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Jan 07th, 2019

Dear Editors and Reviewers,

Happy New Year!

Enclosed please find our revised manuscript, “The patient-derived heterogeneous xenograft model of pancreatic cancer using zebrafish larvae as hosts for comparative drug assessment”, which we are re-submitting to *JOVE*.

We thank you and the reviewers very much for the insightful comments, which were all valid and helped us substantially improve the manuscript, as well as clarify our model. We have attempted to address all of the concerns through new experiments and extensive revisions, and the detailed responses are listed below.

We really thank you for your efforts for helping us with this paper and look forward to your response.

Sincerely,

Xu

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**Editorial comments:**  
The manuscript has been modified and the updated manuscript, **59507\_R0.docx**, is attached and located in your Editorial Manager account. **Please use the updated version to make your revisions.**  
  
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.  
2. Please provide at least 6 keywords or phrases.  
3. For in-text referencing, please superscript the reference number and remove the parentheses before and after the reference numbers.  
4. Please ensure that the references appear as the following:  
Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. **Volume** (Issue), FirstPage – LastPage, (YEAR).  
For more than 6 authors, list only the first author then et al.  
5. Please add a one-line space between each of your protocol steps.  
6. There is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol steps in yellow (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.  
7. Figure 1: Please add a short description of the figure in addition to the figure title in Figure Legend.  
8. Please do not abbreviate journal titles for all references.  
9. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials.  
10. Please use standard SI unit symbols and prefixes such as µL, mL, L, g, m, etc.  
11. Please use greek characters for SI unit prefixed, e.g. use ‘μL’ instead of ‘uL’.

A: Dear Editor, we have fixed all the above problems except No. 8. It turns out that the full name data based in EndNote are not really full names, but relatively Full names. We have referenced the entire manuscript in a “JoVE” style downloaded from EndNote database, and hope it can fit your requirement.

---------------------------------------------------------------------------------------------------------------------------------------------Reviewers' comments:

Reviewer #1 (Remarks to the Author):

The authors underline the advantages of zebrafish larvae as a host for cell and patient derived xenografts compared to mice as a tool for precision medicine. Emphasis is placed on the small scale of resources and time required by this model as well as the favorable fluorescence visualization due to its transparency. They propose to generate xenografts with this model using a co-culture of fluorescently labelled patient derived pancreatic cancer cells and cancer associated fibroblasts to better mimic the tumor microenvironment without compromising the read-out.

Major Concerns:

Q. Stable expression of anti-apoptotic protein (BCL2L1 gene) is used in this model to "enhance the survival time of xenograft cells to prolong the observation window for the studies", however this is also expected to decrease cancer cells and fibroblast drug sensitivity, likely resulting in "false negative" outcomes and compromising the validity of this tool for clinical purposes. Could the authors address this matter?

A：Thank you very much for your comments, and we completely agree with your opinion. However, we think it was necessary to genetically modify the primary cells somehow to improve the usefulness of PDX models as real patient avatars, since most primary cells will naturally become apoptotic or senescent at the early stages of culture and xenograft, and loses their original heterogeneity.

BCL2L1(BCL-XL) was currently proposed as a compromised choice:1) BCL2L1 is endogenously expressed in most cancer cells, and compared to other pro-survival factors (eg. TERT, HPV16, and pro-oncogenic MYC, RAS, MDM2), the effects of BCL2L1 are relatively predictable and specific; 2) BCL2L1 antagonizes mitochondria-apoptosis pathways, and there exist alternative apoptosis pathways that are BCL2L1-independent/insensitive; 3) BCL2L1 does not directly block the damages in cancer cells by chemotherapy, and the proliferation of cancer cells may still be arrested even the cells are not killed immediately.

Nevertheless, to improve the validity of our tool for clinical purpose, we modify our proposal by adding an additional control experiment (Fig. 1): Co-culture the zebrafish PDX models with Navitoclax (ABT-263) (an oral BCL-XL inhibitor) during the drug test procedures, and Compare the results with the drug test results without Navitoclax treatment. Such comparison will provide an assessment of drug resistances particularly caused by BCL2-L1, and help identify the potential “false negative" outcomes (Fig. 4D).

Minor comments:

Q1. The authors wrote "In this paper, we labeled the cells with lenti virus constantly expressing anti apoptosis gene BCL2L1, as well as fluorescent proteins." The BCL2L1 gene encodes both the anti-apoptotic Bcl-xL protein and the pro-apoptotic Bcl-xS protein through alternative splicing [1], can the authors clarify how is this controlled in the proposed model?

A1：Thank you very much for your comments. The cDNA sequence coding BCL2-L1 in our virus is the Bcl-X(L) isoform, and we have emphasized it in the title of step 4 and discussion in the revised manuscript. We also referenced the mentioned paper.

Q2. Have the authors validated the proposed model by demonstrating that it is actually able to screen for therapeutic strategies benefitting patients?

A2. Thank you very much for your comments. To optimize our model to screen for real therapeutic strategies benefitting patients is our final goal, and we have not reached it yet. Currently, we have tested this model in around 20 cases, but the results were not used to affect clinical decisions made for the patients. However, we will combine information from both bench and bedside, and perform retrospective study.

Q3. Could the authors comment on the applicability of this model to other solid cancers?

A3：Thank you very much for your comments. We comment it in the discussion section as:

“The strategy of patient-derived heterogeneous xenograft model of pancreatic cancer for comparative drug assessment can also be applied to other types of solid cancers.”

Q4. Could the authors indicate the desired/expected purity of the two cultures after growth with or without fibroblasts inhibitors for the suggested time?

A4：Thank you very much for your comments. We have inspected the cell morphology in the two cultures, and observed over 98% purity of the expected cell types in the successful cases. We have commented it in Step 3.10 in the revised manuscript.

Q5.The authors suggest mixing equal proportion of cancer cells and fibroblasts; does this reflect the original ratio in the tumour?

A5： Thank you very much for your comments. The original ratio of cancer cells to fibroblasts in primary tissues is highly different. In our project, we started with 1:1 ratio, and will investigate the effects of drug treatment on the same cell composition with different ratio in the future studies. We have commented it in the discussion in the revised manuscript.

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Reviewer #2 (Remarks to the Author):

The manuscript describes an interesting and useful protocol for researchers who are interested to employ zebrafish as a model to assess drug sensitivity. Zebrafish no doubt provides a more economical platform compared to animal models for drug studies. The lenti constructs developed in this study will be useful for other cancer types as well.

Minor Concerns:

Q1.There are typos and grammatical errors throughout the manuscript. Please re-check these errors using a professional editing service.

A1：Thank you very much for your comments. We have gone through the manuscript and fixed some typos and errors, and we are sorry for those mistakes.

Q2. Please provide error bars and statistical significance to the bars provided in Figure 4c.

A2：Thank you very much for your comments. We now performed a new experiment of zPDX drug treatment in four groups (DMSO, gemcitabine, navitoclax (a BCL2-L1 inhibitor), and gemcitabine & navitoclax), and remade the statistics as the new Figure 4D.

Q3. Discussion section (lines 284-294): the discussion provided is irrelevant to the study. Hence, my suggestion would be to discuss future experiments or work with the existing model.

A3：Thank you very much for your suggestions. We now reorganized the entire paper and put the first paragraph in discussion into the introduction, and the new discussion is composed of two sections: 1) the future optimization to improve the survival of xenograft; 2) the future studies on the composition ratios and application of existing protocol in other tumor types.

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Reviewer #3 (Remarks to the Author):

Authors present an interesting protocol to further develop zebrafish PDX in embryos. However, more refinement is needed.

Major Concerns:

Q1. The title gives the impression of a general protocol but the protocol is only tested in pancreatic adenocarcinoma primary cultures. Authors should better explain that their xenografts are derived from primary cultures. If they want to propose a general protocol it should be tested in other cancers. If they are not able to do so the title should include pancreatic cancer derived PDX.

A1: Thank you very much for your comments. We have changed the title as: “The patient-derived heterogeneous xenograft model of pancreatic cancer using zebrafish larvae as hosts for comparative drug assessment”

Q2. Authors should show a comparison of growth rate between primary cultures and same cells infected with the lentivirus. If they are not differences this could be a great model but if they are the validity would be lesser. In the discussion, authors need to analyse the impact on incubation temperature at 32 degrees in the growth rate of cells.

A2：Thank you very much for your comments. We now modify the protocol by adding an additional control experiment (Fig. 1): Co-culture the zebrafish PDX models with Navitoclax (ABT-263) (an oral BCL-XL inhibitor) during the drug test procedures, and Compare the results with the drug test results without Navitoclax treatment in four groups (DMSO, gemcitabine, navitoclax (a BCL2-L1 inhibitor), and gemcitabine & navitoclax). Such comparison will provide an assessment of drug resistances particularly caused by BCL2-L1, and help identify the potential “false negative" outcomes (Fig. 1). In the representative test, the inhibition of BCL2-L1 does not significantly affect the viability of the infected tumor cell population (Fig. 4D).

We also added the temperature question into the discussion as a future work:” Besides, the effects of 32 °C instead of 37 °C incubation on the behaviors of human cells also require detailed comparative studies.”

Q3. There is mention to the use of dyes but to my understanding there are no picture included with their use.

A3： Thank you very much for your comments. We have published the dyes-stained zebrafish CDX model of pancreatic cancer before (Guo et al., 2014), and here we removed the relevant content in this manuscript.

Minor Concerns:

Q1. Line 128, I think authors meant "…immediately transferred"

A1：Thank you very much for your comments, and we have fixed it

Q2. 2.3 why not using casper embryos instead of using PTU. Is PTU affecting cells? Authors should clarify this.

A2：Thank you very much for your comments. There is current no evidence that 0.03% PTU affects human non-melanin tumor cells in short-term incubation. However, we agree that it is a better idea to use Casper line instead of PTU-treated embryos, and we now describe this option in Step 2.3.

Q3. 3.10 Why defer the injection for 1-2 weeks? Could it be done in less time? Authors should explain thoroughly why 2 weeks are needed.

A3. Thank you very much for your comments.

At the beginning of this project, we injected the primary cells immediately after digestion, and found the following issues: 1) many dead and dying cells; 2) many non-cell structure and substance; 3) unknown cell composition. It was impossible to quantify the drug response.

After several tests, the 1-2 weeks stabilizing time in our protocol proves to be a critical step to isolate living cells, label different cell types, and significantly improve the feasibility and reliability of the zPDX system.

Q4. 5.3 please describe volume? fL?

A4. Thank you very much for your comments. We have added the volume (8 nL)

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We thank you again for your consideration and look forward to your response.



Sincerely,

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Young Investigator

Department of Biochemistry and Molecular Biology

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01/07/2019