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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

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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

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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,

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