**TITLE:**

**A method for orthotopic transplantation of lung cancer in mice**

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**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 women1. Less than 4% of metastatic lung cancer patients are alive at 5 years or later after their initial diagnosis2. 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 mutations3-5 and ALK fusion proteins6. 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 use7,8. 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 models10. 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 implantantion11,12 and direct thoracic implantation of lung cancer cells13-17. 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.

# Preparation of instruments

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

1. **Preparation of cells for injection**
   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% CO2.
   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.

* 1. 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.

* 1. 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. 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.
  3. 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 x 105 cells per mouse are to be injected in 15 µL per mouse in 2 mice; thus, 1.2 x 106 cells in 30 µL of 25% ECM gel total are needed (or 4.0 x 104 cells/ µL). The cell count with trypan blue shows a concentration of 2.0 x 105 cells/mL and the total cell volume at 5 mL. The actual cell concentration is 4.0 x 105 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 x 105 cells/mL). A volume of 50 µL of 25% ECM gel will be required (volume = 2.0 x 106 cells ÷ 4.0 x104 cells/ µL).

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

**4. Pre-surgical preparation**

* 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).
  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.
  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.

* 1. In a sterile laminar flow hood, place a sterile, absorbent underpad. Place sterile paper towels on the underpad.
  2. 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.
  3. Dispose of all disposable materials used to shave the mouse.
  4. Clean the hood with 70% ethanol or another disinfectant favored by the institution’s animal facility.

* 1. 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.

1. **Orthotopic Injection**
   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.
   2. Check for the lower edge of the ribs and the scapula using forceps.

* 1. 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.
  2. Confirm that the mouse is anesthetized by checking the toe pinch reflex prior to continuing.
  3. 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.
  4. 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.

* 1. 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.

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

* 1. 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.

* 1. 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.

* 1. 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.

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

* 1. 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.
  2. 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.

1. **Removal of the wound clips**
   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.
   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* bioluminescence18,19, which is suggestive of tumor formation. The lungs were dissected, inflated, and fixed with 4% paraformaldehyde overnight20-22. 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 transplantations14-16. 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. Surgical11 and non-surgical12,22 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 gravity11.

The non-surgical injection of tumor cells into the murine thorax has also been described13,17. 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 cancer14 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 *KrasG12D/+;Trp53fl/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.

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