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TITLE:

Generation of Tumor Organoids from Genetically Engineered Mouse Models of Prostate Cancer

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KEYWORDS:

Genetically engineered mouse models, Cre recombinase, tumor suppressor genes, prostate cancer, tumor dissection, organoids, 3D cell culture

SUMMARY:

We show a method for necropsy and dissection of mouse prostate cancer models, focusing on prostate tumor dissection. A step-by-step protocol for generation of mouse prostate tumor organoids is also presented.

ABSTRACT:

Methods based on homologous recombination to modify genes have significantly furthered biological research. Genetically engineered mouse models (GEMMs) are a rigorous method for studying mammalian development and disease. Our laboratory has developed several GEMMs of prostate cancer (PCa) that lack expression of one or multiple tumor suppressor genes using the site-specific Cre-loxP recombinase system and a prostate-specific promoter. In this article, we describe our method for necropsy of these PCa GEMMs, primarily focusing on dissection of mouse prostate tumors. New methods developed over the last decade have facilitated the culture of epithelial-derived cells to model organ systems in vitro in three dimensions. We also detail a 3D cell culture method to generate tumor organoids from mouse PCa GEMMs. Pre-clinical cancer research has been dominated by 2D cell culture and cell line-derived or patient-derived xenograft models. These methods lack tumor microenvironment, a limitation of using these techniques in pre-clinical studies. GEMMs are more physiologically-relevant for understanding tumorigenesis and cancer progression. Tumor organoid culture is an in vitro model system that recapitulates tumor architecture and cell lineage characteristics. In addition, 3D cell culture methods allow for growth of normal cells for comparison to tumor cell cultures, rarely possible using 2D cell culture techniques. In combination, use of GEMMs and 3D cell culture in pre-clinical studies has the potential to improve our understanding of cancer biology.

INTRODUCTION

Since the late 1980s, the ability to alter genes by homologous recombination has greatly advanced the study of biological systems[1]. Inducible, tissue-, or cell-specific promotor systems and site-specific recombinases, such as Cre-loxP, has advanced genetic studies by facilitating control over genetic modifications both temporally and spatially[2-4]. The combination of these genetic strategies has created a wide array of experimental model systems[5-7].

Genetically engineered mouse models (GEMMs) are an integral tool to assess how individual genes or groups of genes affect mammalian development and disease. In pre-clinical cancer research, GEMMs are the most physiologically-relevant and rigorous method to study cancer development, progression, and treatment[8]. Our laboratory specializes in generating and characterizing cancer GEMMs.

The most highly diagnosed non-cutaneous cancer among men in the United States is prostate cancer (PCa). The majority of patients with PCa have low-risk disease and high likelihood of survival, but survival rates decline drastically when disease is diagnosed at advanced stages or if targeted hormonal therapy induces progression to aggressive, non-curable PCa subtypes[9,10]. Our laboratory has developed GEMMs that utilize floxed alleles of one or more tumor suppressor genes. Recombination and loss of tumor suppressor gene expression occurs specifically in the prostate because we have introduced a transgene with Cre recombinase downstream of the probasin promoter activated only in prostate epithelial cells[11,12]. We have also bred our GEMMs to contain a Cre reporter transgene called *mT/mG,* which induces Tomato fluorescent protein expression in cells lacking Cre and green fluorescent protein (GFP) expression in cells with Cre[13]. While the presentation of this method and our representative results show GEMMs we study in our laboratory, this protocol can be used to generate prostate cancer organoids from any mouse model. However, as discussed in detail in our representative results section, we have observed that certain tumor characteristics are optimal for prostate cancer organoid generation.

In the last decade, new methods of culturing cells from tissues of epithelial origin has led to significant advances in our ability to model organ systems in vitro[14,15]. The term “3D cell culture” has been attributed to the techniques involved in establishing and maintaining organoids, which can be generally defined as structures made up of cells that assemble secondary architecture driven by organ-specific cell lineage characteristics[16]. These new methods are distinct from classic 2D cell culture in that cells do not require transformation or immortalization for long term growth; thus, 3D cultures of normal cells can be compared to diseased cells. This is particularly valuable in cancer research where normal cell control cultures have typically not been available. In addition, organoids spontaneously form secondary tissue architectures with appropriately differentiated cell types, making them a better model system to understand cancer in vitro than 2D cell lines[17]. Our laboratory has created 3D organoid lines from tumor issue isolated from our PCa GEMMs to complement our in vivo data and perform experiments which would not be feasible in GEMMs.

In this article, we present written and visual protocols for the complete necropsy of PCa GEMMs, including dissection of distinct mouse prostate lobes and metastatic lesions. We describe and show a step-by-step method for generating organoids from mouse prostate tumors based on a protocol previously published by Drost et al. for deriving organoids from normal mouse prostate epithelial tissue[18].

PROTOCOL

Animal procedures described here were performed with the approval of the Institutional Animal Care and Use Committee (IACUC) at the Department of Laboratory Animal Resources, Roswell Park Comprehensive Cancer Center, Buffalo, New York.

NOTE: Male mice to be dissected to isolate prostates or prostate tumors for generation of organoids should have at least reached the age of sexual maturity—about 8-10 weeks of age. Specific ages of mice can vary amongst studies. Some factors to consider when choosing age include age-dependent changes in prostate cell populations, age-dependent expression of specific promoter-driven Cre transgenes, and rate of prostate tumor progression in a particular GEMM.

1. Dissection and imaging of mouse prostate tumor and metastatic tumors

1.1. Preparations

1.1.1. Obtain necessary sterile dissection tools. Stage dissection area on clean sterile surface with a 15 cm ruler, precision balance, analytical balance, spray bottle with 70 % ethanol, phospho-buffered saline (PBS), and paper towels.

1.1.2. **Preparation of fixative solutions for visceral organs and bones not to be used for organoid generation**

1.1.2.1. For visceral organs: Prepare 4% paraformaldehyde (PFA) in PBS. For one mouse, make 20 mL of 4 % PFA, aliquot into two 15 mL conical tubes and keep at room temperature until use.

1.1.2.2. For long bones: Aliquot 10 mL of pre-made 10% neutral buffered formalin (NBF) in one 15 mL conical tube and keep at room temperature until use.

1.1.3. Obtain untreated 10 cm dishes and fill with non-sterile PBS—these will serve as temporary containers for organs during fine dissection using a dissection microscope.

NOTE: Sterile tools are used for dissecting tissues for generating organoids. Non-sterile tools are used for the initial incision and dissection of the hind limbs.

1.2. Euthanasia and initial incision

1.2.1. Euthanize the mouse by CO_2 asphyxiation using a 2.0 L/min flow rate for 5 min. Remove the mouse from the cage and perform cervical dislocation. Use the precision balance to measure the mouse's body weight and record.

1.2.2. Place the mouse on top of a paper towel and orient on the dissection surface ventral side up with the mouse's head facing away from the investigator. Affix the mouse to the board by stretching out its limbs and piercing each of the forepaws and hind paws with one disposable needle.

1.2.3. Using a spray bottle, douse the mouse's fur with 70% ethanol. Using non-sterile dissection scissors and straight forceps, pinch just above the mouse's penis and make a small incision through the fur only.

1.2.4. Continue the incision midline up to the mouse's neck through the fur only. Make bilateral incisions from the point of the initial incision through the mouse's ventral plane through the fur only.

1.2.5. Grasp the fur and carefully pull it away from the skin of the mouse. Pin down the fur to the board to allow access to both the abdominal and thoracic cavities.

1.3. Extraction of male urogenital system *en bloc*

1.3.1. Using sterile dissection scissors and straight forceps, carefully cut through the skin about 0.75 cm above the rectum. Continue the incision midline up to the ribcage without disturbing any organs in the abdominal cavity. Carefully pull the skin away and pin to the board to expose the entire abdominal cavity.

NOTE: Do not allow these instruments to touch the outside of the mouse or any surface in the staging area, as these tissues will be used to generate organoids.

1.3.2. Dissect the urogenital system and other organs according to the diagram in **Figure 1A**, with numbers indicating the order the organs will be removed from the mouse.

1.3.3. Find the bladder, then grasp the fat pad to either the left or right and pull upward to expose the testicle. Carefully dissect away the testicle from the rest of the urogenital region and put aside. Do the same procedure on the other side.

NOTE: Achieving the optimal tissue quality and detailed tumor characterization of prostate tumors requires that the entire urogenital region be removed from the mouse *en bloc*[19]. It is recommended that the urogenital region be removed first (**Figure 1A)**.

1.3.4. Grasp the bladder and carefully pull up so that the urogenital region lifts together, exposing the urethra underneath. While holding the bladder, orient the scissors so they are

against the underside of the dorsal prostate and cut the urethra. The entire urogenital region will then release from the abdominal cavity.

1.3.5. Put the urogenital region in a 10 cm dish filled with PBS. If still filled with urine, drain the bladder by making a small incision. Using the analytical balance, weigh the urogenital system and record.

1.4. Extraction of pelvic lymph nodes, spleen, liver, kidney, lung, tibia, and femur

1.4.1. Removing the urogenital system exposes the pelvic lymph nodes, positioned right behind the urogenital system (**Figure 1A**) and on either side of the spine. The lymph nodes will only be visible if they contain metastatic lesions or if there is local inflammation. Orient the straight forceps underneath the lymph node and pull up to remove the lymph node. Do the same procedure on the other side.

1.4.2. Grasp the rectum with the straight forceps and cut. Pull on the rectum to unravel the entire colon and small intestine, looking for metastatic lesions in the mesenteric lymph nodes. When the entire ileum is removed, continue to pull on the duodenum to expose the stomach. Cut the esophagus to completely remove the stomach and discard. If any metastatic lesions in the lymph nodes are observed, carefully dissect away from the intestine and store in a 10 cm dish of PBS.

1.4.3. Exposing and removing the stomach will pull the spleen from the dorsal side of the abdomen (**Figure 1A**). Remove the spleen and place in 4% PFA. The spleen serves as a staining control for hemotoxylin as it is highly cellular.

NOTE: Visceral tissues not to be used for organoid generation will be fixed overnight in 4% PFA, washed with PBS, and then placed in 70 % ethanol.

1.4.4. Remove the liver in the upper part of the abdomen (**Figure 1A**). Based on the metastatic load in the liver, individual lobes can be dissected, or the entire liver can be removed by carefully cutting along the diaphragm. (Do not cut through the diaphragm at this time). Place the liver in a 10 cm dish of PBS.

1.4.5. Removing the liver will fully expose the kidneys on either side of the spine (**Figure 1A**). Remove the kidneys–along with the renal lymph nodes, if the nodes have metastatic lesions—by placing the straight forceps underneath the kidney and pulling up. Do the same procedure for the other side.

1.4.6. To expose the thoracic cavity, carefully cut the diaphragm along the ribcage. Piercing the diaphragm will release the negative pressure in the thoracic cavity and expose the heart and lungs (**Figure 1A**).

1.4.7. Grasp the sternum and pull up to open the thoracic cavity further. Observe the ventral face of the thoracic cavity along the rib cage for metastatic lesions in the thoracic lymph nodes and dissect, if present.

1.4.8. While still holding the sternum, cut away the ventral rib cage to access the heart and lungs. Grasp the heart, pull up and cut underneath the lungs. To fully remove the heart and lungs *en bloc*, cut all anterior blood vessels and the trachea. Place the tissue in PBS and carefully remove the heart without damaging the lung tissue or lung metastatic lesions.

1.4.9. Take the non-sterile straight forceps and dissecting scissors and cut the hind leg at the head of the femur. Carefully grasp the femur and remove the hind leg from the fur.

1.4.10. Place the hind leg on a paper towel and grasp the hind paw. Use the single edge razor blade to scrap/cut away all muscle and connective tissue from the tibia and femur. Remove the femur from the tibia by cutting posterior to the patella and remove the tibia by removing the hind paw. Place the tibia and femur in 10% NBF. Do the same procedure on the other side.

NOTE: For the purposes of examining the long bones of the mouse for metastatic lesions, the fibula does not need to be intact. The long bones will be fixed in 10% NBF for one week, then decalcified using neutral EDTA solution[20]. After three weeks, the bones will be transferred to 70% ethanol.

1.4.11. After removing the hind limbs, take an ear or tail cutting for future genotyping. Discard the mouse carcass and all tissue not to be fixed or used for organoid generation.

1.5. Dissection of prostate tumor

NOTE: Dissection of mouse prostate lobes can only be achieved using a dissecting microscope[19]. However, the prostate tumor load can be so high that individual lobes cannot be distinguished, and dissection can be carried out without a microscope. Nevertheless, the full protocol for dissection of individual prostate lobes is described below.

1.5.1. Place the urogenital region in a 10 cm dish of PBS under a dissecting microscope and orient the ventral side up with the bladder and seminal vesicles facing away from the investigator. All manipulation of the urogenital region should be done using sterile instruments.

1.5.2. Assess the general phenotype of the prostate tumor– most likely, either a fluid-filled or solid tumor will be observed in PCa GEMMs (**Figure 2A, B**).

NOTE: Fluid-filled tumors are often located in the anterior prostate region (**Figure 2A**). Fluid filled tumors are made up of primarily connective tissue with scant epithelial components – thus these tumors are not optimal for organoid generation because of the low number of tumor epithelial cells, as will be discussed in the representative results section.

1.5.3. If fluid filled tumors are present, poke a small hole in the tumor. Place the urogenital region in a new 10 cm dish of PBS.

1.5.4. Take a pair of straight forceps in the non-dominant hand and curved forceps in the dominant hand**.** Flip the urogenital region to its dorsal face. Look for the proximal prostate region (**Figure 1B**), which can be identified by the pink/red color of the urethra. Grasp the urethra and hold firmly so to manipulate the urogenital tissue with the curved forceps.

NOTE: During prostate dissection, always take note of the bladder's location, as this is the simplest way to locate the individual prostate lobes (**Figure 1B**).

1.5.5. Removal of non-prostate tissue from the urogenital region

1.5.5.1. While still on the dorsal face of the urogenital region, find the base of the seminal vesicle. Carefully remove the seminal vesicle and discard. Perform the same procedure on the opposite side.

NOTE: Avoid puncturing the seminal vesicle, as that will release sticky and opaque secretory fluid which interferes with prostate dissection. If the seminal vesicle is punctured, transfer the remaining urogenital region to new 10 cm dish with fresh PBS.

1.5.5.2. Remove and discard the vas deferens and as much fatty and connective tissue as possible using the curved side of the forceps.

1.5.5.3. While still firmly holding the urethra/proximal prostate region, use a pair of fine, pointed scissors to remove the bladder from the urethra.

1.5.6. Isolation of individual prostate lobes

1.5.6.1. Locate the anterior prostate (**Figure 1B**). Ensure to firmly hold the proximal prostate region/urethra with the straight forceps. Remove the anterior prostate region by directly grasping the tissue with the curved side of the forceps and firmly pulling it away from the bladder and the rest of the prostate. Place the tissue in PBS.

NOTE: For all prostate regions, dissecting scissors may be required to remove the tissue, depending on the size of the tumor.

1.5.6.2. Locate the ventral prostate region (**Figure 1B**). Remove the ventral prostate region in the same manner as in step 1.5.7.

1.5.6.3. At this point in the dissection, only the lateral and dorsal prostate regions should be present, while the proximal prostate region is still being grasped by the straight forceps. Assess the lateral and dorsal prostate regions (**Figure 1B**). If the lateral region can be distinguished from

the dorsal region, remove the lateral prostate region as described in step 1.5.7. Do the same procedure on the opposite side.

1.5.6.4. Remove the dorsal prostate region as described in step 1.5.7. Place the proximal prostate region in 4% PFA, as its structure within the muscular tissue of the urethra makes it unsuitable for organoid generation.

2. Generation of 3D organoids from prostate tumor tissue

NOTE: **Figure 3** shows a pictorial description of the procedure for generation of tumor organoids.

2.1. Preparation

2.1.1. Prepare mouse prostate organoid media according to Drost et al.[18] with the following alterations. Use conditioned medium instead of recombinant proteins for Noggin and R-Spondin and use a final concentration of 1% (v/v), instead of 10 %, for both Noggin- and R-Spondin-conditioned medium.

NOTE: HEK293 cells stably transfected with HA–mouse Noggin–Fc or HA–mouse Rspo1–Fc are used to produce Noggin- or R-Spondin- conditioned medium, respectively. These cell lines were a gift from the Calvin Kuo Laboratory at Stanford University.

2.1.2. Prepare digestion solution in a 15 mL tube by diluting 20 mg/mL collagenase II with Advanced DMEM/F12(+++) media to a final concentration of 5 mg/mL. Add Y-27632 Rock Inhibitor to the collagenase II solution at a final concentration of 10 µM.

NOTE: The ratio of digestion buffer to tissue is 1 mL to 50 mg, which we use according to Drost et al.[18].

2.2. Mincing and digestion of tumor tissue

2.2.1. In the cell culture hood, place prostate tumor tissue in a sterile 10 cm culture dish, dissect and discard necrotic tissue.

2.2.2. Mince the remaining prostate tumor tissue into 1 mm^3 cubes by holding the tissue pieces with the sterile curved forceps and cutting with dissection scissors.

2.2.3. Place the minced tumor pieces in the 15 mL tube with the digestion buffer by scooping them up with the curved side of the forceps. Digest the tumor tissue at 37 °C with shaking for 1.5 to 2 h. Check digestion progress every 20 min.

NOTE: At this time, take out at least 2 mL of matrix from -20 °C storage and thaw on ice. 1 mL aliquots of matrix will take approximately 3 h to thaw.

2.2.4. After tissue digestion, centrifuge tube at 175 x *g* for 5 min at 4 °C to form a cell pellet.

2.2.5. Remove the supernatant, flick the tube to loosen the cell pellet, and resuspend the cell pellet in 1 mL of pre-warmed trypsin supplemented with 10 µM Y-27632 Rock Inhibitor. Put the tube in a 37 °C water bath for 5 min.

2.2.6. After incubation, pipette up and down 5 times with a standard P1000 tip. Return the tube to 37 °C water bath for another 5 min and repeat step 2.2.6.

NOTE: At this time in the procedure, warm a sterile 6-well cell culture dish by putting it into a 55 °C incubator.

2.3. Counting cells and resuspension in matrix

2.3.1. Wash the cells by adding 9 mL of cold AdDMEM/F12(+++) and centrifuge the tube at 175 x *g* for 5 min at 4 °C.

2.3.2. After centrifugation, remove the supernatant, flick the tube to loosen the pellet, and wash the cells again by adding 10 mL of cold AdDMEM/F12(+++). Centrifuge the tube at 175 x g for 5 min at 4 °C.

2.3.3. After centrifugation, remove the supernatant, flick the tube to loosen the pellet, resuspend the cells in 1 mL of AdDMEM/F12(+++), and count the number of cells using a hemocytometer according to standard procedure.

2.3.4. Seven-to-eight domes fit in one well of a 6 well dish when organoids are plated in matrix *via* a drop-wise fashion. Approximately 200 µL of matrix will produce 7-8 domes. Decide how many wells of organoids are needed for future experimental purposes and calculate the volume of matrix required. Then, calculate the volume of cell-containing solution needed to have a final concentration of 1.0 x 10^6 cells/mL of matrix.

2.3.5. After counting cells, centrifuge at 175 x *g* for 5 min at 4 °C. Remove the supernatant, flick the tube to loosen the pellet, and resuspend the cells in volume of matrix calculated in step 2.3.4.

NOTE: Matrix remains in liquid form only at 4 °C; keep matrix stock tubes and matrix-cell solutions on ice at all times.

2.4. Plating matrix domes and application of media

2.4.1. Mix matrix-cell solution with a P200 pipet to evenly distribute the cells without introducing bubbles. Remove the 6-well culture dish from the 55 °C incubator.

2.4.2. Carefully pipette 200 µL of matrix-cell solution and quickly drop the solution into a well to create domes.

2.4.3. Repeat step 2.4.2. until volume of matrix-cell solution is spent. Allow the domes to solidify at room temperature for 2 min.

2.4.4. Flip the 6-well dish upside-down and put the dish into a 37 °C incubator to continue solidification for 20 min.

2.4.5. After incubation, add 2 mL of mouse prostate organoid media to each well. Add synthetic androgen R1881 to each well for a final concentration of 1 nM and Y-27632 Rock Inhibitor to a final concentration of 10 µM. Mix carefully and place the plate in a 37 °C incubator for culturing.

NOTE: Organoid culture media needs to be supplemented with 10 µM Y-27632 Rock Inhibitor for only 1 week after organoid generation.

REPRESENTATIVE RESULTS

Representative necropsy images of a mouse with a large fluid-filled primary prostate tumor in the anterior prostate region are shown in **Figure 2A.** In contrast, **Figure 2B**, shows representative necropsy images of a mouse with a large solid primary prostate tumor for which individual prostate regions are indistinguishable. Fluorescent dissection images show the same solid prostate tumor from **Figure 2B** expressing GFP, indicating that the tumors cells express Cre (**Figure 2C**). Tissue that does not express probasin, such as the bladder, express Tomato and thus does not express Cre (**Figure 2C**). The liver and lungs from the mouse from **Figure 2B** have metastatic tumors expressing GFP, showing that they originated from the primary prostate tumor, and are surrounded by normal tissue that expresses Tomato (**Figure 2C**). Finally, the pelvic lymph node from this mouse expresses GFP and not Tomato, indicating that this metastatic tumor has overtaken this organ and no normal tissue remains (**Figure 2C**).

We show images in **Figure 4** of organoids we have generated from a solid prostate tumor. At Day 1, small organoids are forming, as seen in the representative phase contrast images. Fluorescent images on Day 1 show that both Tomato and GFP expressing cells are present in the tumor organoid culture (arrows). However, by Day 7 when prostate tumor organoids have fully formed, these organoids are expressing GFP and not Tomato. These data suggest that these organoids have originated from tumor cells that were expressing Cre and not from normal epithelial cells. These tumor organoids continue to be only GFP-positive as we expand our culture to passage 1 and 2.

In **Figure 5, we** show images of organoids we have generated from a fluid-filled prostate tumor. On Day 1, small organoids are forming, and fluorescent images show that both Tomato- and GFP-expressing cells are present in the organoid culture—similar to our observation at Day 1 for organoids generated from a solid prostate tumor (**Figure 4**). However, organoids from a fluid-filled prostate tumor express either GFP or Tomato at Day 7—indicating that organoids have formed from cells that do not express Cre. This pattern continues at passage 1 and passage 2, where the culture has both Tomato- and GFP-expressing organoids. Further analysis of these organoids is severely limited because the line is a mixture of normal epithelial organoids and

tumor organoids. We believe that fluid-filled prostate tumors are suboptimal in generating tumor organoids simply because there is a greater percentage of normal prostate epithelial cells. Since both normal prostate epithelial cells and prostate cancer cells form organoids, the lines generated from fluid-filled prostate tumors are a mixture of normal and cancer organoids. We obtain pure tumor organoid lines from fluid-filled prostate tumors by flow sorting for GFP-positive cells and generating organoids from that population of cells. Solid prostate tumors are primarily comprised of tumor cells, therefore organoids generated from these tumors are a more pure population of cancer organoids without prior sorting for GFP.

LEGENDS TO FIGURES

Figure 1. Our recommended dissection order for prostate cancer (PCa) genetically engineered mouse models (GEMMs) and anatomy of the mouse prostate. (**A**) The order we recommend in our protocol for dissecting the major organs from a PCa GEMM. 1. Urogenital region. 2. Pelvic lymph nodes. 3. Spleen. 4. Liver. 5. Kidneys. 6. Lungs. 7. Tibia and Femur. (**B**) Map of the mouse urogenital region and prostate anatomy. Fluorescent dissection images of a 12 week old mouse expressing probasin-Cre and the *mT/mG* Cre reporter transgene. Bladder (BL), seminal vesicles (SV), anterior prostate (AP), ventral prostate (VP), lateral prostate (LP), dorsal prostate (DP), and proximal prostate (PP).

Figure 2. Representative dissection images of prostate cancer (PCa) genetically engineered mouse models (GEMMs). (**A**) The abdominal cavity prior to removal of the urogenital region and the urogenital region with a fluid-filled prostate tumor. (**B**) The abdominal cavity prior to removal of the urogenital region and the urogenital region with a solid prostate tumor. (**C**) Representative Tomato and GFP fluorescent images of a solid prostate tumor, liver, lung, and pelvic lymph node from a PCa GEMM that develops metastatic lesions. Bar = 5 mm. Bladder (BL), anterior prostate (AP), and dorsal prostate (DP).

Figure 3. Flow chart of the protocol for generating prostate tumor organoids. After dissecting the prostate tumor, mince the tissue into 1 mm pieces. Digest the tumor pieces in collagenase, collect the cells, and digest in trypsin to obtain a single cell suspension. After counting cells, resuspend in volume of matrix required for a 1.0 x 10^6 cell/mL cell concentration. Plate domes in dish using a drop-wise method.

Figure 4. Representative images from generation of mouse prostate tumor organoids from a solid prostate tumor. Representative phase contrast, Tomato, and GFP fluorescent images from Day 1, Day 7, Passage 1, and Passage 2 of organoids generated from a solid mouse prostate tumor. Bar = 100 µm. Arrows indicate individual cells in fluorescent images.

Figure 5. Representative images from generation of mouse prostate tumor organoids from a fluid-filled tumor. Representative phase contrast, Tomato, and GFP fluorescent images from Day 1, Day 7, Passage 1, and Passage 2 of organoids generated from a fluid-filled mouse prostate tumor. Bar = 100 µm. Arrows indicate individual cells in fluorescent images.

DISCUSSION:

Critical steps within the protocol for prostate tumor dissection and organoid generation

Removal of non-prostate tissue and fine dissection of the mouse prostate tumor is crucial for the optimal generation of cancer organoids since both non-prostate epithelial cells and normal prostate epithelial cells will generate organoids. For solid prostate tumors specifically, it is crucial to isolate areas of viable tumor to remove contamination with necrotic tissue that would reduce the number of viable cells. During organoid generation, tissue digestion with collagenase should be diligently monitored, as prolonged exposure to collagenase will limit cell viability. With organoids derived from cancer GEMMs, it is crucial to fully genotype each line to ensure that all transgenes and modified alleles that were engineered in the mouse are present in the organoids. Repetition of genotyping after prolonged passaging is also necessary to ensure that genetic modifications are maintained.

Modifications and troubleshooting of prostate tumor dissection and organoid generation

We have observed mouse to mouse variability in prostate tumor characteristics, even amongst animals with the same genotype. Therefore, specific modifications to the prostate dissection protocol described here may be necessary for each mouse. In addition, adaptability is necessary when dissecting metastatic tumors since it is difficult to predict the severity of these lesions prior to starting the dissection.

On a few occasions, we have observed excess contamination of our cell pellet with what appears to be connective tissue, even after digestion with both collagenase and trypsin. When this occurs, we resuspend the pellet in at least 2 mL of AdDMEM F12(+++) and use a 40 µm cell strainer to remove the connective tissue. Since there is lot-to-lot variability in the solidification rate of the matrix, increasing or decreasing the time for dome solidification may be necessary prior to application of organoid media.

Limitations in using GEMMs

While GEMMs are the most rigorous method for pre-clinical cancer studies, this approach requires significant time, expense, and training. In addition, mouse to mouse variability can, as in the study of humans, complicate interpretation of data.

Limitations in using 3D cell culture

Compared to 2D cell culture, generating and maintaining organoid lines require increased time and cost. For instance, our tumor organoid lines are passaged every 2-3 weeks, while cell lines can be passaged every 2-3 days. This slower growth rate of organoids increases the time required to complete experiments considerably. Organoid culture media contains several specialized growth factors and reagents, which can be costly depending on source, thus generating and maintaining organoids is more expensive than traditional 2D cell lines. Finally, our laboratory and others have observed lot to lot differences in matrix and other reagents—creating a challenge for maintaining consistency in organoid growth for long term experiments.

Significance in using 3D cell culture with respect to existing/alternative methods

Pre-clinical cancer research has been dominated by 2D cell culture and cell line-derived xenograft models. Cell growth in 2D requires transformation/immortalization–thus both in vitro and

xenograft studies using 2D cultures typically do not have unaltered normal cell lines to serve as non-cancer controls. The last decade of research in 3D organoid culture of normal epithelial-derived tissues has now allowed for the growth of non-cancerous epithelial tissues that can be used to compare to analogous organoids derived from cancer tissue. Cancer organoids can also be used to establish xenografts to further understand tumor development. In addition, non-cancer organoids can be used to generate control xenografts—which was not possible before 3D cell culture methods were developed[16].

Significance in using 3D cell culture in prostate cancer research

In recent studies, organoids have been used to recapitulate GEMM prostate tumor characteristics. Dardenne et al. show that organoids generated using prostate tumors from GEMMs that simultaneously lack the tumor suppressor *Pten* and overexpress the *MYCN* oncogene had greater growth potential than organoids generated using prostates from control GEMMs. In addition, both sequencing and immunohistochemistry showed that tumor organoids recapitulated the expression profiles of prostate tumors both lacking *Pten* and overexpressing *MYCN*[21]. Blattner et al. show that simultaneous prostate overexpression of an oncogenic mutant of Speckle Type BTB/POZ Protein (*SPOP*) and deletion of *Pten* increases the rate of tumorigenesis in GEMMs. When prostate organoids were generated to overexpress mutant *SPOP*, their proliferation was increased compared to control prostate organoids and lineage marker expression recapitulated original prostate tumors[22]. Together, these studies demonstrate that organoids are an optimal model for further study of prostate tumor characteristics in GEMMS.

Organoid culture has also been used as a tool to assess individual subpopulations of prostate tumor cells. Using GEMM tumors that lack *Pten* and both *Pten* and *Trp53* tumor suppressors in prostate epithelial cells, Agarwal et al. fractionated cells into basal and luminal progenitors, propagated these subpopulations as organoids, and further characterized their specific phenotypes[23]. Thereby using 3D cell culture, it is possible to characterize subpopulations of tumor cells which may be limited in abundance within prostate tumors themselves.

As described above, 3D cell culture techniques permit the growth of normal epithelial cells. Thereby, prostate organoids generated from GEMMs lacking a Cre driver provide a unique model for real time monitoring of tumorigenesis by induction of Cre recombinase in vitro. Indeed, Dardenne et al. assessed how *NMYC* overexpression affects growth potential in the context of *Pten* loss over time by ectopically expressing ERT2-Cre and treating with tamoxifen[21]. Additionally, the effect of *NMYC* overexpression on androgen receptor (AR), the major target of therapy for prostate cancer, was assessed after induction of Cre recombinase in organoids generated from GEMMs[21]. The same inducible Cre system was used by Blattner et al. in prostate organoids to measure how overexpression of mutant *SPOP* affects prostate cancer cell proliferation and AR expression[22]. Notably, experiments inducing Cre expression in vitro have a built-in non-cancer control with vehicle-treated organoids.

Specific limitations in using 3D cell culture in prostate cancer research

While organoid growth of normal epithelial cells is an advantage of using 3D cell culture techniques, capacity to grow normal organoids has also presented a challenge in prostate cancer

research studies. As shown in our representative results section, we have observed outgrowth of normal prostate organoids in lines generated from prostate tumors which are less aggressive (**Figure 5**). One way to address this phenomenon is to generate organoids from GEMMs expressing a Cre reporter transgene, such as *mT/mG*. Fluorescent microscopy can be used to assess the relative ratio of normal to tumor organoids by observing expression of Tomato and GFP. In addition, GFP expression can be used to flow sort organoid cells to generate pure prostate tumor organoid lines. Agarwal et al. show a sorting method for separation of normal epithelial cells and cancer cells from GEMM prostate tumors without a Cre reporter. They show that epithelial cell adhesion molecular (EpCAM)-positive cells from prostate tumors did not separate into subpopulations when sorted using either CD24 or Sca-1 cell surface markers[23]—thus, these markers could be employed to exclude normal prostate epithelial cells from GEMM prostate tumors prior to organoid generation. Our laboratory and others have observed that the conditions under which prostate cancer cells form organoids appear to either select for or promote lineage specific gene expression programs characteristic of prostate basal epithelial cells. This is a significant challenge because prostate tumors in both mice and humans are primarily luminal in nature, expressing AR, CK8, and other luminal markers, and rarely express basal lineage markers such as p63 or CK5. While this phenomenon has yet to be published in detail, immunohistochemistry analysis shows that AR is decreased in *Pten*f/+ organoids compared to *Pten*f/+ prostates[21]. The outgrowth of basal epithelial cells in prostate cancer organoids calls into question whether these lines are truly an accurate pre-clinical model of prostate cancer.

While prostate cancer organoids have been documented to model the tumor from which they are derived better than traditional 2D culture, there is potential for organoids to undergo genetic changes in culture, especially after several passages. Currently, we are not aware of any published studies that have documented spontaneous genetic mutations, genetic gains or losses, or epigenetic changes that are common after prolonged passaging of prostate cancer organoids. To limit variability as a result genetic or epigenetic changes that may occur due to prolonged passaging, experiments should be performed in early organoids from early passages (<10) as often as possible.

Future applications of 3D cell culture

While it is impossible to predict all future applications that will be developed using 3D cell culture in cancer research, there are several avenues which appear to have the most potential. As with 2D cell lines, carrying out in vitro genetic modification is relatively straightforward in organoids. Modifying specific genes in either normal or cancer organoids opens up many possibilities in the study of the mechanisms governing tumorigenesis, cancer progression, and treatment—especially when genetically-modified organoids are used to generate organoid xenografts. Genetic modification of organoids is greatly advantageous when GEMMs do not exist for a specific gene or establishing a new GEMM is outside the scope of a particular study.

Cancer organoid culture also has many potential applications for clinical research. A library of relevant tumor subtypes within each organ system from both patients and animal models could be used to quickly assess efficacy of a new drug or new combination of existing drugs. As 3D cell

culture becomes mainstream and increases in efficiency, generating patient-derived organoids for the purpose of personalized medicine has the potential to help tailor treatment for each cancer patient by testing all available drugs and combinations of drugs using his or her individual organoid line[16].

DISCLOSURES

The authors do not have financial relationships to disclose.

ACKNOWLEDGMENTS

The authors would like to thank the Calvin Kuo Laboratory at Stanford University for providing HEK293 cells stably transfected with either HA–mouse Noggin–Fc or HA–mouse Rspo1–Fc. We would also like to thank Dr. Dean Tang for allowing us access the fluorescent dissection microscope in his laboratory. This work was supported by CA179907 to D.W.G. from the National Cancer Institute. Shared resources at Roswell Park Comprehensive Cancer Center were supported by National Institutes of Health Cancer Center Support Grant CA016056.

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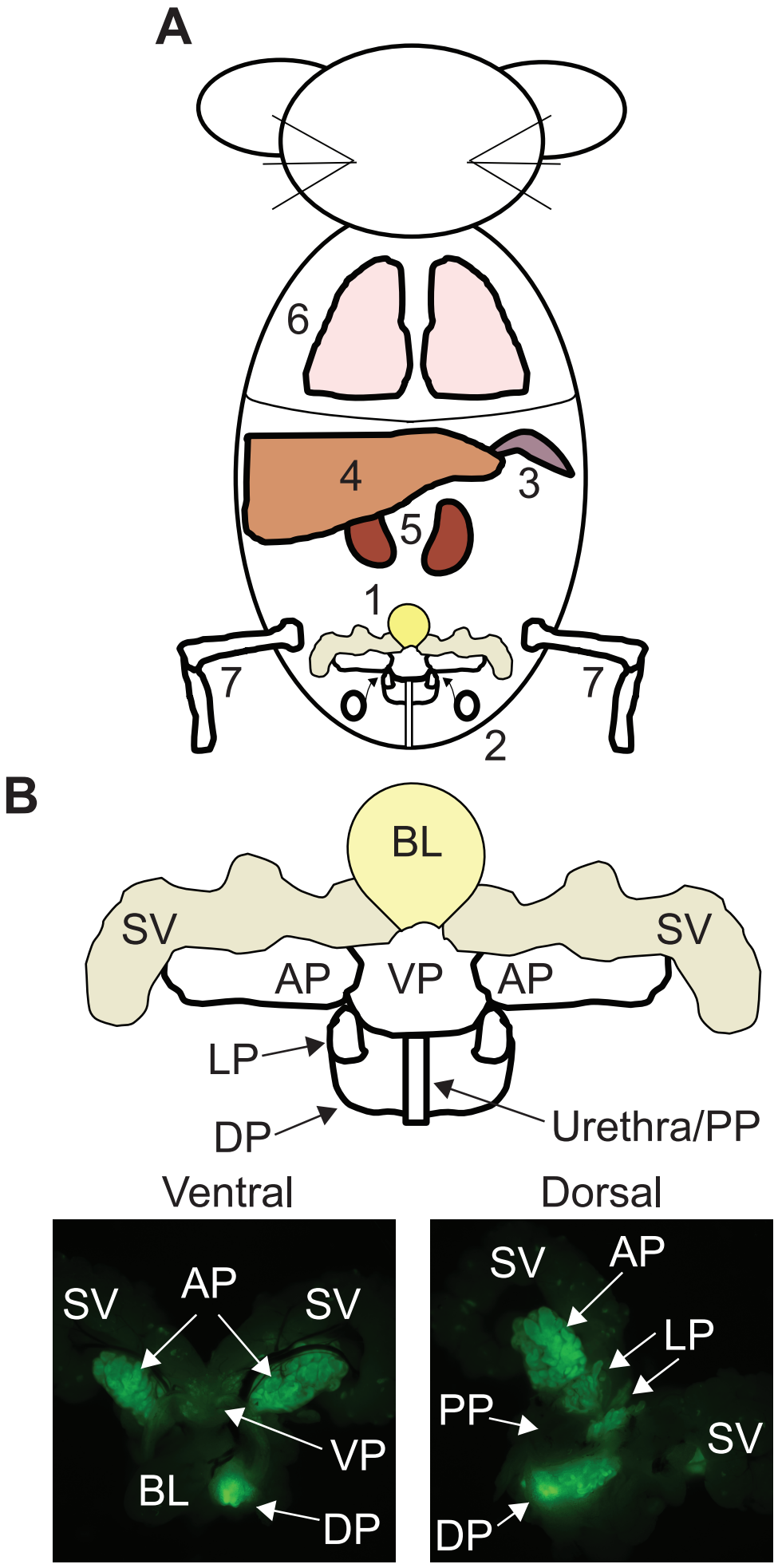

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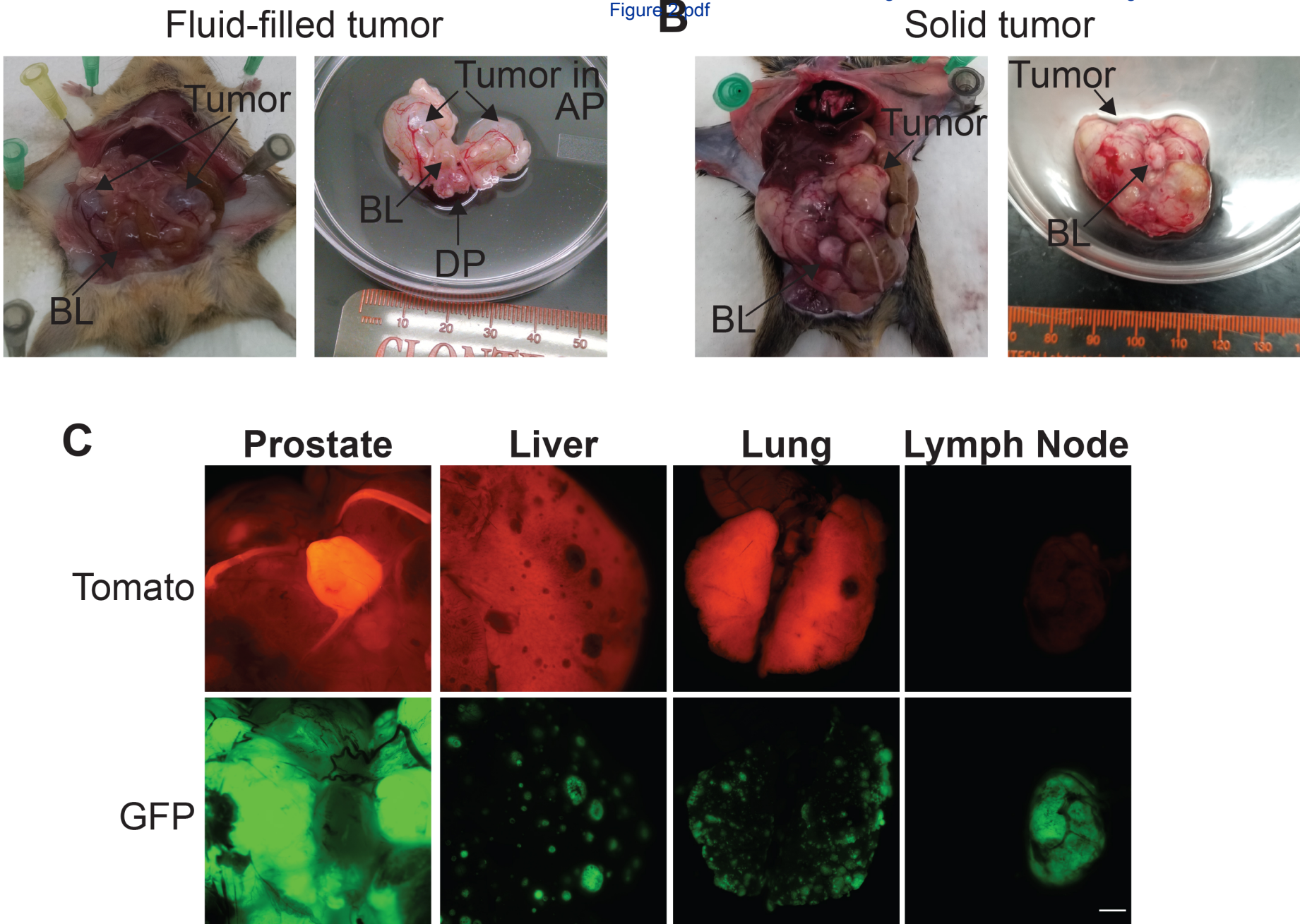

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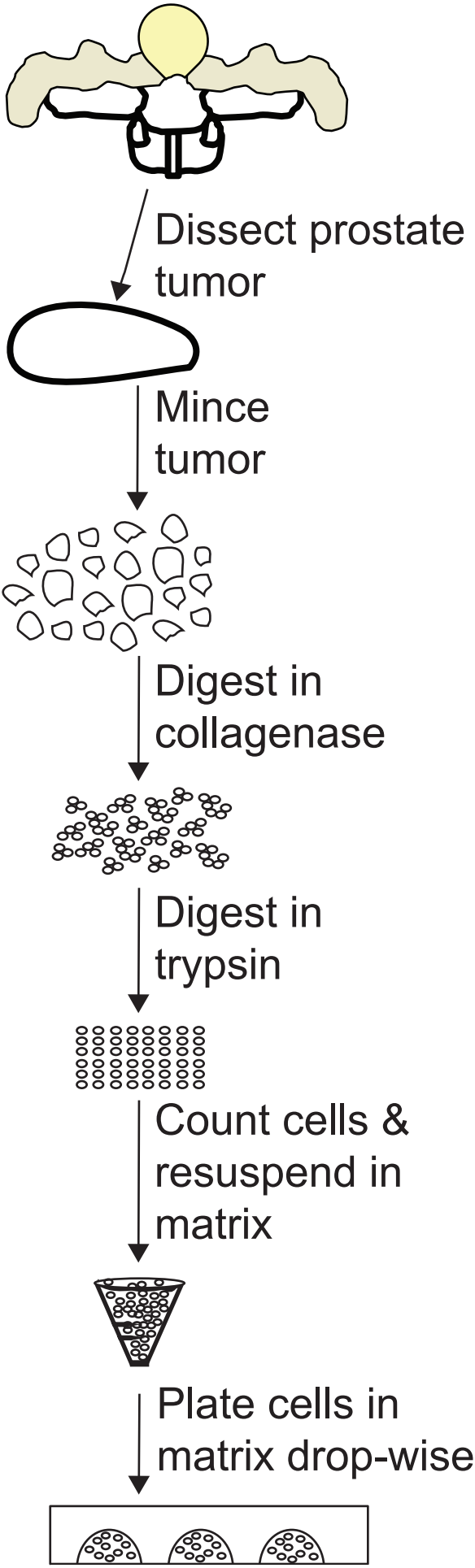

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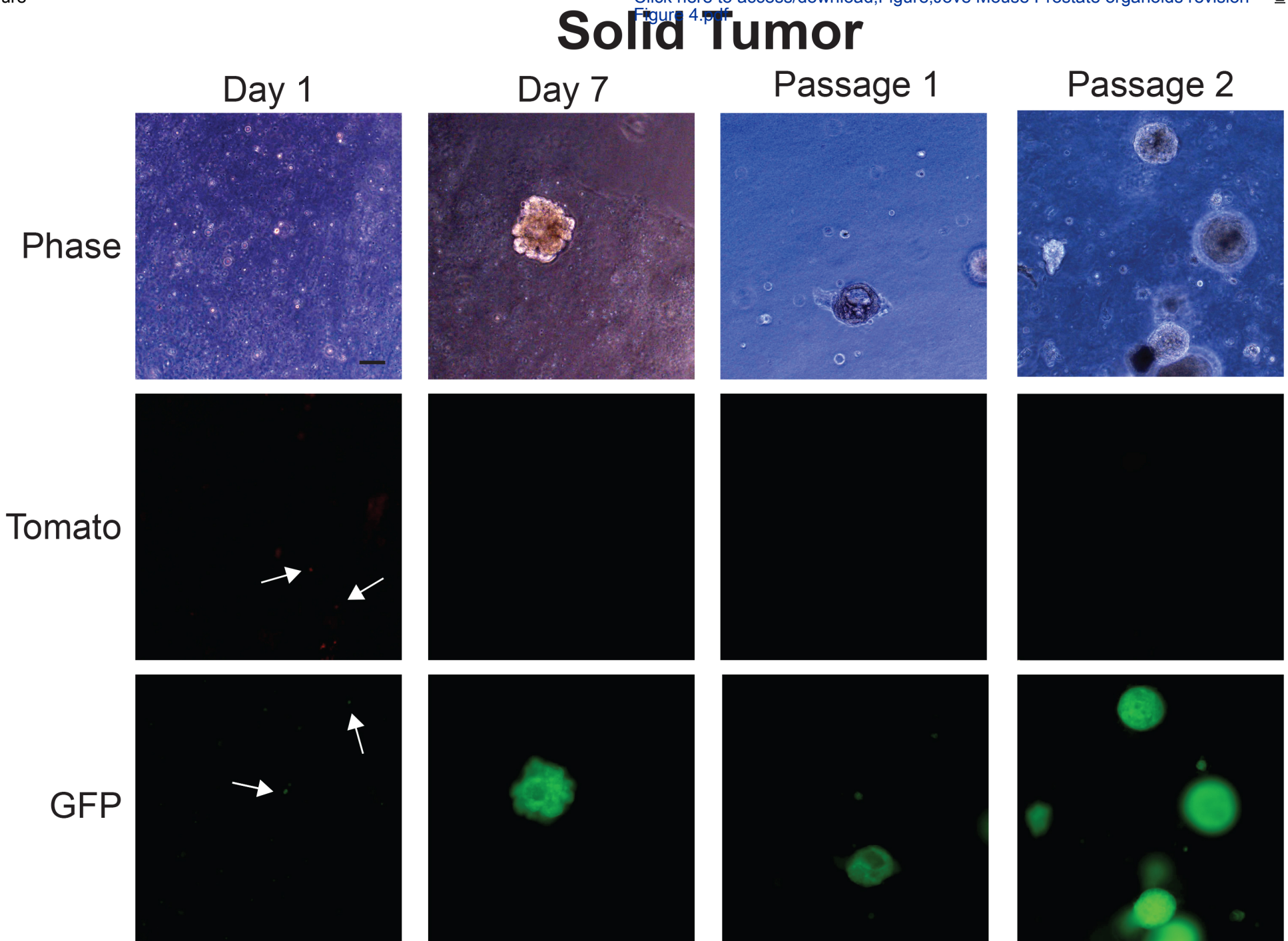

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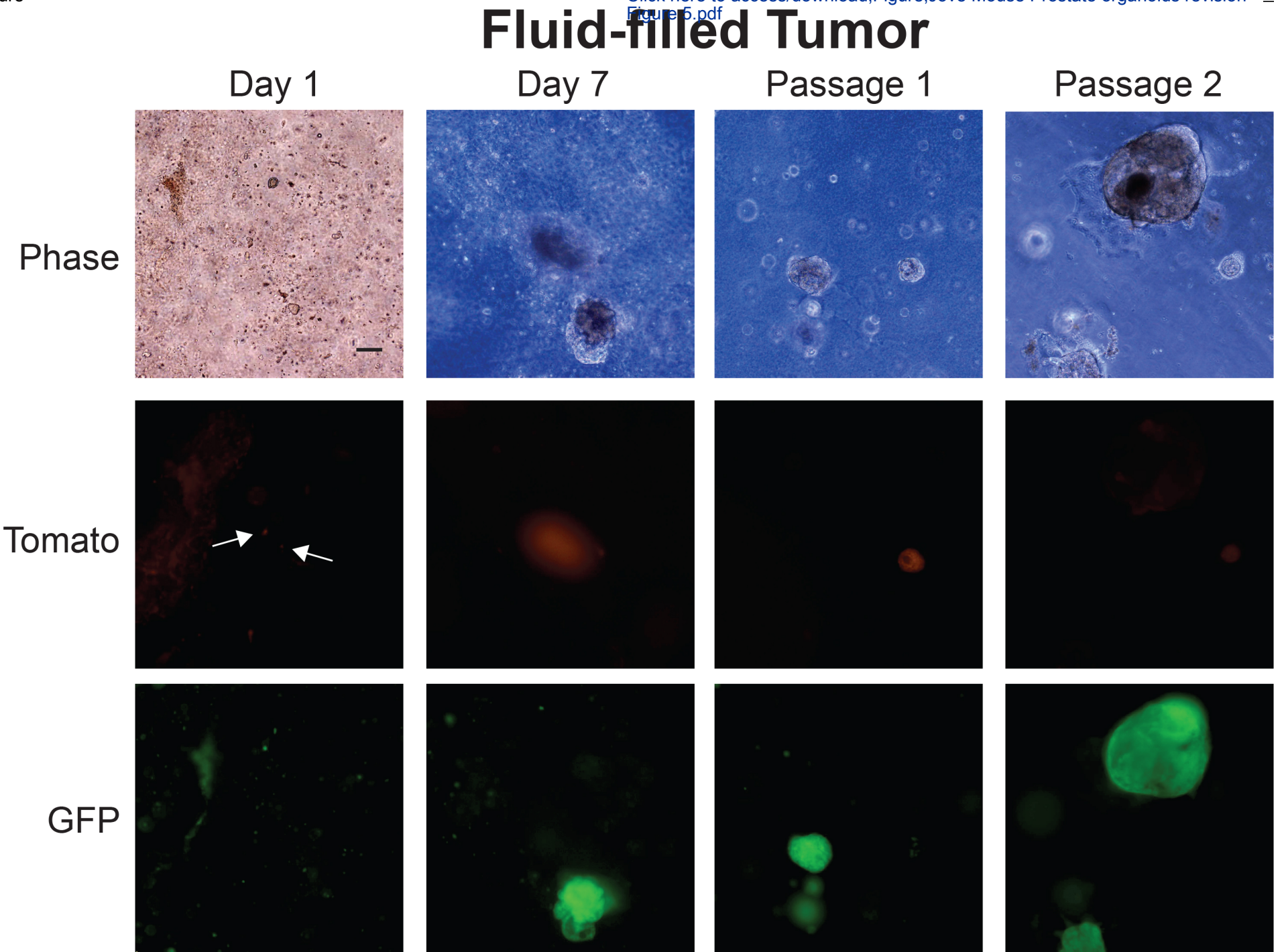

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Name of Material/ Equipment	Company	Catalog Number	Comments/Description
0.25 % Trypsin+2.21 mM EDTA	Sigma	25-053	
1 1/4 in, 23 gauge, disposable syringe needles	Becton Dickinson	Z192430	
10 % neutral buffered formalin	Sigma	HT501128	
32 % paraformaldehyde	Electron Microscopy Services	15714	
A83-01	MedChemExpress	HY-10432	
Advanced DMEM/F12+++	Gibco	12634	
Analytical balance	Mettler Toledo	30216623	
B27 (50X)	Gibco	17504044	
Collagenase II	Gibco	17101015	
Dissecting Board	Thermo-Fisher	36-1	
EHS Sarcoma matrix, Pathclear Lot#19814A10	Manufactured by Trevigen	Requistitioned from the National Cancer Institute at the Frederick National Laboratory	Holder of grants from the National Cancer Institute can request matrix
HEPES (1M)	Sigma	25-060	
human recombinant Epidermal growth factor (EGF)	PeproTech	AF-100-15	
L-glutamine (200 mM)	Sigma	25-005	
N-Acetyl-L-Cysteine	Sigma	A9165	
Penicillin-Streptomycin	Sigma	P4333	
Precision balance	Mettler Toledo	30216561	
Scalpel #23	World Precision Instruments	504176	
Scalpel Handle #7, 16 cm	World Precision Instruments	500238	
Single-edge carbon razor blade	Fisherbrand	12-640	

Stainless steel dissecting scissors, 10 cm, straight	World Precision Instruments	14393
Stainless steel Iris forceps, 10 cm, curved tip, serrated	World Precision Instruments	15915
Stainless steel Nugent utility forceps, straight tip, serrated	World Precision Instruments	504489
Y-276632 (Rock Inhibitor)	APExBIO	A3008

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February 26, 2019

Bing Wu, Ph.D.
Review Editor
JoVE

Dear Dr. Wu,

The authors would like to thank you for the careful and thorough review of our manuscript titled "Generation of tumor organoids from genetically engineered mouse models of prostate cancer" (JoVE59710) by yourself and the four reviewers. The expertise of the reviewers was well suited for our manuscript and we believe the alterations we have made in response to their comments have greatly improved our submission.

In reply to your request for revisions to our submitted manuscript, we have made two major overall changes. At the request of multiple reviewers, we have removed mention of details specific to the prostate cancer (PCa) genetically engineered mouse models (GEMMs) that we use on our laboratory. This edit has generalized the description and discussion of the protocol in our manuscript so that readers can apply it to their own GEMMs.

We have also substantially expanded the Discussion section of our manuscript, as suggested by yourself and multiple reviewers, from 3/4 of a page to 3.5 pages. We believe that our expanded Discussion will provide readers with the necessary depth for understanding both the method of generating organoids from PCa GEMMs and current state of the field in utilizing organoids in PCa research.

Please see below for detailed responses to individual comments from the editor and each reviewer. We have provided the revised manuscript, the original manuscript with tracked changed, and PDF files for Figures 1-5.

Response to comments from the Editor

1. We have thoroughly proofread our manuscript and made changes to reflect appropriate grammar and correct spelling.
2. None of the Figures in our manuscript have been published elsewhere.
3. We have extensively revised and expanded the Discussion section and organized it into the subtitles you have listed (Lines 557-715 of manuscript with tracked changes, Lines 503-644 of revised manuscript).
4. We have downloaded the most updated Endnote file from the JoVE Instructions to Authors

and used this output style to generate our Reference section.
5. We have proofread the Protocol section and removed all personal pronouns.
6. We have replaced all time units in the Protocol section with h, min, and s.
7. We have reworded Step 1.3.2. in the Protocol section using the imperative tense (Lines 187-189 of manuscript with tracked changes, Lines 183-184 of revised manuscript).

Response to comments from Reviewer 1
The authors would like to thank Reviewer 1 for his or her comments, which we have used to revise our manuscript specifically to reflect the capacity of the protocol to apply to any PCa GEMM and the current state of the PCa field in utilizing mouse organoids in research.

Major Concerns:
None

Minor Concerns:
We have removed any discussion of the characteristics or data which are specific to the PCa GEMMs we use in our laboratory. This revision includes removal of Figure 1 from the original manuscript, as this figure depicted the Cre-loxp system and the *mT*/*mG* Cre reporter transgene that are apply specifically to our GEMMs.

We have greatly expanded the Discussion section from 3/4 of a page to 3.5 pages. In our new Discussion, we give several examples of *in vitro* experiments that have been carried out using organoids in PCa research studies—including *in vitro* activation of Cre, expansion of rare cell populations, and analysis of tumor lineage characteristics using immunohistochemistry. In the "Future applications" section of the Discussion, we have highlighted genetic engineering of organoids and its potential for use in mechanistic studies, *in* vivo organoid grafting, and drug screens. We have also emphasized the challenge of mixed prostate organoid cultures that contain normal and cancer organoids and highlighted the FACS sorting approach that was carried out by Agarwal et al. as method to select for tumor cells. We have pointed out in the "Critical steps" section of the Discussion that investigators should genotype organoid to ensure that transgenes expressed in GEMMs, such as probasin-Cre, are maintained in organoid lines.

Finally, we have referenced and discussed all the studies suggested by Reviewer 1; except for PMID 25243035, since we could not locate this reference on Pubmed. If the Reviewer could provide us with the first author, publication year, and journal we will be able to discuss this study as the Reviewer suggested.

Response to comments from Reviewer 2
The authors appreciate the comments made by Reviewer 2 that have benefited our manuscript with revisions that reflect the capacity of the protocol to apply to any PCa GEMM. We agree with Reviewer 2 that specific discussion of the GEMMs we study in our laboratory is irrelevant to the current manuscript. Therefore, we have removed any discussion of characteristics or

data which are specific to the PCa GEMMs we use in our laboratory.

Major Concerns:
None

Minor Concerns:
1. We have added a section in the Introduction section of our manuscript (Lines 90-94 in manuscript with tracked changes, Lines 89-92 in revised manuscript) to emphasize that this protocol is appropriate for any PCa GEMM.

2. As suggested by Reviewer 2, the protocol our manuscript describes is not for creating GEMMs. Consequently, we have removed Figure 1 from the original submission.

3. We have completely removed the paragraph in the "Representative Results" section (Lines 446-454) that described the phenotype of our GEMMs.

Response to comments from Reviewer 3
The comments from Reviewer 3 on our manuscript were detailed and thoughtful—the authors would like to thank him or her, as the revisions made as a results of these suggestions have greatly improved our manuscript.

Major Concerns:
None

Minor Concerns:
1. We have corrected the grammatical errors in Line 177 (Line 191-193 in the manuscript with track changes and Lines 186-188 in the revised manuscript). We have included fluorescent images of the urogenital region from both the ventral and dorsal aspects of a prostate from a 12 week old GEMM which expresses GFP in the prostate.

2. Thanks to this comment, we identified a mistake in the text of this section of the protocol and have corrected it to instruct spinning down the cells suspension, not the matrix, prior to resuspending organoids (Lines 413-418 of the manuscript with tracked changes and Lines 395-402 of the revised manuscript).

3. Both the Pathclear Matrix we describe in our manuscript and the Matrigel (BD Biosciences, cat. no. 356231) from Drost et al. are derived from the soluble basement membrane of a Engelbreth-Holm-Swarm (EHS) mouse sarcoma and are growth factor reduced—these reagents only appear to differ because the companies vary in the naming of their products.

4. We have added a couple sentences to the Discussion commenting on the possibility of genetic or epigenetic changes resulting from prolonged passaging of prostate organoids. As we

write, we are not aware of a published study that has described such changes that occur in organoids over time in culture. We are currently tracking such changes in cancer organoids from our GEMMs, but all of these data are unpublished and yet to be presented.

5. In response to Reviewer 3's comment, we have further explained our distinction between fluid-filled and solid prostate tumors according to our observations in relation to organoid generation (Lines 491-498 in the manuscript with tracked changes, Lines 459-466 in the revised manuscript). We have also added representative images of fluid-filled and solid prostate tumors to Figure 2 and labelled the images for clarity.

6. In response to this comment, we have added a note to the Protocol section that discusses optimal mouse age for isolating prostate tumors for cancer organoid generation (Lines 122-127 in manuscript with tracked changes, Lines 120-125 in revised manuscript). Male mice at least of the age of sexual maturity (8-10 weeks) will be required for expression of many prostate-specific transgenes, including probasin. However, the goal of an individual study and specific *in vivo* characteristics of each PCa GEMM alters the age at which an investigator may isolate prostates for organoid generation.

Response to comments from Reviewer 4

The authors thank Reviewer 4 for his or her detailed comments. As a result of these suggestions, we have made substantial changes to our manuscript that has improved our communication with the reader about aspects of organoid culture that limit the efficacy of this method in PCa research. We have also refined our protocol for dissection of the mouse prostate by adding a section describing removal of non-prostate tissue from the urogenital region.

Major Concerns:

We agree with the Reviewer that our Discussion section was not to the depth required for the purpose of this manuscript. We have expanded the Discussion section of our manuscript from 3/4 of a page to 3.5 pages (Lines 557-715 of manuscript with tracked changes, Lines 503-644 of revised manuscript). We have included a section on outgrowth of normal prostate organoids and selection for basal epithelial cell characteristics over luminal epithelial cell characteristics under prostate organoid culture conditions—the latter of which emphasizes the decreased expression of androgen receptor (AR) in cancer organoid cultures compared to prostate tissue. We have also discussed how mouse organoids have been utilized in recent studies from the PCa field.

Minor Concerns:

1. We have added the duration of CO2 asphyxiation in Section 1.2.1 (Line 157 of the manuscript with tracked changes and Line 153 of revised manuscript).
2. Thank you for catching our proofreading error, we have removed all references to Figure 2C.
3. and 4. We have added an entire section (Step 1.5.5.) describing removal of non-prostate

tissue including the seminal vesicles, vas deferens, bladder, and fatty/connective tissue, to the Protocol section of our manuscript (Lines 307-322 in manuscript with tracked changes, Lines 295-309 of revised manuscript.
5. We have added labels to our representative necropsy images in Figure 3A and 3B of our revised manuscript.

Review of the manuscript has been restricted to the co-authors and colleagues at Roswell Park Comprehensive Cancer. None of the material in the manuscript has been published or submitted for publication elsewhere. All animal work described is approved by the IACUC at Roswell Park and is in accordance with Institutional guidelines.

The authors would like to thank you for considering our revised manuscript for publication in JoVE. Please do not hesitate to contact me with any questions or concerns.

Kindly,

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