Journal of Visualized Experiments

# Establishment and characterization of small bowel neuroendocrine tumor spheroids

--Manuscript Draft--

| | |
|---|---|
| **Article Type:** | Methods Article - JoVE Produced Video |
| **Manuscript Number:** | JoVE60303R1 |
| **Full Title:** | Establishment and characterization of small bowel neuroendocrine tumor spheroids |
| **Keywords:** | Small bowel neuroendocrine tumors, spheroids, synaptophysin, chromogranin A, SSTR2, immunofluorescence, immunohistochemistry, extracellular matrix, rapamycin |
| **Corresponding Author:** | James R Howe, MD<br>University of Iowa College of Medicine<br>Iowa City, IA UNITED STATES |
| **Corresponding Author's Institution:** | University of Iowa College of Medicine |
| **Corresponding Author E-Mail:** | james-howe@uiowa.edu |
| **Order of Authors:** | Po Hien Ear, Ph.D. |
| | Guiying Li |
| | Meng Wu |
| | Ellen Abusada |
| | Andrew Bellizzi |
| | James R. Howe |
| **Additional Information:** | |
| **Question** | **Response** |
| Please indicate whether this article will be Standard Access or Open Access. | Standard Access (US$2,400) |
| Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations. | Iowa City/Iowa/USA |

**TITLE**

# Establishment and Characterization of Small Bowel Neuroendocrine Tumor Spheroids

**AUTHORS & AFFILIATIONS**

Po Hien Ear[1], Guiying Li[1], Meng Wu[2], Ellen Abusada[3], Andrew M. Bellizzi[3], James R. Howe[1]

[1]Department of Surgery, University of Iowa Carver College of Medicine, Iowa City, IA
[2]High Throughput Screening Facility, University of Iowa Carver College of Medicine, Iowa City, IA
[3]Department of Pathology, University of Iowa Carver College of Medicine, Iowa City, IA

**Corresponding Author:**

James R. Howe (james-howe@uiowa.edu)

**Email Addresses of Co-authors:**

Po Hien Ear (pohien-ear@uiowa.edu)
Guiying Li (guiying-li@uiowa.edu)
Meng Wu (meng-wu@uiowa.edu)
Ellen Abusada (ellen-abusada@uiowa.edu)
Andrew Bellizzi (andrew-bellizzi@uiowa.edu)

**KEYWORDS**

Small bowel neuroendocrine tumors, spheroids, synaptophysin, chromogranin A, SSTR2, immunofluorescence, immunohistochemistry, extracellular matrix, rapamycin

**SUMMARY:**

Neuroendocrine tumors (NETs) originate from neuroendocrine cells of the neural crest. They are slow growing and challenging to culture. We present an alternative strategy to grow NETs from the small bowel by culturing them as spheroids. These spheroids have small bowel NET markers and can be used for drug testing.

**ABSTRACT:**

Small bowel neuroendocrine tumors (SBNETs) are rare cancers originating from enterochromaffin cells of the gut. Research in this field has been limited because very few patient derived SBNET cell lines have been generated. Well-differentiated SBNET cells are slow growing and are hard to propagate. The few cell lines that have been established are not readily available, and after time in culture may not continue to express characteristics of NET cells. Generating new cell lines could take many years since SBNET cells have a long doubling time and many enrichment steps are needed in order to eliminate the rapidly dividing cancer-associated fibroblasts. To overcome these limitations, we have developed a protocol to culture SBNET cells from surgically removed tumors as spheroids in extracellular matrix (ECM). The ECM forms a 3-dimensional matrix that encapsulates SBNET cells and mimics the tumor micro-environment for allowing SBNET cells to grow. Here, we characterized the growth rate of SBNET spheroids and described methods to identify SNBET markers using immunofluorescence microscopy and

immunohistochemistry to confirm that the spheroids are neuroendocrine tumor cells. In addition, we used SBNET spheroids for testing the cytotoxicity of rapamycin.

**INTRODUCTION:**

Small bowel neuroendocrine tumors (SBNETs) originate from enterochromaffin cells of the small intestine. Although SNBETs are generally known to grow slowly, they commonly metastasize to the liver[1]. While the surgical removal or tumor ablation can be considered in many cases, recurrence is nearly universal, and, therefore, medical therapy plays an important role in management. Tremendous efforts have been invested to generate new SBNET cell lines for drug testing. However, there has been very little success. Only 6 SBNET cell lines (KRJ-I, CND2, GOT1, P-STS, L-STS, H-STS) have been reported[2-5]; and unfortunately one cell line no longer expresses NET markers[6] and three other SBNET cell lines (KRJ-I, L-STS, H-STS) were determined to be derived from transformed lymphoblasts instead of NETs[7]. In order to accelerate the identification of drugs for targeting SBNETs, alternative methods for in vitro drug testing are needed.

Here, we take an advantage of the availability of resected SBNETs and have established a way to culture these patient derived SBNETs as spheroids growing in ECM. The overall goal of this manuscript is to describe a method to culture SBNET as a three-dimensional (3D) culture and outline procedures to characterize these spheroids for the retention of SBNET markers by immunofluorescence staining and immunohistochemistry.

In addition, we demonstrate how these SBNET spheroids can be used for testing the effect of rapamycin, an anti-cancer drug for NETs[8]. The rationale behind this protocol is to develop a new method to grow SBNET cells in vitro and use them for drug testing. The advantage of this technique over the traditional method of establishing a SBNET cell line is that 3D cultures of SBNETs can rapidly be obtained and drug testing can be done within 3 weeks. SBNET spheroids could potentially be used as a model for performing in vitro drug screens to identify new drugs for SBNET patients. Since SBNET cell lines are not widely available, 3D cultures of SBNET spheroids tacan serve as a new in vitro model for studying SBNETs and can be shared among scientists in the field.

**PROTOCOL:**

All experiments using human neuroendocrine tumor samples have been approved by the University of Iowa Hospital and Clinics IRB committee (Protocol number 199911057). A list of all materials and equipment is described in the **Table of Materials**. A list of growth media and key solutions is found in **Table 1**.

**1. Small bowel neuroendocrine tumor (SBNET) collection and cell dissociation**

1.1. Obtain resected patient SBNET samples after tumor tissues confirmation from the Surgical Pathology Core.

1.2. Cut SBNETs into 5 $mm^2$ cubes and store in 25 mL of DMEM/F12 medium in a conical tube for the transportation to the laboratory.

1.3. Transfer the tumors in DMEM containing 1% FBS, 1% penicillin/streptomycin (Pen/Strep), 1% glutamine (Wash Medium) and incubate in this Wash Medium for 15 min.

1.4. Transfer tumors to a new dish and mince tumors to less than 1 mm pieces using sterile curved scissors.

1.5. Transfer the minced tissues to a new 50 mL tube containing 25 mL of Wash Medium.

1.6. Centrifuge the sample at 500 x *g* for 15 min at 4 °C.

1.7. Discard the supernatant and resuspend the pellets in 10 mL of Wash Medium containing collagenase (100 U/mL) and DNase (0.1 mg/mL).

1.8. Allow the digestion of the minced tumors to occur in a 37 °C incubator with slow shaking (50 rpm) for 1.5 h.

**2. Culture of SBNETs as tumor spheroids in ECM**

2.1. After digestion is completed (step 1.8), centrifuge at 500 x *g* for 15 min at 4 °C, discard the supernatant and resuspend the pellet in 15 mL of Wash Medium.

2.2. Place a 40 µm cell strainer on top of a new 50 mL tube and transfer 10 mL of the Wash Medium over the cell strainer. Swirl the Wash Medium to cover the side of the plastic tube to prevent NET cells from sticking to the side of the plastic tube.

2.3. Filter the cell suspension through cell strainer.

2.4. Centrifuge at 500 x *g* for 15 min at 4 °C, discard the supernatant, resuspend the pellet in 200 µL of Wash Medium (total volume is ~ 250 µL) and place it on ice.

2.5. Transfer 5 µL of cells to 500 µL of Wash Medium (1/100 dilution factor). Use the diluted cells for cell counting using a hemocytometer to obtain the number of cells per milliliter. Multiply by 100 to correct for the dilution factor.

2.6. Multiply the number of cells per mL obtained in step 2.5 by the total volume of cells in suspension obtained in step 2.4 (~ 250 µL).

2.7. Centrifuge at 500 x *g* for 15 min at 4 °C, discard the supernatant, and resuspend the cells in liquid ECM (1 x $10^6$ cells/mL) and keep on ice.

2.8. Transfer 5-20 µL of SBNET spheroids in ECM to a 96-well plate and allow the liquid ECM to solidify by placing the plate in a 37 °C incubator for 5 min.

2.9. Add 200 µL of SBNET culture medium (DMEM/F12 + 10% FBS + 1% PEN/STREP + 1% Glutamine + 10 mM nicotinamide + 10 µg/mL insulin) to each well of the 96-well plate containing the SBNET spheroids in ECM.

2.10. Alternatively, culture SBNET organoids in stem cell media similar to what has previously been described for growing either human liver or pancreatic organoids[9] and listed in the **Table of Materials**.

2.11. Change media every 5-7 days.

**3. Quantification of SBNET spheroid size using ImageJ**

3.1. Take 5-10 pictures of SBNET spheroids at Day 1, 4, 7, 14, 23 and 97 of the culture using a 10x objective.

3.2. Open saved images in ImageJ. Go to the **Analyze** tab, select the **Set Measurements** option and place a check on the **Area** option.

3.3. Use the oval selection tool from the tool bar to generate an ellipsoid or circle around a well-focused spheroid.

3.4. Go to the **Analyze** tab and select the **Measure** option. Repeat steps 3.4 and 3.5 in order to get area measurement from 25-50 SBNET spheroids. The values are provided in pixel square.

3.5. Convert the pixel area to $\mu m^2$ by dividing the size of each pixel with the length of each pixel to µm conversion factor of the microscopy images.

NOTE: SBNET cells grow mainly as spheroids and sometime as ellipsoids.

**4. Characterization of SBNETS spheroids by immunofluorescence**

4.1. Transfer the organoids grown in ECM to a 1.5 mL tube using a P1000 pipette.

4.2. Centrifuge at 1,500 x *g* for 1 min and remove the supernatant.

4.3. Wash the organoid culture by adding 1 mL of PBS, mix, centrifuge at 1,500 x *g* for 1 min and remove the supernatant.

4.4. Fix the organoids by adding 500 µL of 4% paraformaldehyde and incubate for 15 min.

4.5. Wash the culture twice with 1 mL of PBS.

4.6. Permeabilize the culture by adding 500 µL of PBS + 3% BSA + 0.1% Triton X 100 for 5 min.

4.7. Then wash for three times with 1 mL of PBS + 3% BSA.

4.8. Incubate for 1 h with primary antibodies against synaptophysin (SYP)[10] at 1/600 dilution or chromogranin A (CgA)[11] and somatostatin receptor 2 (SSTR2)[12] at 1/400 dilution in antibody buffer (2.5% bovine serum albumin, 0.1% sodium azide, 25 mM Tris pH 7.4, 150 mM sodium chloride).

NOTE: Use 300 µL or more of antibody solution per tube.

4.9. Wash three times with 1 mL of PBS + 3% BSA.

4.10. Incubate with secondary antibodies coupled to FITC at 1/500 dilution in antibody buffer for 1 h. Use 300 µL or more of antibody solution per tube.

4.11. Wash 3 times with 1 mL of PBS + 3% BSA. Make sure to aspirate and discard all the supernatant.

4.12. Add 5 µL of mounting medium containing the nuclear stain DAPI.

4.13. Use a P20 pipette to transfer 5 µL of the SBNET spheroids from step 4.12 to a glass slide and seal with a cover slip.

4.14. Take images using a fluorescent microscope using the 10x, 20x or 40x objectives.

**5. SBNET spheroids characterization by immunohistochemistry (IHC)**

5.1. Transfer SBNET spheroids from the culture plate to a 1.5 mL tube by using a P1000 pipette.

5.2. Centrifuge at 1,500 x *g* for 1 min and remove the supernatant.

5.3. Fix SBNET spheroids by adding 500 µL of 10% formalin to the tube from step 5.2 and incubate at room temperature for 2-5 days prior to paraffin embedding and cut 4 µm thick spheroid sections.

5.4. Deparaffinize, rehydrate, and use heat-induced epitope retrieval in Antigen Retrieval Solution at pH 9 by heating to 97 °C for 20 min using the 3-in-1 automated slide processing station to prepare slides for antibody incubation.

5.5. Block slides and incubate with primary antibodies against SYP[10] at a 1/100 dilution for 15 min, CgA[11] at a 1/800 dilution for 15 min, and SSTR2[12] at a 1/5,000 dilution for 30 min.

5.6. Incubate with the secondary antibody detection system for 15 min for the anti-SYP and CgA staining and 30 min for anti-SSTR2 staining. Take pictures using a 400x objective.

**6. Treatment of SBNET organoids with rapamycin**

6.1. Dissolve rapamycin in DMSO to obtain a 10 mM stock solution.

6.2. Prepare SBNET culture medium with DMSO (e.g., 1 mL of SBNET culture medium + 1 µL of DMSO) and SBNET culture medium with 10 µM of rapamycin (e.g., 1 mL of SBNET culture medium + 1 µL of 10 mM rapamycin stock solution).

6.3. Transfer 200 µL media with SBNET culture medium with DMSO or 10 µM rapamycin to SBNET spheroid cultures.

6.4. Incubate SBNET spheroids for 5 days in 37 °C incubator.

6.5. Add 1 µM of ethidium homodimer and incubate for 30 min. Remove the medium containing ethidium homodimer, wash with 200 µL of PBS, and transfer 200 µL of PBS to each well.

6.6. Take microscopy images of SBNET spheroids with and without drug treatment using the red filter cube with the 10x, 20x, or 40x objectives of a fluorescent microscope.

NOTE: Dead cells stained with Ethidium homodimer appears as red using the red filter cube G of a commercially available microscope (**Table of Materials**). Alternatively, the signal can be captured using a spectrophotometer at 535 nm excitation and 624 nm emission.

**7. Splitting SBNET spheroids**

NOTE: This is done for expansion and for sharing with other researchers.

7.1. Use a P1000 pipette to mechanically break the ECM and aspirate the ECM with SBNET spheroids to a sterile 1.5 mL tube.

7.2. Centrifuge at 1,000 x *g* at 4 °C, remove all the supernatant and place the tube on ice.

7.3. Add 2-4x the volume of new ECM to the pellet. Mix the new ECM with the old ECM and SBNET spheroids by pipetting up and down 10x. Avoiding introducing air bubbles.

7.4. Transfer 5-20 µL of the ECM and SBNET spheroids mixture to a new plate and allow ECM to solidify.

7.5. Cover with new SBNET medium and transfer to the incubator. The recovery rate is approximately 95 to 100%.

7.6. For shipping SBNET spheroids to another lab, transfer SBNET spheroids with new ECM to a T25 flask and allow ECM to solidify.

7.7. Fill the T25 flask with SBNET spheroid medium, screw on the cap tightly, and prepare shipment package.

7.8. Upon receiving an SBNET spheroid culture, remove the culture medium and perform step 7.1 to 7.5 to put SBNET spheroids back in culture.

**8. Cryostorage and recovery of SBNET spheroids**

8.1. Transfer SBNET spheroids from 5-10 small wells to a 15 mL tube. Centrifuge at 500 x *g* for 15 min at 4 °C, remove supernatant, resuspend in freezing medium (90% FBS + 10% DMSO), store in a cell freezing container, and place this at -80 °C.

8.2. Transfer to the liquid nitrogen for longer storage.

8.3. To recover SBNET spheroids, place the frozen vial on ice and wait until the content is completely thawed. Invert the tube and re-place on ice in order to speed up the thawing process.

8.4. Once the sample is thawed, placed inside a pre-chilled centrifuge and spin at 1,000 x *g* for 10 min.

8.5. Remove the supernatant and resuspend the spheroids in ECM. Keep the tube on ice, transfer 20 µL to a new plate, and wait for the ECM to solidify.

8.6. Transfer 200 µL of SBNET culture medium with 10 µM of ROCK inhibitor (Y-27632)[13] and transfer to the incubator.

8.7. Allow SBNET spheroids to recover and grow for 1 week. Remove the old culture medium, replenish with 200 µL of SBNET culture medium and return to the incubator.

8.8. Allow SBNET spheroids to continue to grow.

NOTE: It takes at least 1 week for rapidly growing spheroids to start to recover after cryopreservation[13]. SBNET spheroids take a minimum of 2-4 weeks to start growing. Many SBNET cells will die within the first 2 weeks and the survival rate is less than 10%. Since this is a very time-consuming and low yield process, it is better to share with other researchers SBNET spheroids in culture flasks (as described in step 7). Cryostorage and recovery should only be used as a backup plan in case of a bacterial contamination occurs.

**REPRESENTATIVE RESULTS**

There are currently only 2 SBNET cell lines established and published[2-5] and they are not readily available to many researchers. Here, we propose to culture SBNET as spheroids in ECM and use

this as an alternative model to study SBNET drug sensitivity. Patient-derived tumor from an SBNET that metastasized to the liver was collected, digested to release SBNET cells, and mixed with liquid ECM for establishing an SBNET spheroid culture (**Figure 1A**). The Ki-67 of this SBNET was 4.3%. Although SBNET spheroids have a slow growth rate, their growth can be monitored by microscopic imaging (**Figure 1A**). It takes approximately 14 days for SBNET spheroids to double in size when the culture media is changed once a week (**Figure 1B**). After 14 days in culture, SBNET spheroids do not increase in size. Instead, some SBNET cells will dissociate to a neighboring location and form new spheroids. To propagate the SBNET spheroids culture, harvest the ECM containing SBNET spheroids and reseed them in new culture plate with new culture medium (Step 7).

To confirm that the organoid cultures contain SBNET cells, we describe a simple and fast method to stain the spheroids for SBNET markers such as synaptophysin, chromogranin A, and the somatostatin receptor type 2 (SSTR2) using immunofluorescence (IF) microscopy (Step 4). Using antibodies specific against synaptophysin, chromogranin A and SSTR2, our IF data showed that these markers are localized in the cytoplasm and at the membrane of SBNET cells (**Figure 2A**, in green) after 1 or 9 months in culture (F**igure 2B**). To ensure the specificity of the SYP, CgA and SSTR2 antibodies, we performed the same staining procedures on an organoid line from pancreas tumor that does not express SYP, CgA or SSTR2 (**Figure 2C**) as no green signal was detected. The main advantage of this SBNET spheroid IF experiment is that it can be performed within 4 h and gives similar staining information as the immunohistochemistry (IHC; **Figure 3**). We provide a protocol for performing IHC of the SBNET spheroids in step 5.

Culturing SBNET as spheroids is a valuable technique for identifying drugs that can inhibit SBNET growth. As a proof of principle, we treated SBNET spheroids with rapamycin for 5 days, an mTOR inhibitor, a class of drugs commonly used to treat NETs[8]. In comparison to our control SBNET spheroid, the rapamycin-treated spheroid formed a grape-like structure and became apoptotic or necrotic (**Figure 4A-D**). Dying cells can be detected using Ethidium homodimer to stain DNA and RNA and generate a bright yellow signal[14]. This dye cannot penetrate the cell membrane of live cells.

**FIGURE AND TABLE LEGENDS**

**Figure 1**: **Patient-derived small bowel neuroendocrine tumors (SBNETs) grown in extracellular matrix (ECM) as spheroids.** (**A**) Isolation of tumor cells from a resected SBNET and put in culture by mixing with ECM. Scale bar represents 100 µm. (**B**) Surface area of SBNET spheroids with respect to the number of days in culture quantified using ImageJ. Data were obtained from SBNET spheroids of 1 patient and are represented as the mean area ± standard error of the mean. The surface area of 30 to 60 spheroids were measured for each time point.

**Figure 2: Immunofluorescence (IF) staining of SBNET spheroids.** IF staining of SBNET spheroids after (**A**) 1 month in culture and (**B**) 9 months in culture. (**C**) IF staining of pancreas tumor organoids that do not express SBNET markers as negative controls. Tumor spheroids were fixed in 4% paraformaldehyde and stained using antibodies against synaptophysin (SYP) at 1/600 dilution, chromogranin A (CgA) at 1/400 dilution, and somatostatin receptor 2 (SSTR2) at 1/400.

IF images were taken at 100 ms, 200 ms and 400 ms exposure time for SYP, CgA and SSTR2 staining, respectively using the 10x, 20x or 40x objectives. Scale bar represents 50 µm.

**Figure 3**: **Immunohistochemistry (IHC) staining of SBNET spheroids.** Formalin-fixed and paraffin-embedded SBNET spheroids sections were deparaffinized, rehydrated, blocked and stained with (**A**) SYP, (**B**) CgA, and (**C**) SSTR2 antibodies. Images were taken using the 400x objective. Scale bar represents 50 µm.

**Figure 4: Using SBNET spheroids for drug testing.** (**A**) Bright field image of SBNET spheroids treated with DMSO for 5 days. (**B**) Image of SBNET spheroids treated with DMSO and stained with Ethidium homodimer (Ethidium H). (**C**) Bright field image of a dead SBNET spheroid forming grape-like structure after treatment with 10 µM of rapamycin for 5 days. (**D**) Dead SBNET spheroid stained with Ethidium H appears as red dots. Images of Ethidium H staining were taken using the red filter cube at 100 ms exposure time. Scale bar represents 10 µm.

**Table 1. List of growth media and solution.**

## DISCUSSION

Tumor 3D cultures have become a valuable resource for preclinical drug testing[15]. Various tumor organoid biobanks have recently been established from breast cancer and prostate cancer tumors[16,17]. In this study, we provide a detailed protocol to culture SBNET as spheroids and a simple and fast method to validate the spheroid cultures for NET markers by immunofluorescence and test drug sensitivity. From our experience, SBNET spheroids can grow in various culture media. They grow slightly faster in stem cell media for human pancreas or liver isolation that we adapted from previously published protocols[9]. We chose to grow the SBNETs in DMEM/F12 medium supplemented with insulin and nicotinamide because this is less expensive than stem cell media. Increasing the percentage of fetal bovine serum is another strategy to promote the growth of SBNET organoid cultures; however, this would also increase the overall cost for culture maintenance.

Performing IF staining and imaging with a fluorescent microscopy using the 10x, 20x or 40x objectives is a quick and simple method to test for the expression SBNET markers. However, it does not give a well-defined localization in comparison to IHC (**Figure 2, Figure 3**). For example, the IF data showed that the membrane localization of SSTR2 is difficult to detect. We mainly detect the cytosolic SSTR2, which has previously been reported[18]. In order to obtain a better localization of the marker proteins, we recommend using IF and confocal microscopy. Overall, IF is a useful method to rapidly confirm SBNET markers. Our antibodies (anti-SYP, anti-CgA, anti-SSTR2) did not cross-react with the negative control organoids that do not express SYP, CgA, or SSTR2 (**Figure 2C**) in the IF experiment. This suggestion that the fluorescent signals that we detected are specific to SBNET spheroids.

To increase the yield of SBNET spheroids, the critical steps of this protocol are during the cell filtration step (step 2.2) and mixing the SBNET with the liquid ECM for aliquoting into tissue culture plates (step 2.8). Make sure to cover the cell strainer membrane and the collection tube

with media in order to prevent the SBNET cells from sticking to the plastic. Liquid ECM will rapidly solidify if not placed on ice. Make sure to have a small ice container in the tissue culture hood in order to keep the ECM and SBNET cells cold.

The limitation of this technique is the slow growth rate of the SBNET spheroids. Experiments must be planned efficiently and avoid using an excess amount of SBNET spheroids. Another limitation is the slow recovery after freeze thaw. It takes over 1 month after thawing for the SBNET spheroids to start growing. To overcome this limitation, we suggest to continuously maintain SBNET spheroids in cultures and splitting them as needed (step 7). Even after 9 months in culture, the SBNET spheroids still maintain expression of SBNET markers (**Figure 2B**).

Although 3D culture of SBNET spheroids is more labor intensive than traditional 2D culture of other cancer cell lines, it is an extremely valuable model for in vitro culture of SBNET because many SBNET researchers do not have access to the existing cell lines. With this protocol, scientists and clinicians can establish SBNET spheroid cultures from resected tumors and share them with other laboratories. In addition, the SBNET spheroids could potentially be used to establish SBNET patient-derived xenograft mouse models. Overall, the techniques presented here can be adapted for culturing, characterizing and performing drug testing of other NETs such as pancreatic or lung NETs. Note that the growth rate of the organoids will vary between the different types of NETs and patient samples.

**ACKNOWLEDGMENTS**

This work was supported by NIH grants P50 CA174521 (to J.R. Howe and A.M. Bellizzi). P.H. Ear is a recipient of the P50 CA174521 Career Enhancement Program award.

**DISCLOSURES**

The authors have nothing to disclose.

**REFERENCES**

1 Maxwell, J. E., Sherman, S. K., Howe, J. R. Translational Diagnostics and Therapeutics in Pancreatic Neuroendocrine Tumors. *Clinical Cancer Research.* **22**, 5022-5029 (2016).

2 Pfragner, R. et al. Establishment of a continuous cell line from a human carcinoid of the small intestine (KRJ-I). *International Journal of Oncology.* **8**, 513-520 (1996).

3 Kolby, L. et al. A transplantable human carcinoid as model for somatostatin receptor-mediated and amine transporter-mediated radionuclide uptake. *American Journal of Pathology.* **158**, 745-755 (2001).

4 Van Buren, G. et al. The development and characterization of a human midgut carcinoid cell line. *Clinical Cancer Research.* **13**, 4704-4712 (2007).

5 Pfragner, R. et al. Establishment and characterization of three novel cell lines - P-STS, L-STS, H-STS - derived from a human metastatic midgut carcinoid. *Anticancer Research.* **29**, 1951-1961 (2009).

6 Ellis, L. M., Samuel, S., Sceusi, E. Varying opinions on the authenticity of a human midgut carcinoid cell line--letter. *Clinical Cancer Research.* **16**, 5365-5366 (2010).

7 Hofving, T. et al. The neuroendocrine phenotype, genomic profile and therapeutic sensitivity of GEPNET cell lines. *Endocrine Related Cancer.* **25**, 367-380 (2018).
8 Moreno, A. et al. Antitumor activity of rapamycin and octreotide as single agents or in combination in neuroendocrine tumors. *Endocrine Related Cancer.* **15**, 257-266 (2008).
9 Broutier, L. et al. Culture and establishment of self-renewing human and mouse adult liver and pancreas 3D organoids and their genetic manipulation. *Nature Protocols.* **11**, 1724-1743 (2016).
10 Saito, Y. et al. Establishment of Patient-Derived Organoids and Drug Screening for Biliary Tract Carcinoma. *Cell Reports.* **27**, 1265-1276 (2019).
11 Park, S. J. et al. Detection of bone marrow metastases of neuroblastoma with immunohistochemical staining of CD56, chromogranin A, and synaptophysin. *Applied Immunohistochemisty and Molecular Morphology.* **18**, 348-352 (2010).
12 Clifton-Bligh, R. J. et al. Improving diagnosis of tumor-induced osteomalacia with Gallium-68 DOTATATE PET/CT. *The Journal of Clinical Endocrinology and Metabolism.* **98**, 687-694 (2013).
13 Clinton, J., McWilliams-Koeppen, P. Initiation, Expansion, and Cryopreservation of Human Primary Tissue-Derived Normal and Diseased Organoids in Embedded Three-Dimensional Culture. *Current Protocols in Cell Biology.* **82**, e66 (2019).
14 Markovits, J., Roques, B. P., Le Pecq, J. B. Ethidium dimer: a new reagent for the fluorimetric determination of nucleic acids. *Analytical Biochemistry.* **94**, 259-264 (1979).
15 Weeber, F., Ooft, S. N., Dijkstra, K. K., Voest, E. E. Tumor Organoids as a Pre-clinical Cancer Model for Drug Discovery. *Cell Chemical Biology.* **24**, 1092-1100 (2017).
16 Sachs, N. et al. A Living Biobank of Breast Cancer Organoids Captures Disease Heterogeneity. *Cell.* **172**, 373-386 e310 (2018).
17 Puca, L. et al. Patient derived organoids to model rare prostate cancer phenotypes. *Nature Communication.* **9**, 2404 (2018).
18 Singh, S. P. et al. SSTR2-based reporters for assessing gene transfer into non-small cell lung cancer: evaluation using an intrathoracic mouse model. *Human Gene Therapy.* **22**, 55-64 (2011).

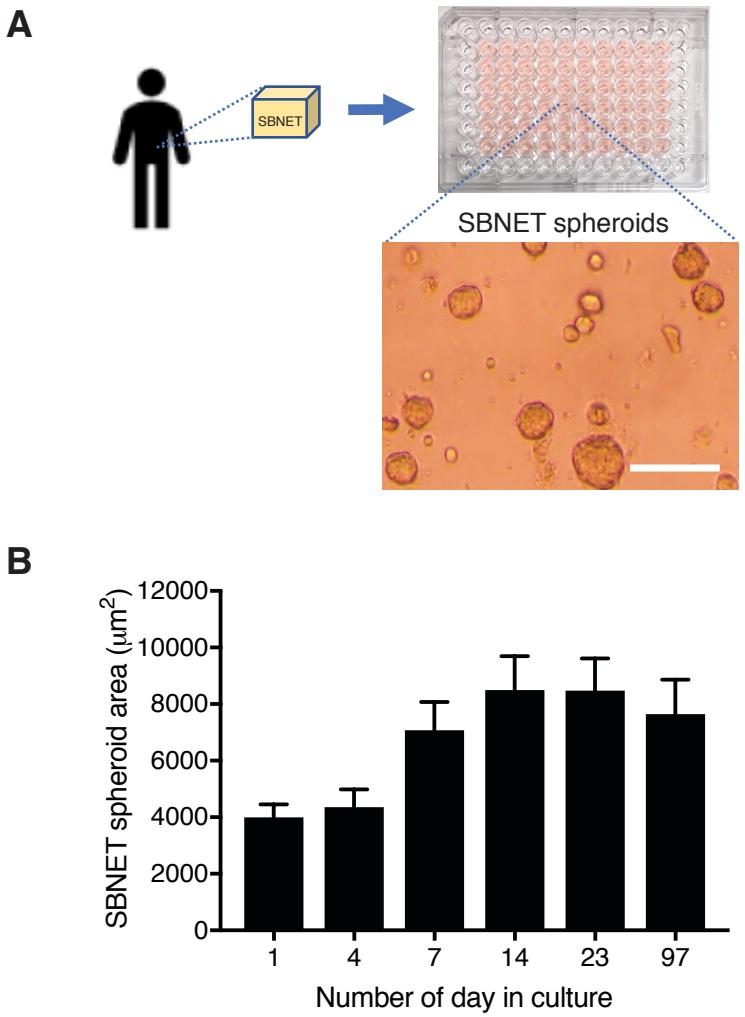


**Figure 1**

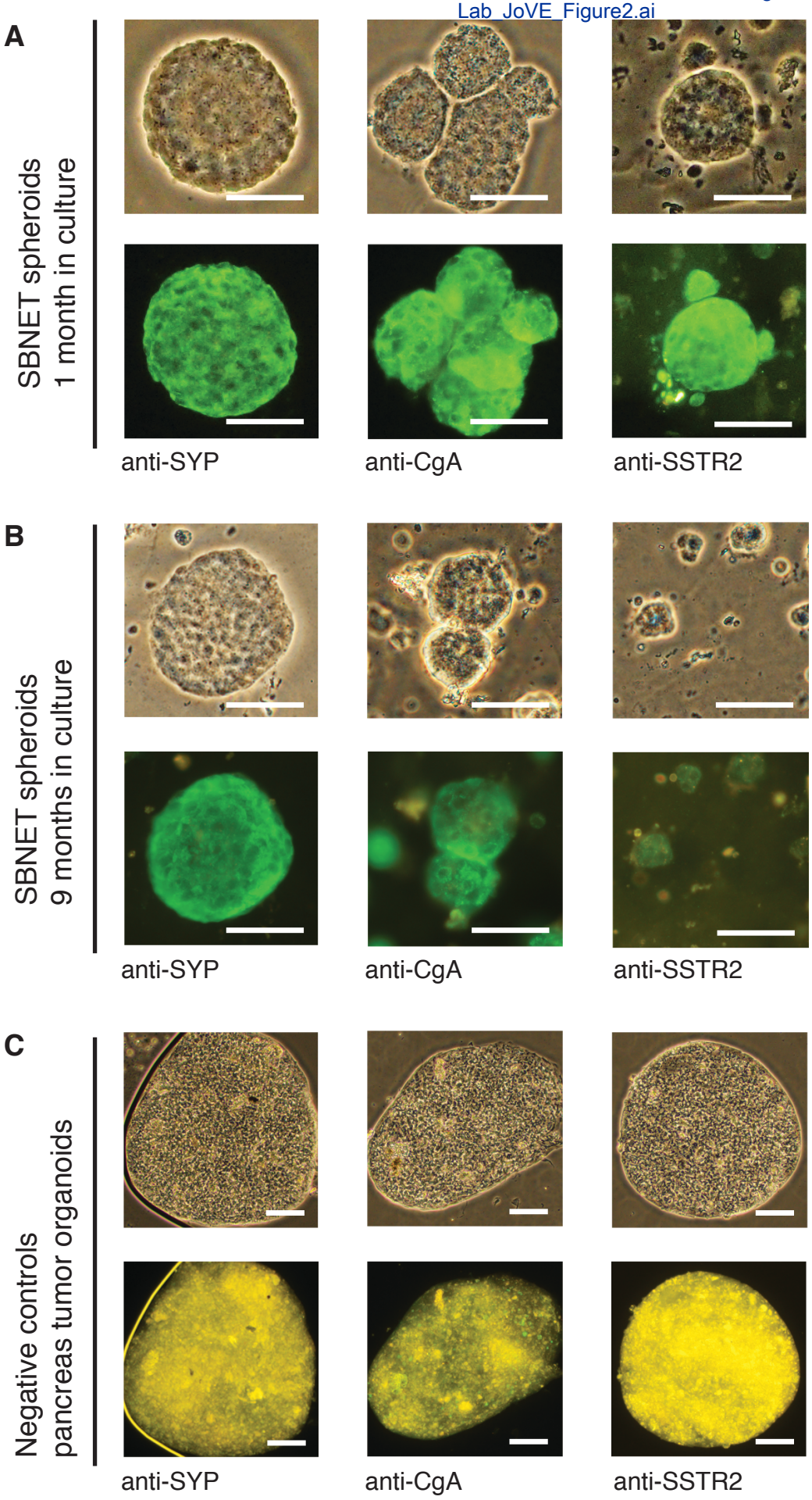


**Figure 2**

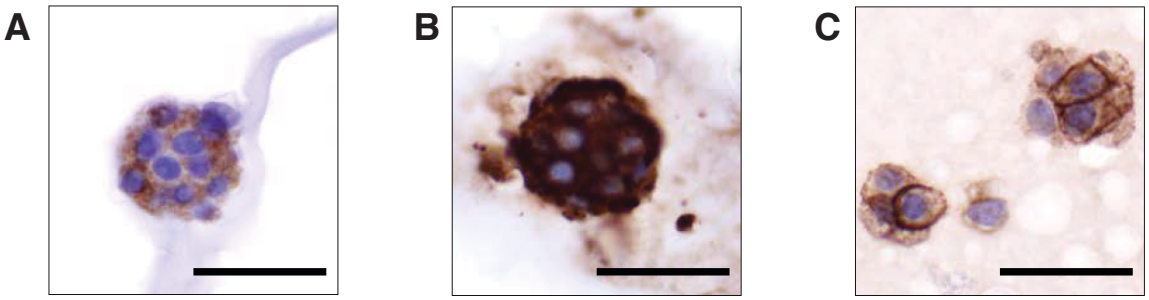


**Figure 3**

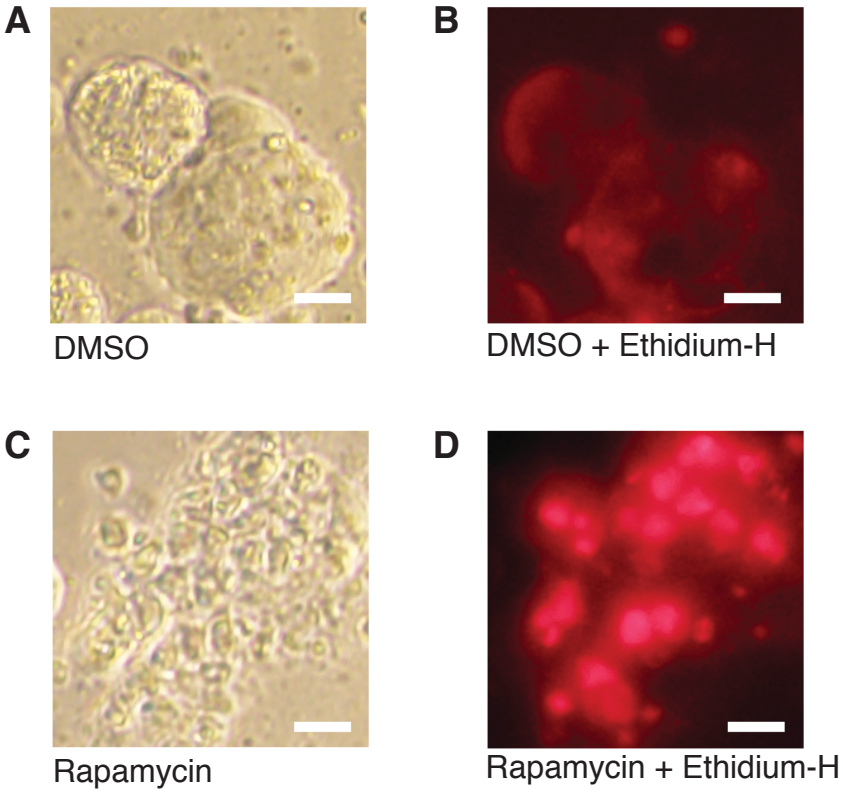


**Figure 4**

**TABLE 1. LIST OF GROWTH MEDIA AND SOLUTIONS**

| Growth media or solution | Composition |
|---|---|
| Wash Medium | DMEM containing 1% FBS, 1% PEN/STREP, 1% Glutamine |
| SBNET culture medium | DMEM/F12 + 10% FBS + 1% PEN/STREP + 1% Glutamine + 10 mM nicotinamide + 10 µg/mL insulin |
| Antibody buffer | 2.5% bovine serum albumin, 0.1% sodium azide, 25 mM Tris pH 7.4, 150 mM sodium chloride |
| Freezing medium | 90% FBS + 10% DMSO |
| Human liver stem cell isolation medium | DMEM/F12 , 1% Pen/Strep, 1% GlutaMAX, 10 mM HEPES, 1/50 B27 Supplement, 1/100 N2 Supplement, 1 mM N-acetylcysteine, 200 ng/mL Rspo 1, 50ng/mL EGF, 100 ng/mL FGF10, 10 mM nicotinamide, 10 uM forskolin, 5uM A83-01 |
| Human pancreatic stem cell isolation medium | DMEM/F12 , 1% Pen/Strep, 1% GlutaMAX, 10 mM HEPES, 1/50 B27 Supplement, 1/100 N2 Supplement, 1 mM N-acetylcysteine, 200 ng/mL Rspo 1, 25 ng/mL Noggin, 50ng/mL EGF, 100 ng/mL FGF10, 10 mM nicotinamide, 10 uM forskolin, 5uM A83-01, 3 uM PGE-2 |

Table of Materials



| Name of Material/ Equipment | Company | Catalog Number | Comments/Description |
|---|---|---|---|
| Anti-rabbit FITC | Jackson ImmunoResearch | 11-095-152 | Secondary antibody couple to a green fluorophore |
| Antigen Retrieval Solution | Agilent Dako | S2367 | Solution at pH 9 for preparing slides for IHC |
| Autostainer Link 48 | Agilent Dako | Not Available | Automated system for antibody staining |
| Cell freezing container | Thermo Scientific | 5100-0001 | Container to for freezing cells |
| CellSence | Olympus | Version 1.18 | Computer software for using fluorescent microscope |
| Chromogranin A antibody | Abcam-45179 | RB-9003-PO | Antibodies for IF |
| Chromogranin A antibody (clone LK2H10) | Thermo Scientific | MA5-13096 | Antibodies for IHC |
| Collagenase | Sigma | C0130 | Enzyme for digesting tumor tissue |
| DMEM | Gibco | 11965-092 | Medium for tissue preparation |
| DMEM/F12 | Gibco | 11320-033 | Medium for organoid cultures |
| DMSO | Sigma | D8418 | Solvent for dissolving drug |
| DNAse | Sigma | DN25 | Enzyme for digesting tumor tissue |
| Ethidium Homodimer | Chemodex | CDX-E0012-T1E | DNA and RNA binding dye |
| FBS | Gibco | 16000044 | Reagent for culture media |
| Fluorescent microscope | Olympus | CKX35 | Microscope for taking pictures of SBENT spheroids |
| Glutamine | Gibco | A2916801 | Reagent for culture media |
| ImageJ | National Institutes of Health | Version 1.51 | Computer software for image analysis |
| Insulin | Sigma | I0516 | Reagent for culture media |

JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com



# ARTICLE AND VIDEO LICENSE AGREEMENT

| | |
|---|---|
| Title of Article: | Establishment and Characterization of Small Bowel Neuroendocrine Tumor Spheroids |
| Author(s): | Po Hien Ear, Guiying Li, Meng Wu, Ellen Abusada, Andrew M. Bellizzi and James R. Howe |

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

[X] Standard Access [ ] Open Access

Item 2: Please select one of the following items:

[X] The Author is **NOT** a United States government employee.

[ ] The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

[ ] The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

## ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

# ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

**CORRESPONDING AUTHOR**

| | |
|---|---|
| Name: | James R. Howe |
| Department: | Surgery |
| Institution: | University of Iowa |
| Title: | Professor |
| Signature: | James Howe |
| Date: | July 26th, 2019 |

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

UNIVERSITY of IOWA
CARVER COLLEGE OF MEDICINE
University of Iowa Health Care

*Department of Surgery*

*Po Hien Ear, PhD*
*Research Scientist*
*University of Iowa Roy J. and Lucille A.*
*Carver College of Medicine*
*375 MERF, Room 4235*
*Iowa City, IA 52242*
*Tel. (319)335-8983*
*E-mail: pohien-ear@uiowa.edu*

July 24th, 2019

**RE:** Rebuttal for Editorial Comments of Manuscript JoVE60303 "Establishment and characterization of small bowel neuroendocrine tumor organoids,"

Dear Dr. Bajaj,

Thank you for expressing interest regarding our manuscript. We have submitted a revised copy of the manuscript addressing all the editorial and reviewers' comments along with figures in Illustrator format. We apologize for the delay! We wanted to make sure that we addressed all the reviewers' comment thoroughly. The current manuscript is now 10 pages long. We hope the editorial team and reviewers will like our modifications and recommend this manuscript for publication.

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

Sincerely,

Po Hien Ear, PhD
Research Scientist
James Howe Laboratory
Department of Surgery
University of Iowa
375 Newton Rd,
MERF Room 4235
Iowa City, IA 52242
Lab phone: (319) 335-8983

**Rebuttal for Editorial Comments:**

Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues. The JoVE editor will not copy-edit your manuscript and any errors in the submitted revision may be present in the published version.

Thanks for pointing this out. We have performed the revision.

2. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Matrigel, Abcam-45179, Abcam-32127, VECTASHIELD, Vector Laboratories, Jackson ImmunoResearch, etc

Ok, all removed.

3. Please ensure that the short abstract/summary is no more than 50 words.

Corrected, it's 49 words now.

4. Please expand the Introduction to include all of the following:
a) A clear statement of the overall goal of this method

Added
b) The rationale behind the development and/or use of this technique

added

c) The advantages over alternative techniques with applicable references to previous studies

added
d) A description of the context of the technique in the wider body of literature.

added

e) Information to help readers to determine whether the method is appropriate for their application. We updated the introduction with more information.

5. Please include an ethics statement before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee. We've included a statement with IRB protocol number. We included the statement and IRB protocol number.

6. Please include a single line space between each step, substep, and note in the protocol section. We included the single lines for spacing.

7. Please ensure that all text in the protocol section is written in the imperative tense as if telling someone how to do the technique (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." Yes, we double checked and inserted a few Notes in the protocol.

8. The Protocol should contain only action items that direct the reader to do something. Yes, confirmed.

9. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please ensure that individual steps of the protocol should only contain 2-3 actions per step. We double checked.

10. Please ensure you answer the "how" question, i.e., how is the step performed? Yes, we checked.

11. 1.1: How and from where the tumors were obtained? We included a statement saying that tumors were resected from patients in the operating room, brought to the surgical pathology core facility for analysis, and a fraction of the tumors were cut into 5 mm piece for storage in DMEM/F12 medium.

Do you perform any wash steps before storing? No, resected tumors were simply cut into smaller pieces and stored in DMEM/F12.

Do you immediately transfer it to DMEM, or this can be stored at -80 C? No storage at -80$^{o}$C. Resected tumor samples are stored at 4$^{o}$C in DMEM/F12 for up to 3 hrs prior to processing. Overnight storage will decrease the yield of viable organoids.

How do you transfer the tumor samples to the lab-based settings? In 50 mL conical tubes.

What is the general size of the tumors obtained? Do you make small pieces before transferring to DMEM? Cut tumors into 5 mm cubes and use 3 or more pieces for processing to ensure enough tumor cells can be obtained.

12. 3, 4: After how many days do you perform the characterization and rapamycin treatment? We have SBNET spheroids growing in culture for up to 9 months now and they still express the NET markers (see new Figure 7). SBNET spheroids can be used for drug treatment experiment at any timepoint. We tested our SBNET spheroids that were in culture for 2 months with rapamycin treatment for 5 days.

Citations for the antibody used as the marker for organoid culture? These antibodies have been used in other cancers and tissue sections but not for neuroendocrine tumors. Hence, our pathologist team has included the detailed conditions IHC in the new Section 5.

13. 3.1: Is it one organoid per tube? We plate ~ 1000 to 10000 spheroids per well (96-well plated).

14. 3.11, 4.6: What is the mangnification used? 10X, 20X, 40X for immunofluorescence and 400X for immunohistochemistry. Images were cropped with appropriate scale bars in ImageJ.

15. 4. Do you perform DMSO control as well? Yes, new figure with 4 conditions.

16. There is a 10-page limit for the Protocol, but there is a 2.75-page limit for filmable content. 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. We have highlighted the 2.5 pages of protocol for the filmable content.

17. Please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]." We haven't published these elsewhere. No copyright permission is needed.

18. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations: We've edited the Discussion section.
a) Critical steps within the protocol

Added

b) Any modifications and troubleshooting of the technique

We added ROCK inhibitor to the culture media to slightly improve cell survival of SBNET spheroids after thawing. In addition, we switched from calling our SBNET "organoids" to "spheroids" since Reviewer#3 pointed out that our 3D cultures are not heterogenous.

c) Any limitations of the technique

Added

d) The significance with respect to existing methods

Added

e) Any future applications of the technique

Added *in vivo* model as future application.

19. Please sort the materials table in alphabetical order.

We sorted the materials/equipment.

20. The signed ALA is for the UK only. Please print and sign the attached Author License Agreement (ALA). Please then scan and upload the signed ALA with the manuscript files to your Editorial Manager account.
We are from the USA. No ALA needed.

UNIVERSITY of IOWA
CARVER COLLEGE OF MEDICINE
University of Iowa Health Care

*Department of Surgery*

*Po Hien Ear, PhD*
*Research Scientist*
*University of Iowa Roy J. and Lucille A.*
*Carver College of Medicine*
*375 MERF, Room 4235*
*Iowa City, IA 52242*
*Tel. (319)335-8983*
*E-mail: pohien-ear@uiowa.edu*

July 24th, 2019

**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,

Po Hien Ear, PhD
Research Scientist
James Howe Laboratory
Department of Surgery
University of Iowa
375 Newton Rd,
MERF Room 4235
Iowa City, IA 52242
Lab phone: (319) 335-8983

**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).