3rd May 2018

Dr. Jaydev Upponi, Ph.D.

Science Editor

JoVE

Dear Dr. Upponi,

Thank you for your e-mail of 16th April 2015 regarding our manuscript entitled “JoVE53172R4” We have corrected all the points raised by the reviewers. The followings are the point-by-point responses to the reviewer’s comments. The changed, or added sentences in the text are highlighted. In addition, I would like to note that the revised manuscript contains direct cell counting using a fluorescent stereo microscope. This process is very simple and is worth being included in the manuscript.

Sincerely,

Takeshi Tomita

Department of Pharmacology

Tokyo Women’s Medical University

8-1 Kawada-cho, Shinjuku-ku,

Tokyo 162-8666, Japan

Editorial comments:

1) Grammar:

-The manuscript would benefit from copy editing for numerous grammatical errors, preferably by a native speaker. In particular, please make sure all grammar in the long abstract is correct.

-Figure 2 legend: please clarify/correct grammar for “red fluorescence possibly does their remnants.”

[Answer1] Thank you for your comments. I sent the manuscript a proofreading service.

2) Highlighting – Under half a page is highlighted for filming (as we cannot film mouse euthanasia). At a minimum, the tail injection from section 1 should also be filmed, as it is the most critical step in the protocol.

[Answer2] I would like to film from step 2.1) (except for euthanization) to step 4.4).

3) Additional detail is required:

-1.3 – Please provide stepwise detail on how the tail injection is performed, given this is a critical step. What steps are taken to minimize leakage or incomplete injection?

-2.2 – How is euthanasia confirmed? This information is required for ethical reasons.

-3.4 – Please provide stepwise detail or a citation for flow cytometry.

-4.1 – Are the lungs still inside the carcass when they are injected with OCT compound? If so, how are the lungs removed?

[Answer3] -Unfortunately, we cannot provide tips for the tail vein injection. Our hands are not as good as those of experienced technicians. We only did repeat practice to improve our technique.

-For euthanasia, we follow the guideline provided by NIH OACU. “The CO2 gas flow is maintained for a minimum of 1 minute after observation of each mouse for lack of respiration and faded eye color.”

-We use FC-500 flow cytometer. Its detailed procedure is unique to the model so that it cannot be applied to the other types of flow cytometer. If this does not matter, I will provide the detail.

-My answer is yes. The lungs are still inside when they receive OCT or PFA/PBS injection. This part is removed in the revised version.

4) Discussion: A citation for the information the second sentence of the last paragraph is necessary.

[Answer4] I added the reference.

5) Unnecessary branding in 3.4 - FC-500.

[Answer5] I removed it.

6) 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.

[Answer6] The format is maintained.

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

[Answer7] Thank you. As abovementioned, the manuscript is checked by a proofreading service.

8) 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.

[Answer8] Our data in this manuscript are original and never published before.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This manuscript describes a quantification method to study the tumor metastasis with intravenous injection of PKH26-labed tumor cells. In general, this manuscript is well written and presented in a logical fashion. The authors elaborated the advantages to studying the tumor metastasis with intravenous injection of tumor cells. However, the limitation of this method should also be discussed.

Major Concerns:

1.How long does the signal of PKH26 last? Is it possible to detect the PKH26 labeled tumor cells 14 days (or even longer) after the intravenous injection of tumor cells?

[Answer#1-1] Thank you for your comments. In our experience, the lifetime of PKH26 label is at least more than a week. Since the PKH26 labeling is diluted in the dividing cells, we consider that tracking PKH26 labeled cells in vivo is impossible. Additionally, labeled cell is a good target for the resident macrophages. Macrophages retain the fluorescence after the intake. Thus, PKH26 labeled cells may be still detected even14 days after the injection, but the signal does not guarantee the presence of initially labeled cells.

2. Page 2, starting on row 82: The circulating tumor cells must be different from the primary tumor cells because some changes have happened before the intravasation (e.g. EMT process, etc). So, the tumor cells directly injected into the blood vessel should have some differences from the naturally found circulating tumor cells. This point is not discussed.

[Answer#1-2] Thank you for your informative comment. We added sentences to describe the difference between circulating tumor cells and artificially injected tumor cells.

Minor Concerns:

1. Page 2, starting on row 70: Many previous reports have studied the pre-metastatic phase using the method of intravenous injection of tumor cells. In the premetastatic stage, Angpt2, MMP3, and MMP10 are up-regulated in the lung which leads to the increased permeability of pulmonary vasculatures and extravasation of circulating tumor cells. This was not explicitly evaluated in the present study which is an unfortunate omission.

[Answer#1-3] Thank you for your comment. I added a sentence with the reference in the text. I believe that the manuscript is improved.

2. Page 2, starting on row 85: The author mentioned that "Behaviors of circulating tumor cells are very important; they determine the ultimate consequences of cancer patients." "Blood is not a good place for the tumor cells to survive." The references support these points should be provided.

[Answer#1-4] Thank you. The references were added in that part.

3. Page 3, row 94: As mentioned above, the phrase "is same as" is inappropriately used.

[Answer#1-5] Thank you for your comment. The tone of the sentence was changed.

4. Page 3, row 121: The relevance of the concentration of intravenously injected tumor cells needs to be shown. What is the reason for choosing the concentration that used in this experiment?

[Answer#1-6] Thank you for your comment. In the standard protocol, 1 x 104-1 x 105 cells are used for the injection. This number is much higher than naturally occurring CTC. However, given the detection limit of this assay system, at least 1 x 104 cells are necessary as input (injection into the tail vein). CTC appears to metastasize more efficiently than the cultured cells.

5. Page 3, row 133: The process of perfusion is not optional but necessary because blood cells would influence the results of following studies especially the fluorescence assay.

[Answer#1-7] Thank you for your suggestion. The perfusion process is incorporated in the main protocol.

6. Page 4, row 141: Is it sufficient to separate the lung tissues into single cells through the methods described in 2.4) and 2.5)? It would be better to cut the lung tissues into pieces with scissors before put it in the syringe.

[Answer#1-8] Thank you for your comment. I changed the protocol.

7. Page 6, row 254: The author mentioned that "This ratio decreases in a time dependent manner". When would the tumor cells begin to regrowth? How the ratio changes in the lung needs further discussion.

[Answer#1-9] Thank you for your comment. The apparent tumor cell regrowth is not observed in 48 hours after the injection. Five days after the injection, micro-size colony formations were seen in the case of LLC inoculation. In the ref 13 paper, it is reported that the tumor microenvironment in the lungs influences the ratio.

Reviewer #2:

Manuscript Summary:

The manuscript "Tumor cell lung recruitment assay" by Tomita et al describes a tail vein injection-based experimental approach to evaluate specific steps of the metastatic cascade.

Major Concerns:

A general point of objection is the statement in abstract, lines 80 and 83 of the introduction, and discussion that injected tumor cells into the bloodstream would be equivalent to circulating tumor cells (CTCs). This is not correct as CTC are a selected sub-population of all cancer cells, often with specific features (EMT, stemnes, ...). I would strongly suggest amending these statements.

[Answer#2-1] Thank you for your informative comments. Another reviewer gave us the almost same comment. In the revised manuscript, this part is amended.

Surprisingly-and disappointingly regarding the scope of the journal-the method section is highly inaccurate and/or misses essential information. A few of these problems are highlighted below:

Are all steps (without specific information) performed with room temperature material and at room temperature?

Line 119: concentration of PKH26?

[Answer#2-2] The protocol provided by the manufacturer recommends that the concentration of PKH26 be 4 x 10-6 M for staining 2 x 107 cells. All procedures, including PKH26 labeling, are performed at room temperature unless otherwise stated.

Line 121: which wash steps are performed?

[Answer#2-3] After the labeling reaction with PKH26, cells were washed with PBS (10 ml) three times.

Line 144-151; steps2.5 and 2.7: confusing as the buffer seems to be in the plunger (2.5) and then in the tube (2.7).

[Answer#2-4] Thank you for your comment. In step 3.3), the lungs were pushed back to a 50 ml tube. I rewrote this part to avoid reader’s confusion.

line 158, step 3.2: composition of red blood cell lysis buffer? How long are samples treated with it? Temperature?

[Answer#2-5] I suppose the composition of the buffer is as following; NH4Cl, 8.26g/L H2O, NaHCO3, 1.19g/L H2O, and EDTA2Na, 0.0378g/L H2O. However, the manufacturer did not disclose the exact composition of the buffer. The red blood cell lysis buffer requires no incubation time after mixing with the sample. The operator can proceed to the next step immediately.

Line 165: step 3.4: how many cells are analyzed? Authors need to include gating strategy as a figure

[Answer#2-6] Thank you for your comment. I rewrote this part.

Line 171, step 4.1: please show histology of 4%PFA/PBS soaked lung; how is the pressure of H2O controlled?

[Answer#2-7] Thank you for your comment. This part is removed in the revised manuscript. Please see a paper “Am J Physiol Lung Cell Mol Physiol 306: L10-L22, 2014”. The pressure of H2O (100-136 cm H2O for the blood vessel perfusion, 20-25 cm H2O for the lung fixation) is controlled by utilizing hydrostatic pressure. A tank with PBS or saline is placed on a stand, the height of which from the operation table is set to be 100-136 cm for the blood vessel perfusion or 20-25 cm for the lung fixation. The tank outlet and a needle are connected with tubing.

Line 181, step 4.4: which microscope was used in this study?

[Answer#2-8] We used Olympus BX51. This part is removed in the revised manuscript. Instead, the new version contains fluorescence stereo microscopy using Leica M205FA.

Line 189: gating conditions: again, needs more detailed gating information: was it lung cells, singlets, positive?

[Answer#2-9] Thank you for your comment. We revised the gating conditions. Doublet removal was not introduced in the flow cytometry analysis.

Line 194: Why are there PKH26-positive signals in non-tumor-bearing mice? The increase (from 0.2-0.3% to 0.4-0.6% (line 195)) is tiny. This needs to be represented in a table or figure with statistical analysis.

[Answer#2-10] Thank you for your comment. The reason why there is PKH26-positive signal in non-tumor-bearing mice is that large number of tumor cells (1 x 105) are injected. The increase earned in the pre-metastatic phase is tiny but it is similar to the values previously reported (references 4, 10, and 12).

Line 198: how can the authors conclude that these are tumor cell remnants? Could it be dormant tumor cells, non-proliferating tumor cells...?

[Answer#2-11] Thank you for your informative comment. As you pointed out, they might be dormant tumor cells, non-proliferating cells, or even resident macrophages digesting labeled cells.

Line 210 see above, type of microscopy

Line 213: is it expected that cells become 2-color positive despite being labelled with (only) PKH26 originally?

[Answer#2-12] In this case, GFP-labeled cells were stained with PKH26 so that the resulting cells show two different colors. The PKH26 label is diluted in the cell regrowth process. On the other hand, GFP fluorescence is maintained because GFP is inherited in the descendent.

Line 222: tail vein injections are not difficult to experienced animal technicians

[Answer#2-13] Thank you for your comment. The tail vein injection is very difficult for the beginners, but there is no way other than doing practice to be a skilled operator. Unfortunately, we are not able to describe tips for the tail vein injection.

Minor Concerns:

Line 220/221: check grammar of sentence

Line 228: spelling form-from

Line 249-251: this is not mentioned in results section-why?

[Answer#2-14] Thank you. All points are corrected in the revised manuscript.