical field with laparotomy drapes after land-marking the top of the pubic bone, leaving a 5 cm x 5 cm area of the lower abdomen exposed.

3.2. Creation of the laparotomy incision and identification of the uterine horns

3.2.1. Put on a surgical cap and face mask. Scrub hands using chlorhexidine or betadine surgical scrub solution. Using sterile technique, put on a sterile gown and sterile surgical gloves.

3.2.2. Using a # 11-blade scalpel, make a 2.5 cm long incision 1 cm cranial to the symphysis pubis through skin and subcutaneous tissue of the rabbit abdomen. Incise the rectus fascia and dissect the rectus muscles laterally to expose the underlying peritoneum. Enter the peritoneum sharply after ensuring the undersurface is clear of bowel or other abdominal organs.

3.2.3. Locate the uterine horns identifying the urinary bladder and sweeping a gloved finger superiorly, posteriorly and laterally over the apex of the bladder.

NOTE: If necessary, the full bladder can be emptied using digital pressure. Once located, bring the uterine horns through the abdominal incision to rest on the abdominal wall.

3.2.4. Using a 3-0 braided absorbable suture, perform a single suture ligation of each uterine horn approximately 1.5-2.0 cm distal to the cervices. Place the suture just medial to the uterine arteries, which run along the lateral aspect of each horn. Tie the sutures snugly to occlude the distal uterine horns (**Figure 1**).

3.3. Myometrial VX2 inoculation

3.3.1. Using a 27-gauge needle, inject 0.5 mL of the previously prepared VX2 cell suspension from either step 2.3.2 (for cultured VX2 model) or 2.4 (for in vivo propagated VX2 model) into the myometrium of each uterine horn proximal to the suture site (between the suture site and the cervix). Inject over 1 minute and ensure that cells are not being injected into the underlying uterine cavity.

3.3.2. Apply pressure to the myometrial injection site for 30 s after injection to minimize leakage of cells. Inspect the injection and suture sites for hemostasis and place the uterine horns back into the abdomen.

3.4. Closing the surgical incision

3.4.1. Deep abdominal wall closure: Identify the apex of the peritoneal incision and grasp the peritoneum, rectus muscle and fascia with a surgical clamp. Suture the abdominal wall en-bloc using a running 3-0 absorbable poly-filament suture.

3.4.1.1. To do this, anchor the suture at one apex of the incision by suturing superficial to deep on one side of the incision and deep to superficial on the other. Tie a knot. Using the attached suture, make running stitches perpendicular to the incision through the layers of the abdominal wall working step-wise along the incision from side to side. Tie the suture and cut.

3.4.2. Superficial abdominal wall closure: Identify the apex of the skin incision and suture the abdominal skin using buried running subcuticular 3-0 absorbable poly-filament suture. Apply surgical glue to the closed incision to oppose the skin edges.

3.4.2.1. To do this, anchor the suture at one apex of the incision, by suturing deep to superficial on one side of the incision and superficial to deep on the other. Tie a knot. Using the attached sutures, make running stitches in the dermal layer parallel to the incision working step-wise along the incision from side to side. Tie the suture and cut.

3.5. Post-operative care

3.5.1. Awakening from anesthesia: Turn off the isoflurane once the incision is closed and let the rabbit breath 21% oxygen while monitoring for signs of awakening (e.g., spontaneous movements, eye opening and chewing motions on the laryngeal mask airway). Extubate the rabbit once these signs are noted and provide 21% oxygen by mask and a warm blanket until the rabbits are alert and able to sit independently.

3.5.2. Post-operative monitoring: Monitor rabbits twice a day for 4 days and daily for an additional 10 days post-operatively. To monitor, assess their general condition, their food and water intake, urine and fecal output, pain assessment, weight, vital signs (heart rate, respiration rate) and their surgical site looking for swelling, erythema, discharge or dehiscence.

3.5.3. Post-operative pain control: Administer Meloxicam daily (0.2 mg/kg SQ) for 48 hours post-operatively and then as needed based on pain assessment. Administer buprenorphine every twelve hours (0.01-0.05 mg/kg SQ) for 24 hours and then as needed based on pain assessment.

3.5.4. Post-operative antibiotics and other treatments: Administer Enrofloxacin daily (5 mg/kg IM) for seven days post-operatively to prevent wound infection. Administer subcutaneous fluids (15 mL of SQ) twice a day as needed for dehydration and soft foods or other dietary supplements are provided daily as needed for weight loss.

4. Tumor Growth Monitoring

4.1. Clinical Monitoring: Monitor rabbits every 2 days for clinical signs of tumor growth

starting on post-operative day 14. Clinical signs of tumor growth can include decreased oral intake, lethargy, abdominal tenderness, palpable abdominal mass and loss of cecotrophy.

4.2. Imaging and invasive monitoring

4.2.1. CT imaging: On post-operative day 21-28, pre-medicate, anesthetize, and intubate rabbits as previously described in 3.1.1-3.1.2. Position rabbits in a dorsal position in a pre-clinical CT scanner and obtain images of the pelvis and abdomen after administering 10 mL of intravenous iohexol contrast agent.

4.2.2. Surgical monitoring: Transfer rabbits to the operating room after imaging while still under general anesthetic. Position, prep and drape rabbits as previously described in step 3.1.4 and perform a repeat laparotomy incision approximately 1.5 cm in length through the previous incision. Identify the and the uterine horns and carefully examine for tumor. Close the incision and provide post-operative care as previously described in step 3.5.2-3.5.4.

4.2.3. Rabbit model use: If rabbits are found to have tumors at the time of surgical monitoring, use rabbit endometrial cancer models for planned experiments. Use rabbits created from in vivo propagated cells for additional experiments at 4-weeks post-inoculation and use rabbit models created from cultured VX2 cells at approximately 5-6 weeks post-inoculation to account for slower tumor growth.

REPRESENTATIVE RESULTS:

Twenty-eight rabbits were used for the creation of the endometrial cancer model. Rabbits had an average weight of 2.83 kg (2.71-3.58 kg) at the time of experiment. Uterine tumors successfully grew in 21 rabbits for an overall model success rate of 75%. Prior to the inclusion of uterine suturing in the protocol, the success rate was 57% compared to 81% after uterine suturing was added. Uterine suturing was added to the protocol after the 7^{th} rabbit in response to the initial low model success rate. Five models were created from cultured VX2 cells (attempted in 8 rabbits, 63% success rate) and 16 rabbit models were created from in vivo propagated cells (attempted in 22, 73% success rate). In models created from in vivo propagated VX2 cells, a cell dose of 5×10^6 per uterine horn was used in all animals and the average number of days from inoculation to experiment was 29 days (range 24-31). In models created from cultured VX2 cells, an escalating dose protocol was used to determine the appropriate inoculation dose. Doses of 2.5×10^6 cells and 5×10^6 cells per uterine horn were unsuccessful and a dose of 10×10^6 cells per uterine horn was successful in one rabbit however, tumor growth was slow at 57 days from inoculation to experiment. A dose of 20×10^6 cells per uterine horn was successful in 4 rabbits in an average time of 45 days (range 36 – 51 days) from inoculation to experiment and was thus determined as the optimal injection dose. This data is summarized in **Table 1**. On PCR analysis after passage 5, the cultured VX2 cells were highly positive for both Rabbit LINE-1 and CRPV-E6 with only trace amounts of mouse LI NE-1 was identified (<0.01 pg/µL). Cells were subsequently grown in cell culture however, growth was slow with an average time of 7 days (6-9 d) to achieve flask confluency.

All models successfully resulted in the metastatic transformation of the retroperitoneal lymph nodes (**Figure 2**). Nineteen rabbits had pathologically confirmed lymph node metastases and 11 had pathologically confirmed extra-nodal abdominal metastases. One rabbit did not have distinct lymph nodes removed; however, it had a high burden of intra-abdominal disease in which lymph node metastases were assumed, and one rabbit died prior to the experiment. Tumors and metastatic lymph nodes from cultured VX2 cells appeared similar to tumors from in vivo propagated VX2 cells on histology (**Figure 3**), with dense hematoxylin stained cells invading muscle and forming glandular-like structures with many pathological mitotic figures.

Using a novel imaging agent, the Porphysome, 81 lymph nodes were identified intra-operatively and surgically removed for histologic analysis. 74 lymph nodes were left pelvic lymph nodes, 5 were right pelvic lymph nodes and 2 were right para-aortic lymph nodes. Lymph nodes removed from rabbits with in vivo propagated VX2 tumors were significantly larger and more necrotic than those removed from rabbits with cultured VX2 tumors with an average volume of 0.99 cm^3 (range 0.12 – 3.89) versus 0.59 cm^3 (0.01 – 2.92) (p=0.037). As well, rabbits with in vivo propagated tumors had larger more necrotic uterine tumors than rabbits with cultured cell tumors with an average length 5.6cm (4-6.8 cm) and average width of 5.2cm (3.3 – 9 cm) versus 3.6cm (2-5 cm) and of 4.56cm (3-7 cm) respectively. Finally, rabbit models made from in vivo propagated VX2 cells had more extra-nodal abdominal metastases than rabbit models made from cultured cells with 91% of all metastases found in in vivo propagated rabbits.

FIGURE AND TABLE LEGENDS:

Figure 1: Uterine suturing. Black arrow = sutures, red arrow = uterine horns.

Figure 2: VX2 tumor (**A**) Intra-uterine tumor. Black arrow = tumor, red arrow = uterine horns (**B**) Metastatic left pelvic lymph nodes. Black arrow = metastatic lymph nodes.

Figure 3: Histology of cultured cell VX2 tumor (**A**) H&E staining demonstrates tumor infiltration of surrounding muscle (10x magnification, scale bar = 300 μm) (**B**) Pancytokeratin staining demonstrates densely staining tumor cells corresponding to areas of tumor on H&E (10x magnification, scale = 300 μm). Black arrow = VX2 tumor.

Table 1: Model data including experimental conditions, the number of animals used, and success rate. 2 rabbits used initially for cultured cell tumors models in which growth was unsuccessful were subsequently used for in vivo tumor models

DISCUSSION:

Herein, we have reported a standardized surgical method for the establishment of a VX2 endometrial cancer model and reported on the first use of cultured VX2 cells to create this model. The tumor take rate of 75% is lower than the 100% percent rate previously reported in the literature[35,37,53,56]; however, thw 90% rate of pathologically confirmed lymph node metastases is consistent with previous studies of this model[35,53].

The inclusion of uterine suturing significantly increased the model success rate from 57% to 81%

and we consider this step to be an integral part of the surgical protocol. Uterine suturing was not initially performed due to conflicting reports of the use of suturing in the literature and concerns regarding uterine horn devascularization from bilateral uterine artery ligation. Given the significant improvement in take rate with the addition of uterine suturing, we hypothesize that leakage of the cell suspension away from the injection site may have contributed to the initial low success rate. Anatomically containing the cell suspension in a small portion of the uterine horn ensures that a high local concentration of cells is exposed to the vasculature of the myometrium which likely improves tumor engraftment. Furthermore, no cases of uterine horn necrosis were noted in the experiment. Ensuring that the cell suspension is injected into the myometrium is also important as intra-uterine or extra-myometrial injections may also increase the loss of cell suspension. The uterine myometrium in rabbits is extremely thin and true intra-myometrial injection is difficult. Because of this, we hypothesize that the use of a cellular matrix scaffold such as Matrigel may improve the take rate of cells that are inadvertently injected into the uterine cavity. Despite these potential limitations, we believe that the cell suspension method is superior to previously reported microsurgical methods[36] in which tumor blocks are grafted onto the uterine myometrium as this method is technically challenging. In comparison, the method here is simple, incorporates commonly used techniques and it is for these reasons, we believe it to be highly reproducible.

The in vivo propagated VX2 rabbit models were injected with a standard dose of 5×10^6 cells per uterine horn which was based upon a collaborator's experience with VX2 rabbit models. This dose is significantly lower than reported in the literature in which cell doses as high as 1×10^8 by Harima et al.[52] and 5×10^9 by both Huang[37,53] and Xu[35] were used. Given the short time frame in which the model was established and the high rate of both lymph node and extra-nodal metastases, we do not believe that the use of a higher dose would have improved the model. A higher dose may have promoted even more rapid and aggressive tumor spread which would impair the utility of the model. 83% of the in vivo propagated VX2 models had extra-nodal disease at the time of experiment and this it is surprising that other groups did not report the development of abdominal metastases at 30 days. A possible explanation for this difference could be inadvertent intra-vascular inoculation[27] due to high pressure or high speed injection which can result in more rapid distant metastatic spread. We thus hypothesize that the speed of injection can be a factor in the rate of metastases which is why we recommend a slow injection speed in the protocol.

Comparatively, despite the higher injection dose (20×10^6 per uterine horn), only 40% of the cultured VX2 model rabbits developed extra-nodal disease and all metastatic deposits were noticeably smaller and less necrotic in these rabbits. We do not have any literature with which to directly compare the results as this is the first reported use of cultured VX2 cells to create this model. However, the findings are consistent with studies of other cultured VX2 tumors in which high inoculation doses were required and tumor growth was noted to be slow[27,31]. Through this experiment, we have identified the optimal injection dose of 20×10^6 cells per uterine horn which resulted in reliable growth and metastases in 80% of rabbits using an escalating dose protocol. It is possible that an even higher dose of cultured VX2 cells would result in quicker metastatic spread however as the VX2 cells grew slowly in culture, it was challenging to culture enough cells

to attempt a higher dose. This is a limitation of the study and have identified this as a potential area for future investigation. However, we consider the slower growth rate in the cultured cell model to be advantageous as we believe it may replicate the clinical scenario of endometrial cancer more reliably, as endometrial cancer is generally a slow growing disease that metastasizes first to the pelvic lymph nodes and results in late distant metastases. The initial choice to propagate the VX2 cells in mice allowed for a faster turnaround, and less expensive maintenance costs; however alternatively, the cells could have been derived directly from the quadriceps muscle of rabbits.

We believe that the close post-operative monitoring for signs and symptoms of tumor growth is an important aspect of the protocol. In our experience, once rabbits develop metastatic disease, they progress rapidly to being clinically unwell, most notably in the in vivo propagated group. This rapid, aggressive growth was highlighted by the death of one rabbit from metastatic disease after a delay of only 2 days from the planned experiment date. While the speed of VX2 model establishment has been considered a strength, as similar mouse models (i.e., HEC-1 endometrial carcinoma model with lymph node metastases in mice) can take up to twice as long to establish[57], these findings also demonstrate that determination of the optimal experimental timing is paramount. The findings correlate with previously studies in which tumor growth and lymph node enlargement increased significantly after post-operative day 21[37,53]; however we believe that there will variability with respect to the VX2 cell line used and encourage groups to understand their specific experimental timing. This timeline does not hold true for our cultured VX2 models and have identified this as an area which requires further study. To be certain about tumor growth, we chose to use both non-invasive and invasive tumor monitoring during the protocol. However, another future direction may be to refine the post-operative imaging protocol to avoid the need for a second invasive procedure.

Overall, we have reported a simple, standardized method to create a model of endometrial cancer with retroperitoneal lymphadenopathy in rabbits. Through this protocol, we have addressed the significant variability within the VX2 literature with respect to cell dose, surgical technique and post-operative model monitoring. We recognize that a further limitation of the study is that in using a VX2 cell line instead of a human xenograft we are not completely mimicking the tumor biology and microenvironment of human cancer. However, we hope other groups will use cultured VX2 cells to create their models as we believe this cell type may model human endometrial cancer more reliably through its slower growth and decreased propensity to metastasize. We encourage other groups to this fast and easy model of uterine derived retroperitoneal lymph node metastases to study novel imaging therapies to help patients with metastatic endometrial cancer.

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The authors have nothing to disclose.

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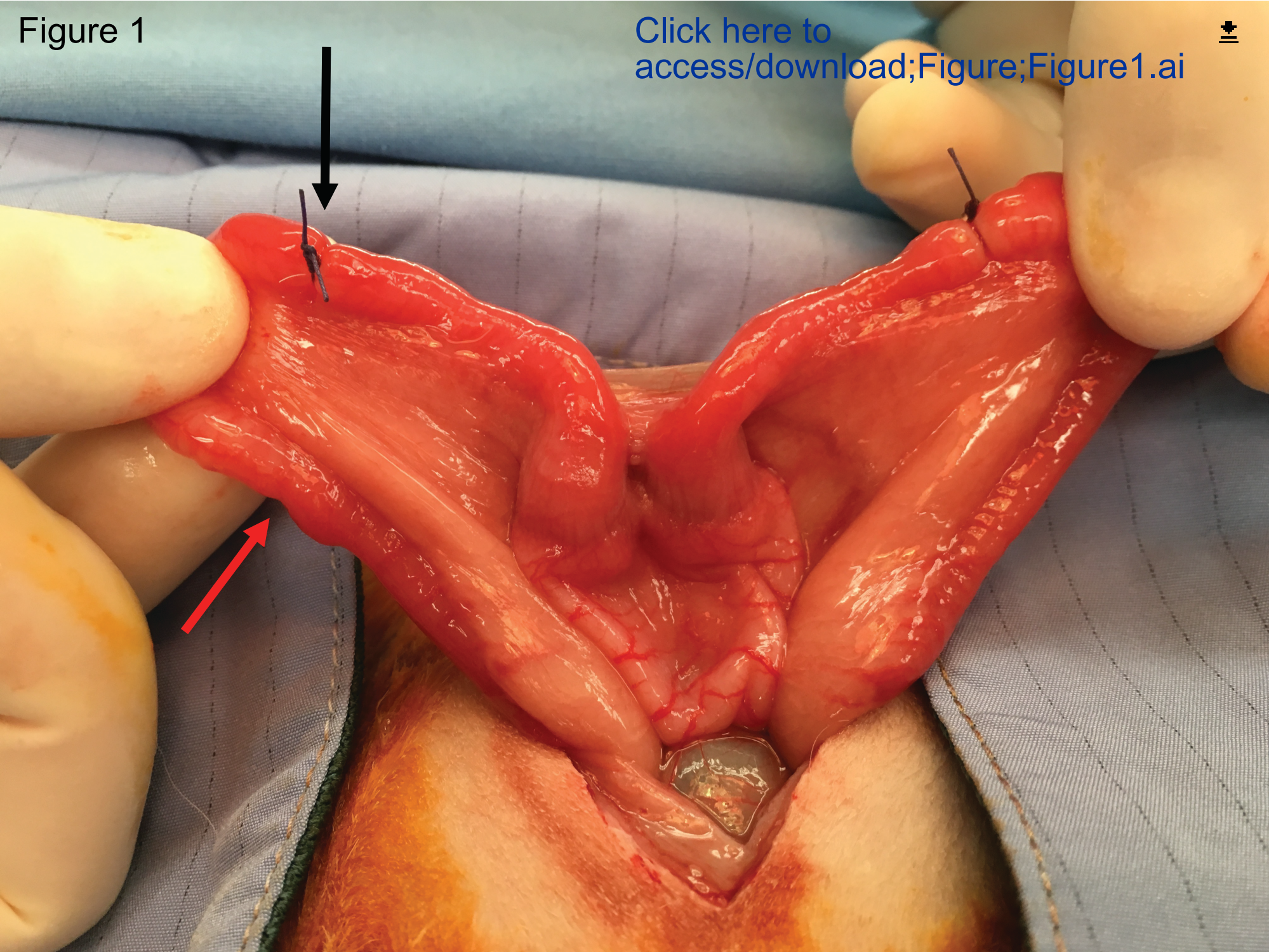

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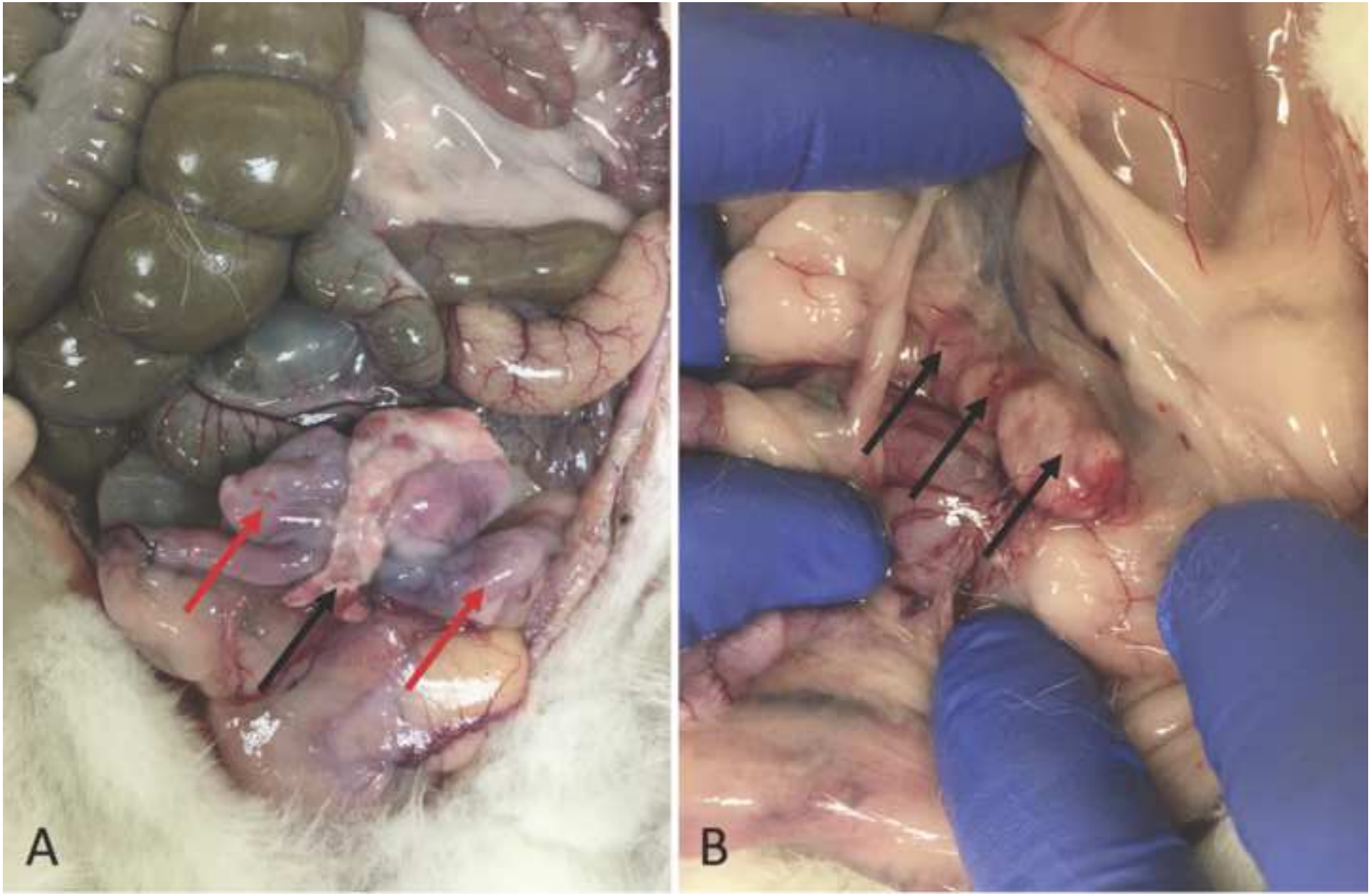

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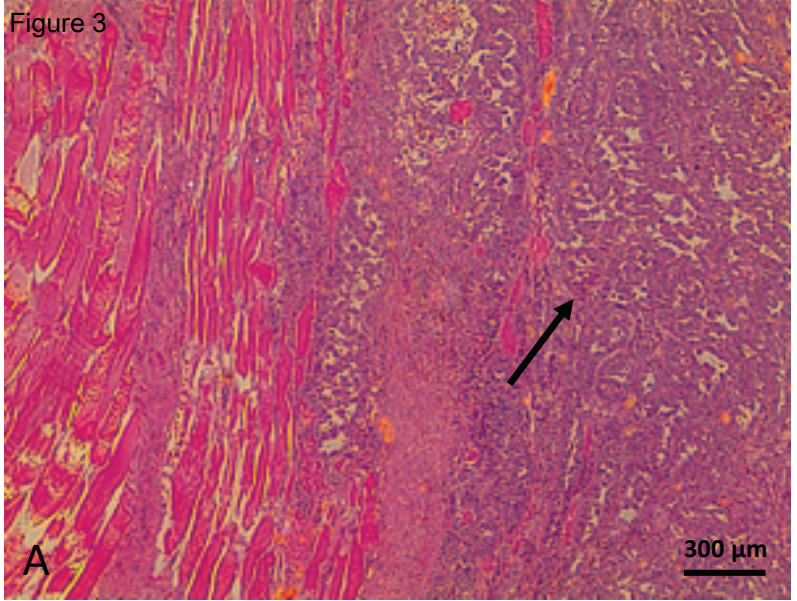

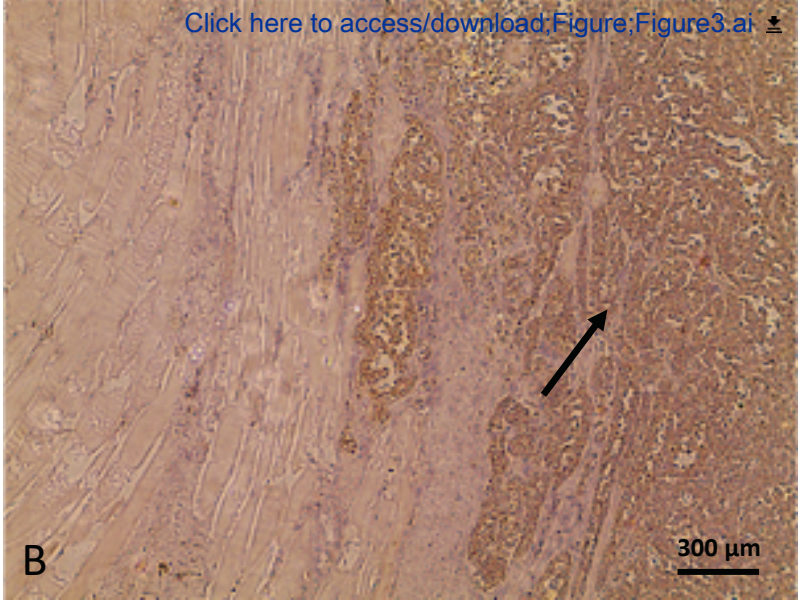

Table

Model Type	In vivo VX2 tumour model	Cultured VX2 tumour model
Number of successful models	16	5
Number of attempted models	22*	8
Model success rate	73%	63%
Time from inoculation to experiment	29 days (24-31)	45 days (36-51)
Successful injection dose	1×10^7 (5×10^6 per uterine horn)	40×10^6 (20×10^6 per uterine horn)
Overall success rate	75%	
Overall Success rate prior to uterine suturing	57%	
Overall Success rate after uterine suturing	81%	

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
11-blade scalpel, Sterile, Disposible	Aspen Surgical (VWR)	80094-086	
22-gague ear vein catheter	CDMV	14332	
3-0 absorbable poly-filament suture (Polysorb)	Covidien	356718	
3-0 braided absorbable suture (Polysorb)	Covidien	356718	
70uM cell strainer, Individually wrapped, Nylon	Falcon	352350	
Acepromazine (Atravet)	CDMV	1047	
Betadine soap (Poviodone iodine 7.5%)	CDMV	4363	
Betadine solution (Poviodone iodine 10%)	UHN Stores	457955	
Buprenorphine	McGill University		
Cefazolin	UHN in-patient pharmacy	No Cat # Needed	
Chlorhexidine solution	CDMV	119872	
Corning BioCoatCellware, Collagen Type I, 100mm dishes	Corning	354450	brand not important
Corning BioCoatCellware, Collagen Type I, 24-well plates	Corning	354408	brand not important
Corning BioCoatCellware, Collagen Type I, 6-well plates	Corning	354400	brand not important
Corning Matrigel Basement Membrane Matrix, *LDEV-free, 10 mL	Corning	354234	
DMEM/HAM F12 1:1	Life Technologies	11320	brand not important
DMSO	Caledon Lab Chem	1/10/4100	
Enrofloxacin (Baytril injectable)	CDMV	11242	
Falcon Tube	Corning Centri-Star	430828	
Fetal Bovine Serum, Qualified, Canadian Origin, 500ml	Life Technologies	12483020	brand/source not importan
Isoflurane	UHN in-patient pharmacy	No Cat # Needed	
Isohexol contrast	GE Healthcare	407141210	
Meloxicam (Metacam 0.5%)	CDMV	104674	

Normal Saline	House Brand (UofT Medstore)	1011	
PBS	Multicell or Sigma	331-010-CL or D8537-500mL	
Penicillin/Streptomycin (100mL; 10000U Penicillin, 10000ug Streptomycin)	Corning-Cellgro	CA45000-652	
Sterile Hanks Balanced Salt Solution (-Ca++, -Mg++, -Phenol Red)	T.C.M.F (Dr Bristow)	28-Jan-11	
Surgical Glue (Tissue Adhesive)	3M Vetbond	14695B	
Trypsin (0.25%), Proteomics Grade	Sigma	T-6567-5X20UG	
Trypsin-EDTA, 0.05%, 100ml	Wisent Inc	325-542-EL	brand not important

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Reviewer #3:
I agree with the authors that a reliable endometrial cancer model with retroperitoneal lymph node metastasis is needed. However, for this to occur, a cancer cell type that closely mimics the uterine cancer seen in humans is required. Even though widely used, the rabbit VX2 cell line not the appropriate cell type to achieve this goal. VX2 cells are very aggressive cancer cells that could form tumors in any organ, and could not only spread rapidly in the rabbit from one organ to another, but also across different animal species. Over the years, VX2 cells have been grown in different organs in vivo, and cells isolated from one organ were used to generate tumors in another organ. As a result, VX2 cell line that is currently used is a chimera of different cell types. As we and others have shown, animal cancer models established with VX2 are far from reliable, and do not mimic human cancers. Unless the authors could demonstrate comprehensively using a range of genomic, transcriptomic, molecular and biochemical approaches, their cancer model is not the appropriate one to study uterine cancer.

Response:

The main point of our protocol is to provide a successful standardized surgical method of VX2 model creation and to report the first use of cultured VX2 cells to create a model of endometrial cancer with retroperitoneal metastases in a rabbit. We agree that the VX2 model presented in our paper does not truly represent human cancer in terms of its exact biology etc. However, the model we present does provide an excellent model of retroperitoneal lymph node metastases derived from primary uterine tumour which is useful when performing preclinical studies for imaging technologies for image guided surgery. In these type of experiments the tumour spread pattern and location of metastatic disease is important and we feel that our model provides a good approximation of endometrial cancer in this way. Furthermore, the use of the cultured cells provided a slower growing model which may be even more useful to study endometrial cancer which is known to be a slower growing tumour. We understand a limitation of our study is that in using a VX2 cell line instead of a human xenograft we are not completely mimicking human cancer. However, our goal was to provide a detailed explanation of a model that is already in use in the literature and not to claim that our model was entirely biologically similar to human cancer. When we were looking to use this model for our imaging experiments we were not able to find a reliable source on the steps of model establishment and the surgical technique. Through this protocol, we have addressed the significant variability within the VX2 literature with respect to cell dose, surgical technique and post-operative model monitoring. We believe this information will be useful to other groups as the steps outlined in our protocol significantly increase the success rate of model establishment. A reference to this limitation was added to our discussion.

Reviewer 3 Comment 3. Authors have studied the tumors produced in rabbits using H&E staining alone. The resemblance of these tumors to uterine cancer should be additionally demonstrated using immunohistochemistry, Western blotting, and RT-PCR.

Response:
As mentioned above, the goal of our study was to provide a surgical method to create a VX2 model of uterine derived retroperitoneal lymph node metastases. As the goal of our study was

not to demonstrate that VX2 cells are similar to human endometrial cancer, we did not perform the studies mentioned above.

Comment e: Figure 3. A representative image (H&E and pancytokeratin staining) of VX2 tumours from "in vivo" propagated cells should be added, in order to compare both type of tumours features.

Response e: I previously replied with the following response which is still the case - We do not have a high enough quality representative image of the same section of an in vivo tumour (both H&E and pancytokeratin staining) to include in the manuscript. It would be good to have this image to compare but unfortunately we did not save any high enough quality images to include in the manuscript

Responses to items that were asked to be included in the manuscript

Comment c with initial response
c. Authors should indicate the parameters evaluate in mice models to guarantee mice welfare and evaluate tumour growth ("clinical monitoring"). Additionally, indicate the tumour formula used for tumour volume estimation.
Response c: The monitoring of the tumours growing in mouse flanks is described in the updated protocol. ARC animal care committee guidelines are adhered to regarding maximum tumour volume/size, and wellbeing of the mice.

Response:
The information about our animal use protocols was already in the manuscript at the beginning of the protocol section. This states that our experiments were carried out according to animal use protocols at our institution and provides the corresponding protocol numbers. I did not feel as though this needed to be added again.