Journal of Visualized Experiments

A method for orthotopic transplantation of lung cancer in mice

--Manuscript Draft--

Manuscript Number:	JoVE55346R2
Full Title:	A method for orthotopic transplantation of lung cancer in mice
Article Type:	Invited Methods Article - JoVE Produced Video
Keywords:	orthotopic transplantation; lung cancer; mouse; intrathoracic injection; tumor microenvironment; xenograft
Manuscript Classifications:	2.1.50.150.900.649.865.635.505.500: Mice; 3.4.588.894.797.520: Lung Neoplasms; 5.4.928: Thoracic Surgical Procedures; 5.5.17: Animal Experimentation
Corresponding Author:	James Kim University of Texas Southwestern Dallas, TX UNITED STATES
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	James.Kim@utsouthwestern.edu
Corresponding Author's Institution:	University of Texas Southwestern
Corresponding Author's Secondary Institution:	
First Author:	Alexandra N. Wilson
First Author Secondary Information:	
Other Authors:	Alexandra N. Wilson
	Baozhi Chen
	Xin Liu
	Jonathan M. Kurie
Order of Authors Secondary Information:	
Abstract:	Lung cancer is the most common cause of cancer death worldwide for which chemotherapy still remains the dominant mode of systemic therapy for most metastatic lung cancers. Preclinical mouse models of lung cancer have been vital experimental tools to elucidate cancer biology and test novel therapeutic regimens. Two main models are most commonly used - genetically-engineered mouse models and xenograft transplantation models. The most common xenograft model employs subcutaneous transplantation of tumor cells. However, the subcutaneous space is a foreign environment to lung cancer cells and does not appropriately model the tumor-stromal interactions of endogenous lung cancers. Here, we present an orthotopic mouse model of lung cancer that utilizes direct injection of cancer cells into the lung parenchyma. The protocol describes this procedure and its potential applications for lung cancer research.
Author Comments:	
Additional Information:	
Question	**Response**
If this article needs to be "in-press" by a certain date, please indicate the date below and explain in your cover letter.	

UT Southwestern Medical Center

James Kim, MD, PhD
Assistant Professor
Dept. of Internal Medicine

Division of Hematology-Oncology
Nancy B. & Jake L. Hamon Center
for Therapeutic Oncology Research

October 6, 2016

Dr. Jaydev Upponi, Ph.D.
Science Editor, *Journal of Visualized Experiments*

Re: Manuscript 55346_R1_090216, "A method for orthotopic transplantation of lung cancer in mice"

Dear Dr. Jaydev Upponi,

We are pleased to submit our revised manuscript 55346_R1_090216, "A method for orthotopic transplantation of lung cancer in mice" for consideration for publication in the *Journal of Visualized Experiments*. We thank you and the reviewers for positive comments and their suggestions for improvement of this work. We have made the editorial modifications as noted in the document, "CoverLetter_Response to Editorial Comments". We believe that we have addressed the reviewers' comments and have made changes in figures (new Figures 2 and 3) and in the text of our manuscript detailed in the document, "CoverLetter_Response to Reviewer's comments". In both documents, our responses are italicized following ">>>". We have uploaded two version of our manuscript. The manuscript where we have tracked changes is in the document, "55346_R1_090216_revised_trackchanges.docx". The revised manuscript without the tracked changes is in "55346_R1_090216_revised_clean.docx."

Thank you again for the opportunity and encouragement to resubmit our manuscript.

Sincerely,

James Kim, MD PhD

5323 Harry Hines Blvd. / Dallas, Texas 75390-8593 / Phone: 214-648-1456 - Fax: 214-648-4940
E-Mail: james.kim@utsouthwestern.edu
www.utsouthwestern.edu

TITLE:
A method for orthotopic transplantation of lung cancer in mice

AUTHORS and AFFILIATION:
Alexandra N. Wilson[1,*], Baozhi Chen[1,*], Xin Liu[2], Jonathan M. Kurie[2], and James Kim[1,3,]

[1]Nancy B. and Jake L. Hamon Center for Therapeutic Oncology Research and Harold C. Simmons Comprehensive Cancer Center, University of Texas Southwestern Medical Center, Dallas, TX 75390
[2]Department of Thoracic/Head and Neck Oncology, University of Texas M.D. Anderson Cancer Center, Houston, TX 77030
[3]Department of Internal Medicine, University of Texas Southwestern Medical Center, Dallas, TX 75390

*Authors contributed equally to this work

CORRESPONDING AUTHOR:
James Kim
5323 Harry Hines Blvd
Hamon Center for Therapeutic Oncology Research
UT Southwestern Medical Center
Dallas, TX 75390-8593
Ph: 214-648-4180
Fax: 214-648-4940
james.kim@utsouthwestern.edu

KEYWORDS:
orthotopic transplantation, lung cancer, mouse, intrathoracic injections, tumor microenvironment, xenograft

SHORT ABSTRACT:
This protocol describes a model for the orthotopic transplantation of lung cancer. This model enables studies of lung cancer biology and therapeutics *in vivo* in a context that more closely resembles the native tumor environment than subcutaneous or renal capsule transplant models.

LONG ABSTRACT:
Lung cancer is the most common cause of cancer death worldwide. Chemotherapy still remains the dominant mode of systemic therapy for most metastatic lung cancers. Preclinical mouse models of lung cancer have been vital experimental tools to elucidate cancer biology and to test novel therapeutic regimens. Two main models are most commonly used: genetically-engineered mouse models and xenograft transplantation models. The most common xenograft model employs subcutaneous transplantation of tumor cells. However, the subcutaneous space is a foreign environment to lung cancer cells and does not appropriately model the tumor-stromal interactions of endogenous lung cancers. Here, we present an orthotopic mouse model of lung cancer that utilizes the direct injection of cancer cells into the lung parenchyma. The protocol describes this procedure and its potential applications for lung cancer research.

INTRODUCTION:

Lung cancer is the most common cause of cancer death worldwide for both men and women[1]. Less than 4% of metastatic lung cancer patients are alive at 5 years or later after their initial diagnosis[2]. Lung cancer is broadly divided into two categories: small cell and non-small cell lung cancer (NSCLC), which accounts for ~85% of lung cancers and consists mostly of adenocarcinoma and squamous cell carcinoma histologies. For metastatic lung adenocarcinoma, oral targeted therapies are now available for those cancers with activating EGFR mutations[3-5] and ALK fusion proteins[6]. However, these two mutations account for only ~20% of lung adenocarcinomas and are predominantly found in non-smokers. For all other lung cancers, chemotherapy remains the only systemic treatment option in the first-line setting. Immune-checkpoint therapies against the PD-1/PD-L1 axis have shown prolonged survival in subsets of metastatic NSCLC patients. Single agent nivolumab (anti-PD1 antibody) and pembrolizumab (anti-PD-L1 antibody) are now approved by the U.S. Food and Drug Administration (FDA) for second-line use[7,8]. Active research is underway to identify biomarkers that will predict those patients who will respond to these therapies. Therefore, given the lethality of the disease and the need for improvements in therapy, preclinical mouse models are vital in lung cancer research in order to elucidate *in vivo* tumor biology and to test new therapeutic regimens.

Two forms of preclinical mouse models of cancer are primarily used: xenograft transplantation models and autochthonous models of either genetically-engineered mouse models (GEMM)[9] or carcinogen-induced models[10]. A discussion of the advantages and disadvantages of these models is beyond the scope of this article. Here, we will focus on transplantation models.

Xenograft transplant of human and murine cancer cells into immunocompromised mice—typically nude or NOD-SCID—have been used for decades. Subcutaneous transplantation has been the most common mode of generating lung cancer xenografts, whether the cells are established cell lines or primary cancer cells. This approach has provided many insights into lung cancer biology and has established many therapeutic regimens that have been translated into human studies. However, a major concern of the subcutaneous model is that tumor implantation and growth occur in non-native environments and therefore will not accurately model all of the appropriate tumor-stromal interactions of lung cancers. Orthotopic models of lung cancer attempt to abate these issues. The two main forms of orthotopic models include endobronchial implantantion[11,12] and direct thoracic implantation of lung cancer cells[13-17]. Here, we present a modified version of a direct intrapulmonary implantation model that is meant to be easily accessible and reproducible across a large cohort of mice.

PROTOCOL:

All procedures below have been approved by the Institutional Animal Care and Use Committee (IACUC) at the University of Texas Southwestern Medical Center at Dallas and results shown have been performed in accordance with its policies. Performance of the following procedure should be done in accordance with the individual IACUC of each institution.

1. Preparation of instruments

1.1) Mark the needle of the liquid microsyringe to be used for the orthotopic injection with a line 4 mm from the tip using a solvent-resistant marker.

1.2) Autoclave all surgical tools, gloves, tissue paper, paper towels, and bench pads at 250 °F for 20 min.

1.3) With 70% ethanol, thoroughly clean the surface of a portable fiber-optic light source to be placed in the hood during the surgical procedure. Dilute 100 mg/mL ketoprofen to a 0.5 mg/mL solution in 0.9% sterile saline.

2. Preparation of cells for injection

2.1) Grow the lung cancer cells of interest in the growth medium appropriate for the cell line. For many cancer cell lines, use RPMI media without L-glutamine supplemented with 5% fetal bovine serum, 1X penicillin-streptomycin, and 1X L-glutamine (or glutamine alternative). Maintain the cells at 37 °C in a humidified atmosphere with 5% CO_2.

2.2) On the evening prior to or the day of the procedure, thaw frozen growth-factor reduced extra-cellular matrix (ECM) gel on ice until it is a free-flowing liquid.
NOTE: Depending on the volume of the ECM gel, it may take several hours or the whole night for the ECM gel to thaw completely.

2.3) Dilute the thawed ECM gel with ice-cold, sterile phosphate-buffered saline (PBS) to a concentration of 25% by volume in a sterile laminar flow hood.
NOTE: It is important to keep the ECM gel on ice so that it maintains its liquid form. ECM gel will begin to solidify as the temperature is increased.

2.4) When the cells are ~80% confluent, trypsinize them for 3-5 min with the appropriate amount of 0.25% trypsin-EDTA for the flask. Neutralize the trypsin by adding complete growth medium (typically 5-10x the trypsin volume) and triturate the cells into a single-cell suspension.

2.5) Centrifuge the single-cell suspension at 100 x g for 5 min, aspirate the supernatant, and re-suspend the cells in 5-10 mL of growth medium.

2.6) Mix a volume of the resuspended cells with an equal volume of trypan blue and count the number of viable cells using a hemocytometer or an automated cell counter. Calculate the volume of 25% ECM gel needed to deliver the desired number of cells in a volume of 15-25 µL per mouse.

NOTE: The cell size and total number of cells per injection need to be considered when calculating the volume to inject per mouse. If the cell size is large or a large number of cells (> 1 million) are being injected, then we recommend using injection volumes of 20-25 µL per mouse. Example: 6.0×10^5 cells per mouse are to be injected in 15 µL per mouse in 2 mice; thus, 1.2×10^6 cells in 30 µL of 25% ECM gel total are needed (or 4.0×10^4 cells/ µL). The cell count with trypan blue shows a concentration of 2.0×10^5 cells/mL and the total cell volume at 5 mL. The actual cell concentration is 4.0×10^5 cells/mL, as an equal volume of trypan blue is added to the cell suspension and thus dilutes the cells by a factor of 2. Thus, the total number of cells is 2.0 million cells (5 mL x 3.0×10^5 cells/mL). A volume of 50 µL of 25% ECM gel will be required (volume = 2.0×10^6 cells ÷ 4.0×10^4 cells/ µL).

2.7) Centrifuge the single-cell suspension in growth medium from step 2.5 at 100 x g for 5 min, aspirate off the supernatant, and re-suspend the cells in ice-cold 25% ECM gel (growth factor reduced)/PBS solution to the desired concentration of cells. Mix gently and place the cell suspension on ice.

2.8) Fill one 15-mL conical centrifuge tube with 10 mL of PBS and put it on ice.

4. Pre-surgical preparation

4.1) Perform the procedure in a sterile laminar flow hood to maintain a sterile environment. Wipe the hood with 70% ethanol or the disinfectant provided by the institution (*e.g.*, quaternary ammonium disinfectant).

4.2) Place the mouse in a vented induction chamber to induce anesthesia. Induce anesthesia with 5% isoflurane in oxygen at a flow rate of 2.0 L/min delivered by a precision vaporizer.

4.3) Check the level of anesthesia by pinching the toes and monitor for a withdrawal reflex; proper anesthesia has been obtained when there is a lack of toe withdrawal and a decreased breath rate. When the mice have reached a sufficient depth of anesthesia, lower the isoflurane to 2-3% for anesthesia maintenance.

NOTE: If metered isoflurane is unavailable, anesthesia may be induced using other approved agents such as ketamine/xylazine. Please refer to your institutional animal resource facility and IACUC for guidance on the use and doses of alternative anesthetics.

4.4) In a sterile laminar flow hood, place a sterile, absorbent underpad. Place sterile paper towels on the underpad.

4.5) Place the anesthetized mouse on the sterile paper towels, with its snout in the nasal cone delivering isoflurane 2-3% in the right lateral decubitus position. If using mice with fur, shave the fur from left thorax in an approximately 2.5 x 2.5 cm area using an electric clipper or trimmer. After shaving, return the animal to the anesthesia chamber. Repeat this step until all mice are shaved. Skip to step 5.1 if using nude mice.

4.6) Dispose of all disposable materials used to shave the mouse.

4.7) Clean the hood with 70% ethanol or another disinfectant favored by the institution's animal facility.

4.8) Place new autoclaved bench pads in the hood. Open the autoclaved surgical tools and lay them in the hood, being careful not to touch the tools. Place the autoclaved gloves in the hood. Place the fiber-optic light source (surfaces cleaned with 70% ethanol) in the laminar hood and direct the light over the surgical area.

5. Orthotopic Injection

5.1) Transfer the mouse from the anesthesia chamber to the sterile hood. Place the mouse in the right lateral decubitus position and keep it under anesthesia using a nasal cone delivering

isoflurane. Apply ophthalmic ointment lubrication to each eye to prevent eye desiccation during anesthesia.

5.2) Check for the lower edge of the ribs and the scapula using forceps.

5.3) Swab the surgical area with povidone-iodine beginning at the center of the surgical field and moving in a circular motion until the distal boundaries are reached. Remove the povidone-iodine solution by wiping the area with 70% ethanol pads. Repeat two more times.

5.4) Confirm that the mouse is anesthetized by checking the toe pinch reflex prior to continuing.

5.5) Using forceps, pull up the skin of the left lateral thorax away from chest wall to avoid injury to the chest wall. Using surgical scissors, make a ~1-cm transverse incision ~0.5 cm below the inferior border of the left scapula along the midline long axis of the left lateral side of the mouse chest.

5.6) Make additional incisions through the subcutaneous fat and fascia until the chest wall is visualized. Take care to avoid cutting or nicking any visible blood vessels. Separate and widen the incision area using curved hemostatic forceps to ensure that all the layers have been cut through and that the ribs are visualized. Observe the respiring lung as a pale pink structure under the rib cage. Do not penetrate the chest wall.

5.7) Wash the needle of the microsyringe with ice-cold, sterile PBS to keep the needle cold. Mix the suspended cell solution gently and take 15-25 µL into the microsyringe, depending on the desired number of cells to inject.

5.8) Sterilize the gloved left index and middle fingers with a 70% ethanol wipe and use these two fingers to hold the incision area open.

5.9) The lowermost rib seen through the incision should be rib 8. Visually count the ribs cranially until ribs 6 and 5 are reached. Hold the needle at a 90° angle to the chest wall surface and inject the needle 4 mm into the midline of the left lateral side, just above rib 6 and inferior to rib 5 (the mouse lung is wider and thicker at this level).
NOTE: It is critical to inject the needle just above rib 6 to avoid the nerve and vessel bundles that line the inferior aspect of rib 5.

5.10) Slowly inject the cells into the lung and hold the needle in place for a few seconds afterwards to ensure that all cells have been properly injected.
NOTE: The injection of the cells into the lung parenchyma is usually visible.

5.11) Close the incision site by lining up the underlying tissue and skin. Place two staples with a wound clip applier. Clean the incision site with sterile PBS.

5.12) Inject 5 mg/kg of ketoprofen intraperitoneally. Administer a second dose of ketoprofen 24 h after surgery to minimize animal pain and discomfort.

5.13) Place the mouse in the left lateral decubitus position, with the incision site facing downwards, alone in a warmed cage. Monitor the mouse until it is fully recovered from anesthesia, and then return it to the company of other mice.

5.14) Wipe the surgical tools with 70% ethanol and repeat steps 5.1-5.12 for each mouse. Perform steps 4.7-4.8 and wash the instruments with 70% ethanol to maintain a sterile environment.

6. Removal of the wound clips

6.1) After 10-14 days, anesthetize the mice, as described in steps 4.2-4.4, and remove the wound clips with a wound clip remover. Allow the mice to recover from anesthesia, as described in step 5.13.

6.2) Determine the time of tumor formation empirically, as the growth of tumor cells varies from tumor to tumor. Evaluate metastases using non-invasive imaging methods including bioluminescence, computed tomography, and magnetic resonance.

REPRESENTATIVE RESULTS:

The surgical tools for the procedure are shown in Figure 1. Three million HCC515 lung adenocarcinoma cells stably expressing firefly luciferase (HCC515-FL) in 25% growth-factor reduced ECM gel were injected into the left lung according to the protocol. A sharp signal in the left lung (Figure 2A) was detected by *in vivo* bioluminescence[18,19], which is suggestive of tumor formation. The lungs were dissected, inflated, and fixed with 4% paraformaldehyde overnight[20-22]. The left lung showed a visible mass (Figure 2B) where the tumor cells had been injected. The left lung from Figure 2B was processed and embedded in paraffin, and 5-µm sections were cut per standard protocols. A hematoxylin and eosin stain showed the tumor to be a poorly differentiated adenocarcinoma (Figure 2C). These results verify the implantation and growth of HCC515-FL tumor cells within the lung parenchyma.

In another experiment, we injected 1 million HCC515-FL lung adenocarcinoma cells into the left lung per the described protocol. Four weeks after injection, the lungs were dissected, and gross metastases were identified in the mediastinal lymph nodes (Figure 3A-C). An H&E image of left lung revealed two distinct nodules of adenocarcinoma (Figure 3C, arrowheads), with one that had invaded through the pleura (Figure 3C, red arrowhead). Figure 3B shows a representative image of mediastinal lymph nodes in which tumor cells had taken over most of the lymph node (Figure 3B), with only small areas of lymphocytes and residual lymph node left (Figure 3B, arrowheads). These results suggest that the tumor cells injected into the left lung formed tumors that metastasized to the mediastinal lymph nodes.

FIGURE LEGENDS:

Figure 1. Instruments for the orthotopic injection of lung cancer cells. The necessary instruments are: (1) an autoclip wound clip applier; (2) an autoclip wound clip remover; (3) VICI Pressure-LOK C-160 liquid microsyringe; (4) Shandon Halstead's curved hemostatic mosquito forceps; (5) dissecting extra-fine-pointed splinter forceps, 4.5 in; (6) Shandon broad-point dressing thumb forceps, 5 in; (7) straight-blade operating scissors; and (8) straight-blade

operating scissors, blunt/sharp, 5.5 in. Please refer to the "Table of Materials/Equipment" for details.

Figure 2. Orthotopic transplant of human lung cancer. (A) An orthotopic tumor of HCC515 lung adenocarcinoma cells stably expressing firefly luciferase in the left lung of nude mouse cells is detectable by *in vivo* bioluminescence from the right decubitus and prone positions. (B) Gross tumor nodules (arrowhead) are visible in left lung of the mouse from panel A. (C) H&E image of the left lung of panel B shows poorly differentiated adenocarcinoma (arrowheads). The scale bar is 500 µm.

Figure 3. Metastasis of lung cancer in an orthotopic transplant model. (A) Metastases to mediastinal lymph nodes (green arrowheads) are found four weeks after the injection of 1 million HCC515 lung adenocarcinoma cells into left lung of nude mice. The primary tumor is shown in a dashed red circle. The heart is not shown. (B) Representative H&E image of the mediastinal lymph nodes. The arrowheads indicate areas of lymphocytes and the remaining lymph node. The scale bar 100 µm. (C) H&E image of the left lung tumors (arrowheads), highlighted by the dashed red circle in panel A. One tumor has invaded through the visceral pleura (red arrowhead). The scale bar is 500 µm.

DISCUSSION:

We recommend that researchers dedicate at least 10-15 min per mouse for the most accurate injections, the best survival rates, and the most consistent results. For consistent tumor growth across cohorts of mice, researchers should prepare cell suspensions in small batches and mix the suspensions thoroughly before each injection. The use of a stereotactic apparatus (not described here) to control the injection of tumor cells may also increase the consistency of injections from mouse to mouse.

We highly recommend rinsing the autoclaved glass microsyringe thoroughly with 70% ethanol in water followed by sterile water between injections to minimize cross-contamination between animals. Finally, as this technique requires minor surgery, the sterility of all tools (Figure 1) and equipment is critical to minimize post-surgical infections (especially if using immunocompromised animals) and to optimize surgical outcomes. We recommend that all surgical instruments be autoclaved prior to use and that the procedure be performed in a sterile environment.

The efficacy of this orthotopic model highly depends on the characteristics of the cancer cells and their ability to grow *in vivo*. In our experience, the growth of tumor cells in culture often does not correlate with their growth *in vivo.* To ensure optimal experimental results, a pilot experiment should be performed where tumor cells are implanted orthotopically in several mice and followed over the course of weeks or months to give an understanding of the kinetics of *in vivo* tumor growth for a given cell line or set of cancer cells. *In vivo* imaging—such as bioluminescence (Figure 2), computed tomography (CT), or magnetic resonance imaging (MRI)—may aid in establishing the kinetics of tumor growth.

We have modified the amount of ECM gel and the volume of injected tumor cells from other reports of lung orthotopic transplantations[14-16]. The volume of injected tumor cells has been

reduced to 15-25 µL in order to minimize the lung volume in which the tumor cells will spread and to generate a more compact tumor. Secondly, 25-30% ECM gel in PBS was found to be the highest concentration of ECM gel in the small tumor volumes that allowed for the smooth injection of cells without too much resistance from the viscosity of the ECM gel. Because we use a diluted ECM-cancer cell suspension, the microenvironment of our protocol is not entirely "native." However, as the cancer cells grow, they will interact with the lung microenvironment, including fibroblasts, immune cells, and vessels. We also recommend use of "growth-factor reduced" versions of commercially available ECMs to minimize the effects of these growth factors found in the regular versions of ECM on tumor cell biology.

Orthotopic methods of cancer transplantation aim to establish exogenous cancer cells within their analogous native environment. The direct injection of cancer cells into the lung parenchyma has several advantages over other methods for orthotopic lung transplantation techniques. Surgical[11] and non-surgical[12,22] methods of intrabronchial/intratracheal administration of lung cancer cells have been described. These models can induce tumor growth in all of the lung lobes, although, in practice, most of the tumors are found in the right inferior lobe due to bronchial anatomy and gravity[11].

The non-surgical injection of tumor cells into the murine thorax has also been described[13,17]. Although this is a non-surgical technique, we have found a wide variability of established cancers in the lung parenchyma and frequent cancers in the pleural cavity and chest wall. With the method described here, we occasionally find small masses on the chest wall, distinct from the lung tumors late in the evolution of tumor growth. These findings most likely reflect some seeding of tumor cells during the injection process. However, the vast majority of tumors cells are placed directly within one lobe (left lung), with minimal seeding of the pleural cavity.

Although the procedure described here is for orthotopic implantation in the left lung, the basic procedure can be modified for the right lung. The left lung was chosen for simplicity, as there is only 1 lobe. The right lung contains 3 lobes and an accessory lobe. The lobes can intersect the plane of the needle injection, depending on the location and depth of injection. Thus, these two factors will be need to be optimized prior to the injection of cells into the right lung.

Our experience with this protocol has primarily been non-small lung cancer cell lines, although it can be easily adapted to other cell types, including small cell lung cancer[14] and primary human cancer cells, to establish patient-derived xenografts. Additionally, we have successfully applied this technique to inject particles of adenovirus-expressing Cre recombinase directly into the lung parenchyma of $Kras^{G12D/+};Trp53^{fl/fl}$ mice and have generated unilateral tumors (data not shown). The use of adenoviral particles that express Cre recombinase under lineage-specific promoters (*e.g.*, *Sftpc-cre* for alveolar type II cells or *CC10-cre* for club and bronchioalveolar junction cells)[23] will be even more advantageous. Such viral particles will limit the expression of Cre recombinase lung epithelial cells, excluding stromal and immune cells. Unilateral tumors generated from the technique described here are amenable for therapeutic radiation studies and for metastasis studies that faithfully model many aspects of human lung cancer (Figure 3). Thus, the lung orthotopic transplantation model described in this protocol is a powerful tool to elucidate the biology of lung cancer cells and their response to therapeutics.

DISCLOSURES:
The authors have no competing financial interests.

ACKNOWLEDGEMENTS:
This work was supported in part by the University of Texas Southwestern and the American Cancer Society (ACS-IRG-02-196), the Bonnie J. Addario Lung Cancer Foundation (Young Investigator Award), and the Communities Foundation of Texas to JK.

REFERENCES:

1 Siegel, R., Ma, J., Zou, Z. & Jemal, A. Cancer statistics, 2014. *CA Cancer J Clin.* **64** (1), 9-29, doi:10.3322/caac.21208, (2014).
2 http://seer.cancer.gov/statfacts/html/lungb.html.
3 Lynch, T. J. *et al.* Activating mutations in the epidermal growth factor receptor underlying responsiveness of non-small-cell lung cancer to gefitinib. *The New England Journal of Medicine.* **350** (21), 2129-2139, doi:10.1056/NEJMoa040938, (2004).
4 Paez, J. G. *et al.* EGFR mutations in lung cancer: correlation with clinical response to gefitinib therapy. *Science.* **304** (5676), 1497-1500, doi:10.1126/science.1099314, (2004).
5 Pao, W. *et al.* EGF receptor gene mutations are common in lung cancers from "never smokers" and are associated with sensitivity of tumors to gefitinib and erlotinib. *Proc Natl Acad Sci U S A.* **101** (36), 13306-13311, doi:10.1073/pnas.0405220101, (2004).
6 Mosse, Y. P., Wood, A. & Maris, J. M. Inhibition of ALK signaling for cancer therapy. *Clinical cancer research : an official journal of the American Association for Cancer Research.* **15** (18), 5609-5614, doi:10.1158/1078-0432.CCR-08-2762, (2009).
7 Administration, U. S. F. a. D. *FDA approves Keytruda for advanced non-small cell lung cancer*, <http://www.fda.gov/NewsEvents/Newsroom/PressAnnouncements/ucm465444.htm> (2015).
8 Administration, U. S. F. a. D. *FDA expands approved use of Opdivo in advanced lung cancer*, <http://www.fda.gov/newsevents/newsroom/pressannouncements/ucm466413.htm> (2015).
9 Richmond, A. & Su, Y. Mouse xenograft models vs GEM models for human cancer therapeutics. *Dis Model Mech.* **1** (2-3), 78-82, doi:10.1242/dmm.000976, (2008).
10 Vikis, H. G., Rymaszewski, A. L. & Tichelaar, J. W. Mouse models of chemically-induced lung carcinogenesis. *Front Biosci (Elite Ed).* **5** 939-946 (2013).
11 McLemore, T. L. *et al.* Comparison of intrapulmonary, percutaneous intrathoracic, and subcutaneous models for the propagation of human pulmonary and nonpulmonary cancer cell lines in athymic nude mice. *Cancer Res.* **48** (10), 2880-2886 (1988).
12 Curtis, S. J. *et al.* Primary tumor genotype is an important determinant in identification of lung cancer propagating cells. *Cell Stem Cell.* **7** (1), 127-133, doi:S1934-5909(10)00281-X [pii]10.1016/j.stem.2010.05.021, (2010).
13 Onn, A. *et al.* Development of an orthotopic model to study the biology and therapy of primary human lung cancer in nude mice. *Clin Cancer Res.* **9** (15), 5532-5539 (2003).

14 Isobe, T. *et al.* Evaluation of novel orthotopic nude mouse models for human small-cell lung cancer. *J Thorac Oncol.* **8** (2), 140-146, doi:10.1097/JTO.0b013e3182725ff9, (2013).
15 Weiss, I. D. *et al.* In the hunt for therapeutic targets: mimicking the growth, metastasis, and stromal associations of early-stage lung cancer using a novel orthotopic animal model. *J Thorac Oncol.* **10** (1), 46-58, doi:10.1097/JTO.0000000000000367, (2015).
16 Wu, W. *et al.* Targeted therapy of orthotopic human lung cancer by combined vascular endothelial growth factor and epidermal growth factor receptor signaling blockade. *Mol Cancer Ther.* **6** (2), 471-483, doi:10.1158/1535-7163.MCT-06-0416, (2007).
17 Onn, A. *et al.* Epidermal growth factor receptor tyrosine kinase inhibitor does not improve paclitaxel effect in an orthotopic mouse model of lung cancer. *Clin Cancer Res.* **10** (24), 8613-8619, doi:10.1158/1078-0432.CCR-04-1241, (2004).
18 Zhang, Z. *et al.* Effective Rat Lung Tumor Model for Stereotactic Body Radiation Therapy. *Radiat Res.* **185** (6), 616-622, doi:10.1667/RR14382.1, (2016).
19 Rehemtulla, A. *et al.* Rapid and quantitative assessment of cancer treatment response using in vivo bioluminescence imaging. *Neoplasia.* **2** (6), 491-495 (2000).
20 Jackson, E. L. *et al.* Analysis of lung tumor initiation and progression using conditional expression of oncogenic K-ras. *Genes Dev.* **15** (24), 3243-3248, doi:10.1101/gad.943001, (2001).
21 Braber, S., Verheijden, K. A., Henricks, P. A., Kraneveld, A. D. & Folkerts, G. A comparison of fixation methods on lung morphology in a murine model of emphysema. *Am J Physiol Lung Cell Mol Physiol.* **299** (6), L843-851, doi:10.1152/ajplung.00192.2010, (2010).
22 Limjunyawong, N., Mock, J. & Mitzner, W. Instillation and Fixation Methods Useful in Mouse Lung Cancer Research. *J Vis Exp.* (102), e52964, doi:10.3791/52964, (2015).
23 Sutherland, K. D. *et al.* Cell of origin of small cell lung cancer: inactivation of Trp53 and Rb1 in distinct cell types of adult mouse lung. *Cancer cell.* **19** (6), 754-764, doi:10.1016/j.ccr.2011.04.019, (2011).

Figure 1

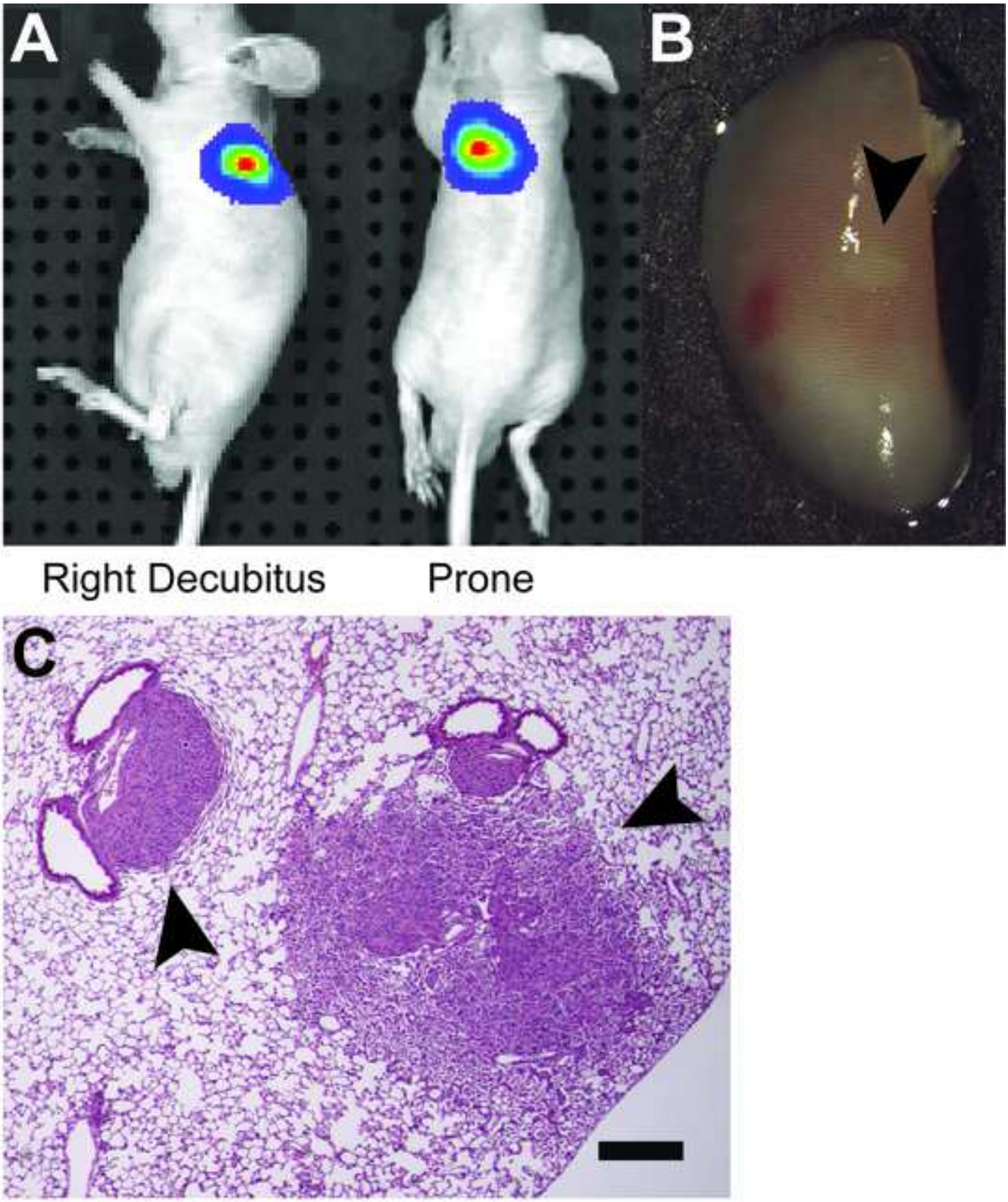

Figure 2.

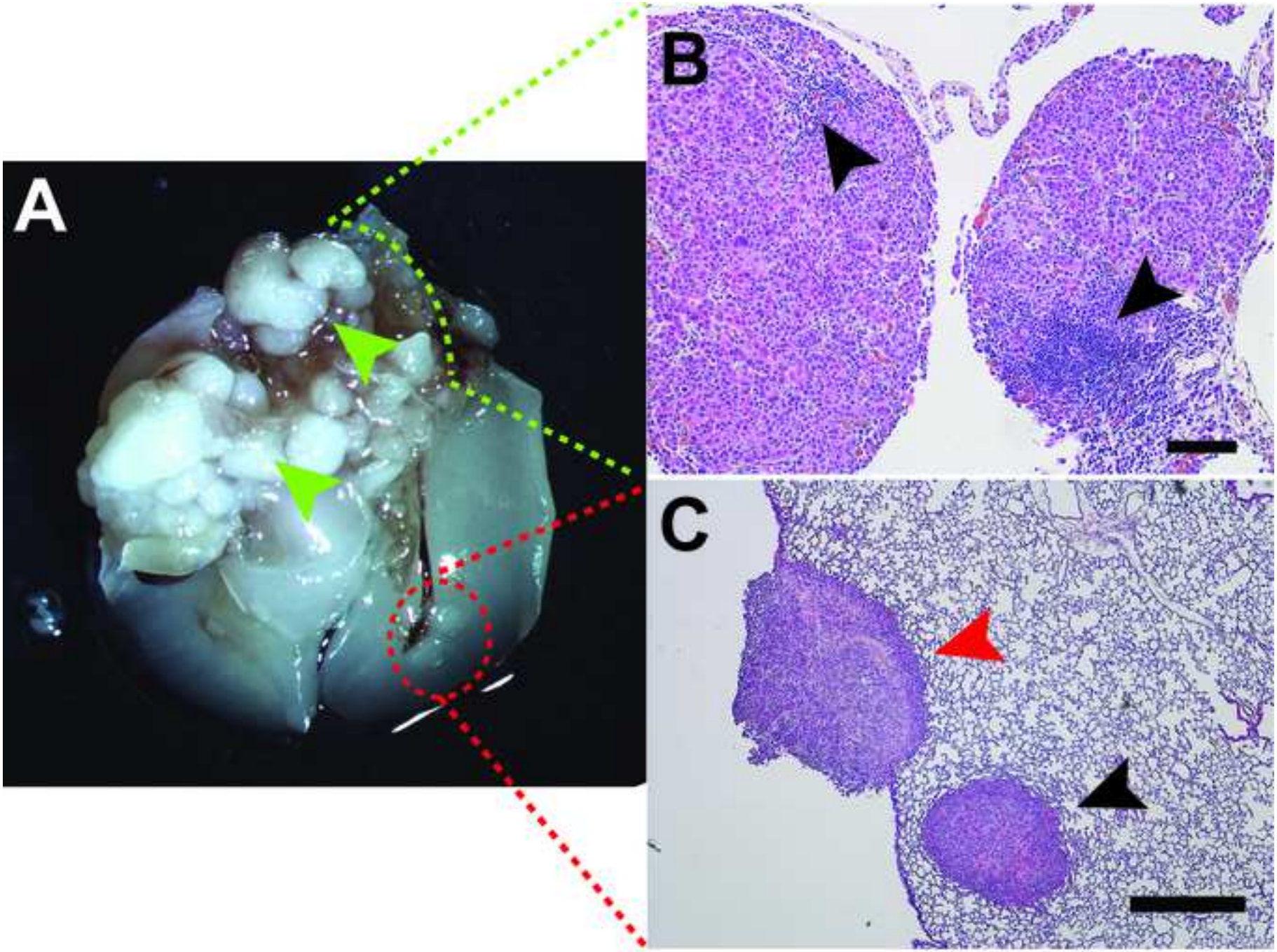

Figure 3.

Excel Spreadsheet- Table of Materials/Equipment

Click here to download Excel Spreadsheet- Table of Materials/Equipment Materials_Table_Wilson_Revised.xlsx

Name	Company	Catalog Number
RPMI Medium 1640	HyClone	R0883-500ml
Phosphate Buffered Saline	HyClone	SH30256.01
Penicillin-Streptomycin (10,000 U/mL)	Fisher Scientific	15140122
GlutaMAX (100X)	Gibco	35050-061
Corning Matrigel Membrane Matrix GFR	Fisher Scientific	CB-40230C
Fetal Bovine Serum	Atlanta biologicals	S11150
1/2CC U-100 Insulin Syringe	BD Biosciences	39461
VICI Pressure-LOK C-160 Liquid microsyringe, 50 ㎖	Sigma	Z170879-1EA
Povidone-Iodine Antiseptic Swabsticks	MEDLINE	MDS093902
Sterile Alcohol Prep Pads	Fisher Scientific	22-363-750
Thermo Scientific Shandon Halstead's Hemostatic Mosquito Forceps, Curved, Standard 5 in.	Fisher Scientific	507
Fisherbrand Straight-Blade Operating Scissors, Blunt/Sharp, 5.5 inch	Fisher Scientific	13-808-2

Fisherbrand Dissecting Extra-Fine-Pointed Splinter Forceps 4.5 inch	Fisher Scientific	13-812-42
Thermo Scientific Shandon Broad Point Dressing Thumb Forceps - Fine serrated tip, standard, 5 in	Fisher Scientific	63-03
Fisherbrand Straight-Blade Operating Scissors	Fisher Scientific	13-806-4
VETEQUIP Inhalation Anesthesia Systems IMPAC	VETEQUIP	
BD Autoclip Wound Closing System (Wound Clip Remover)	Fisher Scientific	01-804-15
BD Autoclip Wound Closing System (Wound Clip Applier)	Fisher Scientific	01-804
BD Autoclip Wound Closing System (Wound Clip)	Fisher Scientific	01-804-5
Ketoprofen	Pfizer	NADA 140-269
Absorbent Bench Underpads	VWR	56617-014
Fisher Scientific LED Gooseneck Illuminator	Fisher Scientific	12563501
Pocket Pro Pet Trimmer	Braintree Scientific	CLP-9931
IsoThesia (Isoflurane)	Henry Schein	50033
Artificial Tears Ointment	Henry Schein	48272
Process NPD One Step Disinfectant	Steris	639002

Comments
Without L-glutamine
Also sold by a number of other vendors
Thaw on ice
The needles should be autoclaved
Should be autoclaved
Should be autoclaved

Should be autoclaved
Should be autoclaved
Should be autoclaved
0.5mg/ml diluted in a sterile 0.9% saline solution
Should be autoclaved
Eye lubricant during anesthesia
Quaternary ammonium disinfectant diluted 1:256 for working solution

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

ARTICLE AND VIDEO LICENSE AGREEMENT

Title of Article: A method for orthotopic transplantation of lung cancer in mice

Author(s): Alexandra N. Wilson, Baozhi Chen, Xin Liu, Jonathan M. Kurie, and James Kim

Item 1 (check one box): The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via: ■ Standard Access ☐ Open Access

Item 2 (check one box):

■ The Author is NOT a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

ARTICLE AND VIDEO LICENSE AGREEMENT

1. Defined Terms. As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of *The Journal of Visualized Experiments;* "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. Background. The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. Grant of Rights in Article. In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. Retention of Rights in Article. Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. Grant of Rights in Video – Standard Access. This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. Grant of Rights in Video – Open Access. This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this Section 6 is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. Government Employees. If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. Likeness, Privacy, Personality. The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

9. Author Warranties. The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

10. JoVE Discretion. If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have

full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

11. Indemnification. The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in *JoVE* or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

12. Fees. To cover the cost incurred for publication, JoVE must receive payment before production and publication the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

13. Transfer, Governing Law. This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement required per submission.

CORRESPONDING AUTHOR:

Name:	James Kim
Department:	Dept. of Internal Medicine, Div. of Hematology-Oncology
Institution:	University of Texas Southwestern
Article Title:	A method for orthotopic transplantation of lung cancer in mice
Signature:	Digitally signed by James Kim Date: 2016.08.10 10:05:24 -05'00'
Date:	08/10/2016

Please submit a signed and dated copy of this license by one of the following three methods:

1) Upload a scanned copy of the document as a pfd on the JoVE submission site;
2) Fax the document to +1.866.381.2236;
3) Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02139

For questions, please email submissions@jove.com or call +1.617.945.9051

UT Southwestern Medical Center

James Kim, MD, PhD
Assistant Professor
Dept. of Internal Medicine

Division of Hematology-Oncology
Nancy B. & Jake L. Hamon Center
for Therapeutic Oncology Research

October 6, 2016

Dr. Jaydev Upponi, Ph.D.
Science Editor, *Journal of Visualized Experiments*

Re: Manuscript 55346_R1_090216, “A method for orthotopic transplantation of lung cancer in mice”

Dear Dr. Jaydev Upponi,

Thank you for the invitation to resubmit the above manuscript. We appreciate the editorial comments to improve this manuscript. In the enclosed revised manuscript, I believe we have addressed the all of the editorial comments in regard to this manuscript. Our responses are below each comment and is italicized following “>>>”.

Editorial Comments:
•Please keep the editorial comments from your previous revisions in mind as you revise your manuscript to address peer review comments. For instance, if formatting or other changes were made, commercial language was removed, etc., please maintain these overall manuscript changes.

>>> Previous changes in response to editorial comments have been retained.

•Animal use note: JoVE veterinary reviewers recommend using a scalpel, not scissors, to make initial incisions for survival procedures.

>>> As noted in previous version, we have discussed this with our veterinarians at UTSW who noted that using scissors as described is safe. Due to the small incision that our procedure requires and the thinness of the skin, we feel that if safer for the mouse and operator if we pull up on the skin with forceps to elevate it away from chest wall and cut a small incision. Thus, this limits the risk of damage to the underlying chest wall and ribs.

•The manuscript should receive copyediting for grammar and formatting issues. Some examples are listed here, although this is not a comprehensive list:
-Ranges of values, such as "3-5 min" or "5-10 mL" should be formatted as having a single hyphen and no spaces. Currently, the formatting varies from step to step (see, for example, steps 2.4 and 2.5).
>>> The text has modified to the requested formatting changes.

-SI units should be used consistently throughout (e.g. mL rather than ml, h rather than hrs, etc.).
>>> The text has modified to the requested formatting changes.

-Grammatical articles (e.g. a, an, the) are often missing throughout the Protocol section.

5323 Harry Hines Blvd. / Dallas, Texas 75390-8593 / Phone: 214-648-1456 - Fax: 214-648-4940
E-Mail: james.kim@utsouthwestern.edu
www.utsouthwestern.edu

>>> The text has modified to the requested formatting changes.

-The Short Abstract should probably begin, "This protocol..." rather than "The protocol..." Likewise, "This model" rather than "The model."
>>> The text has modified to the requested formatting changes.

-First sentence of Long Abstract:"Lung cancer is the most common cause of cancer death worldwide for which chemotherapy still remains the dominant mode of systemic therapy for most metastatic lung cancers."
>>> We are not clear as to what needs to be modified in this comment.

-Step 4.3: "Check the level of anesthesia by pinching the toes and monitor for withdrawal reflex."
>>> We are not clear as to what needs to be modified in this comment.

-Step 4.5: "Place anesthetized-mouse..." This hyphen is not necessary.
>>> The text has modified to the requested formatting changes.

-Step 5.1: "Place the mouse on the right lateral decubitus position" should be "in" rather than "on."
>>> The text has modified to the requested formatting changes.

-Step 5.12: The second sentence should be re-written in imperative tense.
>>> The text has modified to the requested formatting changes.

•Additional detail or clarification is needed:
-Step 4.5 instructs to use an electric clipper or trimmer to shave the fur of the mouse. Step 4.6 then instructs to "dispose of all materials used to shave the mouse." Do the authors really dispose of an electric trimmer after each shaving?
>>> Sentence has been modified such that all "disposable" items be disposed.

-Step 5.14 refers to steps 4.7-4.12, but section 4 ends with step 4.8.
>>> The references in step 5.14 has been corrected.

•If your figures and tables are original and not published previously, please ignore this comment. For figures and tables that have been published before, please include phrases such as "Re-print with permission from (reference#)" or "Modified from.." etc. And please send a copy of the re-print permission for JoVE's record keeping purposes.
>>> All figures are original.

•JoVE reference format requires that the DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.

5323 Harry Hines Blvd. / Dallas, Texas 75390-8593 / Phone: 214-648-1456 - Fax: 214-648-4940
E-Mail: james.kim@utsouthwestern.edu
www.utsouthwestern.edu

>>> We have been unable to find doi numbers for references #10, 11, and 19 including the actual articles. References 2, 7, and 8 are web pages and do not have doi numbers. All other references have doi numbers.

•IMPORTANT: Please copy-edit the entire manuscript for any grammatical errors you may find. The text should be in American-English only. This editing should be performed by a native English speaker (or professional copyediting services) and is essential for clarity of the protocol and the manuscript. Please thoroughly review the language and grammar prior to resubmission. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.

>>> We have copy-edited our manuscript for any grammatical errors.

•NOTE: Please include a line-by-line response letter to the editorial and reviewer comments along with the resubmission.

>>> Please see "CoverLetter_Response to Reviewers' Comments"

Thank you again for the comments and the opportunity to resubmit our manuscript.

Sincerely,

James Kim, MD PhD

5323 Harry Hines Blvd. / Dallas, Texas 75390-8593 / Phone: 214-648-1456 - Fax: 214-648-4940
E-Mail: james.kim@utsouthwestern.edu
www.utsouthwestern.edu

UT Southwestern Medical Center

James Kim, MD, PhD
Assistant Professor
Dept. of Internal Medicine

Division of Hematology-Oncology
Nancy B. & Jake L. Hamon Center
for Therapeutic Oncology Research

October 6, 2016

Dr. Jaydev Upponi, Ph.D.
Science Editor, *Journal of Visualized Experiments*

Re: Manuscript 55346_R1_090216, "A method for orthotopic transplantation of lung cancer in mice"

Dear Dr. Jaydev Upponi,

Thank you for the invitation to resubmit the above manuscript. We appreciate the reviewers' positive comments about this work, and their suggestions for improvement. In the enclosed revised manuscript, I believe we have addressed the reviewers' concerns with figures and additions to the text of the manuscript. Specific comments are reproduced below in the order in which they are raised, followed by our responses. Our responses are below each comment and is italicized following ">>>".

Reviewer #1: *Manuscript Summary:* This is an excellent description of a transplantation method critical for the lung cancer research. The orthotopic method has been described in a number of published papers over many years. However, the previous papers often lacked details of the method, failing to promote widespread use. The authors described the method in a concise-but-sufficiently detailed manner, so that anyone will be able to follow.
Major Concerns: N/A
Minor Concerns: In the discussion, the authors suggest extended use of the same method to inject Adeno-CMV-Cre virus into genetically engineered mouse models. This is an interesting idea that could address the difficulty in generating localized, unilateral tumors in the mouse model. However, the authors should be aware of several pitfalls associated with random infection of the Cre virus in fibroblast and immune cells, etc. The induction of the same oncogenic mutations may result in sarcoma and lymphoma in mice. This could be avoided using epithelial promoter-driven Cre virus.
Additional Comments to Authors: N/A

>>> We thank Reviewer 1 for their kind comments. Reviewer 1 makes an insightful comment regarding the use of adeno-cre virus driven by a constitutive promoter as we have described it. We have amended the text to suggest that a lineage-specific driver to drive Cre recombinase will be a better method as only lung epithelial cells will express Cre recombinase.

Reviewer #2: *Manuscript Summary:* This is a very nice description of this method that could be employed by many labs.

A) I presume that they had to include matrigel to keep the cells from spilling out into the pleural cavity but it seems a little peculiar that they push so hard in the abstract that these cell grow in

5323 Harry Hines Blvd. / Dallas, Texas 75390-8593 / Phone: 214-648-1456 - Fax: 214-648-4940
E-Mail: james.kim@utsouthwestern.edu
www.utsouthwestern.edu

their entirely nature environment with interaction with the correct environment etc but really they initiated growth in matrigel - authors should include something about this in abstract.

>>> We thank Reviewer 2 for their kind comments on our manuscript. We agree with Reviewer 2 that that use of matrigel is not entirely native. As we noted in the text, matrigel is used to confine the injected tumor cells to a region of the lung rather than spread throughout the whole left lung as would be the case without matrigel. However, we believe that this is primarily for the implantation and initial growth. As the tumor grows, it will interact with surrounding lung stromal cells, including fibroblasts, immune cells, blood and lymphatic vessels, that are more native than those in other tissue contexts such as subcutaneous or renal capsule spaces. We also use growth factor reduced matrigel, which has minimized levels of EGF, FGF, and other growth factors found in regular matrigel, to limit the impact of these growth factors on tumor growth. So while the use of matrigel is not entirely "native", the growth of lung cancer cells in the lungs will certainly be more native than if grown in other commonly used spaces. We have modified the text in the Discussion to note that because we use matrigel, it cannot be completely a native environment.

B) The authors might also want to address whether they detected pleural metastases (perhaps just from leakage during cancer cell injection)

>>> We do occasionally see masses in the left chest wall likely secondary to some leakage from the injection of cancer cells. We have noted this in the text of the Discussion.

C) and whether they confirmed by histology that the LN metastases are actually in LN.

>>> We thank the Reviewer 2 for their comment regarding the mediastinal lymph node metastases. We have modified Figure 3 to include H&E micrographs of left lung tumors and mediastinal lymph nodes to show the tumors. We have not included right lung micrographs as the tissue was poorly fixed. Thus despite the masses that are noted grossly, the morphology was too poor to identify the cells within the right lung masses as similar to the cancer cells seen in the left lung and mediastinal lymph nodes. We still believe that the right lung masses are tumors but without histological confirmation (see discussion in point D below), we cannot make this case. We have removed the previous Fig. 3B and mention of right lung metastases.

D) They suggest that the tumors in the other lung lobes is a metastases which might lead a reader to think that it went into the blood and circulated around and seeding the lung but they have no evidence of this, the cancer cell could just have easily got there through cancer cells spreading within the lung through the airways.
Major Concerns: N/A
Minor Concerns: N/A
Additional Comments to Authors: N/A

>>> Reviewer 2 makes a good point that cancer cells could have spread through the airways. In the text of the manuscript, we have removed mention of right lung metastases as noted in point B. But we would like to address this comment. Reviewer 2's point may be

5323 Harry Hines Blvd. / Dallas, Texas 75390-8593 / Phone: 214-648-1456 - Fax: 214-648-4940
E-Mail: james.kim@utsouthwestern.edu
www.utsouthwestern.edu

possible as the lung cancer cells were injected in a moderately viscous fluid (2% matrigel in PBS) and the mouse was placed in a right lateral decubitus position, allowing for cells to travel to the right lung by gravity. This would be an inherent issue with the technique as described here. However, we do not believe that was the case. If lung cancer cells did travel through the airways from the left lung to the right lung, it would have had to so without colonizing the larger airways, including the main stem left and right bronchi, as we see more nodules in the periphery of lungs than proximal portions. However, given the viscosity of the cell suspension mixture, we would expect the tumor cells to colonize the bronchial airways on their way to the right lung leading to medial tumors in left and right lungs, which we do not see. Also, as an inherent issue with the technique, we should consistently see some bioluminescence in the right lung early in tumor evolution as well. Again, we do not see this. In the experiment for Fig. 2, BLI imaging 1 week after tumor cell injection shows bioluminescence in left lung only at site of injection (see new Fig. 2A which now shows the bioluminescence from right decubitus and prone positions). We have consistently found only bioluminescence in the left lung early (1-2 weeks after injection) across 5 cell lines tested thus far.

Also, tumors in the mediastinal lymph nodes are distinct from the left lung with no medial left lung or right lung tumors attached to the mediastinum suggesting that tumor cells from the lungs did not invade the mediastinum. Rather, tumor cells more likely metastasized through the lymphatic vessels. This raises the possibility that tumor cells may also metastasize via a hematogenous route.

We again thank the reviewers for their suggestions regarding this work.

Sincerely,

James Kim, MD PhD

5323 Harry Hines Blvd. / Dallas, Texas 75390-8593 / Phone: 214-648-1456 - Fax: 214-648-4940
E-Mail: james.kim@utsouthwestern.edu
www.utsouthwestern.edu