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February 26, 2019

Bing Wu, Ph.D.
Review Editor
JoVE

Dear Dr. Wu,

The authors would like to thank you for the careful and thorough review of our manuscript titled "Generation of tumor organoids from genetically engineered mouse models of prostate cancer" (JoVE59710) by yourself and the four reviewers. The expertise of the reviewers was well suited for our manuscript and we believe the alterations we have made in response to their comments have greatly improved our submission.

In reply to your request for revisions to our submitted manuscript, we have made two major overall changes. At the request of multiple reviewers, we have removed mention of details specific to the prostate cancer (PCa) genetically engineered mouse models (GEMMs) that we use on our laboratory. This edit has generalized the description and discussion of the protocol in our manuscript so that readers can apply it to their own GEMMs.

We have also substantially expanded the Discussion section of our manuscript, as suggested by yourself and multiple reviewers, from 3/4 of a page to 3.5 pages. We believe that our expanded Discussion will provide readers with the necessary depth for understanding both the method of generating organoids from PCa GEMMs and current state of the field in utilizing organoids in PCa research.

Please see below for detailed responses to individual comments from the editor and each reviewer. We have provided the revised manuscript, the original manuscript with tracked changed, and PDF files for Figures 1-5.

Response to comments from the Editor

1. We have thoroughly proofread our manuscript and made changes to reflect appropriate grammar and correct spelling.
2. None of the Figures in our manuscript have been published elsewhere.
3. We have extensively revised and expanded the Discussion section and organized it into the subtitles you have listed (Lines 557-715 of manuscript with tracked changes, Lines 503-644 of revised manuscript).
4. We have downloaded the most updated Endnote file from the JoVE Instructions to Authors

and used this output style to generate our Reference section.
5. We have proofread the Protocol section and removed all personal pronouns.
6. We have replaced all time units in the Protocol section with h, min, and s.
7. We have reworded Step 1.3.2. in the Protocol section using the imperative tense (Lines 187-189 of manuscript with tracked changes, Lines 183-184 of revised manuscript).

Response to comments from Reviewer 1
The authors would like to thank Reviewer 1 for his or her comments, which we have used to revise our manuscript specifically to reflect the capacity of the protocol to apply to any PCa GEMM and the current state of the PCa field in utilizing mouse organoids in research.

Major Concerns:
None

Minor Concerns:
We have removed any discussion of the characteristics or data which are specific to the PCa GEMMs we use in our laboratory. This revision includes removal of Figure 1 from the original manuscript, as this figure depicted the Cre-loxp system and the *mT*/*mG* Cre reporter transgene that are apply specifically to our GEMMs.

We have greatly expanded the Discussion section from 3/4 of a page to 3.5 pages. In our new Discussion, we give several examples of *in vitro* experiments that have been carried out using organoids in PCa research studies—including *in vitro* activation of Cre, expansion of rare cell populations, and analysis of tumor lineage characteristics using immunohistochemistry. In the "Future applications" section of the Discussion, we have highlighted genetic engineering of organoids and its potential for use in mechanistic studies, *in* vivo organoid grafting, and drug screens. We have also emphasized the challenge of mixed prostate organoid cultures that contain normal and cancer organoids and highlighted the FACS sorting approach that was carried out by Agarwal et al. as method to select for tumor cells. We have pointed out in the "Critical steps" section of the Discussion that investigators should genotype organoid to ensure that transgenes expressed in GEMMs, such as probasin-Cre, are maintained in organoid lines.

Finally, we have referenced and discussed all the studies suggested by Reviewer 1; except for PMID 25243035, since we could not locate this reference on Pubmed. If the Reviewer could provide us with the first author, publication year, and journal we will be able to discuss this study as the Reviewer suggested.

Response to comments from Reviewer 2
The authors appreciate the comments made by Reviewer 2 that have benefited our manuscript with revisions that reflect the capacity of the protocol to apply to any PCa GEMM. We agree with Reviewer 2 that specific discussion of the GEMMs we study in our laboratory is irrelevant to the current manuscript. Therefore, we have removed any discussion of characteristics or

data which are specific to the PCa GEMMs we use in our laboratory.

Major Concerns:
None

Minor Concerns:
1. We have added a section in the Introduction section of our manuscript (Lines 90-94 in manuscript with tracked changes, Lines 89-92 in revised manuscript) to emphasize that this protocol is appropriate for any PCa GEMM.

2. As suggested by Reviewer 2, the protocol our manuscript describes is not for creating GEMMs. Consequently, we have removed Figure 1 from the original submission.

3. We have completely removed the paragraph in the "Representative Results" section (Lines 446-454) that described the phenotype of our GEMMs.

Response to comments from Reviewer 3
The comments from Reviewer 3 on our manuscript were detailed and thoughtful—the authors would like to thank him or her, as the revisions made as a results of these suggestions have greatly improved our manuscript.

Major Concerns:
None

Minor Concerns:
1. We have corrected the grammatical errors in Line 177 (Line 191-193 in the manuscript with track changes and Lines 186-188 in the revised manuscript). We have included fluorescent images of the urogenital region from both the ventral and dorsal aspects of a prostate from a 12 week old GEMM which expresses GFP in the prostate.

2. Thanks to this comment, we identified a mistake in the text of this section of the protocol and have corrected it to instruct spinning down the cells suspension, not the matrix, prior to resuspending organoids (Lines 413-418 of the manuscript with tracked changes and Lines 395-402 of the revised manuscript).

3. Both the Pathclear Matrix we describe in our manuscript and the Matrigel (BD Biosciences, cat. no. 356231) from Drost et al. are derived from the soluble basement membrane of a Engelbreth-Holm-Swarm (EHS) mouse sarcoma and are growth factor reduced—these reagents only appear to differ because the companies vary in the naming of their products.

4. We have added a couple sentences to the Discussion commenting on the possibility of genetic or epigenetic changes resulting from prolonged passaging of prostate organoids. As we

write, we are not aware of a published study that has described such changes that occur in organoids over time in culture. We are currently tracking such changes in cancer organoids from our GEMMs, but all of these data are unpublished and yet to be presented.

5. In response to Reviewer 3's comment, we have further explained our distinction between fluid-filled and solid prostate tumors according to our observations in relation to organoid generation (Lines 491-498 in the manuscript with tracked changes, Lines 459-466 in the revised manuscript). We have also added representative images of fluid-filled and solid prostate tumors to Figure 2 and labelled the images for clarity.

6. In response to this comment, we have added a note to the Protocol section that discusses optimal mouse age for isolating prostate tumors for cancer organoid generation (Lines 122-127 in manuscript with tracked changes, Lines 120-125 in revised manuscript). Male mice at least of the age of sexual maturity (8-10 weeks) will be required for expression of many prostate-specific transgenes, including probasin. However, the goal of an individual study and specific *in vivo* characteristics of each PCa GEMM alters the age at which an investigator may isolate prostates for organoid generation.

Response to comments from Reviewer 4

The authors thank Reviewer 4 for his or her detailed comments. As a result of these suggestions, we have made substantial changes to our manuscript that has improved our communication with the reader about aspects of organoid culture that limit the efficacy of this method in PCa research. We have also refined our protocol for dissection of the mouse prostate by adding a section describing removal of non-prostate tissue from the urogenital region.

Major Concerns:

We agree with the Reviewer that our Discussion section was not to the depth required for the purpose of this manuscript. We have expanded the Discussion section of our manuscript from 3/4 of a page to 3.5 pages (Lines 557-715 of manuscript with tracked changes, Lines 503-644 of revised manuscript). We have included a section on outgrowth of normal prostate organoids and selection for basal epithelial cell characteristics over luminal epithelial cell characteristics under prostate organoid culture conditions—the latter of which emphasizes the decreased expression of androgen receptor (AR) in cancer organoid cultures compared to prostate tissue. We have also discussed how mouse organoids have been utilized in recent studies from the PCa field.

Minor Concerns:

1. We have added the duration of CO2 asphyxiation in Section 1.2.1 (Line 157 of the manuscript with tracked changes and Line 153 of revised manuscript).
2. Thank you for catching our proofreading error, we have removed all references to Figure 2C.
3. and 4. We have added an entire section (Step 1.5.5.) describing removal of non-prostate

tissue including the seminal vesicles, vas deferens, bladder, and fatty/connective tissue, to the Protocol section of our manuscript (Lines 307-322 in manuscript with tracked changes, Lines 295-309 of revised manuscript.
5. We have added labels to our representative necropsy images in Figure 3A and 3B of our revised manuscript.

Review of the manuscript has been restricted to the co-authors and colleagues at Roswell Park Comprehensive Cancer. None of the material in the manuscript has been published or submitted for publication elsewhere. All animal work described is approved by the IACUC at Roswell Park and is in accordance with Institutional guidelines.

The authors would like to thank you for considering our revised manuscript for publication in JoVE. Please do not hesitate to contact me with any questions or concerns.

Kindly,

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