December 12, 2018

Dr. Vineeta Bajaj,

Associate Editor

JoVE

Dear Dr. Bajaj,

Attached please find a revised copy of our manuscript entitled " The Chick Chorioallantoic Membrane In Vivo Model to Assess Perineural Invasion in Head and Neck Cancer” (Manuscript #: JoVE59296), which as indicated in your correspondence of November 27, 2018 could be submitted as a revised manuscript after addressing the reviewers’ comments.

The changes are detailed in the attachment and are summarized below. We thank the reviewers for the suggestions, which strengthened the manuscript. We look forward to hearing about the acceptability of the manuscript for publication in JoVE.

We appreciate the reviewer’s positive feedback about the manuscript. Below, we have responded in detail to the Editor’s and reviewers’ comments, which are in italics to help in differentiating from our responses. All the changes implemented in the revised manuscript are highlighted in red.

***Editorial comments:*** *Changes to be made by the author(s) regarding the manuscript:*

*Comment #1: Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.*Response: Thank you. We have proofread the revised manuscript for spelling and grammar issues.

*Comment #2: Please revise lines 71-75 to avoid previously published text.*

Response: Thank you. These changes have been made in the revised manuscript.

*Comment #3: Please include a space between all numerical values and their corresponding units: 15 mL, 37 °C, 60 s; etc.*Response: These changes have been made in the revised manuscript.

*Comment #4: Please move the ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.*Response: This change has been made in the revised manuscript.

*Comment #5: Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.*Response*:* These changes have been made in the revised manuscript.

*Comment #6: 2.2: Please describe how to harvest DRG from cervical and thoracic regions. As this step is highlighted for filming, we need specific details.*

Response: We added 6 sub-steps to section 2.2 to increase clarity on harvesting DRGs. However, it is still a summarized version of the entire published protocol from Sleigh *et al. BMC Res Notes (2016) 9:82* and cited in the current manuscript.

*Comment #7: 2.4: Please specify incubation temperature.*Response: This change has been made in the revised manuscript.

*Comment #8: 4.1: How large is the culture dish?*

Response: This information has been added to the revised manuscript.

*Comment #9: 5.3: Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).*Response: This change has been made in the revised manuscript.

*Comment #10. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.*

Response: This change has been made in the revised manuscript.

*Comment #11. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.*Response: This change has been made in the revised manuscript.

*Comment #12. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.*Response: This change has been made in the revised manuscript.

*Comment #13. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.*Response: This change has been made in the revised manuscript.

*Comment #14. References: Please do not abbreviate journal titles.*Response: This change has been made in the revised manuscript.

*Comment #15. Table of Materials: Please remove trademark (™) and registered (®) symbols and sort the items in alphabetical order according to the name of material/equipment.*Response: This change has been made in the revised table of materials.

***Reviewers' comments:******Reviewer #1:*** *Manuscript Summary:  
The paper of Schmitd et al. (The chick chorioallantoic membrane in vivo model to assess perineural invasion in cancer) describes a co-culture model of CAM between rat dorsal root ganglia and human head and neck squamous cell carcinoma. The experiments are clearly presented and the co-culture model of interest.  
  
Major Concerns:  
Comment #1. The only problem is that the specificity of the dorsal root ganglia should be presented in a more convincing way, with a statistical approach, in order to demonstrate that any cells type (for example untransformed foreskin fibroblasts) are not able to invade the ganglia.*Response: We understand the concern about the specificity of the DRGs in attracting only cancer cells. However, it is unlikely that DRGs possess specificity for cancer cells and it is not our intent to imply this in the current manuscript. Nerves are implicated in many physiological processes, including regulation of stem cells in the skin1 and are needed for regeneration and embryonic development of many tissues2. For these reasons, we do not expect that the DRG will specifically attract only cancer cells. However, the purpose of this model is to evaluate the impact on perineural invasion of specific proteins in tumor cells. Therefore, we are presenting a protocol for tumor cells. It is foreseeable that this model could be adapted to investigate the impact of the DRG on other cell types.

*Minor Concerns:  
Comment #2. In addition, why not to use GFP expressing cells in order to perform fluorescent labelling on histological sections?*Response: Thank you for this question. We used GFP-labeled cells in the CAM model with the same success as cells labeled with cell tracker (unpublished data) However, we have not used GFP-labeled cells for the CAM-DRG model, which is why we omitted this information in the protocol. It is very likely that GFP-labeling would work in the CAM-DRG model as well as it works on the CAM model and we have added this information in the representative results section.

***Reviewer #2:*** *In this investigation Schmitd et al. developed a chorioallantoic membrane-dorsal root ganglia (CAM-DRG) model in which a mammalian DRG is isolated and grafted onto the surface of the upper CAM. After the DRG becomes incorporated into the CAM, head and neck cancer cells are grafted near the DRG and allowed to interact with the DRG before the entire in vivo system is harvested and analyzed. This system allows ex-vivo visual observation of both the DRG and the tumor by fluorescent labelling, therefore, I believe that it is a very interesting model to evaluate the ability of cancer cells to invade the neural component in vivo. Therefore, I recommend the publication of this paper after minor revisions as follows:  
  
Comment #1- Is there any possibility to quantify the invasion ability of cancer cells?*

Response: Invasion of cancer cells through the basement membrane of the CAM is quantified by counting the number of invasive tumor islands in the connective tissue of the CAM. The quantification of tumor islands inside the DRG is not practical because of sampling issues on histologic sections. While tumor islands may be observed inside the DRG, this is infrequent, likely due to the location of sectioning. We believe that since the analysis is based on a few 5 m sections of a 4-5 mm tissue, it is hard to capture the exact site at which PNI occurs. That is the reason why we rely on the fluorescence images to view PNI rather than histologic sections to quantify PNI. This quantification is performed by measuring how many samples had a directional migration of tumor cells toward the DRG and also by measuring distances between cancer cells and the DRG. We have increased clarity on how the analysis is done in the representative results section.

*Comment #2- The histology pictures A, B and C of figure 4 should be replaced by other pictures of better quality*

Response: Thank you for the comment. We think the reviewer is referring to Figure 5. Figure 5A is a low magnification image of the entire DRG grafted onto the CAM. We replaced the previous Figure 5B with a higher magnification image to add more detail and show integration of the DRG in the CAM connective tissue and the DRG tissue. Figure 5C is a low magnification of tumor grafted onto the CAM. We replaced the previous Figure 5D with a higher magnification image to highlight the tumor islands invading the connective tissue.

*Comment #3- In the discussion section the authors should compare their method with other methodologies used to assess the ability of cancer cells to invade the neural component (example using invasion xenograft mouse model).*

Response: Thanks for the comment. The use of tumor xenografts is not a consistent method to assess PNI. In our experience and based on published literature, tumor xenografts generate very low rates of PNI, which may explain why the tumor xenograft model has not been reported in the literature as a method for investigating PNI.

***Reviewer #3:*** *Manuscript Summary:  
This manuscript presents a very exciting technique that involves leveraging the chick chorioallantoic membrane to model perineural invasion in head and neck squamous cell carcinoma. The authors have identified the paucity of models that can mimic the early events in the progression of perineural invasion and propose a model that can address this gap by serving as an excellent scaffold for rat dorsal root ganglia and human head and neck squamous cell carcinoma cells. They detail a series of steps that are required to employ this model in order to assess the ability of cancer cells to invade the neural component in vivo.  
  
Major Concerns:  
  
Comment #1. In figure 5H, the cancer cells seeded on the CAM appear closer to the DRG than in figure 5G, which makes it unclear whether the more extensive invasion occurred as a consequence of the proximity to the DRG or if it really was a phenotype from the genetic manipulation of the cell line. An H & E stained slide of the both control and GALR2 CAM tissue would also help to corroborate the invasion differences.*Response: We agree that in this specific picture the tumor-DRG distance is smaller than the representative control. Cells are grafted at equal distances in all groups. Since it is technically difficult to ensure completely equal distances across samples, we emphasize the need for several replicates in each group. It is also possible that the SCC1-GALR2 cells are closer because of the greater attractive potential they have towards the nerve. We agree that the image choice was not the most representative and have changed it.

Regarding differences in the invasive phenotype of the GALR2 cells, there are two types of invasion that can be assessed by the model: invasion through the basement membrane (shown in Fig. 5C-D) for which we use HE or IHC stain, and invasion toward the nerve component (Fig. 5G-H), for which we use the fluorescence images. We did not include HE images for the invasion of GALR2 cells because we would like to avoid confusion between these two distinct processes. However, you are right to assume that the GALR2 cells would present more invasion through the basement membrane. They are in fact more invasive, as shown in our previous publication3. Perineural invasion is difficult to quantify inside the DRG due to sampling issues on histologic sections. While tumor islands may be observed inside the DRG, this is infrequent, likely due to the location of sectioning. We believe that since the analysis is based on a few 5 m sections of a 4-5 mm tissue, it is hard to capture the exact site at which PNI occurs. This is similar to the challenge of detecting PNI in human biopsy specimens of cancer. In the CAM-DRG model, we rely on fluorescence images rather than histologic sections to quantify PNI. This quantification is performed by measuring how many samples had a directional migration of tumor cells toward the DRG and also by measuring distances between cancer cells and the DRG.

*Comment #2. This manuscript only used head and neck cancer cell lines, therefore, the title should reflect this by changing the word "cancer" in the title to "head and neck cancer." Care should be exercised throughout the manuscript to avoid generalizing the utility of this PNI model in multiple cancers until a wider range of cancer cell lines have been tried on the CAM.*Response: Thank you for this comment. We have revised the title accordingly.

*Minor Concerns:  
  
Comment #1. Lines 117 and 129 include the timing, however, this can be omitted since every person performing the technique will likely require different time.*

Response: This is a good comment about variations between individuals. The time was mentioned to provide an estimate for planning purposes for a person using this model for the first time. Therefore, we changed it to “estimated timing”.

*Comment #2. Figure 2H has an arrow pointing at a DRG, but it is not entirely clear that this represents a reduced integration of the DRG in the CAM as the Note in line 147 suggests.*

Response: We apologize for this confusion. The goal of figure 2H was only to show the DRG with the axon bundle attached to it and not to show integration. Figure 2H is a high magnification picture of an extracted DRG before it is grafted on the CAM. To avoid confusion, we changed the place where we mention figure 2H in the text. We also added another arrow to point the axon bundle to increase clarity.

*Comment #3. The authors mention a decontamination period, but it is not specified what this incubation is decontaminating. Perhaps a sentence on this would help.*

Response: We changed the wording of this part of the protocol. It is not a decontamination, but a prophylaxis with an increased dose of Pen/Strep (2% instead of 1%), to avoid potential bacterial contamination of the DRGs due to the harvesting process.

*Comment #4. Section 3, line 165 should omit the timing portion.*

Response: We changed it to “estimated timing” for planning purposes. Please see response to Comment #1 above.

*Comment #5. In section 3.1, line 168 should contain exclusion of non-fertilized eggs.*

Response: This information has been added to the revised manuscript.

*Comment #6. An arrow that points to the air sac in figure 3A would help the reader see the naturally-occurring air sac.*

Response: This change has been made in the revised manuscript.

*Comment #7. In section 3.3, the authors should indicate what tool they will use to drill the egg shell in the marked square.*

Response: This change has been made in the revised manuscript.

*Comment #8. Section 3.4 needs to emphasize that this procedure should be performed carefully since applying too much pressure can damage or destroy the egg.*

Response: This information has been added to the revised manuscript.

*Comment #9. Section 3.5, line 194 needs to indicate that this step should be performed while holding the egg against the light source.*

Response: It is not necessary to have the direct light source when perforating the membrane in the square opening; this step can be performed with room lighting.

*Comment #10. Section 3.6 should mention that this step may require multiple attempts before succeeding.*

Response: This information has been added to the revised manuscript.

*Comment #11. Section 3.8 needs to specify what tool will be used to drill.*

Response: This information has been added to the revised manuscript.

*Comment #12. Section 4, line 222 should omit the timing portion.*

Response: We changed it to “estimated timing” for planning purposes. Please see response to Comment #1 above.

*Comment #13. Section 4.3, line 230 should indicate that sterile forceps should be used.*

Response: This information has been added to the revised manuscript.

*Comment #14. Figure 4D should have an arrow pointing towards all openings that should be covered.*

*Response:* This change has been made in the revised figure.

*Comment #15. Section 5, line 248 should omit timing portion.*

Response: We changed it to “estimated timing” for planning purposes. Please see response to Comment #1 above.

*Comment #16. Section 6, line 283, omit timing portion.*

Response: We changed it to “estimated timing” for planning purposes. Please see response to Comment #1 above.

*Comment #17. Section 6.3 needs better explanation and/or images because it is not entirely clear what the reader needs to be doing.*

Response: We added more information to increase clarity to this step. We have also edited Figure 4I-K to better illustrate this step.

*Comment #18. Paragraph three in the "representative results" section needs to be improved with a clearer explanation of the analysis. Perhaps some examples would help.*

Response: This change has been made in the revised manuscript.

*Comment #19. In figure legend for fig. 3, line 375 lists adding 30 ul of water, but in the manuscript is mentions using HBSS, please be consistent.*

Response: Thank you for pointing out the discrepancy, which we have corrected in the figure legend. We use HBSS and not water.

REFERENCES:

1 Peterson, S. C. *et al.* Basal cell carcinoma preferentially arises from stem cells within hair follicle and mechanosensory niches. *Cell Stem Cell.* **16** (4), 400-412, doi:10.1016/j.stem.2015.02.006, (2015).

2 Boilly, B., Faulkner, S., Jobling, P. & Hondermarck, H. Nerve Dependence: From Regeneration to Cancer. *Cancer Cell.* **31** (3), 342-354, doi:10.1016/j.ccell.2017.02.005, (2017).

3 Scanlon, C. S. *et al.* Galanin modulates the neural niche to favour perineural invasion in head and neck cancer. *Nat Commun.* **6** 6885, doi:10.1038/ncomms7885, (2015).