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Using the Chicken Chorioallantoic Membrane In Vivo Model to Study Gynecological and Urological Cancers

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TITLE:

Using the Chicken Chorioallantoic Membrane In Vivo Model to Study Gynecological and Urological Cancers

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SUMMARY:

We present the chicken chorioallantoic membrane model as an alternative, transplantable, in vivo model for the engraftment of gynecological and urological cancer cell lines and patient-derived tumors.

ABSTRACT:

Mouse models are the benchmark tests for in vivo cancer studies. However, cost, time, and ethical considerations have led to calls for alternative in vivo cancer models. The chicken chorioallantoic membrane (CAM) model provides an inexpensive, rapid alternative that permits direct visualization of tumor development and is suitable for in vivo imaging. As such, we sought to develop an optimized protocol for engrafting gynecological and urological tumors into this model, which we present here. Approximately 7 days postfertilization, the air cell is moved to the vascularized side of the egg, where an opening is created in the shell. Tumors from murine and human cell lines and primary tissues can then be engrafted. These are typically seeded in a mixture of extracellular matrix and medium to avoid cellular dispersal and provide nutrient support until the cells recruit a vascular supply. Tumors may then grow for up to an additional 14 days prior to the eggs hatching. By implanting cells stably transduced with firefly luciferase, bioluminescence imaging can be used for the sensitive detection of tumor growth on the membrane and cancer cell spread throughout the embryo. This model can potentially be used to study tumorigenicity, invasion, metastasis, and therapeutic effectiveness. The chicken CAM model requires significantly less time and financial resources compared to traditional murine models. Because the eggs are immunocompromised and immune tolerant, tissues from any

organism can potentially be implanted without costly transgenic animals (e.g., mice) required for implantation of human tissues. However, many of the advantages of this model could potentially also turn into limitations, including the short tumor generation time and immunocompromised/immune tolerant status. Additionally, although all tumor types presented here engraft in the chicken chorioallantoic membrane model, they do so with varying degrees of tumor growth.

INTRODUCTION:

Mice have served as the classic model organism for the study of human diseases, including malignancy. As mammals, they share many similarities with humans. Their high degree of genetic similarity has permitted transgenic manipulation of the mouse genome to provide enormous insight into the genetic control of human diseases[1]. Extensive experience in the handling of and experimentation with mice has resulted in their being the model of choice for biomedical research. However, in addition to the ethical and scientific concerns regarding murine models, they can also be quite costly and time consuming to keep[2,3]. The development of tumors can take weeks or even months. The housing at a typical institution alone can run in the hundreds to thousands of dollars while tumors are developing. Ovarian cancer is an example of this drawback, because its growth in murine models can easily take months. Delays in research progress potentially impact ovarian cancer patients' persistently low 5-year survival rate of only 47% (i.e., an increase in survival of only 10% over 30 years)[4]. Similarly, urological cancers (kidney, prostate, and bladder cancers) constitute 19% of all cancer cases in the United States and 11% of cancer-related deaths[4]. Thus, a novel in vivo approach to study gynecological and urological cancers could save a laboratory considerable time, labor, and money, even if this model is only applied to initial screening experiments. Additionally, the resulting acceleration of research findings could significantly impact the 177,000 individuals diagnosed with these cancers annually.

The chicken CAM model offers many advantages that address the aforementioned issues. A popular model to study angiogenesis[5,6], tumor cell invasion[7,8], and metastasis[7,9], the chick embryo CAM model has already been used to study many forms of cancers, including glioma[10-12], head and neck squamous cell carcinoma[13,14], leukemia[15,16], pancreatic cancer[17], and colorectal cancer[18]. Additionally, CAM models have been generated for neuroblastoma[19], Burkitt lymphoma[20], melanoma[21], and feline fibrosarcoma[22]. Prior studies have also presented engraftment of bladder cancer[23] and prostate cancer cell lines[24], but with limited protocol details. Not only are eggs much cheaper than mice, but they also produce highly reproducible results[25,26]. They show fast vasculature development, and tumor engraftment can occur in as quickly as a few days and be visualized longitudinally through the open window. With the 21 day time frame between egg fertilization and hatching, experiments can be completed within a few weeks. Furthermore, the low cost, limited housing needs, and small size readily permit large-scale experiments that would be prohibitive for mouse studies.

Therefore, we sought to optimize the CAM model for the engraftment of gynecological and urological cancers. Due to the immunocompromised status of the early chicken embryo[27], both mouse and human cells can be readily implanted. As such, we have successfully engrafted ovarian, kidney, prostate, and bladder cancers. For each of these tumor types, the CAM readily

accepts established murine and/or human tumor cell lines. Importantly, freshly harvested primary human tumor tissues can also engraft from either digested cells or pieces of solid tissue with high rates of success. Each of these cancer types and cell sources requires optimization, which we share here.

PROTOCOL:

All of the experiments presented herein were reviewed and approved by the appropriate ethical committees at the University of California, Los Angeles (UCLA). The use of deidentified, primary human tumors has been approved by the UCLA Institutional Review Board (Protocol numbers 17-000037, 17-001169, and 11-001363). At UCLA, Animal Research Committee review is not required for experiments using chicken embryos; protocol approval is only required when the eggs will be hatched. However, best practices, such as the AVMA Guidelines for the Euthanasia of Animals, were used to handle chicken embryos ethically and to avoid pain as much as possible. Researchers are urged to verify the oversight requirements at their institution prior to initiating studies using CAM models.

1. Preparing the eggs

1.1. Before receiving the eggs, assemble and equilibrate the egg incubator to 37.8 °C (100 °F) with 60–70% humidity following the manufacturer's instructions.

NOTE: To minimize contamination risks, autoclaved water may be used to control the humidity.

1.2. Upon receiving fertilized, Rhode Island Red chicken eggs from a certified laboratory-grade egg supplier, dry wipe the surface of the shells with paper towels. A lightly dampened paper towel may be used to remove adhered material. Dry immediately.

NOTE: Wetting the shell with liquid below 43.3 °C can introduce bacteria into the egg. If one chooses to wash or disinfect the eggs, the temperature of the liquid must be between 43.3–48.9 °C. Higher temperatures can boil the eggs. The choice of disinfectant should be made based on the microorganisms causing concern.

1.3. Use a pencil or marker to label the date on the egg. This is considered development day 0.

1.4. Place the eggs into the egg incubator and incubate for at least 7 days with rotation to permit CAM development. An automatic rotator may be used, or the eggs may be rotated 180° 2–3x per day.

2. Opening the eggs

NOTE: Opening of the eggs should be done when the CAM has fully developed. This is typically on development day 7 or 8.

2.1. Disinfect a biosafety cabinet with 70% ethanol. Similarly disinfect and place into the biosafety cabinet an egg rack, egg candler, marker, cordless rotary tool with a silicon carbide grinding stone and circular cutting wheel, 18 G needle, pipet controller with quarter-inch vacuum tubing, packing tape, office scissors, curved scissors, Semken (or similar) forceps, cotton balls, and 6 x 7 cm transparent film dressing. Whenever possible, use sterile, disposable, or autoclaved tools.

2.2. Turn off the egg rotator. Place approximately 1–3 eggs into the biosafety cabinet on the egg rack. While in the dark, place the egg candler against the eggshell to identify the air cell. Mark the location of the air cell.

NOTE: The intensity of illumination from the egg candler is crucial for visualizing the interior of the egg. If the air cell or vasculature is consistently difficult to make out, try replacing the egg candler batteries.

2.3. Move the egg candler over the shell to find a large blood vessel network. Rotate the egg if necessary. An ideal vasculature will be branching near the middle of the egg. Use a marker to draw over the vasculature to be used for implantation.

2.4. Turn on the light in the hood. Using a cordless rotary tool fitted with a silicon carbide grinding stone, drill a small hole in the shell directly over the center of the air cell. Drill until most of the shell has been removed, but the white, inner membrane is intact.

NOTE: Drilling over the air cell before targeting the vasculature permits determining the thickness of that particular eggshell over the air cell rather than over the CAM and vasculature. If the CAM or vasculature is disrupted, then the egg is not useable for implantation.

2.5. Drill and open another small hole where the vascular window will be opened as was done for the air cell (step 2.4).

2.6. Using an 18 G needle, gently pierce through the white, inner membrane over the air cell and vasculature. If unable to penetrate the shell, then carefully drill a little more. Ensure that the white, inner membrane (but not the CAM) is disrupted throughout the entirety of the drilled area. Removal of the membrane piece is not necessary.

NOTE: If the CAM or vasculature is disrupted during this step, discard the egg. Damage is evident if there is blood or albumin leaking from a drilled hole.

2.7. Turn off the hood light. Using the egg candler, verify that the air cell was transferred from the end of the egg to the area over the vasculature. If necessary, place the vacuum tubing inserted into a pipet controller around the hole over the original air cell and gently apply suction in short bursts to move the air cell.

2.8. Use a marker to outline the new location of the air cell approximately 0.5 cm inside the air-

CAM boundary. Affix a piece of packing tape just over the new air cell. If necessary, use standard office scissors to trim the tape to an appropriate size.

NOTE: Tape can disrupt airflow through the shell and should not be larger than necessary to fully cover the air cell.

2.9. Return the eggs to the incubator to warm without rotation with the new air cell facing up. Repeat steps 2.2–2.9 for the remaining eggs to be opened.

NOTE: The protocol may be paused here. If unsure of the quality of CAM development, a small number of eggs should proceed through step 2.16 prior to opening the remaining eggs.

2.10. Place approximately 1–3 eggs with relocated air cells into the biosafety cabinet on the egg rack.

NOTE: Care should be taken to avoid introducing contamination into the opened egg. Therefore, always open eggs inside a biosafety cabinet, and use sterile tools and equipment whenever possible.

2.11. Using a cordless rotary tool fitted with a circular cutting wheel, cut a small line over the air cell boundary drawn in step 2.8. Ensure that this is approximately 0.5 cm inside the actual air-CAM boundary to avoid disrupting the CAM or vasculature. Cut completely through the shell but be careful not to penetrate deeply enough to disrupt the CAM or vasculature.

2.12. Using curved scissors, cut around the remaining air cell to create a window in the shell.

NOTE: If blood or membrane is present on the removed shell, then the egg is not suitable for implantation. If a high proportion of the eggs are not cleanly opened, consider waiting at least 1 additional day to open the remaining eggs to permit better CAM formation. If the shells of the remaining eggs have not been pierced, rotation of the eggs within the incubator should be resumed.

2.13. Verify the viability of the embryo. Viable embryos will display extensive vasculature, clear albumin, embryo movement, or a visible heartbeat.

2.14. Using Semken forceps, pull small pieces of cotton from a sterile cotton ball. Gently blot the CAM surface to remove the shell dust and debris.

NOTE: The removal of debris must be performed on the same day that the eggs are opened.

2.15. Cover the shell opening with a one-quarter piece of 6 x 7 cm transparent film dressing.

2.16. Return the eggs to the incubator. Ensure that the egg sits securely with the opened window facing up and the CAM is not touching the transparent film dressing. Use a piece of egg rack, the

edge of the egg rotator, or another suitable item to prop any eggs that keep rolling.

2.17. Repeat steps 2.10–2.16 for all remaining eggs to be opened.

NOTE: The opening of the eggs does not need to be completed on the same day as implantation. Waiting at least 1 day can help eliminate eggs whose viability was compromised by opening the shell.

3. Preparing the cancer cell suspension for transplantation (option 1)

NOTE: This is to be completed just prior to the implantation, which should ideally take place between days 7 and 10. Please see notes at the beginning of step 5 or 6 for further information concerning the implantation date. This approach was used for all the cell lines and cultured kidney cancer tumor digests.

3.1. Thaw the extracellular matrix solution on ice.

3.2. Using mechanical and/or enzymatic digestion, obtain a single cell suspension using a method appropriate for the cell type being implanted.

3.3. Resuspend the total number of cells to be implanted in an appropriate medium for implantation. Implants of 1–2 x 10^6 cells per egg are typical.

NOTE: The medium used for the implantation of the cell lines is typically the complete medium used for culturing the cells, containing FBS or serum replacement. The implantation of tumor digests typically uses the complete medium used for culturing cell lines of the same cancer type. However, adjustments to the medium formulation may be made if needed experimentally. The effects on tumor development and growth would need to be empirically determined.

3.4. Pellet the cells using a centrifuge speed and time appropriate for the cells being used. Typical speeds are 250–300 x *g*, and times are 5–10 min.

3.5. Remove the supernatant from the pelleted cells by pipetting. Mechanically resuspend the cells in the residual medium via flicking or pipetting. Place on ice to cool. Measure the volume of cells and medium using an appropriately sized pipet.

3.6. Calculate implantation volumes such that 1–2 x 10^6 cells are implanted in a volume of 20–100 µL per egg with a final extracellular matrix concentration of 2.7–4 mg/mL protein. Add medium, any desired growth factors or additives, and extracellular matrix solution according to this calculation. Keep on ice until ready to implant.

NOTE: For the calculations in steps 3.3 and 3.6, an extra volume of at least one-half egg implant should be incorporated to ensure an adequate volume for all eggs in the group. For implantation of the ovarian cancer cell lines (i.e., SKOV3 and ID8), 10^6 cells per egg were implanted. For

implantation of the renal cell carcinoma cell line RENCA, cultured cells derived from digested primary human renal cell carcinoma, prostate cancer cell lines (i.e., CWR, C4-2, and MyC-CaP), and bladder cancer cell lines (i.e., HT-1376 and T24), 2 x 10^6 cells were implanted.

4. Preparing tumor pieces for implantation (option 2)

NOTE: This is to be completed just prior to implantation, which should ideally take place between days 7 and 10. Please see notes at the beginning of step 5 or 6 for further information concerning the implantation date. Primary ovarian and bladder cancers were implanted as tumor pieces.

4.1. Thaw extracellular matrix solution on ice. Dilute to a final protein concentration of 2.7–4 mg/mL in an appropriate medium containing any desired growth factors or additives. Keep on ice.

NOTE: Implantation of tumor pieces typically uses the complete medium used for culturing cell lines of the same cancer type. However, adjustments to the medium formulation may be done if needed experimentally. The effects on tumor engraftment and growth would need to be empirically determined.

4.2. Using a scalpel or scissors, excise pieces from the fresh tumor. Ideal sizes range from 2–5 mm on each side. Keep tissues immersed in the medium until ready to implant.

5. Implantation using a nonstick ring (option 1)

NOTE: Cells may be implanted beginning on development day 7 if the CAM is fully developed. Implantation can occur any time prior to hatching that permits adequate time for tumor development and the desired experiment, but note that the embryo's immune cells begin to be present around day 10 postfertilization[27]. Tumor growth rate varies considerably by cell type and needs to be empirically determined for the cell type of interest. The ovarian cancer and the prostate cancer cells were implanted using the nonstick ring method. Note that when a nonstick ring is not available, a pipet tip may be cut to a similar size and used.

5.1. Use 70% ethanol to disinfect a biosafety cabinet and all the required tools: an egg rack, curved iris forceps, nonstick rings (1/4 inch inner diameter), glass stir rod, appropriate volume pipets and tips, 6 x 7 cm transparent film dressing, office scissors, and marker or pencil. Whenever possible, sterile disposable or autoclaved tools should be used.

5.2. Place eggs to be implanted on an egg rack in a biosafety cabinet. Select a manageable number of eggs. Up to six is typical. Avoid allowing the eggs to cool substantially while working with them.

5.3. Remove transparent film dressing from the shell by rolling the edges towards the open window to avoid pulling away pieces of shell. Check that the eggs are viable and healthy. Ideal eggs will have a large vessel in the center of the opened area with smaller vessels branching from

it.

5.4. Using curved iris forceps, place a sterile, nonstick ring onto the CAM over the vessel, ideally over a branch point. Use a sterile glass stir rod to gently abrade the CAM.

5.5. Pipet the cell suspension from step 3.6 into the center of the nonstick ring. Alternatively, use forceps to place a tumor piece from step 4.2 into the center of the nonstick ring and cover with 20–50 μL of the extracellular matrix solution generated in step 4.1.

5.6. Seal the opening with a one-quarter piece of 6 x 7 cm transparent film dressing. Label the eggs with an appropriate implant designation.

NOTE: Numbering the eggs within a group facilitates longitudinal observations.

5.7. Return the egg to the incubator. Ensure that the opening of the shell sits upright and that the egg is secure.

NOTE: Eggs can be returned to an egg incubator without rotation. Alternatively, high postimplant viability can be obtained using a 37–38 °C cell incubator with the CO_2 deactivated and a hygrometer to monitor the humidity, which should be 50%–80%.

6. Implantation without a nonstick ring (option 2)

NOTE: Cells may be implanted beginning on development day 7 if the CAM is fully developed. Implantation can occur any time prior to hatching that permits adequate time for tumor development and the desired experiment but note that the embryo's immune cells begin to be present around day 10 postfertilization[27]. This method was used for implanting the renal cell carcinoma cells and the bladder cancer cells.

6.1. Use 70% ethanol to disinfect a biosafety cabinet and all required tools: appropriate volume pipets and tips, sterile 10 cm tissue culture dishes, egg rack, 6 x 7 cm transparent film dressing, office scissors, and marker.

6.2. Aspirate an inoculation volume of the cell suspension generated in step 3.6 into an appropriately sized pipet tip (200 μL is typical). While holding the top portion of the tip, carefully eject the pipet tip and place horizontally into a sterile, 10 cm tissue culture dish.

6.3. Repeat step 6.2 for all samples within a group with one dish for each group.

6.4. Place tips into a 37 °C incubator for 15–30 min to allow the extracellular matrix to partially polymerize.

6.5. After 15 min of incubation, begin checking for polymerization. A small amount of liquid typically leaks out of the tip when placed onto the dish. Polymerization of this liquid can be used

to estimate the extent of polymerization of the liquid within the tip.

6.6. Place the eggs to be implanted on an egg rack in a biosafety cabinet. Select a manageable number of eggs. Up to six is typical. Avoid allowing the eggs to cool substantially while working with them.

6.7. Remove the transparent film dressing from the shell by rolling the edges towards the open window. This avoids pulling away pieces of shell.

6.8. Check that the eggs are viable and healthy. Ideal eggs will have a large vessel in the center of the opened area with smaller vessels branching from it.

6.9. Place one pipet tip onto an appropriately sized pipet. Depress the plunger to force the partially polymerized cell suspension onto the CAM over a large, well-developed vessel, ideally over a branch point.

6.10. Seal the opening with a one-quarter piece of 6 x 7 cm transparent film dressing.

6.11. Label the egg with an appropriate implant designation.

NOTE: Numbering the eggs within a group facilitates longitudinal observations.

6.12. Return the egg to the incubator. Ensure that the opening of the shell sits upright and that the egg is secure.

NOTE: Eggs can be returned to a dedicated egg incubator without rotation. Alternatively, high postimplant viability can be obtained using a 37–38 °C cell incubator with the CO_2 deactivated and a hygrometer to monitor the humidity, which should be 50%–80%.

6.12. If the implanted cells were stably transduced with the gene encoding firefly luciferase or other imaging factors, then the resulting tumors may be visualized using bioluminescence imaging.

7. Bioluminescence imaging of firefly luciferase marked tumors

NOTE: If the implanted cells were stably transduced with the gene encoding firefly luciferase or other imaging factors, then the resulting tumors may be visualized using bioluminescence imaging. Fluorescence imaging is not recommended on intact eggs due to high background from the eggshell. This is endpoint analysis, as the opening of the shell drastically reduces survival. Tumors may be imaged at any time that is appropriate for experimental needs and the speed of tumor growth. However, on average, eggs hatch 21 days postfertilization. Therefore, development day 18 is an appropriate endpoint to avoid unwanted hatching.

7.1. If necessary, transport eggs to the imaging facility. Tape eggs onto 10 cm tissue culture

dishes. Return them to a 37.8 °C (100 °F) egg incubator with the rotation mechanism removed or deactivated. Humidity is not crucial for transport and imaging.

7.2. Using curved iris forceps, gently push the CAM away from the shell until the CAM is flush with the albumin and embryo. Using wide-tipped specimen forceps, break away pieces of the shell to expand the shell opening adequately for tumor visualization.

7.3. Inject a minimum of 50 μL of 30 mg/mL D-luciferin into the egg albumin. A similar volume of D-luciferin may also be pipetted into the nonstick ring or onto the surface of the CAM in the area containing the implanted cells. This ensures optimal bioluminescence in the absence of an ideal vascular supply. Incubate for 8 min.

7.4. Inject 20–50 μL of isoflurane into the albumin of the egg to anesthetize the egg. Incubate for an additional 2 min. Alternatively, the egg may be placed into an induction chamber containing 2–2.5% vaporized isoflurane. Adequate anesthesia depth is obtained when embryonic movement ceases.

NOTE: Larger volumes of isoflurane (≥100 μL) can be used to euthanize the egg.

7.5. Place eggs into a bioluminescence imaging device and image using appropriate settings as determined by the manufacturer's instructions. For this study, an exposure time of 1 min was used.

7.6. To image the remaining CAM and embryo, open the egg into a 10 cm tissue culture dish.

7.6.1. Grasp the egg with the fingers of both hands on the underside of the egg near the middle of the egg and the thumbs on either side of the shell opening.

7.6.2. Gently pull apart the two halves of the egg with the thumbs while gently pressing into the egg with the fingers.

7.6.3. When the shell is approximately half-separated from the CAM and embryo, flip the egg upside down over a 10 cm tissue culture dish.

7.6.4. Continue to separate the shell halves. If the CAM sticks to the inside of the shell, gently use the fingers to push the CAM away from the shell.

7.6.5. The embryo may be flipped into another dish if desired.

7.6.6. If necessary, additional isoflurane may be administered as in step 7.4.

8. Tumor harvesting

NOTE: Tumors may be harvested at any time that is appropriate for the experimental needs and

the speed of tumor growth. However, on average, eggs hatch 21 days postfertilization. Therefore, development day 18 is an appropriate endpoint to avoid unwanted hatching.

8.1. Grasp tumor with forceps. Using scissors (spring iris scissors work well) or a scalpel, carefully excise tumor from CAM.

8.2. If the tumor has been transduced with the firefly luciferase gene, reimaging may be performed to verify successful removal of difficult-to-visualize tumors.

NOTE: Excised tumors may be analyzed by any method appropriate for a particular experiment. Tumors may also be reimplanted into CAM or mice. To confirm tumor identity, tumors may be fixed, paraffin embedded, and examined with hematoxylin and eosin or immunohistochemical staining.

REPRESENTATIVE RESULTS:

Thus far, we have found this method of implantation to be successful for ovarian, kidney, prostate, and bladder cancers. Each was optimized to identify specific conditions for implantation, although there may be flexibility. Of the tested tumor types, ovarian cancer growth was much less pronounced and typically not visible without the assistance of bioluminescence imaging (**Figure 1**). However, a stiffening of the CAM could be felt with forceps in the area of implantation. This may help identify possible tumor growth in the absence of bioluminescence imaging, though further validation would be required via histology or another suitable method. We achieved successful engraftment of both human and murine cell lines that show morphologies consistent with those seen in other in vivo models (**Figure 1A** and **1B**). In addition, the implantation of tumor pieces was possible (**Figure 1C**, blue arrow indicates tumor). The implanted tumor in this case came from a patient with high-grade, metastatic, ovarian serous carcinoma. The resulting tumors morphologically resembled their tumors of origin and expressed human-specific proteins, such as cytokeratin 8/18, that readily permit their differentiation from CAM and chicken embryonic tissues.

When optimizing tumor engraftment and growth, appropriate growth factors or hormones may be added at the time of implantation. For example, a subtle, nonsignificant increase in tumor size (as measured by total radiance flux) was observed in ID8 cells when supplemented with three units (U) of human follicle stimulating hormone (FSH) at the time of implantation (**Figure 1D**). The selection of FSH for ovarian cancer growth stemmed from the expression of the FSH receptor in ID8 cells and the high levels of FSH typically found in postmenopausal women, who constitute the majority of ovarian cancer cases. Similar approaches may be adopted for other, difficult-to-grow cancer types to enhance the utility of the CAM model. Furthermore, FSH was only added at the time of cell implantation. Replenishing the growth factor or hormone could be accomplished by pipetting an appropriate amount of the additive in the medium into the nonstick ring at appropriate intervals, which could enhance the effect.

For kidney cancer, implantation of 2×10^6 clear cell renal cell carcinoma cells from established cell lines, such as RENCA, produced rapid, robust tumor formation, though lower cell doses may

also be used (**Figure 2A**, 10 days postimplantation). Resulting tumors morphologically resembled those obtained through standard mouse in vivo models[28]. Implantation of RENCA cells marked with firefly luciferase permitted bioluminescence imaging. Primary human tumors may also be implanted. The pieces of tumor persisted and recruited vasculature without showing significant growth. Implantation of digested cells originating from a primary, clear cell renal cell carcinoma that were expanded in vitro, however, grew similarly to the established cell lines (**Figure 2B**). Renal cell carcinoma cells may be seeded using either the nonstick ring method or the method without the nonstick ring.

Multiple human and murine prostate cancer cell lines were tested for CAM engraftment (**Figure 3**). Each grew well when 2 x 10^6 cells were implanted using the nonstick ring. Histological evaluation of the resulting tumors was consistent with expectations for each cell line. Additionally, implantation of cells stably marked with firefly luciferase permitted tumor identification with bioluminescence imaging (**Figure 3A**).

Bladder cancer was established in the CAM model from established cell lines and pieces of primary human tissue (**Figure 4**). Cell lines grew well when implanted with 2 x 10^6 cells without the nonstick ring (**Figure 4A and 4B**), although implantation with the ring was successful. Primary human tumor may be implanted from pieces of tissue (**Figure 4C**). The presented case originated from a patient with high grade, non-muscle-invasive, urothelial carcinoma. Implantation of digested cells has not yet been attempted due to ongoing tumor digestion optimization. Cancer cells of the resulting CAM tumors retained the morphology of the original tumor, but with an altered stromal component. Tumor growth was less than for renal cell carcinoma and prostate cancer, though still readily visible.

To ensure a high number of viable, assayable eggs at the endpoint, care must be taken when incubating and opening the eggs. If the incubator conditions are suboptimal either before or after implantation, then embryo viability may be compromised. If significant embryonic death occurs, troubleshoot the incubator conditions according to the manufacturer's instructions. In our experience, temperature and humidity stability appear to be more crucial than their exact values. Therefore, we found that incubating the inoculated eggs in a modified, cell-culture, CO_2 incubator achieved superior viability over retaining the eggs in small, dedicated egg incubators that require more frequent opening to replenish the water that controls the humidity. Minimizing the frequency with which the inoculated eggs are removed for tumor examination may also enhance embryo viability.

An additional concern of CAM implantation is the integrity of the CAM at inoculation. If the CAM is not intact after opening the shell, then the nonstick ring and implanted cells will sink into the albumin of the embryo (see the note after step 2.12). This causes cancer cell dispersal. Some tumors may still form, but cancers that receive significant growth and survival signaling from neighboring cancer cells, such as ovarian cancer, will not reliably form tumors under such conditions. If nonstick rings are used for implantation, the failure of the CAM can be identified by the sinking of the ring into the albumin. This must be distinguished from cases in which the ring and implanted cells were moved to the bottom of the egg by embryonic movement. In those

cases, the ring will be found between the CAM and the underside of the shell. Embryonic movement cannot be controlled. However, ring placement can make it more difficult for the embryo to disrupt the implanted cells. Rings placed at the edges of the air pocket generated in step 2.7 are more likely to be moved by the embryo than those placed at the center of the field.

FIGURE AND TABLE LEGENDS:

Figure 1: Representative tumor development from ovarian cancer. Ovarian cancer cells successfully implanted include: (**A**) the human SKOV3 cell line, (**B**) the murine ID8 cell line, and (**C**) primary tumors. Representative hematoxylin and eosin staining is presented along with bioluminescence imaging, as appropriate. For the CAM tumor resulting from the implantation of a primary tumor piece (**C**) hematoxylin and eosin staining of both the CAM tumor and primary tumor are included, along with cytokeratin 8/18 (CK 8/18) staining of the resulting CAM tumor. The blue arrow in the first panel indicates the tumor. (**D**) Tumor size of ID8 implants with and without 3U FSH, calculated as the total flux resulting from bioluminescence imaging (n = 3 for untreated and n = 4 for FSH-supplemented).

Figure 2: Representative tumor development from clear cell renal cell carcinoma. Tumors resulting from implantation of (**A**) the murine renal cell carcinoma cell line, RENCA, or (**B**) cultured cells derived from a digested human primary tumor. The measurement scale adjacent to the excised tumor in (**B**) shows 1 mm markings. Corresponding histology in (**A**) and (**B**) shows hematoxylin and eosin staining. In (**A**), the representative bioluminescence imaging of firefly-luciferase-marked RENCA cells is also shown.

Figure 3: Representative tumor development from prostate cancer cell lines. Representative tumors and hematoxylin and eosin staining resulting from the implantation of the human prostate cancer cell lines (**A**) CWR and (**B**) C4-2 along with the murine cell line (**C**) MyC-CaP. Bioluminescence imaging of the resulting tumors from firefly luciferase marked CWR cells is shown in (**A**). The measurement scale adjacent to the excised tumors shows 1 mm markings.

Figure 4: Representative tumor development from bladder cancer. Representative tumors resulting from the implantation of the established human bladder cancer cell lines (**A**) HT-1376, (**B**) T24, and (**C**) primary human bladder tumor. In (**C**), hematoxylin and eosin staining of the resulting CAM tumor and the primary tumor are shown. The measurement scale adjacent to the excised tumors shows 1 mm markings.

DISCUSSION:

Tumor expansion and engraftment using the CAM model permits more rapid and directly observable tumor growth than existing in vivo animal models. In addition, costs are significantly lower once the initial purchase of equipment is complete, especially when compared to the cost of immunocompromised mice. The initial, immunocompromised state of chicken embryos readily permits engraftment of human and murine tissue. Even with these strengths, the CAM model does have limitations. The short time that can be a benefit could also be a detriment if long-term treatment studies are warranted. The immunocompromised/immune tolerant status of the CAM model could complicate studies of tumor-immune interactions. For these studies, coimplantation

of immune cells of interest may be necessary, possibly with replenishment of the immune compartment. Furthermore, although all the tumor types we presented engraft into the CAM model, they do so with varying degrees of growth. For example, renal cell carcinoma rapidly forms large tumors, but ovarian cancer tumors are difficult to visualize without the assistance of bioluminescence imaging. Evaluating tumor growth and speed would need to be assessed for a particular tumor type before determining if CAM would be a suitable model for the experimental needs.

Successful tumor engraftment requires the careful completion of specific steps of the protocol. First, eggs need to be incubated under ideal conditions to have optimal embryo survival and CAM formation prior to engraftment. Due to the natural variability in batches, we suggest obtaining excess eggs from the specific egg supplier. We have also found that cooler, rainier weather can lead to fungal growth in the eggs. During wetter weather, more eggs may need to be engrafted to obtain adequate yields at the endpoint. This seasonal variation is likely to be region-specific. Adequate CAM formation is essential for successful tumor engraftment. Any indications of the CAM remaining attached to the shell when opening should not be ignored. If several eggs fail to have intact CAM when opening the first batch, then we recommend delaying the opening of the remaining eggs for at least 1 additional day.

If poor viability or engraftment are obtained, several steps could be at fault. First, the embryo viability from the supplier may be poor. Absence of an air cell and visible vasculature indicates a nonviable egg. Sometimes embryo movement can be visualized to confirm viability. First, though, ensure that fresh batteries are in the egg candler, because a strong light is needed for visualization. The float test may also be done to assess viability (a variety of instructions and videos are available online). Another possible explanation for poor viability is the incubator. Using an independent hygrometer and thermometer, ensure that the settings are accurate and stable. Manufacturer's instructions for the incubator should contain detailed troubleshooting instructions to assess proper settings. Improper incubator settings could also compromise CAM development. Using tape and/or a marker, determine if the egg rotator is actually spinning the eggs. If nonstick rings sink into the albumin in a high proportion of the engrafted eggs, then the CAM did not adequately develop. Finally, we have found that frequent checking of the engrafted egg, leading to temperature and humidity fluctuations, may decrease postimplant viability. If implanted eggs are initially viable, but the viability decreases throughout the engraftment period, the eggs should be checked less frequently. This decrease in viability could also be due to inaccurate or inappropriate incubator settings after implantation.

When optimizing this method for a new cell type, several factors can be controlled. The first is cell number. We typically implant 5×10^5–2×10^6 cells from cell lines. Implanted tumor pieces are typically 2–5 mm on each side. These amounts may be adjusted to improve engraftment or size. However, we have found that tumor growth lessens above a certain threshold, which is cell type dependent. Additional engraftment parameters include the presence or absence of a nonstick ring. This choice is typically made based on whether the ring will hinder the endpoint analysis, although it may also influence successful engraftment. The presence of the nonstick ring may also permit covering the nascent graft with medium at desired intervals to improve survival prior

to vascular recruitment, if so desired. The choice of extracellular matrix type, concentration, and medium in which the matrix is diluted may also impact engraftment. The addition of growth factors or hormones may further improve tumor take rate or size. The selection of additives and concentrations would need to be done based on what would be appropriate for the specific cell type and not interfere with the experiment. These would also have the potential to be replenished at intervals if a nonstick ring is used.

Future applications of this model system depend on the hypothesis to be tested. For example, these tumor grafts could be used to test novel therapeutic approaches to reduce tumor size or deplete a subpopulation of the tumor. Co-implantation with cells from the host tumor microenvironment could permit studies of the influence of these cells on a variety of parameters, including tumor growth and treatment resistance. This model could also serve as an opportunity to incrementally expand primary human tumors prior to establishing xenografts in immunocompromised animal models. The initial adaptation to CAM engraftment could perhaps facilitate tumor take rate in murine models. These applications have yet to be tested, but warrant further exploration.

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DISCLOSURES:

The authors have nothing to disclose.

REFERENCES:

1. Kersten, K., de Visser, K. E., van Miltenburg, M. H., Jonkers, J. Genetically engineered mouse models in oncology research and cancer medicine. *EMBO Molecular Medicine.* **9** (2), 137–153 (2017).
2. Jackson, S. J., Thomas, G. J. Human tissue models in cancer research: looking beyond the mouse. *Disease Models & Mechanisms.* **10** (8), 939–942 (2017).
3. Cheon, D.-J., Orsulic, S. Mouse Models of Cancer. *Annual Review of Pathology: Mechanisms of Disease.* **6** (1), 95–119 (2011).
4. Howlader, N. et al. SEER Cancer Statistics Review, 1975-2016. *National Cancer Institute. Bethesda, MD, https://seer.cancer.gov/csr/1975_2016/, based on November 2018 SEER data submission, posted to the SEER web site, April 2019.* (2019).
5. Ribatti, D. Chicken chorioallantoic membrane angiogenesis model. *Methods Molecular*

Biology. **843** 47–57 (2012).
6. Nowak-Sliwinska, P., Segura, T., Iruela-Arispe, M. L. The chicken chorioallantoic membrane model in biology, medicine and bioengineering. *Angiogenesis.* **17** (4), 779–804 (2014).
7. Lokman, N. A., Elder, A. S., Ricciardelli, C., Oehler, M. K. Chick chorioallantoic membrane (CAM) assay as an in vivo model to study the effect of newly identified molecules on ovarian cancer invasion and metastasis. *International Journal of Molecular Science.* **13** (8), 9959–9970 (2012).
8. Xiao, X. et al. Chick Chorioallantoic Membrane Assay: A 3D Animal Model for Study of Human Nasopharyngeal Carcinoma. *PLoS ONE.* **10** (6), e0130935 (2015).
9. Deryugina, E. I., Quigley, J. P. Chick embryo chorioallantoic membrane model systems to study and visualize human tumor cell metastasis. *Histochemistry and Cell Biology.* **130** (6), 1119–1130 (2008).
10. Shoin, K. et al. Chick Embryo Assay as Chemosensitivity Test for Malignant Glioma. *Japanese Journal of Cancer Research.* **82** (10), 1165–1170 (1991).
11. Hagedorn, M. et al. Accessing key steps of human tumor progression in vivo by using an avian embryo model. *Proceedings of the National Academy of Science U S A.* **102** (5), 1643–1648 (2005).
12. Kavaliauskaitė, D. et al. The Effect of Sodium Valproate on the Glioblastoma U87 Cell Line Tumor Development on the Chicken Embryo Chorioallantoic Membrane and on EZH2 and p53 Expression. *BioMed Research International.* **2017**, 12 (2017).
13. Liu, M. et al. The Histone Methyltransferase EZH2 Mediates Tumor Progression on the Chick Chorioallantoic Membrane Assay, a Novel Model of Head and Neck Squamous Cell Carcinoma. *Translational Oncology.* **6** (3), 273–281 (2013).
14. Rudy, S. F. et al. In vivo Wnt pathway inhibition of human squamous cell carcinoma growth and metastasis in the chick chorioallantoic model. *Journal of Otolaryngology - Head & Neck Surgery.* **45** (1), 26 (2016).
15. Canale, S. et al. Interleukin-27 inhibits pediatric B-acute lymphoblastic leukemia cell spreading in a preclinical model. *Leukemia.* **25**, 1815 (2011).
16. Loos, C. et al. Amino-functionalized nanoparticles as inhibitors of mTOR and inducers of cell cycle arrest in leukemia cells. *Biomaterials.* **35** (6), 1944–1953 (2014).
17. Rovithi, M. et al. Development of bioluminescent chick chorioallantoic membrane (CAM) models for primary pancreatic cancer cells: a platform for drug testing. *Scientific Reports.* **7**, 44686 (2017).
18. Majerník, M. et al. Novel Insights into the Effect of Hyperforin and Photodynamic Therapy with Hypericin on Chosen Angiogenic Factors in Colorectal Micro-Tumors Created on Chorioallantoic Membrane. *International Journal of Molecular Science.* **20** (12), 3004 (2019).
19. Swadi, R. et al. Optimising the chick chorioallantoic membrane xenograft model of neuroblastoma for drug delivery. *BMC Cancer.* **18** (1), 28 (2018).
20. Klingenberg, M., Becker, J., Eberth, S., Kube, D., Wilting, J. The chick chorioallantoic membrane as an in vivo xenograft model for Burkitt lymphoma. *BMC Cancer.* **14** (1), 339 (2014).
21. Avram, S. et al. Standardization of A375 human melanoma models on chicken embryo chorioallantoic membrane and Balb/c nude mice. *Oncology Reports.* **38** (1), 89–99 (2017).
22. Zabielska-Koczywas, K. et al. 3D chick embryo chorioallantoic membrane model as an in vivo model to study morphological and histopathological features of feline fibrosarcomas. *BMC*

Veterinary Research. **13** (1), 201 (2017).
23. Skowron, M. A. et al. Applying the chicken embryo chorioallantoic membrane assay to study treatment approaches in urothelial carcinoma. *Urologic Oncology: Seminars and Original Investigations.* **35** (9), 544.e511–544.e523 (2017).
24. Jefferies, B. et al. Non-invasive imaging of engineered human tumors in the living chicken embryo. *Scientific Reports.* **7** (1), 4991 (2017).
25. Taizi, M., Deutsch, V. R., Leitner, A., Ohana, A., Goldstein, R. S. A novel and rapid in vivo system for testing therapeutics on human leukemias. *Experimental Hematology.* **34** (12), 1698–1708 (2006).
26. Strojnik, T., Kavalar, R., Barone, T. A., Plunkett, R. J. Experimental model and immunohistochemical comparison of U87 human glioblastoma cell xenografts on the chicken chorioallantoic membrane and in rat brains. *Anticancer Research.* **30** (12), 4851–4860 (2010).
27. Ribatti, D. The chick embryo chorioallantoic membrane as a model for tumor biology. *Experimental Cell Research.* **328** (2), 314–324 (2014).
28. Hu, J. et al. A Non-integrating Lentiviral Approach Overcomes Cas9-Induced Immune Rejection to Establish an Immunocompetent Metastatic Renal Cancer Model. *Molecular Therapy - Methods & Clinical Development.* **9**, 203–210 (2018).

Figure 1

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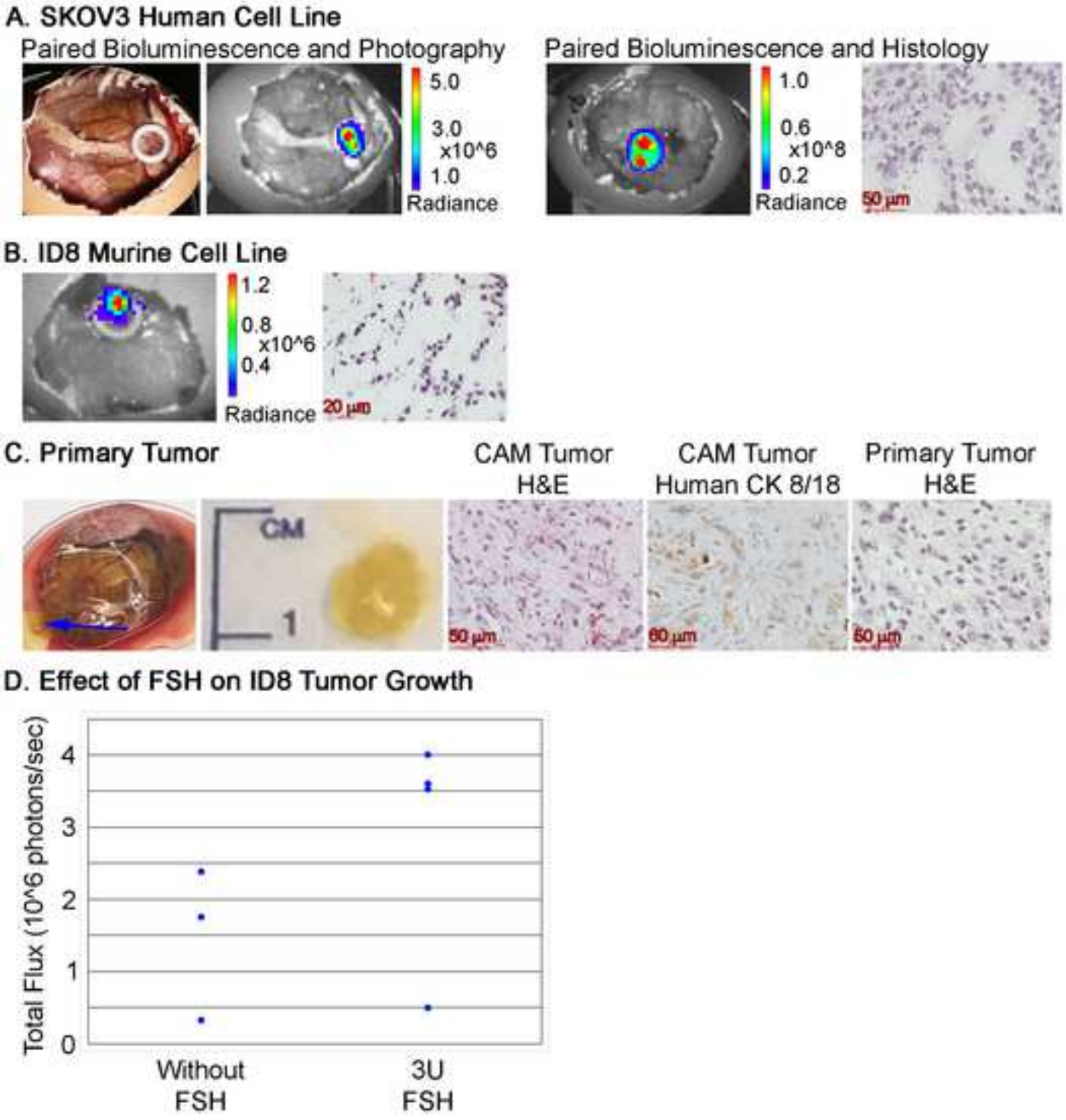

Figure 2

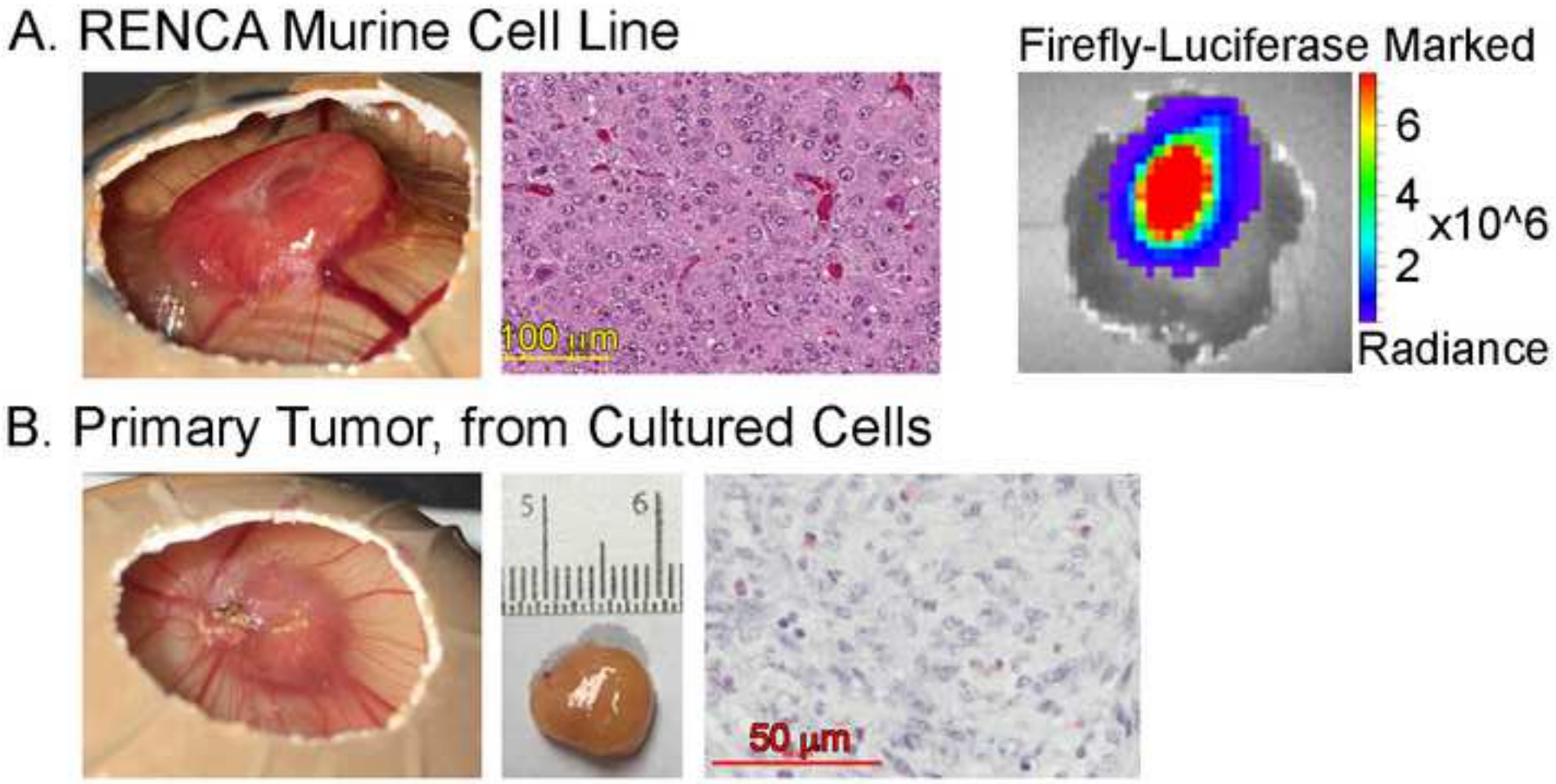

Figure 3

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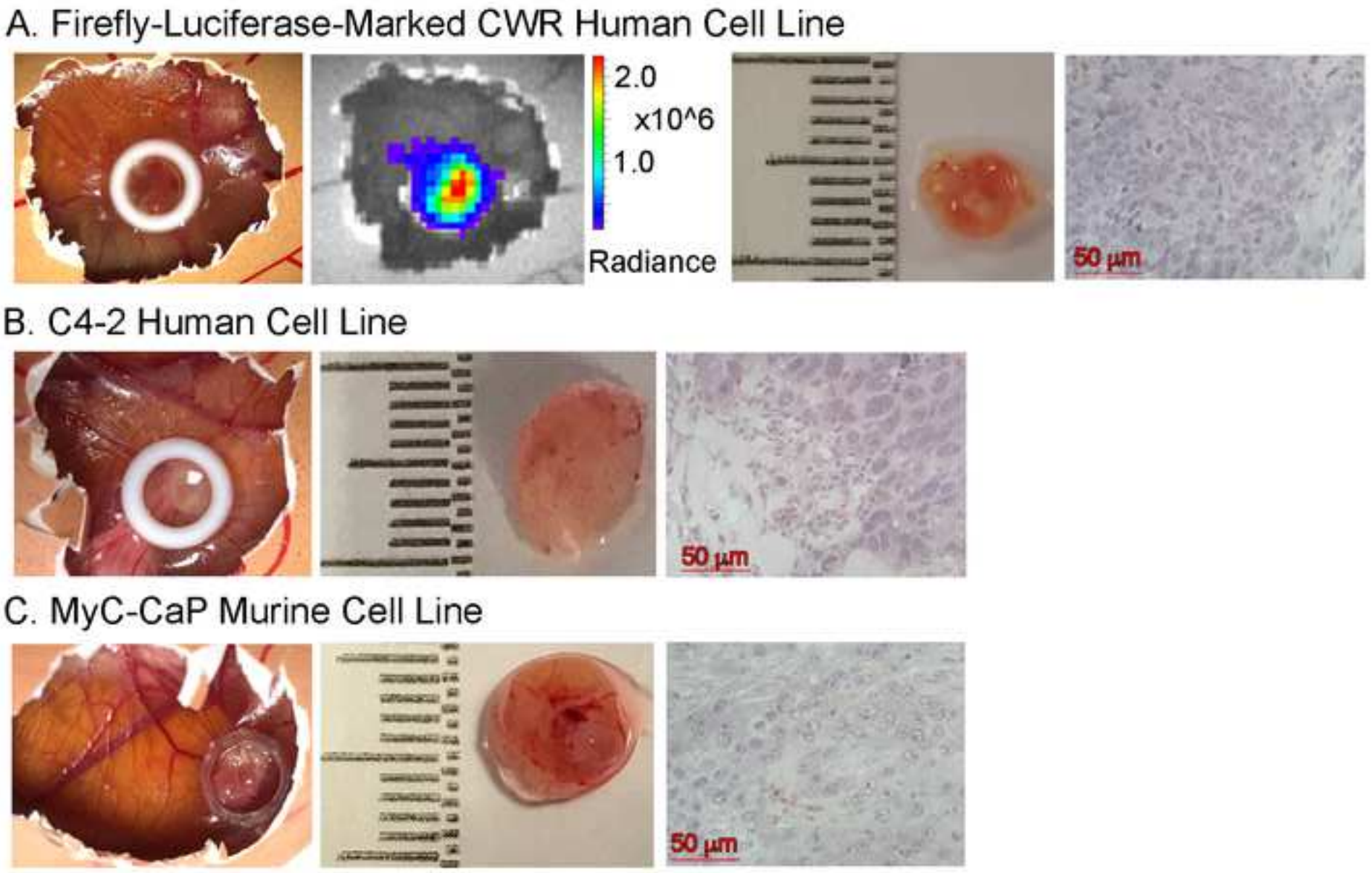

Figure 4

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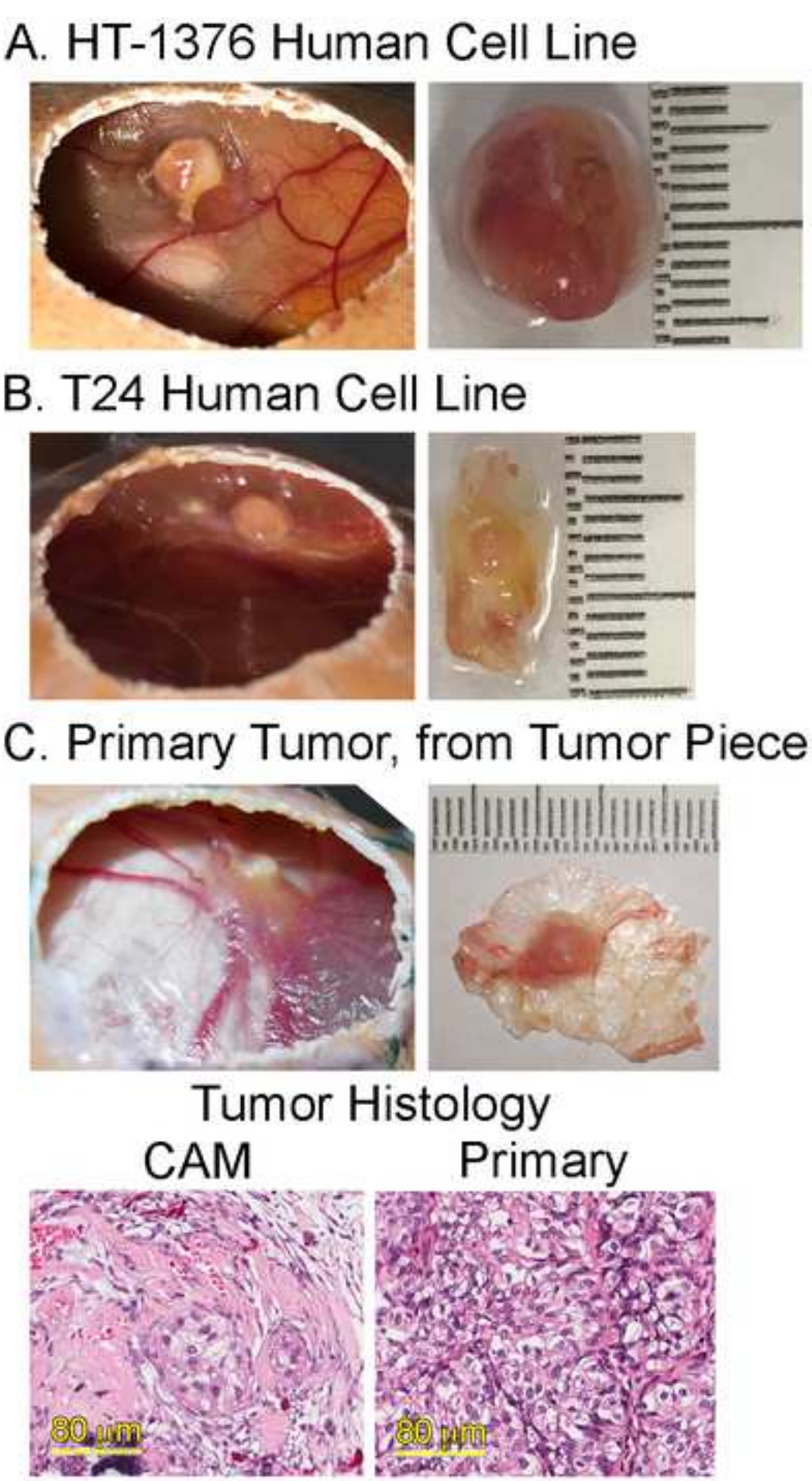

Name of Material/ Equipment

-010 Teflon (PTFE) White 55 Duro Shore D O-Rings

C4-2

CWR22Rv1

Cytokeratin 8/18 Antibody (C-51)

D-Luciferin Firefly, potassium salt

Delicate Operating Scissors; Curved; Sharp-Sharp; 30mm Blade Length; 4-3/4 in. Overall Length

Dremel 8050-N/18 Micro 8V Max Tool Kit

Fertilized chicken eggs (Rhode Island Red - Brown, Lab Grade)

HT-1376

Hovabator Genesis 1588 Deluxe Egg Incubator Combo Kit

ID8

Incu-Bright Cool Light Egg Candler

Iris Forceps, 10cm, Curved, Serrated, 0.8mm tips

Isoflurane

IVIS Lumina II In Vivo Imaging System

Matrigel Membrane Matrix HC; LDEV-Free

MyC-CaP

Portable Pipet-Aid XP Pipette Controller

PrecisionGlide Hypodermic Needles

RENCA

Semken Forceps

SKOV3

Specimen forceps

Sterile Cotton Balls

Stirring Rods with Rubber Policeman; 5mm diameter, 6 in. length

T24

Tegaderm Transparent Dressing Original Frame Style 2 3/8" x 2 3/4"

Tissue Culture Dishes, 10 cm diameter

Tygon Clear Laboratory Tubing - 1/4 x 3/8 x 1/16 wall (50 feet)

Company	Catalog Number
The O-Ring Store	TEF010
ATCC	CRL-3314
Novus Biologicals	NBP2-44929-0.02mg
Goldbio	LUCK-1G
Roboz Surgical	RS6703
Dremel	8050-N/18
AA Lab Eggs Inc.	N/A
ATCC	CRL-1472
Incubator Warehouse	HB1588D-NONE-1102-1588-1357
Incubator Warehouse	1102
World Precision Instrument	15915
Clipper Distributing	0010250
Perkin Elmer	
Corning	354248
ATCC	CRL-3255
Drummond Scientific	4-000-101
BD	305196
ATCC	CRL-2947
Fine Science Tools	11008-13

ATCC	HTB-77
Electron Microscopy Sciences	72914
Fisherbrand	22-456-885
United Scientific Supplies	GRPL06
ATCC	HTB-4
Moore Medical	21272
Corning	353803
Tygon	AACUN017

Comments

Nonstick ring for cell seeding. 1/4"ID X 3/8"OD X 1/16"CS Polytetrafluoroethylene (PTFE).

Human prostate cancer cell line.

CWR cells were the kind gift of Dr. David Agus (Keck Medicine of University of Southern California)

Used at a dilution of 1:100 for immunohistochemical analysis of human ovarian CAM tumors.

This is provided as an example. Any similar curved scissors would work as well.

This kit contains all necessary tools.

A local egg supplier would need to be identified, as this supplier only delivers regionally.

Human bladder cancer cell line.

Other egg incubators may be used, but their reliability would need to be verified. After implantation, a cell incubator with the CO2 disabled may also be used.

Not commercially available, please see PMID: 10753190.

Other candlers may be used; however, this is preferred among those that we have tested. This candler is included in the aforementioned incubator kit.
This is provided as an example. Any similar curved forceps would work as well. Multiple brands have been used for this method.

Extracellular matrix solution

Murine prostate cancer cell line.

Any similar pipet controller would be appropriate.

This is provided as an example. Any 18G needle would work similarly.

This is provided as an example. Any similar forceps or another style that suits researcher preference would be appropriate.

Human ovarian cancer cell line.

This is provided as an example. The forceps used for pulling away the shell for bioluminescence imaging are approximately 12.8 cm long with 3 mm-wide tips.

This is provided as an example. Any sterile cotton balls would suffice.

This is provided as an example. Any similar glass stir rods would work as well.

Human bladder cancer cell line.

This is provided as an example. Any similar, sterile 10-cm dish may be used. Tissue culture treatment is not necessary.

This is provided as an example. Any similarly sized tubing would work as well.

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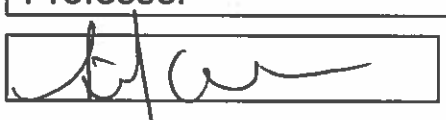
Signature:	
Date:	Aug 7, 2019

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We would like to take this opportunity to thank the editor and four reviewers for the insightful comments and suggested corrections that have helped to strengthen our manuscript. Major changes include the restructuring of the Representative Results and Figure Legends to prioritize the findings of the study as well as ensure that information is included in the appropriate areas. At the direction of Reviewers #1 and #4, additional references have been added to the Introduction. Detailed responses to each comment are included below in italics. We hope that we have appropriately addressed all of the recommended improvements.

Editorial comments:

Changes to be made by the Author(s):

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

We appreciate this final opportunity to copyedit our manuscript.

2. Please include an ethics statement to show that the study follows the guidelines of institutional ethics committee.

Information concerning IRB and ARC guidelines and oversight have been added to the beginning of the Protocol section.

3. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Dremel Micro rotatory kit, IVIS Lumina II In Vivo Imaging 251 System,

Thank you for detecting those oversights. We thought that we avoided all brand names, but clearly missed a few. Dremel Micro has been corrected to cordless rotary tool, the specific imaging system has been removed from the protocol, and Tygon tubing has been corrected to vacuum tubing.

4. Please revise the protocol text to avoid the use of any personal pronouns in the protocol (e.g., "we", "you", "our" etc.).

Instances in which "we" was used have been appropriately revised.

5. Please add more details to your protocol steps. Please ensure you answer the "how" question, i.e., how is the step performed?

The protocol has been checked and any steps that appeared to insufficiently address the "how" question have been expanded upon.

6. 1. Please specify these are chicken eggs in the protocol section.

Step 1.2 has been revised to incorporate this information.

7. 1.4: Please include the speed of rotation.

The rotation speed of our automatic rotators is not provided by the manufacturers. However, specific instructions for manual rotation have been provided in an effort to address this question.

8. 3.2: Please include the source of the cells to be implanted. How do you obtain single cell suspension?

Thank you for identifying this oversight. Sources of cell lines have been added to the Materials List. The single cell suspension generation should be tailored to the cells to be used, such as the specific cell line or generating a tumor digest. Step 3.2 has been expanded to specify mechanical and/or enzymatic digestion; however, the specifics for all of the cell types presented in this protocol would exceed the maximal permissible protocol length.

9. 3.3. Please provide the total number of cells used in your case.

Cell numbers varied by cell lines, which is discussed in more detail in the Representative Results section. The typical range used has been added to step 3.3 of the protocol.

10. 3.4: Please include the centrifuge speed and time.

These parameters vary by cell type, but typical ranges have been added to step 3.4 of the protocol.

11. 3.8: Please include volumes and concentrations of all the solutions/reagents used

We apologize that the phrasing here was unclear as to what volumes and concentration were used. This step has been revised to improve clarity.

12. 6.9: Which vessel?

This step has been clarified to indicate that the cells should be implanted over a "large, well-developed vessel."

13. 7: Please include the time of incubation prior to bioluminescence imaging.

The note at the beginning of step 7 has been expanded to discuss what timings for imaging are appropriate.

14. 7.4: How long do you incubate the cells for after D luciferin injection?

The eggs are incubated for a total of 10 minutes: 8 minutes in step 7.4 and 2 minutes in step 7.5.

15. 7.5: how do you check the depth of anesthesia?

The following information has been added to step 7.5: "Adequate anesthesia depth is obtained when embryonic movement ceases."

7.6: What is the appropriate setting? Please include all the button clicks, the knob turns if any. If using the settings provided as per manufacturer's guidelines, please mention.

We have included the phrase "as determined by manufacturer instructions" to step 7.6. Further details would likely be dependent on the specific manufacturer of the imaging device. The exposure time used was added, though, to aid with adapting this protocol to any desired imaging system.

17. 7.7: We cannot have a paragraph of text in the protocol section. Please make substeps.

The content previously contained in step 7.7 has been expanded into substeps 7.7.1 through 7.7.6.

18. 8: After how many days tumor harvesting is performed?

The following information has been added to a note at step 8: "Tumors may be harvested at any time that is appropriate for one's experimental needs and the speed of tumor growth. However, eggs hatch 21 days postfertilization, on average. Therefore, day 18 postfertilization is an appropriate maximal time to avoid unwanted hatching."

19. Please describe the result with respect to your experiment, you performed an experiment, how did it help you to conclude what you wanted to and how is it in line with the title. e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc? The text should refer to all the figures. Data from both successful and sub-optimal experiments can be included.

The representative results section has been edited to prioritize the successful engraftment of the varied cell types, the results of this analysis.

20. Each Figure Legend should include a title and a short description of the data presented in the Figure and relevant symbols. The Discussion of the Figures should be placed in the Representative Results. Details of the methodology should not be in Figure Legends, but rather the Protocol

Thank you for the clarification, specific methodology contained in the Figure Legends has been moved to the appropriate locations within the Protocol Section. Any discussion of findings that had previously been included in the Figure Legends have been moved to the Representative Results section.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

In this manuscript the authors describe the use of the CAM model in different settings for monitoring tumor growth.

Minor Concerns:

Since there are several groups working with this model in genitourinary cancer, it might be of benefit to the reader to learn more about cell lines that form tumors and variations of this systems that have been used for prostate and bladder cancer already. I would like to ask the authors to include the

following or other suitable publications: PMID: 28551413, PMID: 28694510, PMID: 11547121

We thank Reviewer #1 for identifying these publications for addition to our introduction. Two of these have been added. We elected not to include the third as the full-text was not available for us to read.

Reviewer #2:

Manuscript Summary:

Overall, a well written manuscript with different method of implantation into the CAM was described in great detail. The strength of the paper is also looking at different tumour types and perform comparison with established cell lines, primary cell and primary tumour. The technical difficulties and challenges in the CAM experimental setup was also discussed throughout the manuscript. A few major and minor concerns could be address to further improve the manuscript.

Major Concerns:

1. A supplementary figure with the images of the steps for preparing and opening eggs procedure will be helpful to the readers.

We appreciate Reviewer #2's suggestion for a supplementary figure and agree that visualization of the steps will be important. However, we believe that the visual representation of the protocol will be much clearer in the video professionally produced by JoVE. As much of the work is done in the dark, our photographs would not successfully show the necessary information.

2. Figure 1C, 2B and 4C: primary tumour: Need to include information in regards to the source and clinicopathological detail (tumour type, stage etc) of patient which the tumour was derived from? Need to include details of human ethics application details as well.

Thank you for identifying this oversight. The available clinical details of the tumors have been added to the Representative Results section. UCLA IRB approval has been added to the beginning of the Protocol section.

3. Details of which day the cells on tumour was implanted and harvested should be mention in the figure legend for all Figure 1-4 or in the text. The duration of the treatment for the FSH should be mention as well in the paper and how as the end time point chosen?

Implantation time is discussed in the note at the beginning of protocol section 5. The endpoint for analysis has been added to notes at the beginning of Protocol sections 7 and 8. All data presented in this paper is in keeping with these guidelines. The duration of FSH supplementation is discussed in the Representative Results section in stating that the hormone was only supplemented at implantation. Unfortunately, the half-life of the hormone in the CAM setting is unknown, making us unable to specify the duration of exposure of the cells to FSH.

Minor Concerns:

1. Line 78: Need to mention what type/species of chicken eggs? Fertilized white leghorn chicken eggs?

The species, Rhode Island Red, has been added to step 1.2.

2. Could include details at which embryonic development day that the transplantation and implantation could be perform. A timeline summary as a supplementary figure will be helpful as well.

Details of ideal implantation times have been added to notes at the beginning of Protocol sections 5 and 6.

3. Could comment about conducting experiment in a sterile environment with autoclave tools or autoclave water added for the incubator? Mention the possibility of eggs could be infected during the incubation?

Use of sterile, autoclaved tools has been added to steps 2.1 and 5.1. The use of autoclaved water for the incubator has been added to a note after step 1.1. The importance of not introducing contamination into the opened egg is now mentioned in a note after step 2.13.

4. Comment of animal ethics required to conduct the CAM experiment?

The ethical and regulatory framework for conducting CAM experiments has been added to the beginning of the Protocol section.

5. Line 150: Give examples of extracellular matrix solution that can be used here ie. Matrigel, geltrex?

As per JoVE guidelines, all commercial brands are omitted from the protocol. We have only used Matrigel for these implants. As such, the specific Matrigel formulation used in these experiments is listed in the Materials List.

6. Line 152: 'Appropriate medium' need more clarification, does this refer to growth media with FCS and antibiotics?

We appreciate the identification of this oversight. Notes have been added after steps 3.3 and 4.1 to clarify that this is complete growth medium.

7. Line 303: details of antibody for cytokeratin 8/18, dilution for IHC and company?

As JoVE guidelines expressly prohibit including company and brand information in the manuscript, these details have been added to the Materials List.

8. Line 347: require n=? Details for how many embryo per treatment group?

The numbers of embryos per group have been added to the figure legend.

9. Line 353: Histology for figure 2B. Is it an IHC?

and

10. Figure 3 legend: histology CK8/18 IHC?

Histology images in both Figures 2 and 3 are hematoxylin and eosin stains. We apologize for the confusion and have clarified in all figure legends that the histology presented are hematoxylin and eosin staining with the exception of the cytokeratin 8/18 staining presented in Figure 1.

Reviewer #3:

Manuscript Summary:

The authors have claimed to optimize the CAM model for the engraftment of gynaecological and urological cancers more specifically ovarian, prostate, kidney and bladder cancers. The authors comprehensively describe the method of implantation of cancer cells and tumors pertaining to the aforementioned cancers. Although previously published manuscripts cite the use of Chorioallantoic Membrane (CAM) assay in respect to these cancers [1-3], there is a general lack of knowledge as to the exact procedure of the experiments because the manuscripts fail to mention the exact details and precautions to be taken during the experiments which the authors have successfully addressed. The present manuscript will add to the field in terms of exact protocols that can be followed by researchers for implantation of cells/tumors with respect to CAM model for ovarian, prostate, kidney and bladder cancers, the need for which has only been addressed for hepatocellular carcinoma[4] and sarcoma[5]. Although the manuscript adds to the existing knowledge in the field but they have not provided any experimental knowledge of the most common endpoint experiments such as metastasis and angiogenesis for which the CAM model is generally used. The manuscript would benefit immensely by the detailed description of accessing metastasis and angiogenesis using CAM model for these cancers. All in all, the manuscript will add to existing lack of knowledge in the field and should be considered for publication.

References:

1. Lokman NA, Elder AS, Ricciardelli C, Oehler MK. Chick chorioallantoic membrane (CAM) assay as an in vivo model to study the effect of newly identified molecules on ovarian cancer invasion and metastasis. International journal of molecular sciences 2012; 13: 9959-9970.

2. Fergelot P, Bernhard J-C, Soulet F, Kilarski WW, Léon C, Courtois N et al. The experimental renal cell carcinoma model in the chick embryo. Angiogenesis 2013; 16: 181-194.

3. Reuter A, Sckell A, Brandenburg L-O, Burchardt M, Kramer A, Stope MB. Overexpression of MicroRNA-1 in Prostate Cancer Cells Modulates the Blood Vessel System of an In Vivo Hen's Egg

Test-Chorioallantoic Membrane Model. in vivo 2019; 33: 41-46.
4. Li M, Pathak RR, Lopez-Rivera E, Friedman SL, Aguirre-Ghiso JA, Sikora AG. The in ovo chick chorioallantoic membrane (CAM) assay as an efficient xenograft model of hepatocellular carcinoma. JoVE (Journal of Visualized Experiments) 2015: e52411.
5. Sys GM, Lapeire L, Stevens N, Favoreel H, Forsyth R, Bracke M et al. The in ovo CAM-assay as a xenograft model for sarcoma. JoVE (Journal of Visualized Experiments) 2013: e50522.

Major Concerns:
Provide more detailed description of accessing metastasis and angiogenesis using CAM model for these cancers.

We agree that the CAM model has a great history in being used to study angiogenesis, which has been widely reported, leaving little opportunity to contribute to this field. As such, angiogenesis was not an objective of this study, and we did not conduct the analyses necessary to address angiogenesis in these models, although vascular recruitment to the tumor is clearly visible in the gross images. We agree with Reviewer #3's belief as to the importance of addressing metastasis using the CAM model. We felt that this important topic warranted a manuscript specifically addressing metastasis, which is currently under review by JoVE.

Minor Concerns:
None.

Reviewer #4:
Manuscript Summary:
Presented paper entitled "Using the Chicken Chorioallantoic Membrane In Vivo Model to Study Gynecological and Urological Cancers" by Sharrow et al., describes the process of preparing the solid tumors in chorioallantoic membrane of chicken embryo in a suitable way. However it is important to refill some details and improve the quality of some materials (details are specified below). The authors describe the process of experimental tumors establishment. Moreover, the paper is enriched with the protocols informing about the depiction of the tumor cells by antibodies and bioluminiscence imaging. All mentioned information determines the quality and utility of the manuscript. Methods article is useful and after the revisions described below (part : Major Concerns) can be published in Journal of Visualized Experiments.

Major Concerns:
- In the part Introduction, lines 59-60, the authors stated only partial information about utilization of chorioallantoic membrane for cancer research, so it is important to include the latest, most recent results in the text. Some recommendations are put in the brackets in form of PMID code: (24884418, 28304379, 28651614, 26107941, 29301505, 28642877,31248208, 28535001).

We thank Reviewer #4 for the kind suggestions for additional references to include. Those not previously referenced within the manuscript have been added.

- In the part 5. it is appropriate to specify the preferential diameter of nonstick ring used for implantation of the cells

The inner diameter of the nonstick ring has been added to step 5.1 and to the Materials List.

- The quality of the images must be improved. The resolution of the images is actually very low. Moreover, in some cases it is not appropriate to use the red colour font for the pictures scale description, specifically if the pictures have a lot of red colour as it is for example in hematoxylin and eosin staining in figure 4, so I suggest to substitute the colour of the font. In relation to numbers, please check the scale number in figure 2 because there are some little numbers.

The resolution of all figures is 300dpi, which is standard. Perhaps the figures underwent size reduction or file conversion when supplied to the reviewers? With respect to the color of the scale bars in the histology images, there does not appear to be an option for changing the

color in the imaging software that we used. In cases where the red font was obviously disruptive, Figures 2A and 4, Photoshop was used to change the color while retaining the integrity of the scale bar as best as possible. We felt that it was best to not edit those figures in which the red color was clearly visible. An additional software challenge is that we are unable to change the font size of the scale bar. Therefore, larger fonts with the size of the scale bar were added in Photoshop. There was no way to remove the smaller, illegible numbers originally inserted by the software without obscuring part of the image.