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Ultrasound guided orthotopic implantation of murine pancreatic ductal adenocarcinoma

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Dr. Alisha DSouza
Senior Science Editor
Journal of Visualized Experiments
1 Alewife Center, Suite 200
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Dear Dr. DSouza:

We very much appreciated the comments from both you and the Reviewers regarding our *Journal of Visualized Experiments* manuscript #60497, entitled "Ultrasound guided orthotopic injections of murine pancreatic ductal adenocarcinoma" by Hay, et al.

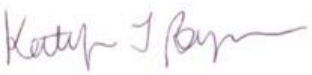
We were elated that the Reviewers found our manuscript to be "novel," "well-written" and "well-described," with "excellent figures." The Reviewers also commented that the protocol was "timely," which we agree with given the interest in many fields in addressing aggressive, treatment-resistant tumors such as pancreatic cancer. The Reviewers and the Editors also raised a number of concerns regarding the protocol, and we have thoughtfully and carefully addressed each one in this revision. We believe the Reviewers' comments have significantly improved the overall presentation and organization of the protocol, and we hope that it is felt to be ready for publication.

As a result of the Reviewers' and Editor's comments, we have made significant changes to the text, as well as added a new panel to Figure 2.

We have attached a revised manuscript with changes to the text tracked, as well as a point-by-point response to each comment provided by the Reviewers and the Editors.

We thank you for your help with this manuscript.

Yours sincerely,



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TITLE:

Ultrasound-Guided Orthotopic Implantation of Murine Pancreatic Ductal Adenocarcinoma

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KEYWORDS:

pancreatic, ductal, adenocarcinoma, ultrasound, orthotopic, preclinical model

SUMMARY:

We describe a protocol for the ultrasound-guided implantation of murine-derived pancreatic ductal adenocarcinoma cell lines directly into the native tumor site. This approach resulted in pancreatic tumors detectable by ultrasound scanning within 2-4 weeks of injection, and significantly reduced the proportion of tumor cell seeding on the peritoneal wall as compared to surgical orthotopic implantation.

ABSTRACT:

The recent success of immune checkpoint blockade in melanoma and lung adenocarcinoma has galvanized the field of immuno-oncology as well as revealed the limitations of current treatments, as most patients do not respond to immunotherapy. Development of accurate preclinical models to quickly identify novel and effective therapeutic combinations are critical to address this unmet clinical need. Pancreatic ductal adenocarcinoma (PDA) is a recognized example of an immune checkpoint blockade-resistant tumor, with only 2% of patients responding to immunotherapy. The genetically engineered $Kras^{G12D+/-;}Trp53^{R172H+/-}$;Pdx-1 Cre (KPC) mouse model of PDA recapitulates human disease and is a valuable tool for assessing therapies for immunotherapy-resistant cancer in the preclinical setting, but time-to-tumor onset is highly variable. Surgical orthotopic tumor implantation models of PDA maintain the immunobiological hallmarks of the KPC tissue-specific tumor microenvironment (TME) but require a time-intensive

procedure and introduce aberrant inflammation. This study uses an ultrasound-guided orthotopic tumor implantation model (UG-OTIM) to noninvasively inject KPC-derived PDA cell lines directly into the mouse pancreas. UG-OTIM tumors grow in the endogenous tissue site, faithfully recapitulate histological features of the PDA TME, and reach enrollment-sized tumors for preclinical studies by 4 weeks after injection with minimal seeding on the peritoneal wall. The UG-OTIM system described here is a rapid and reproducible tumor model that may allow for high throughput analysis of novel therapeutic combinations in the murine PDA TME.

INTRODUCTION:

Pancreatic ductal adenocarcinoma (PDA) is a notoriously aggressive disease that is refractory to current treatments, with a dismal 5-year survival rate of 9%[1]. PDA recently surpassed breast cancer to become the third-leading cause of cancer-related mortality in the U.S. and is projected to become the second-leading cause behind lung cancer by the year 2030[2]. Some features characteristic to the immunologically 'cold' PDA tumor microenvironment (TME), including high infiltration of immunosuppressive myeloid cell populations[3-7], dense stromal deposition[8-11], and a dearth of T cells[5,12,13], contribute to the failure of immunotherapies in PDA[14]. To this end, the use of a clinically relevant animal model is an essential tool for investigating the efficacy of novel drug combinations for immunologically cold tumors in vivo.

The genetically engineered $Kras^{G12D+/-;}Trp53^{R172H+/-}$;Pdx-1 Cre (KPC) mouse model of PDA faithfully recapitulates salient clinical aspects of human PDA, including the molecular drivers of disease and histopathological features[15]. KPC tumors develop spontaneously in fully immunocompetent mice, allowing for examination of therapeutic approaches including chemotherapy[16,17], immunotherapy[18-21], and stroma-targeting therapy[9,11,22] in vivo prior to the administration of these drugs in a clinical trial setting. Despite its many strengths as a preclinical model of PDA, the use of KPC mice is disadvantaged by the highly variable progression of spontaneous tumor development because tumor onset can range from 4−40 weeks, thus requiring the maintenance of a large breeding colony[15]. Additionally, KPC mice have the potential for polyclonal primary tumors[23], and there is a rapid decline in animal health and increase in comorbidities, including cachexia and ascites, as the disease progresses[15].

One alternative to the spontaneous KPC mouse model is to use an orthotopic implantation model of PDA[24]. The direct surgical implantation of tumor cell lines into the native tissue site is a more cost-effective and predictable method of recapitulating the tissue-specific tumor microenvironment (TME) of PDA. Tumor implantation allows for injection of clonal tumor cell lines to genetically backcrossed mice[5], allowing for host mice with additional genetic manipulations that would be time-consuming to breed in to the KPC mouse model. However, pancreatic tumor implantation requires a labor-intensive surgical procedure that introduces aberrant inflammation at the suture site in the abdominal wall[24-26], and often includes a lengthy postoperative recovery[27-29].

Technological advances in ultrasound imaging using rodent-specific probes provides high resolution images in real-time. Guided by the ultrasound imaging of the syringe movement in the peritoneal cavity, tumor cells can be implanted into the pancreas, leveraging the benefits of

orthotopic tumor injections in the absence of surgical implantation and associated inflammation. This approach, called the ultrasound-guided orthotopic tumor implantation model (UG-OTIM), has been previously established in xenograft models of pancreatic cancer[30] as well as in several other cancer models, including Ewing's sarcoma, neuroblastoma, and bladder cancer[31,32].

Here, a detailed protocol for performing ultrasound-guided injections of tumor cell lines into the murine pancreas is described. The resultant tumors recapitulate the histological and immunological features of the KPC TME and can therefore be used to investigate novel therapeutic combinations, including immunotherapies, to rapidly reveal the most promising treatments to move forward into clinical trials.

PROTOCOL:

Animal protocols were reviewed and approved by the Institution of Animal Care and Use Committee at the University of Pennsylvania. Female 5-to-6-week-old C57Bl/6 mice were purchased (see **Table of Materials**) and used after 1−3 weeks of rest. The University Laboratory Animal Resources oversaw animal care.

1. Preparation of PDA tumor cell lines for injection

1.1. Grow the KPC-derived PDA cell line in tumor cell (TC) media. Use Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), 2mM L-glutamine, and 83 μg/mL gentamicin.

1.2. Allow the cells to grow to 80%−85% confluency in flasks maintained at 37 °C and 5% CO_2.

1.3. Once the cells reach ideal confluency, decant the media from the flasks and wash 2x with warm (37 °C), sterile phosphate-buffered saline (PBS). Use enough PBS to cover the monolayer of adherent cells. Pipette off the remaining PBS after the final wash.

1.4. Add warm (37 °C) 0.05% trypsin-EDTA solution (0.5% stock trypsin-EDTA diluted 1:10 in Hanks'-based enzyme-free cell dissociation buffer) to cover the monolayer in each flask and incubate at 37 °C for 3−5 min or until cells detach with tapping on the sides of the flask.

1.5. Add 10−25 mL of cold (4 °C), sterile TC media into each flask to stop the trypsinization reaction. Pour the cell suspension into a 50 mL conical tube, and fill to 50mL with cold TC media.

1.6. Centrifuge at 300 x *g* for 5 min at 4 °C. Discard supernatant and resuspend pellet in cold, serum-free, sterile DMEM. If multiple conical tubes were used to collect the cells, all the pellets can be combined to a single conical tube at this step.

1.7. Centrifuge at 300 x *g* for 5 min 4 °C.

Repeat steps 1.6−1.7 2x. In the final wash, take an aliquot of cells for counting. A cell viability of ≥90% is recommended for in vivo injections.

Prepare a uniform suspension of the PDA tumor cells at the desired concentration. This study used 10−20 x 10^6 cells/mL (250,000−500,000 cells/25 µL) in the appropriate amount of sterile, cold DMEM or PBS, but each cell line should be titrated in vivo.

1.8. Keep cells cold on ice until ready to inject.

2. Pre-surgical preparation of mice

NOTE: It is recommended that this step be performed 24 h prior to the procedure.

2.1. Place the cages containing the experimental mice on a warming platform set to 37 °C.

2.2. Obtain new, clean cages and place on a second warming platform set to 37 °C.

2.3. Thoroughly clean the biological safety cabinet, the induction chamber, and the ultrasound (US) stage with sterilant (see **Table of Materials**).

2.4. Turn the warming function of the US stage to 37 °C.

2.5. Turn on the anesthesia machine. Adjust the dials on the tube splitter so that airflow is restricted to the induction chamber only. Turn on the oxygen tank and set the flow rate to 1 L/min. Turn on the isoflurane vaporizer to 2%−3%.

2.6. Place a single mouse into the induction chamber. Monitor the mouse until it is no longer mobile and breathing has slowed.

2.7. Adjust the dials on the splitter so that airflow enters both the induction chamber and the nose cone. Quickly move the mouse from the induction chamber and lay it in ventral recumbency on the warm US stage with its muzzle in the nose cone.

2.8. Test the level of induction by observing any reflexive response to toe pinching. If there is none, the mouse is ready for hair removal.

2.9. Place a small amount of eye lubricant (see **Table of Materials**) on both eyes to prevent tissue dehydration. Turn the mouse so that it lays in dorsal recumbency on the stage. Gently adhere the upper and lower extremities to the stage with paper tape in order to maximize exposure of the abdomen and secure the mouse to the stage.

2.10. Use a sterile cotton tip applicator to apply a generous layer of depilatory cream to the upper left quadrant of the abdomen. The depilatory should be applied in the general area of the spleen and extend towards the midline. Allow to sit for about 1 min.

2.11. Test the degree of hair removal by gently using the opposite end of the cotton tip applicator to wipe away the depilatory cream. Once the hair comes off easily, wipe the abdomen clean with a dry gauze pad. Wet a clean layer of gauze with a small amount of warm saline and wipe the area once more to completely remove the depilatory agent.

CAUTION: In order to reduce the chance of chemical burns, do not exceed 2 full min of direct contact with the depilatory agent on the mouse skin.

2.12. Once the hair has been sufficiently removed from the abdomen, return the mouse to a new, clean cage on a warmer.

2.13. While the first animal is undergoing hair removal on the US stage, the next animal can be added to the induction chamber.

2.14. Before anesthetizing the next cage of mice, turn off the isoflurane and flush the induction chamber with oxygen. Clean the induction chamber and US stage/nose cone with sterilant. Repeat steps 2.5–2.14 until all cages and mice have undergone hair removal.

NOTE: Consider having the animals fast by temporary withdrawal of food for a period of 12–24 hours prior to implantation to minimize the visual obstruction of the abdominal organs due to undigested food in the stomach and intestines. Water restriction is not recommended. If the animals fast, it is recommended that they be treated with an intraperitoneal injection of 1 mL warm (37 °C), sterile saline following the tumor injection in order to prevent dehydration.

3. Ultrasound-guided implantation of PDA cells

NOTE: All ultrasound procedures are performed using an ultrasound imaging machine and software (see **Table of Materials**). The transducer has a center frequency of 40 MHz and a bandwidth of 25–55 MHz.

3.1. Adjust the ultrasound platform so that the platform surface is parallel to the floor and the investigator faces the left side of the animal, with the animal's head to the right. Adjust the transducer position so that a transverse abdominal image will be obtained (**Figure 1A**).

3.2. Anesthetize the mouse to be injected as described in steps 2.1–2.8. Stabilize the anesthetized mouse on the US platform as described in step 2.9.

3.3. Apply a generous about of warm (37 °C) ultrasound gel to the hairless section of the mouse abdomen.

3.4. Gently lower the transducer to contact the mouse abdomen. Adjust the transducer as needed until the pancreas is clearly visible. Locate the left kidney and spleen in order to provide accurate orientation in the abdominal cavity.

NOTE: Because the transducer position has been changed to allow access to the left side of the abdomen, the X and Y axes on the stage controls are now inverted.

3.5. Load a 29 G x ½" insulin syringe (see **Table of Materials**) with 25 µL of the tumor cell suspension. Wipe the needle tip with a sterile alcohol prep pad prior to injection in order to minimize tumor cell seeding in the abdominal wall.

3.6. Using blunt-edge forceps, grasp the mouse skin and peritoneal wall to increase tension at the desired injection site. Holding the syringe at approximately a 25°−45°angle to the ultrasound platform surface, slowly advance the needle through the skin and the peritoneal wall. Confirm the needle has punctured through the peritoneal wall before proceeding to the next step. A small pop should be felt as the needle pierces the peritoneal wall.

3.7. Under ultrasound visualization, guide the needle directly into the pancreas (**Figure 1B**,**C**). Confirm the needle is within the pancreas tissue by gently moving the syringe barrel up and down. If the placement is correct, the needle tip will remain within the pancreas tissue while the syringe barrel is moving.

3.8. Slowly inject the tumor cells and confirm the cells are being implanted in the desired location by the formation of a fluid bolus within the pancreas, which should be visible on the ultrasound screen (**Figure 1D**).

NOTE: Some resistance should be felt while depressing the plunger. Do not pierce the pancreas multiple times, as this increases the likelihood of leakage into the abdominal cavity.

3.9. Once the full volume of suspension has been injected and a fluid bolus can be seen in the pancreas, keep the needle very still for several seconds. Slowly retract the needle from the mouse abdomen, taking great care not to disturb the injected cells.

3.10. Place the mouse in a clean, warm cage and ensure that the mouse fully recovers from anesthesia. Repeat this process for all animals.

3.11. Before anesthetizing the next mouse, clean the induction chamber and US stage/nose cone with sterilant.

3.12. Repeat steps 3.2−3.11 until all mice have been injected.

REPRESENTATIVE RESULTS:

The goal of this report was to provide a detailed protocol for performing ultrasound-guided implantation of KPC-derived PDA cell lines. The representative experiments shown in **Figure 2**,

Figure 3, and **Figure 4** confirm that the UG-OTIM tumors grow at a consistent rate and in a dose-dependent manner. Furthermore, the UG-OTIM tumors recapitulate the salient immunological and histological features of the KPC TME. Thus, the UG-OTIM system is a preclinical PDA mouse model that can be used in a high-throughput manner to rapidly screen new treatment combinations in vivo.

Using the UG-OTIM protocol outlined here, the mice were prepared for implantation, secured to the heated ultrasound platform, and the positions of both the platform and the transducer were adjusted for the procedure as shown in **Figure 1A**. High-resolution ultrasound imaging was used to identify an injection site within the mouse pancreas that could be targeted without perforation of either the kidney or spleen (**Figure 1B**). Under ultrasonographic visualization, the needle was carefully introduced to the abdominal cavity through the peritoneal wall and guided into the mouse pancreas (**Figure 1C**). After correct placement of the needle was established, the tumor cell suspension was injected very slowly into the pancreas. A successful implantation was confirmed by the presence of a bubble within the pancreas (**Figure 1D**). In early experiments, the mouse was sacrificed and efficacy of the procedure was verified by directly visualizing a fluid bolus in the gross pancreas tissue (**Figure 1E**).

Tumor implantation and growth rate were monitored by weekly ultrasound imaging. Successful implantations produced tumors that were contained within the borders of the pancreas throughout the time of the experiment (**Figure 2A**). The ultrasound software used (see **Table of Materials**) allowed for tumor area and volume to be determined for each time point as well as for three-dimensional (3D) mapping of measured tumors to be generated (**Figure 2B**), and the 3D images were confirmed at the time of mouse necropsy (**Figure 2C**). An improper cell injection during the UG-OTIM procedure can result in the development of a peritoneal wall tumor (see **Figure 2D**). Mice that present with peritoneal tumors can be excluded from further studies.

To determine the concentration of cells optimal for use in preclinical studies, a C57Bl/6 KPC-derived PDA cell line (4662)[9] was injected in to naive, wild type C57Bl/6 mice across six independent experiments. This cell line (passaged in vitro six times) was fully backcrossed (>10 generations, confirmed by speed congenics[19]) to prevent molecular histocompatibility complex mismatch tumor rejection antigens. Tumor cells were injected at a low titer (1.25×10^6 cells/25 µL) and at a high titer (5×10^6 cells/25 µL) dose. The high titer tumor injections resulted in a larger proportion of tumor-bearing animals 2 weeks after injections compared to the low titer cohort (**Figure 3A**). Despite the delay in tumor onset, the overall tumor growth rate was not significantly different between the two doses (**Figure 3B**). Similarly, while the survival rate between the two cohorts was not significantly different, the data trend towards slightly improved survival in the low titer cohort (**Figure 3C**). The high titer cohort also produced a greater proportion of mice with tumors that were enrollable in preclinical studies (designated at ≥20 mm^3 tumor volume) by 4 weeks postinjection than the low titer cohort (**Figure 3D**). The majority of mice from both high and low titer cohorts presented with enrollable tumors by day 25 and developed end-stage disease symptoms including ascites (data not shown). Metastases, which do not occur using 4662 at the cell doses in this protocol, may be modeled with different cell lines or doses[33].

The UG-OTIM method requires fine motor skills to accurately localize the desired injection site, which was challenging when performing the experiments. For this reason, a table depicting the number of animals that developed tumors within the pancreas (successful implantations) compared to the total number of animals that underwent ultrasound-guided tumor implantation (**Table 1**) is included. Animals were eliminated from future analyses if tumors developed in an undesired location (e.g., kidney) or if there was no evidence of tumor by 6 weeks postinjection, as indicated. The weekly progression of the proportion of mice bearing enrollable pancreatic tumors (≥20 mm^3 tumor volume) from each experiment is also shown in **Table 1**.

To determine if there were other benefits to using the UG-OTIM approach rather than the traditional surgical orthotopic model, we compared the seeding of PDA tumors in the mouse peritoneal wall after each procedure. We found that only 2/31 mice (6.5%) developed unintended peritoneal wall tumors after UG-OTIM injections, compared to 7/15 mice that developed peritoneal wall tumors after surgical injection (46.6%, $p < 0.0029$) (**Table 1**). Thus, the rate of seeding additional tumors in the peritoneum is greatly reduced in the UG-OTIM method compared to surgical implantation.

Upon sacrifice of mice bearing UG-OTIM tumors, the gross anatomy of the tumors was similar to spontaneous KPC tumors (**Figure 4A**). Histological analysis demonstrated a pattern of abnormal ductal structures that was similar in both models and that recapitulated the morphology of the human disease (**Figure 4B**). To investigate the immune infiltrate within both KPC and UG-OTIM tumors, representative histological samples were stained for CD3 (expressed by T cells) and F4/80 (expressed by macrophages). In both models, straining patterns revealed tumors that were poorly infiltrated by T cells (**Figure 4C**), but highly infiltrated by macrophages (**Figure 4D**). This finding is consistent with the immunologically cold phenotype of most human and KPC PDA samples[5,7,12].

FIGURE AND TABLE LEGENDS:

Figure 1: Ultrasound-guided implantation of PDA cells into the murine pancreas. (**A**) Orientation of the mouse, ultrasound stage, and ultrasound probe used to obtain a high-resolution image of the abdominal organs. Note that the stage and platform have been turned 90° from the standard orientation to allow easy access to the upper left quadrant of the mouse abdomen. (**B**) Ultrasonographic image depicting the identification of kidney, spleen, and pancreas. Here the syringe is positioned against the mouse abdomen. (**C**) Ultrasonographic image depicting the syringe within the mouse pancreas. (**D**) Ultrasonographic image depicting the bubble at the injection site (outlined in blue) following the controlled injection of tumor cells into the pancreas. (**E**) Laparotomy revealing the fluid bolus containing PDA cells in the pancreas after ultrasound guided injection.

Figure 2: Monitoring tumor growth after UG-OTIM injection. (**A**) Representative ultrasonographic image of UG-OTIM tumor (outlined in blue) at 2, 3, and 5 weeks postinjection, as indicated. (**B**) Representative reconstructed 3D image of UG-OTIM tumor 5 weeks postinjection using the ultrasound software. (**C**) Representative gross anatomy of UG-OTIM tumor 5 weeks postinjection upon mouse necropsy. (**D**) Representative ultrasonographic image

of a peritoneal wall tumor growing in the subcutaneous layer after improper cell injection at 7 weeks postinjection.

Figure 3: Dose-dependent onset of tumors after ultrasound-guided injection of PDA cells. (**A**) Proportion of tumor-bearing mice at indicated time points after injection as described in **Figure 1** with a high titer (500,000 cells/25 µL) or low titer (125,000 cells/25 µL) of tumor cells, as indicated. (**B**) Tumor growth kinetics of mice from (**A**). Each symbol represents a group of mice. The error bars indicate standard error of the mean (SEM). (**C**) Proportion of mice from (**A**) alive at the indicated time points after injection. The mice were euthanized or censored if tumors were >1,000 mm^3 or the body condition was poor due to tumor comorbidities. (**D**) Time to the clinically relevant tumor onset for mice from (**A**). Clinically relevant tumors are considered to be ≥20 mm^3 in volume. Data representative of four independent experiments with n = 8–20 mice per experiment.

Figure 4: UG-OTIM tumors recapitulate the KPC tumor microenvironment. Representative images are shown. Top row = KPC tumors. Bottom row = UG-OTIM tumors at 5 weeks postimplantation. (**A**) Gross anatomy of excised tumors upon necropsy. (**B**) Haemotoxylin and eosin staining (H&E) (10x, bar = 100 µm) (**C**) Immunohistochemistry staining for CD3 (brown) (10x; bar = 100 µm). (**D**) Immunofluorescent staining of F4/80 (red) and DAPI (blue) (4x; bar = 500µm).

Table 1: Number of successful UG-OTIM tumors compared to surgical implantation. Combined data from two to four independent experiments per high or low titer condition with n = 5–10 mice per experiment. “Surgical injection” indicates mice that received an abdominal laparoscopic surgical injection of 125,000 tumor cells. “Tumor-bearing” indicates mice with a pancreatic tumor. “Clinically Relevant” indicates the proportion of tumor-bearing mice at each time point with a tumor volume >20 mm^3. “Seeded Peritoneal Tumors” indicates the total proportion of tumor-bearing mice that had concomitant seeding of tumors on the peritoneal wall from tumor cell injection. Mice with tumors presenting incorrectly injected tissues (e.g., kidney) were excluded from “Tumor-Bearing” populations but included in total injected mice. ND = not determined. Frequency of peritoneal wall seeding comparing high and low titer groups combined versus surgical implantation, $p < 0.0029$ via two-tailed T test with Mann-Whitney post-test.

DISCUSSION:

The use of high-resolution ultrasonography to direct implantation of murine PDA cell lines to the autochthonous tissue site is a reliable alternative to both the KPC and surgical orthotopic model systems. UG-OTIM produces biologically relevant tumors that retain the immunopathological features of PDA with a shortened time frame to tumor diagnosis and reliable tumor growth kinetics. Ultrasound-guided injection can therefore serve as a useful tool for rapid production of mice bearing orthotopically implanted PDA tumors, allowing for investigation of therapeutic combinations in a clinically relevant model.

Ultrasound-guided implantation offers important improvements over standard models of preclinical investigation. First, this procedure eliminates the time-intensive monitoring of KPC

mice for the development of spontaneous tumors. Second, similar to traditional surgical orthotopic injections, the UG-OTIM approach allows for control over the cell line injected, including selection of a monoclonal tumor cell line and/or ex vivo manipulation of the cell line, as well as control over the host receiving the tumor cell implantation. Third, this minimally invasive technique avoids the arduous labor of survival surgery and bypasses the complicated postoperative recovery period for the animals as well as inflammatory signals from surgical wound healing. Finally, similar to surgical implantation, UG-OTIM tumors recapitulate the TME observed in the KPC mice, including low T cell infiltration and high macrophage infiltration. Thus, the UG-OTIM model retains key features of the KPC tumors without the additional complications that delay therapeutic investigations in the spontaneous KPC model.

A number of critical steps in the protocol need to be mastered for the success of the technique. Expertise in murine ultrasound imaging is essential for this procedure, and the manual dexterity required to successfully implant cells in the pancreas is a skill set that must be developed independently. For mice on a 12-hour light/dark cycle, having the animals fast overnight ensured that the stomach and intestines were cleared of any undigested food that could block view of the pancreas, kidney, and spleen by ultrasound. Additionally, each cell line used for orthotopic injection should be titrated prior to further experiments to understand its growth kinetics and determine the metastatic potential[33]. During injection, the use of forceps to pinch the skin at the injection site created the tension needed to gently puncture through both the skin and peritoneal wall. A key step in the procedure is to carefully guide the needle into the pancreas without perforating the tissue or puncturing an off-target site such as the spleen or kidney. Confirmation of a fluid bolus was the best indicator of successful tumor cell injection in the proper tissue. After injection, the needle should be withdrawn slowly so as not to disturb the fluid bolus. A series of trial injections using either DMEM or Trypan Blue helped master the fine motor skills needed for the injections.

During troubleshooting of this procedure, a number of factors were identified that impacted the success of the protocol. In trial experiments, the most frequent error was perforating the kidney during implantation, which occurred more frequently in early experiments, suggesting that regular exercise of injection skills improves proficiency. Additionally, confirming the presence of a fluid bolus via both ultrasound and direct visualization at necropsy during the troubleshooting phase after a tumor cell injection improved successful injection technique. If the formation of a bubble is not confirmed by ultrasound during the injection, the location of the needle can be adjusted before fully depressing the syringe to release the remaining bolus of tumor cells. Suspension volumes injected too quickly resulted in the spilling of tumor cells into the peritoneal cavity or collapse of the fluid bolus in the pancreas in some mice. Generally, these animals went on to develop pancreatic tumors with the exception of n = 7 animals that showed no evidence of tumor 4 weeks postinjection. This result was reported only in the first attempts (and 6/7 animals were injected with a low titer of tumor cells). Mice that have questionable tumor cell injections, or requiring repositioning of the needle, should be closely monitored for the development of tumors outside of the pancreas.

The foremost limitations of the ultrasound-guided method are the availability of the required instruments and the technical skill associated with tumor implantation. The procedure is not sterile, as the mouse is injected on an unsterilized ultrasound platform, with the syringe and needle tip passing through the ultrasound gel. Although we have seen no evidence of infection in n = 148 mice across a total of eight independent experiments since initiating these studies, it is possible that an infectious agent could enter the pancreas through the syringe during injection. As such, all tools required for the protocol, including gloves, ultrasound surfaces, and ice boxes, should be sprayed with disinfectant or 70% ethanol to reduce the potential exposure to pathogens. An additional limitation of the current protocol was the lack of metastasis using the 4662 cell line at the current dilutions. Each cell line used in the UG-OTIM system should be titrated for the desired growth rates as well as metastatic potential[33]. Finally, the current protocol established techniques for injecting tumor cells in a single-cell suspension. However, the addition of an extracellular matrix substrate could be added to potentially enhance tumor establishment and prevent tumor cell leakage, as used in surgical implantation models[27,30-32]. Thus, many of the limitations of UG-OTIM can be overcome with appropriate testing of the cell lines being used in the orthotopic injections.

In summary, the UG-OTIM model is a precise method of tissue-directed injection of tumor cells into the murine pancreas. This minimally invasive implantation technique benefits both the investigator and the animals by reducing procedure time, minimizing post-surgical complications, and improving the accuracy of injection. Tumors arising from UG-OTIM injections retain the characteristic immunobiological features of spontaneous KPC tumors, have reliable time to tumor onset, and have reproducible tumor growth kinetics. Thus, the UG-OTIM model can be used in a relatively high-throughput manner to interrogate therapeutic combinations in a preclinical setting to reveal novel treatments for patients with the unmet clinical needs.

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DISCLOSURES:

The authors have nothing to disclose.

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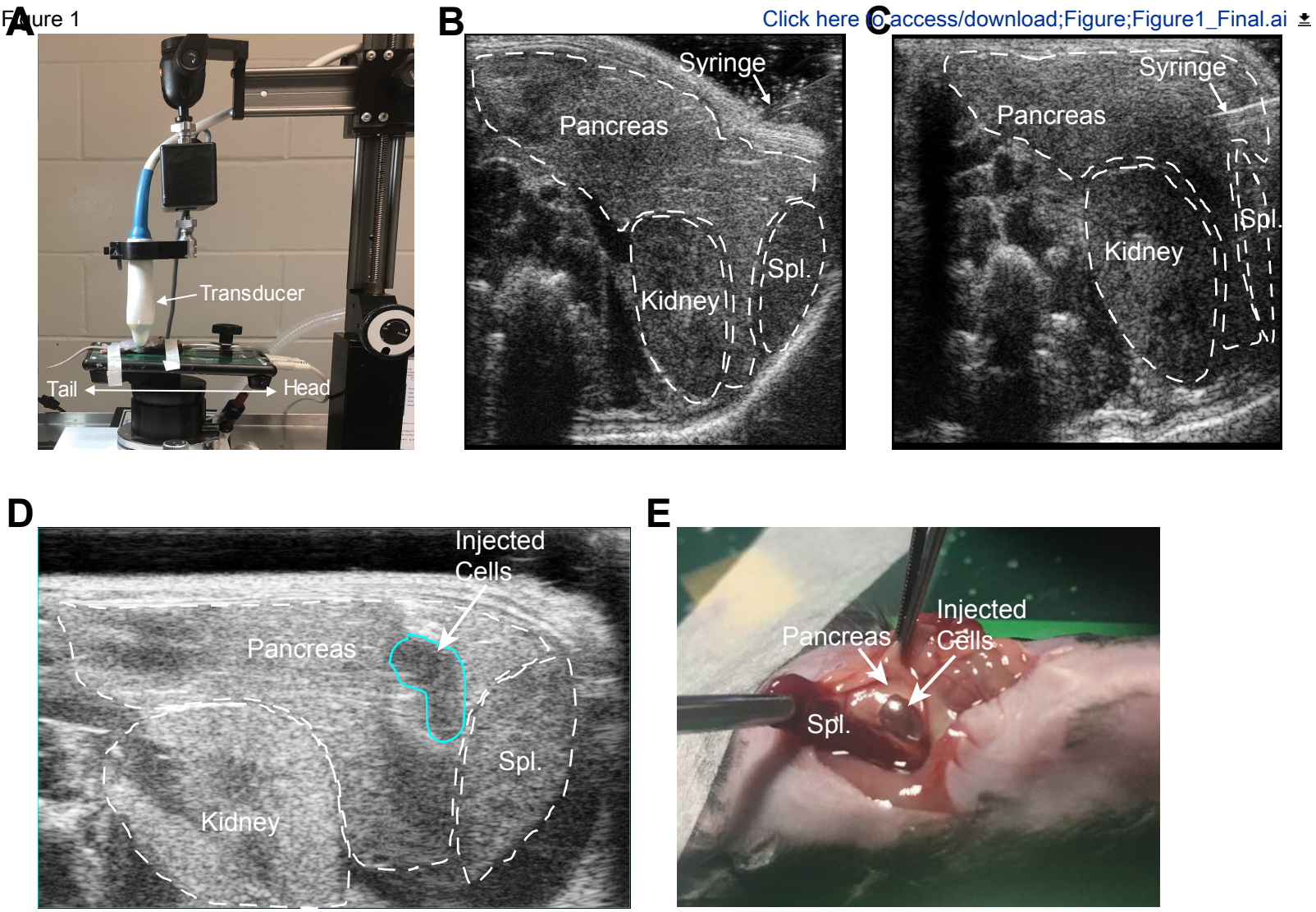
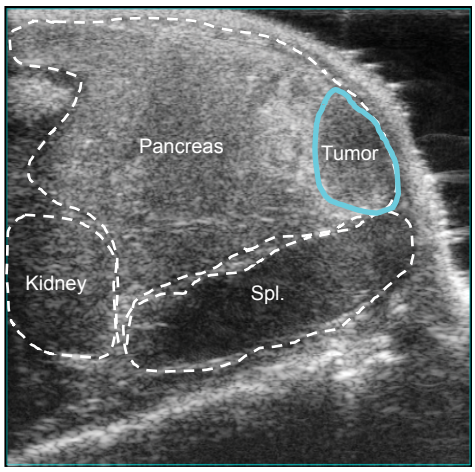


Figure 2

A



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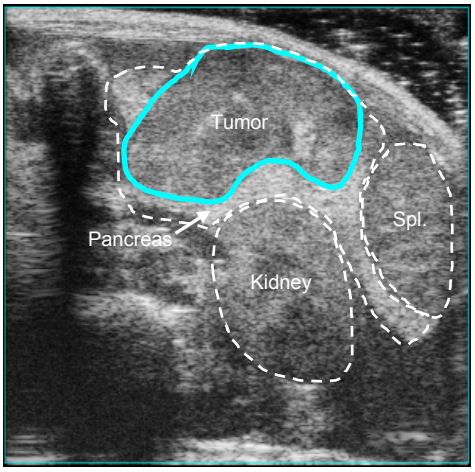

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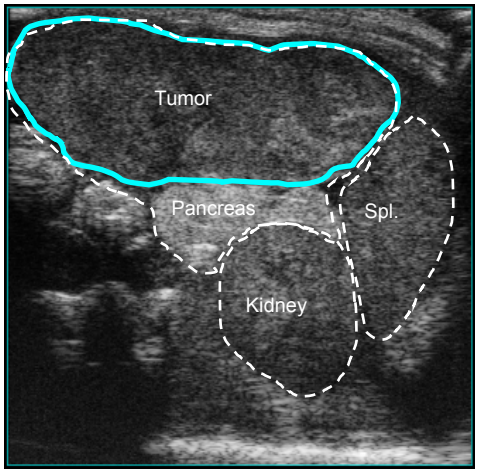
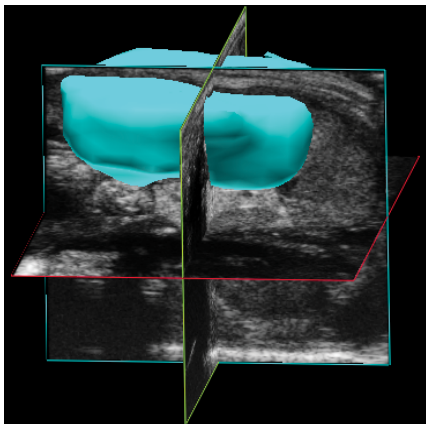


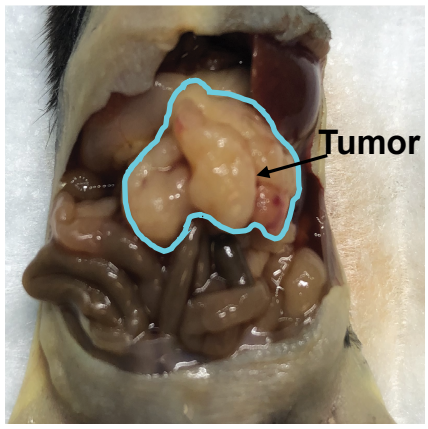
5 weeks

B



5 weeks

C



5 weeks

D

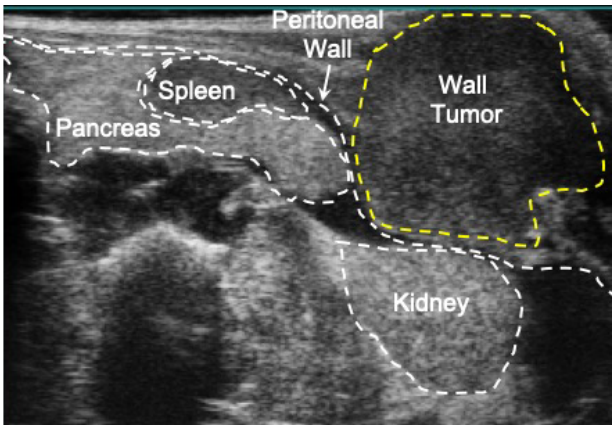


Figure 3

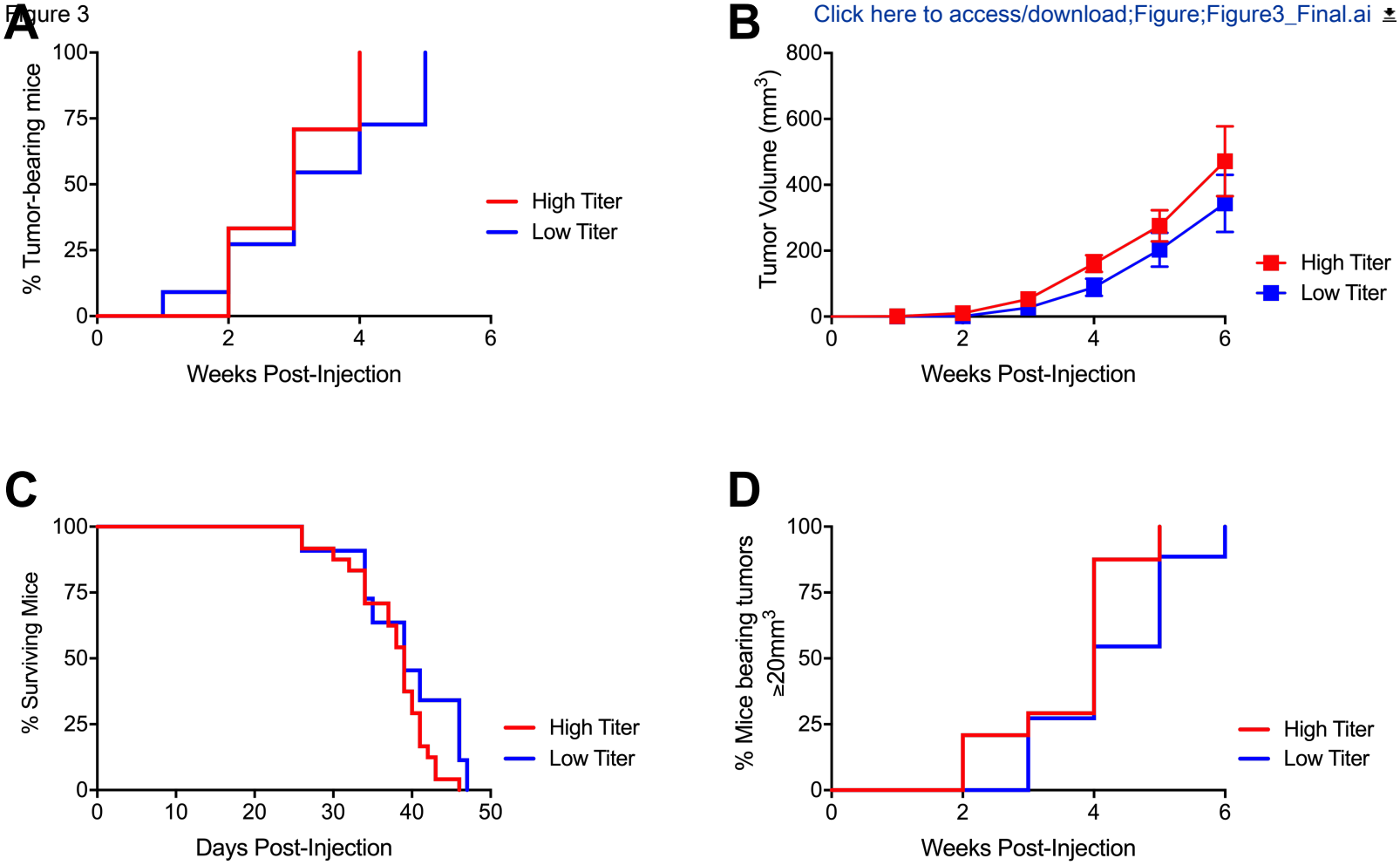


Figure 4

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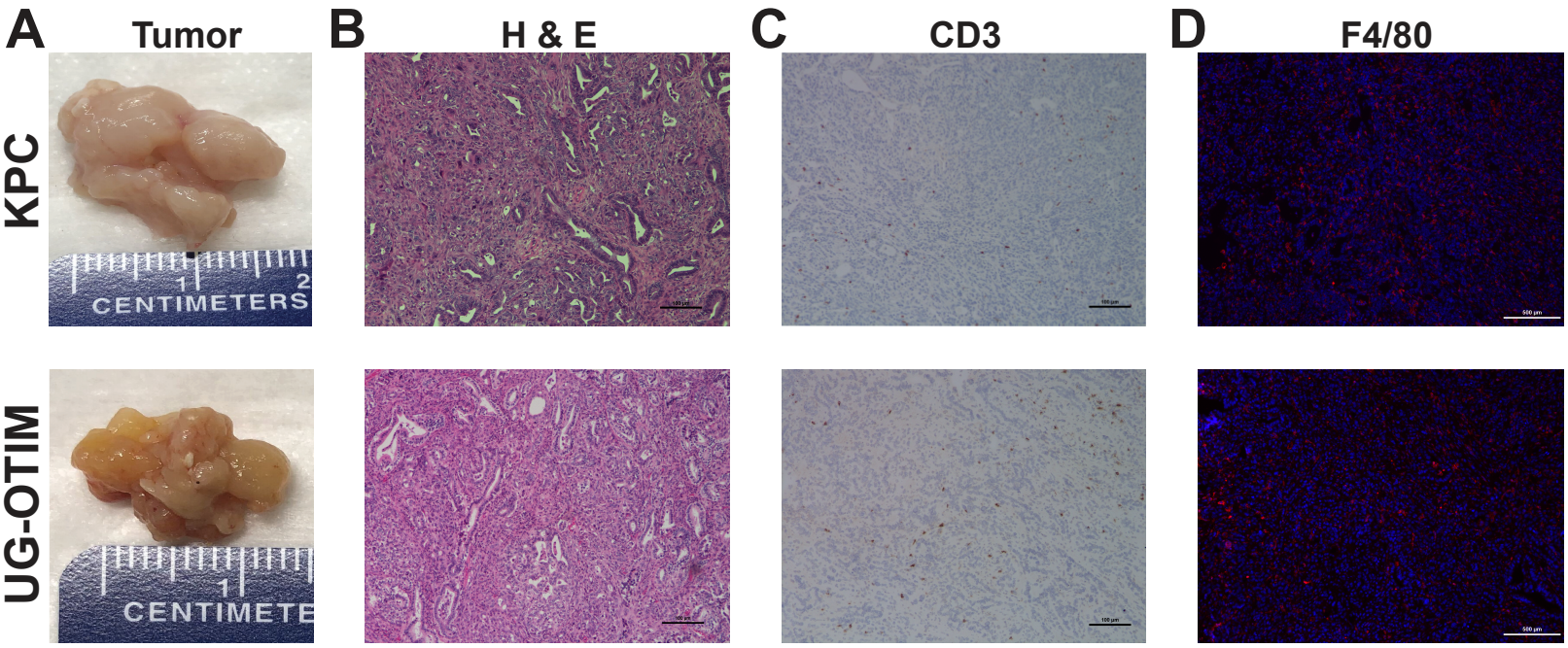

Table

Experiment	Pancreas-tumor bearing/total injections	Week 1 (Enrollable)	Week 2 (Enrollable)	Week 3 (Enrollable)	Week 4 (Enrollable)
High Titer	24/31	ND	8/24 (3/24)	15/24 (7/24)	23/23 (20/23)
Low Titer	11/17	ND	3/11 (0/11)	6/11 (3/11)	9/11 (5/11)
Surgical Injection	15/17	ND	ND	15/15 (9/15)	15/15 (14/15)

Seeded Peritoneal Tumors
2/20
0/11
7/15

Name of Material/ Equipment	Company	Catalog Number
50 mL Conicals	Thomas Scientific	2602A26
Blunt edged forceps	Fine Science Tools	11000-12
Cell Dissociation Buffer	Thermo-Fisher	13151014
Cotton Tipped swabs	Thermo-Fisher	19062614
Covidien Monoject 3/10mL, 29G X 1/2	Thermo-Fisher	8881600145
Depilatory Agent	Amazon	Nair Body Lotion
DMEM	Thermo-Fisher	10-566-016
FBS	Gemini Bio-oroducts	100-106
Flask	Sigma-Aldrich	CLS430825
Forceps (blunt edge)	Fine Science Tools	11000-12
Gauze	Fisher	13-761-52
Gentamicin	Thermo-Fisher	15750060
Induction Chamber	VetEquip	941444
Isofluorane	Penn Vet Supply	VED1350
Isofluorane Vaporizer	VetEquip	911103
L-glutamine	Thermo-Fisher	25030081
Optixcare	MidWest Veterinary Supply	052.50310.3
Paper Tape	Medline	MMM1530Z5
PBS	Thermo-Fisher	14-190-250
Slide warmer	C&A Scientific	XH-2001
Sterilant (Clidox-S)	Fisher Scientific	NC0332382 (activator) NC9189926 (base)

Sterile Alcohol prep pad	Covidien	6818
Trypsin	Thermo-Fisher	15090046
Ultrasound gel	Thermo-Fisher	03-34-1LT
Visualsonics Ultrasound Vevo 2100	Visual Sonics	Vevo 2100

Comments/Description

Needs to be combined according to manufacturer's instructions

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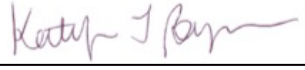
Signature:	
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Dear Editors and Reviewers,

We thank both the Editors and the Reviewers for their insightful comments and questions, which have led to improvements on our manuscript, JoVE 60497. We believe the Reviewers were positive about the manuscript and the contribution our protocol may make to the field of cancer biology and tumor immunology. As a result of the concerns raised here, which we have replied to on a point-by-point basis below, we have added an additional panel in Figure 2 and significant alters in the text to clarify our findings.

Editorial Comments:

• Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammatical errors.

Reply: We have corrected spelling and grammatical errors in our manuscript.

• Protocol Highlight: Please do not highlight any steps describing anesthesia or euthanasia as these will not be filmed.

Reply: We thank the Editor for pointing this out, and have removed the highlighting from steps 2.5-2.8, 2.14 and 3.2 to exclude steps involving anesthesia.

• Discussion: JoVE articles are focused on the methods and the protocol, thus the discussion should be similarly focused. Please ensure that the discussion covers the following in detail and in paragraph form (3-6 paragraphs): 1) modifications and troubleshooting, 2) limitations of the technique, 3) significance with respect to existing methods, 4) future applications and 5) critical steps within the protocol.

Reply: We have altered the text on page 10 to emphasize these topics in our Discussion.

• References: Please spell out journal names.

Reply: We have fixed our references to spell out journal names.

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Reply: All figures and tables are original.

Reviewers' comments:

Reviewer #1:
Manuscript Summary:
Injection of mouse pancreatic cancer cells. Our lab has done this and we get a better percentage of tumors using matrigel when we inject the tumor cells.

Reply: We agree with the Reviewer that Matrigel can be used as a substrate to enhance the tumor establishment and growth for orthotopic tumor injections. However, for our purposes, we developed a technique that was minimally invasive with high fidelity to the endogenous tissue site at baseline, so we preferred to exclude Matrigel for the establishment of this protocol. Other groups may find the addition of Matrigel to be helpful so we have added text on page 9 to address the potential of using Matrigel in this protocol.

Major Concerns:
None

Reviewer #2:
Manuscript Summary:
This is a very well written manuscript describing a novel technique of ultrasound guided injection of pancreatic cancer cells to establish pancreatic tumors in a transgenic mouse model. The technique is well described and the figures are excellent. The technique has some advantages over surgical orthotopic injection.

Reply: We thank the Reviewer for these positive comments.

Major Concerns:

None

Minor Concerns:
None

Reviewer #3:
Manuscript Summary:
This is a well-written and timely manuscript with an important approach to document for the field. The use of ultrasound-guided injection of tumor cells into the pancreas, although quite challenging and requires likely quite a bit of trial and error at first likely, could help others use this model, rather than injecting the cells subcutaneously (which has many additional problems) and could overcome the requirement for surgery.

Reply: We thank the Reviewer for these positive comments.

There are several key issues however, that should be addressed to improve clarity, accuracy and precision of what has and has not been demonstrated in this study as well as cited studies as follows:

Major Concerns:
Since the needle is puncturing the skin and the abdominal wall, and presumably the needle is also going through ultrasound gel, this seems like it is not sterile and could potentially also introduce contaminating ultrasound gel into the mouse abdomen and potentially infection into the pancreas. Is this the case?

Reply: We appreciate the Reviewer raising this issue, and the Reviewer is correct that the needle passes through the (non-sterile) ultrasound gel during the injection. It is therefore theoretically possible that the gel could lead to infection in the pancreas or in the abdomen. However, we have performed this technique in n=8 independent experiments with n=147 total mice with no evidence of infection, including during the monitoring phase after the UG-OTIM procedure (3-10 weeks of weekly assessment of the mice), so in our experience, the chance of infection appears to be extremely low. To make readers aware of this potential risk of infection, we have highlighted the potential issue raised by injecting through the non-sterile gel in the discussion section on page 9.

In 3.8, they state that if the "formation of the bubble cannot be confirmed, stop the injection, adjust the location of the needle tip and try again." It seems likely that the user then, must have injected the tumor cells elsewhere, likely IP, and that this would be a concern in enrolling that mouse for further study. The authors should indicate this as a possibility and consider not using that mouse for a study.

Reply: We thank the Reviewer for pointing out this confusing statement. Our current protocol is to assess the incorrect seeding of peritoneal wall tumors by ultrasound and exclude the mouse if a tumor develops outside of the pancreas. However, for clarity in this protocol, we have omitted the sentence in 3.8, and we have adjusted the text in the

discussion on page 9 to address this point.

An issue at large is how far backcrossed is the KPC cell line is, which seems to be glossed over a bit. The authors should include exact details as to how far backcrossed this line is, discuss why this is important, as well as if this line is available for other investigators.

Reply: We agree with the Reviewer that the background of the cell line and the mouse the cells are being injected in to is critical for proper interpretation of the tumor growth data. The KPC cell line has been backcrossed for >10 generations on the C57BL/6 background, and this background has been confirmed by SNP analysis at DartMouse (reported in Byrne and Vonderheide, Cell Reports, 2016). To clarify these points for the reader, we have altered the text on page 6. As previously reported, the 4662 cell line was generated in a laboratory supported by NIH funding, so this cell line is available to other investigators (Lo et al., Cancer Research, 2015).

The authors state that they only use "low passage" cells. What is "low passage"? How many passages are acceptable? And what passage number is this 4662 line which has been used for several years now for these studies?

Reply: We appreciate the Reviewer raising this important question. The 4662 KPC cell line used in these studies has been passaged *in vitro* 6 times, with previous studies using the 4662 cell line with a passage number between 3-5 (Bayne, Cancer Cell, 2012., Olive, Science, 2009 and Byrne, Cell Reports, 2016). While we consider this to be a low passage number, we understand that the difference between high and low passage numbers is open to interpretation and also dependent on individual cell lines. To avoid confusion regarding this point, we have included a more detailed description of our cell line on page 6 and removed the term 'low passage.'

The authors show that with their surgeries, they observe more peritoneal seeding, which they attribute loosely to the actual surgical procedure (Table 1). Their results are unexpected because it seems surgery would ensuring that the tumor cells are injected specifically into the pancreas rather than injecting the needle through the pancreas. Although anecdotal, to our knowledge, the fact that the surgery procedure causes peritoneal seeding has not been shown rigorously by others. Even the paper they cite to support this finding (citation #29, Erstad et al, 2018) fails to show that this is due to the surgical procedure (although it is possible it is buried somewhere in the paper and we somehow overlooked it). It seems more likely it is injection error or a highly aggressive cell line that is poised to be metastatic (see Erstad et al, 2018) that causes peritoneal metastases, liver metastases, etc....throughout the animal and is independent of the procedure itself.

Reply: We appreciate the Reviewer bringing to our attention the need for clarification on this issue. We agree that we are also not aware of a rigorous comparison of the two orthotopic techniques by other groups, however, we included the data regarding the peritoneal wall implantation from the two techniques in our experience here in Table 1.

In the manuscript by Erstad et al, 2018, the authors state that “An erroneous injection of tumor cells in the retroperitoneal space… is likely to result in the extravasation of the contents in the peritoneal cavity, resulting in carcinomatosis (Figure 1C).” In the figure, the authors include an image of tumor cells “…adhered to the anterior abdominal wall…” which they argue is the result of technical error regarding the angle of tumor cell injection. Thus, the metastatic potential of the cell lines used in the orthotopic implantation is not the only contributing factor to tumor seeding on the peritoneal wall, and the actual injection and surgical technique used can impact the seeding of the tumor cells. This issue with cell leakage during injection is also referred to in the citations by Qiu and Su (Methods in Molecular Biology, 2013) and Moreno et al. (Journal of Visualized Experiments, 2014), in the “Footnotes” and Discussion sections, respectively. Thus the improper seeding of the peritoneal cavity (as opposed to metastatic cell dissemination as part of tumor progression) is a known issue with orthotopic tumor implantation, despite the lack of extensive numbers for this process in previous publications. Here, we show that using the UG-OTIM system, we were better able to limit the occurrence of seeding to the peritoneal wall due to technical error as compared to surgical implantation of the tumor cells. As an indirect contributing factor, many groups have shown that wound healing, and the associated inflammatory signals, are known to contribute to tumor progression (Foster et al., JCI Insight, 2018., Kasper et al., PNAS 2010., Stuelten et al., Cancer Research 2008.). To better clarify that the actual injection technique of the tumor cells—and not simply the wound caused by surgical implantation—contributes to the development of improper peritoneal tumor implantation, we have altered the text on pages 1 and 9.

First, the authors should show images of what peritoneal seeding at the surgical site looks like as it would help readers identify if this is indeed due to the surgical procedure rather than an inaccurate injection of cells into the peritoneum.

Reply: We agree with the Reviewer that an image of a peritoneal wall tumor would be helpful for differentiating a pancreatic tumor from a subcutaneous tumor by ultrasound. We have added a new panel in Figure 2 that shows a peritoneal wall tumor (highlight in yellow, Figure 2D) and adjusted the text on page 6 and 7.

One potential explanations for the abnormally high peritoneal seeding using surgery in their study is that they are injecting logs more KPC tumor cells (>10^6) compared to other studies (typically ~ 10^4 -10^5). This could be why they see peritoneal seeding in the surgical model and an artifact of having to inject so many tumor cells with this particular cell line.

Reply: We thank the Reviewer for pointing out this important point. We failed to clarify in the text that our surgical implantation injections were performed using 1.25^10^5 cells/mouse, a log lower than the volumes injected in the UG-OTIM procedure we have presented here. Therefore, we used a lower, not a higher, cell count in the surgical model where we observed increased tumor seeding in the peritoneal wall as compared to UG-OTIM. We have updated the text on page 8 to specify the number of cells used in surgical orthotopic injections.

The other possibility is that they are not using matrigel to keep tumor cells within the pancreas. The authors should indicate that these are possibilities for their abnormally high rate of peritoneal seeding using surgery in the discussion. They should also indicate if matrigel can be used in the UG-OTIM approach and why they chose not to use it to help contain tumor cells at the site of injection.

Reply: We agree with the Reviewer that the addition of Matrigel may be a useful tool to combine with UG-OTIM. However, Matrigel provides an artificial substrate which theoretically can impact the structure and organization of the stroma during tumor development. Investigators have the option of using Matrigel, but this specific protocol had the aim of perturbing the native tissue site as little as possible, so as such we did not troubleshoot using Matrigel. We have added sentences regarding the use of Matrigel in the discussion on page 9.

Further, they should more accurately cite the studies that show surgery causes peritoneal seeding because our review of the papers they cite (#24, 25 and 29) don't show this.

Reply: We regret the confusion to the Reviewer regarding these citations. We do find that all three citations refer to the leakage of cells during the surgical process results in seeding of the peritoneal spread, as we highlighted above. Additionally, the surgery (or 'wounding') itself may contribute to the tumor progression in the event of tumor cell leakage during tumor injection (Foster et al., JCI Insight, 2018., Kasper et al., PNAS 2010., Stuelten et al., Cancer Research 2008.). We have edited the manuscript to reflect this distinction on pages 2 and 11.

Their "low titer" (1.25 x 10^6) and "high titer" (5 x 10^6) are not that different- both are high numbers of PDAC cells, yet they still only have ~70% success rate in tumor take using ultrasound guided injection (Table 1) and which is much lower than the surgical approach. They should discuss this limitation of the UG-OTIM approach, and actually titer robustly (10^4 - 10^5 - 10^6) tumor cells using surgery vs. UG-OTIM because their results raise the possibility that there is something unusual about this particular KPC cell line if it requires so many tumor cells for tumor establishment. Rigor and reproducibility would be added to this study if they show experiments with at least one other cell line.

Reply: We agree with the Reviewer that this is not an extensive range of cell numbers for injection, however, each murine pancreatic tumor cell line should be titrated for appropriate cell number to achieve desired growth kinetics *in vivo*. While 4662 grew at the desired growth rate with >1 x 10^6 cells, other cell lines may require far fewer cells. We have begun the process of titrating other cell lines in the laboratory currently. We have adjusted the text to reflect the need to titrate each cell line used in OTIM studies on page 6.

Is this model metastatic? One advantage of the KPC model is it metastatic, albeit not

uniformly. The authors should discuss this limitation and how it could be overcome potentially.

Reply: We thank the Reviewer for raising this important point. While the KPC mouse model has been reported to be grossly metastatic in a subset of mice, the 4662 cell line is not metastatic under the orthotopic conditions that we have tested. We find that the rate of primary tumor growth may prevent a time course that would allow for the establishment of gross metastatic tumors, however, as Aiello et al. (Cold Spring Harbor Protocols, 2016) have reported, a lower cell injection number may result in higher metastatic tumor rate. These points regarding metastases have been added in the text on page 10.

Minor Concerns:
It is unclear what the authors mean on lines 291-293, 294, regarding "clinically relevant" tumor size of 20mm^3. They use the word "clinically" again on line 303. Why is this particular tumor size "clinically relevant"? It seems like they should remove the ambiguous terms "clinically, and clinically relevant" in this methods report.

Reply: We agree with the Reviewer that this terminology was confusing and have removed these statements from the text on pages 1, 6, 10.

In lines 389-391, the authors state the UG-OTIM recapitulates KPC tumors, yet this is not unique to UG-OTIM. This is just a reflection of implanting a cell into the pancreas and this should be indicated here.

Reply: The Reviewer’s point is well taken that other models can also recapitulate salient features of the KPC microenvironment. We have adjusted the text to more accurately reflect this fact on page 9.