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RE: REBUTTAL FOR REVIEWERS' COMMENTS OF JoVE60303 MANUSCRIPT
"Establishment and characterization of small bowel neuroendocrine tumor organoids,"

Dear Dr. Bajaj,

We re-submitted a revised version of our manuscript. We would like to thank your editorial team and all the reviewers for pointing out important controls and many constructive comments. We have incorporated all the requested information in our revised manuscript. Overall, we think that the constructive comments have allowed us to significantly improve this manuscript. We hope that you would consider our paper for publication in JoVE.

Please see below for our point by point responses to the reviewers' comments.

Please let me know if you need any further information.

Sincerely,

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Reviewers' comments:
Reviewer #1:
Manuscript Summary:
The manuscript by Po Hien Ear et al. describes the establishment of SBNET organoids, their cryoconservation as well as their approval of SBNET markers by IF and IHC.

Major Concerns:
- Two different concentrations are given for insulin in the medium (line 98). Corrected, it should be 10 µg/mL of insulin.

It´s also not clear to which stem cell medium it is referred as reference 10 contains different media for murine or human liver or pancreas. We tried both human liver and human pancreas stem cell media with slight modifications. We're providing a list of media used in the new Table 2.

- Regarding the treatment with rapamycin: Which wavelengths were used to detect this Ethidium Homodimer? For microscopy, we used the red filter cube G (Olympus microscope CKX53 model). Ethidium homodimer can be detected using the spectrophotometer at 535 nm excitation and 624 nm emission.

The DMSO control should also be shown with the Ethidium Homodimer due to the fact that it can be toxic for cells as well. Therefore it would be nice to show another negative control with organoids only in medium. Please see new Figure 3 with DMSO control.

Also, the fluorescence signal is not necessarily caused by apoptosis. It could be also necrosis or another signaling pathway leading to permeable cell membranes. Thanks for pointing this out. We will edit our text accordingly.

Therefore a more precise assay would be necessary. In general, a viability assay (like e.g. Presto Blue or Cell Titer Glo 3D) with IC50 calculation would be more meaningful for using the organoids as tool for personalized drug screening. We agree. We are optimizing conditions for Cell Titer Glo 3D for systematic drug testing in the near future.

- Growth/ lifespan: As the authors mentioned in the introduction drug screening with existing SBNET cell cultures is limited by the slow doubling time and resulting less material. The growth of their organoids was shown by "organoid areas" (Figure 1b). How were the organoid areas determined? By Image J. We have included a new section describing how to quantify SBNET spheroid surface area (New Section 3).

How many organoids were calculated per day? 30 to 60 spheroids were measured for each time point.

The diagram looks confusing with these different coloured colums and frames. We agreed, we have now changed the columns to the same color.

In general, the lifespan of all established organoids should be mentioned, especially because we experienced NET organoids to die after 6-10 passages. Are they all expandable to 97 d? We are experiencing more difficulty with NET spheroids from the pancreas. Some of our PNET spheroids died after multiple passages and some were outgrown by fibroblasts. However, for the SBNET spheroids, we have been able to keep them growing for over 9 months now. It looks like they are healthy growing in the conditions that we describe in this manuscript.

When they write "After 14 days in culture, SBNET organoids do not increase in size. Instead, some SBNET cells will dissociate to a neighboring location and form new organoids." (line 151f.) - Can they be split like it is usual for organoids or how was the Matrigel change performed? That has to be described. Yes, they can be split. We split the spheroids by recovering the old Matrigel with all the cells and mixing with new Matrigel and transferring to a new plate. Please see new Section 7 of the Protocol.

Minor Concerns:
- It's not explained how the IHC was performed (reference or protocol). We have included a new section 5 on IHC.

- In general, white balance while taking photographs should have been used (Fig 1a, 3). Good suggestion! We will start using this in the future. Initially, we did not want to lose any information and therefore did not want to filter out anything.

Reviewer #2:
Manuscript Summary:
This is a review of the manuscript entitled "establishment and characterization of small bowel neuroendocrine tumor organoids." In this manuscript, the authors first document the difficulties in studying small bowel neuroendocrine tumors (SBNETs) and subsequently describe organoid culture with a view to using this system for drug discovery. The goal of this paper is to clearly explain this new system so that others could follow this line of research

Major Concerns:
1) The authors state that 1 X 10(6) cells are resuspended in 1ml of Matrigel and then media is changed every 5-7 days. In Figure 1B, they show organoids can be cultured for 97 days. Given that Matrigel will degrade over time, how are these organoids passaged into new Matrigel? Yes we agree. We the cells become too crowded after 2-4 weeks in culture, we split them. Organoids with Matrigel were harvested and transferred to a 1.5 microtube, centrifuge at 1000 g and remove any excess media. New Matrigel is added to the pellet and aliquot into new plate. Please see new Section 7 of the protocol.

Are they passaged every 14 days? No, SBNET spheroids only needs to be passaged every 2-4 weeks because they are slow growing.

During the passaging process, are the organoids disassociated or passaged whole? Passaged whole since we do not see SBNET spheroids growing bigger than 150 μm in diameter.

This is important and needs to be explained so others can follow their work. We agree, we have incorporated all the information in our resubmission. Please see new Section 3,5, and 7. Our Protocol has in total 8 sections now.

2) Regarding storage and recovery of SBNET organoids, it can be difficult to remove organoids from old Matrigel when reseeding into new Matrigel. Centrifugation will not remove organoids from Matrigel in my experience so how do the authors overcome this problem? We agree that it is very difficult to remove all old Matrigel because we have also tried doing this. However, we noticed that the SBNET spheroids can still grow very well when they are simply mixed with new Matrigel.

3) The authors state in the introduction that one of the original SBNET lines failed to continue to express NET markers, presumably after prolonged cell culture. At what timepoint were the images in Fig. 2 obtained? Sorry for the confusion, we were referring to an organoid line from a pancreas tumor. These PNET line seemed to be outgrown by fibroblast and other stromal cells and the NET markers can no longer be detected.

Was it after 97 days or far earlier? Do they express the necessary markers after 97 days? Yes, our SBNET spheroids continue to express NET markers after 97 days. We have included new data showing that SBNET spheroids expressing NET markers after 9 months in culture although the level of SSTR2 seems to be decreased. It appears that the Matrigel microenvironment helps to preserve the expression of NET markers.

4) Similarly, how long were the SBNET organoids cultured before they were incubated with rapamycin and is there an optimal time for assaying responses? We usually culture our SBNET spheroids for 5 days or more to make sure the SBNET cells are growing before using for a drug testing. For the rapamycin experiment, those SBNET spheroids were grown for 2 months and used for drug treatment.

Minor Concerns:
In the protocol section 1.1, should resected tumors be stored in 25ml of DMEM/F12 at room temp/on ice/at 4C etc. How long can this tissue be stored for until tumors are processed? We usually process our tumors within 3 hrs after tumor resection. We have tried to process tumors after storage in DMEM/F12 at 4^{o}C for 24 hrs. Some SBNET spheroids were obtained but there were many dead cells in the Matrigel. We estimate that the efficiency decreases by 50-80% after 24 hrs incubation in DMEM/F12 at 4^{o}C.

Reviewer #3:
Manuscript Summary:
The authors present a detailed step by step description of the methods they used to successfully culture small intestinal neuroendocrine tumors following surgical resection in

humans. In addition, the authors present data results describing the appearance, growth rate, and immunohistochemical detection of known tumor marker genes confirming that they have in fact cultured cells from the tumor itself. The materials and equipment needed are included in the protocol. The steps are complete, clearly explained and should allow the successful culturing of these tumors. The practical application of these procedures should be useful to investigators with access to these patient's tumors and interest in understanding their abnormal growth as well as test new growth inhibiting therapies.

Major Concerns:
The title, abstract and manuscript refer to tumor organoids. It is inaccurate to call these organoids because the data presented indicates that these are uniform tumor cells and there is no data to suggest the presence of normal small bowel epithelium implied in the term organoid, such as a mixture of Lgr5 stem cells, paneth cells, goblet cells and enterocytes. Furthermore, no data is presented indicating tumor heterogeneity. All cells are monotonously chromogranin A, synaptophysin and SSTR2 positive. It would be more accurate to simply call them small bowel neuroendocrine tumor cultures. This is a good point. We have not seen Paneth and goblet cells in our culture, but we do see some fibroblasts. Since these are predominantly SBNET cells selected out from other cell types we have changed our descriptor to “spheroids” rather than “organoids”.

Introduction:
1. KRJ-1, L-STS and H-STS celline are now found to be naturallly-occurring EBV transformed lymphoblasts (ref #8). Authors cited this reference, the information regarding small bowel neuorendocrine tumor cell lines should be correctly updated. Thank you. We’ve made the correction.

Methods:
Line 98. Insulin, different concentrations were listed twice. We have corrected this.
Line 99. Authors stated tumors can be cultured in stem cell media. If so, these components should be listed in the methods section in the supplement. We have included a new Excel sheet with the growth media composition in our revised manuscript.
Results.
1. Are the representative cultured tumors derived from a single cell or a fragment of the original tumor? From a fragment of the original tumor.

Can tumors form from single cells or fragments derived from larger cultured tumors? We have not tried dissociating all the tumors cells into single cells. We noticed that by using the cell strainer with 40 µm pore cutoff, we obtained a mixed population of single cells to groups of ~6 cells.

2. Are the cultured tumors cyropreservable? Yes, however we have only been able to recover up to 10% of our SBNET organoids after 1 month. Not all cells will survive

cryopreservation and thawing but some will and can be used to restart the culture. However, this process takes months. We recommend just keeping the culture going by splitting them approximately once very 2 weeks or so. Please see new Section 7.

3. Data should be presented regarding culture efficiency (success rate for culturing tumors per tumor (if multiple tumors are present) and per patient). We have been able to generate cultures of 8/9 resected SBNETs. The SBNET that had poor viability was the tumor that was left in DMEM/F12 for 24 hrs at 4^{o}C prior to processing. We will be reporting these finding in a separate manuscript. In this manuscript, our goal is to provide a detailed protocol for our colleagues in the NET field to be able to generate and grow SBNET spheroids in their own laboratories.

4. Figure #1:A. Include some information about the patients source of tumor (primary vs. sporadic tumor, tumor grade, and ? multiple tumors/patient, location mets etc.) and how many tumors the graph represents (N=?). The tumor sample was from an SBNET that metastasized to the liver (n=1 patient). The Ki-67 was 4.3%.

B. ? Mean +/_ SEM, and how many tumors from how many patients? We are showing only SBNET of 1 patient since this is a protocol paper. We will include other patient samples in a different paper.

5. Figure#2: Please rule out autofluorescence by including a control staining. Yes, thank you for pointing this out. We've added new panels to Figure 2 consisting spheroids that were derived from a pancreas tumor but do not express SYP, CgA, or SSTR2.

6. Figure#3: Please show figures from both before and after treatment with Rapamycin. Thanks for pointing this out. We have added new panels to this figure (now Figure #4).