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TITLE:

Creating Matched In vivo/In vitro Patient-Derived Model Pairs of PDX and PDX-Derived Organoids for Cancer Pharmacology Research

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KEYWORDS:

In vitro cancer model, animal tumor model, morphology, histopathology, genomic profiling, chemotherapy

SUMMARY:

A method is described to create organoids using patient-derived xenografts (PDX) for in vitro screening, resulting in matched pairs of in vivo/in vitro models. PDX tumors were harvested/processed into small pieces mechanically or enzymatically, followed by the Clevers' method to grow tumor organoids that were passaged, cryopreserved and characterized against the original PDX.

ABSTRACT:

Patient-derived tumor xenografts (PDXs) are considered the most predictive preclinical models, largely believed to be driven by cancer stem cells (CSC) for conventional cancer drug evaluation.

A large library of PDXs is reflective of the diversity of patient populations and thus enables population based preclinical trials ("Phase II-like mouse clinical trials"); however, PDX have practical limitations of low throughput, high costs and long duration. Tumor organoids, also being patient-derived CSC-driven models, can be considered as the in vitro equivalent of PDX, overcoming certain PDX limitations for dealing with large libraries of organoids or compounds. This study describes a method to create PDX-derived organoids (PDXO), thus resulting in paired models for in vitro and in vivo pharmacology research. Subcutaneously-transplanted PDX-CR2110 tumors were collected from tumor-bearing mice when the tumors reached 200-800 mm^3, per an approved autopsy procedure, followed by removal of the adjacent non-tumor tissues and dissociation into small tumor fragments. The small tumor fragments were washed and passed through a 100 µm cell strainer to remove the debris. Cell clusters were collected and suspended in basement membrane extract (BME) solution and plated in a 6-well plate as a solid droplet with surrounding liquid media for growth in a CO_2 incubator. Organoid growth was monitored twice weekly under light microscopy and recorded by photography, followed by liquid medium change 2 or 3 times a week. The grown organoids were further passaged (7 days later) at a 1:2 ratio by disrupting the BME embedded organoids using mechanical shearing, aided by addition of trypsin and the addition of 10 µM Y-27632. Organoids were cryopreserved in cryo-tubes for long-term storage, after release from BME by centrifugation, and also sampled (e.g., DNA, RNA and FFPE block) for further characterization.

INTRODUCTION:

Cancers are a collection of diverse genetic and immunological disorders. Successful development of effective treatments is highly dependent on experimental models that effectively predict clinical outcomes. Large libraries of well-characterized patient-derived xenografts (PDX) have long been viewed as the translational in vivo system of choice to test chemo- and/or targeted therapies due to their ability to recapitulate patient tumor characteristics, heterogeneity and patient drug response[1], thus enabling Phase II-like mouse clinical trials to improve clinical success[2,3]. PDXs are generally considered as cancer stem cell diseases, featuring genetic stability, in contrast to cell line derived xenografts[2]. Over the last few decades, large collections of PDXs have been created throughout the world, becoming the workhorse of cancer drug development today. Although widely used and with great translational value, these animal models are intrinsically costly, time consuming and low throughput, inadequate for large scale screening. PDX are also undesirable for immuno-oncology (IO) testing due to an immune-compromised nature[4]. It is thus impractical to take full advantage of the available large library of PDXs.

Recent discoveries, pioneered by the Hans Clevers' laboratory [5], have led to the establishment of in vitro cultures of organoids generated from adult stem cells in most human organs of epithelial origin[5]. These protocols have been further refined to allow the growth of organoids from assumed CSCs in human carcinomas of various indications[6,7]. These patient-derived organoids (PDOs) are genomic-stable[8,9] and have been shown to be highly predictive of clinical treatment outcomes[10-12]. In addition, the in vitro nature of PDOs enables high-throughput screening (HTS)[13], thus potentially offering an advantage over in vivo models and leveraging large organoid libraries as a surrogate of the patient population. PDOs are poised to become an important discovery and translational platform, overcoming the many limitations of PDXs described above.

Both PDO and PDX are patient-derived and CSC-driven models, with the ability to evaluate therapeutics in the context of either personalized treatment or clinical trial format. Existing large libraries of PDXs, like the proprietary collection of >3000 PDXs[14-17], are therefore suitable for the rapid generation of libraries of tumor organoids (PDX-derived organoids, or PDXO), resulting in a matched library of paired PDX and PDXO models. This report describes the procedure to create and characterize colorectal cancer PDXO-CR2110 in relation to its parental PDX-CR2110 model[16].

PROTOCOL:

All the protocols and amendment(s) or procedures involving the care and use of animals were reviewed and approved by the Crown Bioscience Institutional Animal Care and Use Committee (IACUC) prior to conducting the studies. The care and use of animals was conducted in accordance with AAALAC (Association for Assessment and Accreditation of Laboratory Animal Care) International guidelines as reported in the Guide for the Care and Use of Laboratory Animals, National Research Council (2011). All animal experimental procedures were under sterile conditions at SPF (specific pathogen-free) facilities and conducted in strict accordance with the Guide for the Care and Use of Laboratory Animals from different government institutions (e.g., The National Institutes of Health). The protocols were approved by the Committee on the Ethics of Animal Experiments at the facility institution (e.g., institutional IACUC Committee).

1. Preparation for tumor transplantation

1.1. Animal housing

1.1.1. House Balb/c nude mice (n=5) in individual ventilated cages, at 20-26 °C, 30-70% humidity, and a lighting cycle of 12-h light/12-h dark, with corn cob bedding changed weekly, and irradiation sterilized dry granule food plus sterile drinking water ad libitum.

1.2. Donor tumor fragment preparation

1.2.1. Closely monitor tumor-bearing donor mice for body weight (BW, via weighing balance), and tumor volume (TV, by caliper measurement).

1.2.2. When TV reaches 800-1000 mm^3, euthanize the tumor-bearing animals in a biohazard hood.

1.2.2.1. Place mice in a euthanasia chamber with a lid that delivers CO_2 gas into the chamber. Discharge gas into the chamber at a flow rate that produces rapid unconsciousness with minimal distress to the animal. The optimum flow rate for CO_2 is around 2-2.5 Lpm.

1.2.2.2. Ensure euthanasia by observing that the animals do not regain consciousness.

1.2.2.3. After apparent clinical death, maintain gas flow for >1 min to minimize the

possibility that an animal may recover.

1.2.3. Sterilize the skin around the tumor using iodophor swabs. Collect tumors by removing adjacent non-tumor tissues and placing in a Petri dish containing 20 mL of PBS, pre-chilled (4 °C) prior to euthanasia.

1.2.4. Wash the tumors with PBS in another Petri dish to remove blood components followed by cutting in half and removing any extra skin, blood vessels, calcification and/or necrosis.

1.2.5. Place only intact tumor pieces into a sterile 50 mL centrifuge tube with 20 mL of PBS, prior to transporting to a separate animal room for transplantation.

2. Subcutaneous tumor growth

2.1. Subcutaneous inoculation of tumor piece into immunocompromised mice

2.1.1. Cut the PDX tumor into 2 mm pieces with a scalpel, and place each into trocars for subcutaneous (SC) implantation.

2.1.2. Anesthetize the recipient animals with 5% isoflurane (maintained by a nose cone at 1%). The animals relax, losing their righting reflex and eventually becoming immobile, until not responding to pain. Fix the anesthetized mice onto an experiment board in the right lateral position and sterilize with iodophor swabs, particularly the areas surrounding the site of tumor inoculation.

2.1.3. On left flank skin just cranial to the hip, make a 0.5 cm incision with a scalpel and a tunnel under the skin towards the forelimb created, 2-3 cm, using blunt forceps.

2.1.4. Aseptically transfer one cube of tumor fragment per inoculation site from the medium and place deep inside the subcutaneous tunnel. Visually confirm position of the fragment prior to closure of wound with wound clips.

2.1.5. Monitor the tumor implanted mice (5) until they become conscious to maintain sternal recumbence, and then return them to their cage only after their full recovery from anesthesia.

2.2. Tumor-bearing mice health monitoring

2.2.1. Check water and food consumption daily and record body weight weekly.

2.2.2. Check the mice during routine monitoring. Record any effects on mobility, breathing, grooming and general appearance, food and water consumption, BW gain/loss, ascites, etc.

2.2.3. Measure TV twice weekly using calipers and express in mm^3 per: $TV = 0.5\ a \times b^2$, where a and b are the length and width of the tumor, respectively.

2.2.4. Sacrifice animals to collect samples when any of the following signs appear: BW loss >20%; impaired mobility (not able to eat or drink); unable to move normally due to significant ascites or enlarged abdomen; effort in respiration; death.

3. Necropsy and tumor harvest

3.1. When tumor volume reached 200-800 mm^3, euthanize mice per approved protocols (see step 1.2.2).

3.2. Collect tumor tissues by removing adjacent non-tumor tissues, followed by placing the tissue in a 50 mL plastic tube with AD+++ (on ice) before dissociation.

4. Preparation for PDX-derived organoid culture

NOTE: All the following steps were performed inside a biosafety cabinet per tissue culture standard guidelines. Keep pre-warmed stocks of 96-, 24-, and 6-well plates in a 37 °C incubator before use.

4.1. Reagent preparations

4.1.1. Prepare basement membrane extract (BME) solution (growth factor reduced, Phenol Red-free). Keep 10 mL of BME in a 4 °C refrigerator overnight. Once thawed, swirl the BME bottle to ensure dispersion.

4.1.2. Prepare base media/washing buffer AD+++. Add 5 mL of 200 mM L-glutamine, 1 M HEPES and Pen/Strep to Advanced DMEM/F12 media by pipetting with a 5 mL pipette.

4.1.3. Prepare organoid culture media. Prepare colon organoid medium as described by Sato et al.[18] through supplementing base media with N-Ac (1 mM), A83-01 (500 nM), B27 (1x), EGF (50 ng/mL), Noggin (100 ng/mL), Nicotinamide (10 mM), SD202190 (10 nM), R-spondin (500 ng/mL), L-glutamine (2 mM), HEPES (10 µM), penicillin-streptomycin (1x) and Y-27623 (10 µM)[19].

4.1.4. Prepare AD+++Digestion medium: 10 mL of 1x organoid culture media with 500 µL of collagenase type II (20 mg/mL) and 10 µL of RhoKI Y-27632 (10 mM).

4.2. Tumor Dissociation[20]

4.2.1. Transfer the tumor in a 50 mL plastic tube to a 10 cm Petri dish. Take a macroscopic photograph alongside a ruler and record a description of its conditions (i.e., size, fat tissues, vascularisation, necrosis, etc.).

4.2.2. Remove excess AD+++ by aspiration, and cut the tumor tissue into small pieces by scissors followed by transferral of 1-2 pieces into a 2 mL microtube for snap freezing on dry ice. Store at -

80 °C for genomic profiling. Mince the remaining pieces into finer pieces by scissors before transferral to a 50 mL plastic tube using AD+++.

4.2.3. Wash minced tissue 2-3 times by 35 mL of AD+++, followed by addition of 10 mL of digestion medium (see step 4.1.4) and placement on an orbital shaker at 4 x *g* for 1 h at 37 °C.

4.2.4. Homogenize the digested tissue by pipetting up and down using a 5 mL sterile plastic pipette, followed by addition of 20 mL of AD+++ and filtration by 100 µm cell strainer.

4.2.5. Wash the pass-through twice with AD+++ (spin at 450 x *g* for 5 min), followed by resuspension in BME (add 4x volume of BME the pellet for suspension) and keep on ice[19].

4.3. Preparation of organoid culture

4.3.1. Add the BME cell suspension into 6-well plate in multiple drops to a total of 200 µL per well.

4.3.2. Transfer the plate to a 37 °C incubator. After 30 min, the gel drops solidify.

4.3.3. Add 2 mL of organoid media to each well, with the representative drops recorded by microscopic photography before transferring to an incubator (37 °C and 5% CO_2).

4.3.4. Maintain the organoid cultures with medium change every 3-4 days, and passage at a 1:2 ratio every 7 days or depending on their growth and density.

5. Histopathology and next generation sequencing (NGS) analysis

5.1. Histopathology

5.1.1. Collect organoids from the well with the existing medium using a P1000 pipette, followed by centrifugation (spin at 450 x *g* for 5 min), washing with PBS and fixation in 10% formalin for 1 h.

5.1.2. Place the fixed organoids in 100% gelatin at the bottom of 50 mL conical tube, followed by routine tissue processing and embedding.

5.1.3. Perform haematoxylin–eosin (H&E) staining using standard protocols on 4 mm paraffin sections.

5.2. RNAseq and whole exome sequencing

5.2.1. Collect organoids from the well with the existing culture medium using a P1000 pipette, followed by centrifugation using a microcentrifuge at maximum speed (12,000 x *g*) for 5 min at 4 °C.

5.2.2. Collect the pellet by removing the medium supernatant to ensure no visible BME was present, followed by snap freezing in a microtube (dry ice) and then transfer to a -80 °C freezer.

5.2.3. Extract tRNA or DNA using standard procedure from manufacturers and perform NGS analysis for both RNAseq and whole exome sequencing (WES).

6. IC_{50} assay[20]

6.1. Organoid seeding in 384-well plate for IC_{50} assay

6.1.1. Dissociate organoids in BME drops from each well (digesting BME) by adding 20 µL of 100x dispase solution to each well (6-well plate), which contained 2 mL of organoid medium, followed by 10 min of incubation at 37 °C.

6.1.2. Pipette digested organoids from all wells through a 70 µm filter into a 50 mL plastic tube to collect the organoids.

6.1.3. Count the organoids under microscopy for the determination of organoid concentration. Suspend the organoids using culture medium, before adding BME to reach final concentration of 5% (v/v) on ice.

6.1.4. Add 50 µL of the organoid suspension into each 384-well plate with the liquid dispenser, according to the plate map with a seeding density of 200 CR2110 PDXOs per well in corresponding organoid culture medium.

6.2. Cisplatin and irinotecan treatment

6.2.1. Use cisplatin (with the highest concentration of 10 µM) and “irinotecan” (with the highest concentration of 10 µM. Add SN-38 (a metabolite of irinotecan, as opposed to irinotecan which is usually used for in vivo studies) to each well according to the drug dilution scheme for 9 doses, in serial dilution by digital dispener.

6.2.2. Create the platemap using the digital dispenser software tool. Include a negative control vehicle with 100% viability and postive control of 5 µM starurosporine, which showed 0% viability.

6.2.3. Place the drug-treated 384-well plates back into 37 °C incubator.

6.3. Determination of organoid cell viability after drug treatment

6.3.1. At the end of the 5 day of drug treatment, determine organoid cell viability using luminescent cell viability reagents as per the manufacturer’s recommended procedure. Add luminescent reagent into each well with the liquid dispener and mix for 5 min on a plate shaker, followed by a 30 min incubation at room temperature in dark.

6.3.2. Record the luminescent signal on a luminescence multi-well platereader.

6.3.3. Calculate the normalized viabilities of each well using the raw readings from the plate reader and create a dose-response curve and IC_{50} values by nonlinear curve fitting.

REPRESENTATIVE RESULTS:

Morphology of PDXOs, typical of organoids under light microcopy, and consistent with parental PDX per H&E staining

Under light microscopy, PDXO-CR2110 demonstrates typical cystic morphology (**Figure 1A**), as described previously for patient-derived organoids (PDO), evidence supporting the similarity between PDXO and PDO under the same culture conditions.

Histopathological examination by H&E staining reveals that the tissue structures and cell types of PDXO-CR2110 (**Figure 1B**) are reflective of the original PDX-CR2110 (**Figure 1C**), supporting that the PDX and PDXO were developed from the same origin: CR2110. This observation provides histopathology evidence to support the similarity of PDXO to its parental PDX.

Transcriptome expression and whole exome sequencing demonstrates high correlation between PDXO-CR2110 and the parental PDX-CR2110

PDX-CR2110 tumors have been previously genomically-profiled using transcriptome sequencing (RNAseq, mRNA)[16] and whole exome (WES, DNA) sequenced. We now have similarly profiled the corresponding PDXO-CR2110. The genomic profile comparisons of the corresponding matched PDX and PDXO (**Figure 2**) demonstrate a high correlation of 94.92% in transcriptome (mRNA) expression (epigenetic) and a high concordance of 97.67% of DNA mutations (WES) (genetic), suggesting an overall genomic similarity between this pair of models.

Similarities observed for the pharmacological properties between in vitro PDXO-CR2110 and in vivo PDX-CR2110

Drug sensitivity assays were performed on PDXO-CR2110 in 384-well plates, with results shown in **Figure 3A**. PDXO-CR2110 was sensitive to irinotecan and resistant to cisplatin, consistent with PDX treatment results (**Figure 3B**) where TGI (tumor growth inhibition) at dose levels of 100 mg/kg, i.p., Q3Dx3 for irinotecan and 5 mg/kg, i.p., Q4Dx4 for cisplatin is 84.63% and 6.61% respectively. This observed pharmacology consistency supports the potentially biological equivalence of both models, and can be used for complementary pharmacology studies in vitro and in vivo.

FIGURE AND TABLE LEGENDS:

Figure 1. Morphology of PDXO-CR2110. (**A**) Morphology of under light microscopy (cystic type). (**B,C**) Histopathology of PDXO-CR2110 and PDX-CR2110, respectively.

Figure 2. Genomic profiles of PDXO-CR2110 vs. PDX-CR2110, WES and RNAseq. Upper panel: global mRNA expression correlation between PDX-CR2110 and PDXO-CR2110 per RNAseq. Lower

panel: table of both mRNA expression correlations per RNAseq and DNA mutation concordance per WES: PDX- vs PDXO-CR2110.

Figure 3. Pharmacological properties of both PDXO-CR2110 in vitro and SC PDX-CR2110 in vivo. (**A**) PDXO-CR2110 in vitro dose-response to the test compounds. (**B**) Tumor growth inhibition induced by the same test compounds on CR2110 in vivo.

DISCUSSION:

The preliminary data for PDX-/PDXO-CR2110 in this report supports the biological equivalence between PDX and its derivative, PDXO, with regards to genomics, histopathology and pharmacology, since both models represent the disease forms derived from the original CSC of patient. Both models are patient-derived disease models, potentially predictive of the clinical response of patients[10-12,21]. The matched pair of in vitro and in vivo models can complement each other for in vitro screening and validation in vivo, improving the success rate of drug discovery and potentially reducing attrition rates in clinical development. It is worth noting that PDXO can now enable HTS to take advantage of available large patient-derived organoid libraries, where PDXs fail due to high in vivo costs and longer timelines. Needless to say, a matched PDX-PDXO library would likely become the platform of choice to support drug discovery and translational research in the near future.

Converting the existing library of annotated PDX models could be a fast and productive approach to building a practical organoid library by employing an industrial process. This report converted one PDX to PDXO to explore the feasibility of such a process, and the method used could be a foundation to support large scale process to build an extensive PDXO library. Practically, the methods to create PDXO are generally similar to the widely described method for the generation of PDO[18], with the exception of the source of the patient tissue being mice.

There are critical steps to ensure that PDXOs are successfully created: 1) the fresh PDX tumors are fragmented to small pieces; 2) the culture conditions described for organoid culture as described by Clevers and colleagues are faithfully implemented[18,22], but may be adjusted for different organoids; 3) different organoids have different growth rates, impacting the duration for organoid culture and health, as well as drug treatment duration; 4) an effective assay to determine mouse content vs. human content is absolutely critical to ensure the cultures are largely human organoid, since some cultures may inevitably have persistent mouse tissue/cell contamination (in the case reported here, there is minimal mouse tissue contamination, data not shown). Mouse contamination could be one of the important limitations in creating PDXO biobanks if effective detection and removal methods are not used.

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DISCLOSURES:

All authors are the current full-time employees of Crown Bioscience, Inc.

REFERENCES:

1 Tentler, J. J. et al. Patient-derived tumour xenografts as models for oncology drug development. *Nature Reviews Clinical Oncology.* **9** (6), 338-350 (2012).

2 Gao, H. et al. High-throughput screening using patient-derived tumor xenografts to predict clinical trial drug response. *Nature Medicine.* **21** (11), 1318-1325 (2015).

3 Yang, M. et al. Overcoming erlotinib resistance with tailored treatment regimen in patient-derived xenografts from naive Asian NSCLC patients. *International Journal of Cancer.* **132** (2), E74-84 (2013).

4 Li, Q. X., Feuer, G., Ouyang, X., An, X. Experimental animal modeling for immuno-oncology. *Pharmacology & Therapeutics.* **173**, 34-46 (2017).

5 Sato, T. et al. Single Lgr5 stem cells build crypt-villus structures in vitro without a mesenchymal niche. *Nature.* **459** (7244), 262-265 (2009).

6 Drost, J., Clevers, H. Organoids in Cancer Researchearch. *Nature Reviews Cancer.* **18** (7), 407-418 (2018).

7 Muthuswamy, S. K. Organoid Models of Cancer Explode with Possibilities. *Cell Stem Cell.* **22** (3), 290-291 (2018).

8 Sachs, N. et al. A Living Biobank of Breast Cancer Organoids Captures Disease Heterogeneity. *Cell.* **172** (1-2), 373-386 e310 (2018).

9 Weeber, F. et al. Preserved genetic diversity in organoids cultured from biopsies of human colorectal cancer metastases. *Proceedings of the National Academy of Sciences of the United States of America.* **112** (43), 13308-13311 (2015).

10 Vlachogiannis, G. et al. Patient-derived organoids model treatment response of metastatic gastrointestinal cancers. *Science.* **359** (6378), 920-926 (2018).

11 Yao, Y. et al. Patient-Derived Organoids Predict Chemoradiation Responses of Locally Advanced Rectal Cancer. *Cell Stem Cell.* **26** (1), 17-26 e16 (2020).

12 Ganesh, K. et al. A rectal cancer organoid platform to study individual responses to chemoradiation. *Nature Medicine.* **25** (10), 1607-1614 (2019).

13 van de Wetering, M. et al. Prospective derivation of a living organoid biobank of colorectal cancer patients. *Cell.* **161** (4), 933-945 (2015).

14 Yang, J. P. et al. A novel RNAi library based on partially randomized consensus sequences of nuclear receptors: identifying the receptors involved in amyloid beta degradation. *Genomics.* **88** (3), 282-292 (2006).

15 Zhang, L. et al. A subset of gastric cancers with EGFR amplification and overexpression respond to cetuximab therapy. *Scientific Reports.* **3**, 2992 (2013).

16 Chen, D. et al. A set of defined oncogenic mutation alleles seems to better predict the response to cetuximab in CRC patient-derived xenograft than KRAS 12/13 mutations. *Oncotarget.* **6** (38), 40815-40821 (2015).

17 Guo, S. et al. Molecular Pathology of Patient Tumors, Patient-Derived Xenografts, and Cancer Cell Lines. *Cancer Research.* **76** (16), 4619-4626 (2016).

18 Sato, T. et al. Long-term expansion of epithelial organoids from human colon, adenoma, adenocarcinoma, and Barrett's epithelium. *Gastroenterology.* **141** (5), 1762-1772 (2011).

19 Tiriac, H., French, R., Lowy, A. M. Isolation and Characterization of Patient-derived Pancreatic Ductal Adenocarcinoma Organoid Models. *Journal of Visualized Experiments.* (155) (2020).
20 Kopper, O. et al. An organoid platform for ovarian cancer captures intra- and interpatient heterogeneity. *Nature Medicine.* **25** (5), 838-849 (2019).
21 Corcoran, R. B. et al. Combined BRAF and MEK Inhibition With Dabrafenib and Trametinib in BRAF V600-Mutant Colorectal Cancer. *Journal of Clinical Oncology.* (2015).
22 Huch, M. et al. Long-term culture of genome-stable bipotent stem cells from adult human liver. *Cell.* **160** (1-2), 299-312 (2015).

Figure

Fig.1

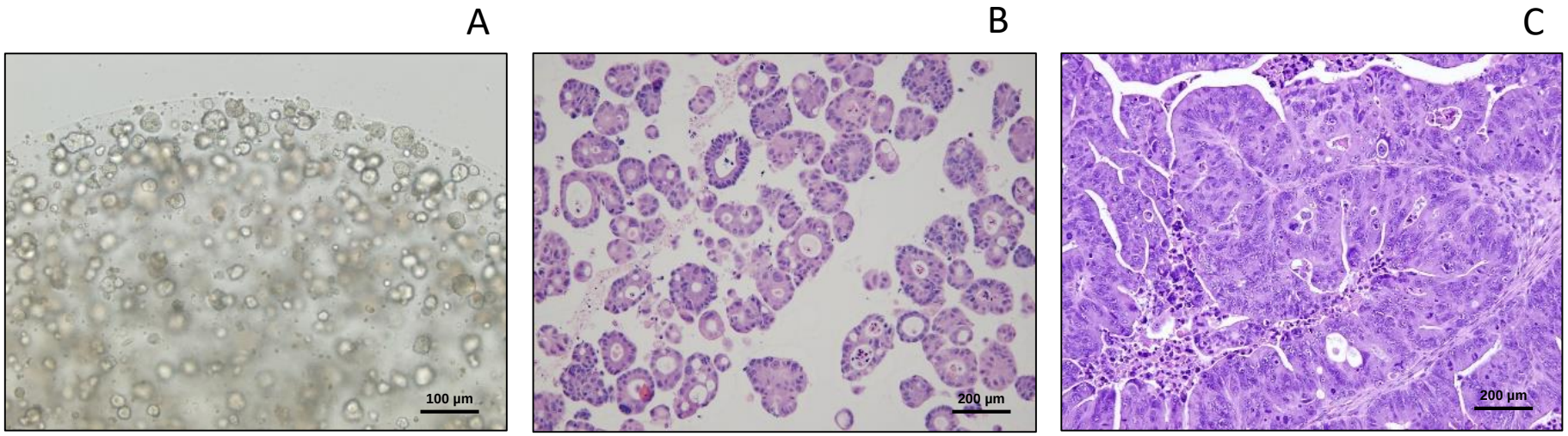
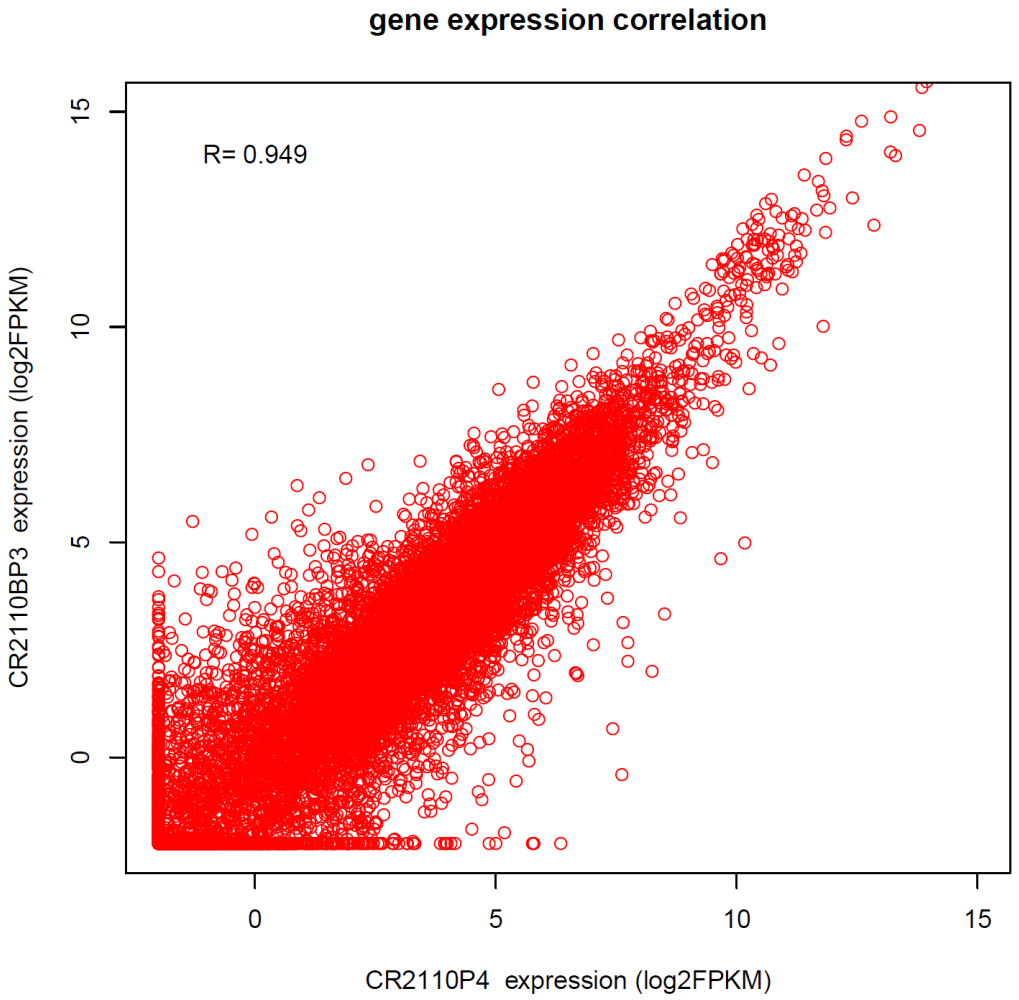


Figure 2

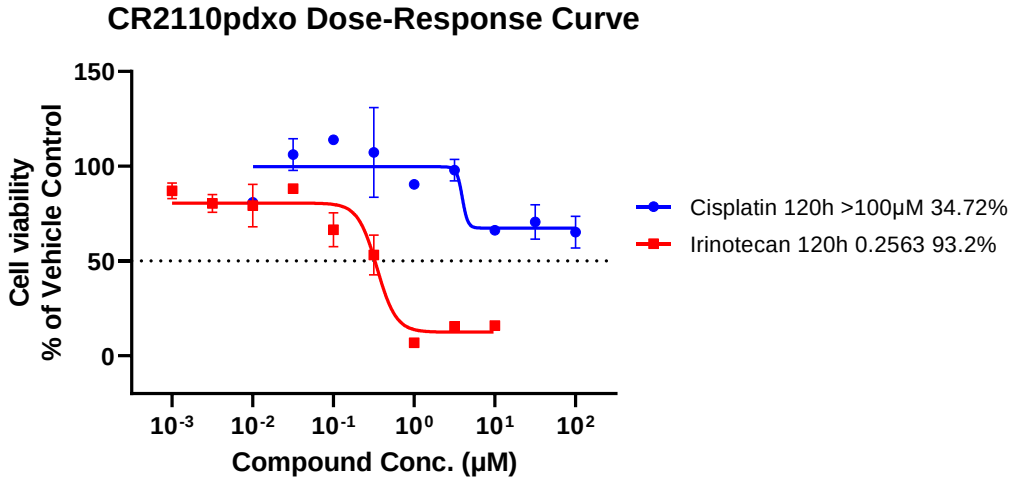


Model	mRNA expression correlation	DNA mutation concordance
CR2110	R=0.9492	97.67%

Figure 3.

A

Cell_Line_Name	Test_Article	IC50(μM)	Comment
CR2110pdxo	Cisplatin	>100	Resistant
CR2110pdxo	Irinotecan	0.2563	Sensitive



B

Model_Name	Test_Article	TGI(%)	Comment
CR2110pdx	Cisplatin	6.61%	Resistant
CR2110pdx	Irinotecan	84.63%	Sensitive

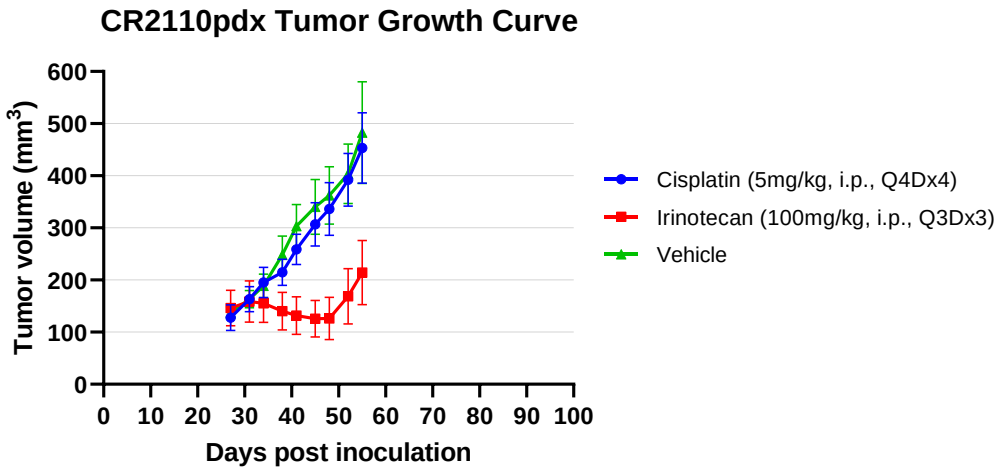


Table of Materials

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Name of Material/Equipment	Company	Catalog Number	Comments/Description
Advanced DMEM/F12	Life Technologies	12634028	Base medium
DMEM	Hyclone	SH30243.01	Washing medium
Collagenese type II	Invitrogen	17101015	Digest tumor
Matrigel	Corning	356231	Organoid culture matrix (Bas
N-Ac	Sigma	A9165	Organoid culture medium
A83-01	Tocris	2939	Organoid culture medium
B27	Life Technologies	17504044	Organoid culture medium
EGF	Peprotech	AF-100-15	Organoid culture medium
Noggin	Peprotech	120-10C	Organoid culture medium
Nicotinamide	Sigma	N0636	Organoid culture medium
SB202190	Sigma	S7076	Organoid culture medium
Gastrin	Sigma	G9145	Organoid culture medium
Rspondin	Peprotech	120-38-1000	Organoid culture medium
L-glutamine	Life Technologies	35050038	Organoid culture medium
Hepes	Life Technologies	15630056	Organoid culture medium
penicillin-streptomycin	Life Technologies	15140122	Organoid culture medium
Y-27632	Abmole	M1817	Organoid culture medium
Dispase	Life Technologies	17105041	Screening assay
CellTiter-Glo 3D	Promega	G9683	Screening assay (luminescen
Multidrop dispenser	Thermo Fisher	Multidrop combi	Plating organoids/CellTiter-G
Digital dispener	Tecan	D300e	Compound addition
Envision Plate reader	Perkin Elmer	2104	Luminescence reading
Balb/c nude mice	Beijing HFK Bio-Technology Co		
RNAeasy Mini kit	Qiagen	74104	tRNA purification kit
DNAeasy Blood & Tissue Kit	Qiagen	69506	DNA purification kit
Histogel	Thermo Fisher	HG-4000-012	Organoid embedding

ement Membrane Extract, growth factor reduced)

t ATP indicator)
lo 3D addition

May 1, 2020

Vineeta Bajaj, Ph.D.
Review Editor
JoVE
617.674.1888

Dear Dr. Bajaj,

Thank you very much for reviewing our manuscript entitled "Create matched *in vivo/in vitro* patient derived model pairs of PDX and PDX-derived organoids for cancer pharmacology research" by Xu *et al*, and the suggested revisions. I sincerely appreciate the time and the thorough review by the three reviewers. We are in complete agreement with reviewers' critiques. We have now completed the revision of the manuscript per editor/reviewers' suggestions and are submitting the final version for publication. We have also addressed the reviewers' comments item-by-item, as shown in Red bold font as below. In addition, we have carefully edited the text and hope that it is now acceptable for publication.

The paragraphs of dimmed fond are suggested for videotaping.

Please do not hesitate to contact me if you have any question regarding this submission. Thank you again, and I look forward to hearing from you.

Sincerely,

Henry Q.X. Li, Ph.D.
Chief Scientific Officer, Crown Bioscience, Inc.
henryli@crownbio.com

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.

We have carefully proofread and edited the text accordingly.

2. Please format the manuscript as: paragraph Indentation: 0 for both left and right and special: none, Line spacings: single. Please include a single line space between each step, substep and note in the protocol section. Please use Calibri 12 points

We have now formatted it accordingly.

3. Please remove redundancy (words like methods, etc) from the title and make it concise to reflect the protocol. Please do not use abbreviations in the title.

We change accordingly now.

4. Please define all abbreviations during the first-time use.

Done accordingly.

5. Please provide an email address for each author.

Xiaoxi Xu (xuxiaoxi@crownbio.com); Limei Shang (shanglimei@crownbio.com); Phillip Wang (shuzong.wang@crownbio.com); Jun Zhou (jun1.zhou@crownbio.com); Xuesong Ouyang (Davy.Ouyang@crownbio.com); Meiling Zheng (zhengmeiling@crownbio.com); Binchen Mao (binchen.mao@crownbio.com); Likun Zhang (zhanglikun@crownbio.com); Bonnie Chen (bonnie.chen@crownbio.com); Jingjing Wang (wangjingjing1@crownbio.com); Sheng Guo (guosheng@crownbio.com); Yujun Huang (yujun.huang@crownbio.com) and Qi-Xiang Li (henryli@crownbio.com)

6. Please rephrase the Short Abstract/Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Presented here is a protocol …”

Rephrase accordingly.

7. Please ensure that the long Abstract is within 150-300 word limit and clearly states the goal of the protocol.

We revised it and ensure it is aligned.

8. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s).

Yes, we reformat it accordingly

9. JoVE cannot publish manuscripts containing commercial language. 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: Beijing HFK Bio-Technology Co, Matrigel, Corning, Multidrop dispenser (Multidrop Combi, Thermo Fisher, MA, D300e, Tecan, Switzerland, Tecan digital dispenser, Tecan D300e software tool (D300eCONTROL, HP Development Company, AR), CellTiter-Glo 3D (CTG assay), Promega, WI, Multidrop Combi, Thermo Fisher, MA, Envision Plate Reader (2104, Perkin Elmer, MA, PRISM Graphpad, Graphpad Software, CA., etc.

We have now removed all commercial languages accordingly.

10. JoVE policy states that the video narrative is objective and not biased towards a particular product featured in the video. The goal of this policy is to focus on the science rather than to present a technique as an advertisement. Please use generic terms and include some limitations of the technique presented.

Yes, we have and will use generic terms as much as possible.

11. Please reword lines 129-131, 136-137, 141-143, 151-153, 160-161, 172-174 as it matches with previously published literature.

We have reworded these lines accordingly.

12. Please move the ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

Yes, we have moved it.

13. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets, alphabets, or dashes.

Yes, we have revised accordingly.

14. Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).

Revised accordingly.

15. 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.”

We edited accordingly.

16. The Protocol should contain only action items that direct the reader to do something in complete sentences.

We have edited it to ensure the complete sentences were used.

17. Please ensure that individual steps of the protocol should only contain 2-3 actions sentences per step.

We have edited accordingly to ensure this.

18. Please ensure you answer the “how” question, i.e., how is the step performed?

Yes.

19. 2.2.2: How was this done for your experiment? What kind of tumor is being used?

should be the same of the procedure in 2.2 (new version)

20. 3: We cannot have paragraph of texts in the protocol section. Please make substeps and include how each step is performed.

We have revised accordingly.

21. Cisplatin and Irinotecan: Please include the volume/concentration used.

We have added accordingly.

22. Please remove the embedded Table from the manuscript. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file. Each table must be accompanied by a title and a description after the Representative Results of the manuscript text.

We have deleted the table accordingly with edits in text.

23. 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.

Ans:

24. Please describe the result in paragraph style with respect to your experiment, you performed an experiment, how did it help you to conclude what you wanted to and how is it in line with the title. Please describe the result with respect to your experiment, you performed an experiment, how did it help you to conclude what you wanted to and how is it in line with the title.

We have added contents according to support the conclusions in relation to the title and intended objective of the manuscript.

25. 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].”

No such figure included in the current manuscript.

26. Each Figure Legend should include a title and a short description of the data presented in the Figure and relevant symbols.

Reworded accordingly.

27. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:

    a) Critical steps within the protocol

    b) Any modifications and troubleshooting of the technique

    c) Any limitations of the technique

    d) The significance with respect to existing methods

    e) Any future applications of the technique

We have revise the discussion sections to reflect aspects of suggested.

28. Please number the citations in the reference section in order of appearance in the manuscript. 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.

We have used JOVE reference format accordingly now.

29. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .pdf, .svg, .eps, .psd, or .ai file. Please combine all panels of one figure into a single image file.

We have now ensured to update figure in these formats.

30. Please upload all tables separately as .xlsx file to your editorial manager account.

Yes, we have now ensured to update the right format.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

The manuscript by Li et al. is a technical summary about developing patient-derived organoids from PDX as a rapid and efficient source for therapeutic screenings. The protocol is well written and useful for the scientific community.

Major Concerns:

However multiple concerns are raised while reading the manuscripts since a lot of details are missing and figures are presented in a very approximate way.

In details:

-A key question is: at which PDX passage organoids are derived? This is a crucial point to avoid that organoids diverge from the corresponding tumor as it has been shown after multiple passages of tumor in PDXs.

The PDXO-CR2110 was derived from PDX-CR2110 P10. Due to our experience, the PDX tumor remain largely consistent.

-The digestion medium (Line 201) is not specified, it is an important step of the protocol and should be precisely written.

Thanks for pointing it out. Now added in the manuscript.

-Matrigel: How much Matrigel is used? Is it a mix with media? If yes at which ratio? Do they form droplets with it? It would be well specified since it is a key component of the 3D culture.

Thanks and yes, we revised the corresponding steps according to your questions, and added citation as well.

-When tumor is extracted, and organoids prepared (line 208) how you get read of mouse fibroblasts or normal keratinocytes growing within the culture? In other words, how are you sure to grow only tumor? Did you stain them to make sure that they are only tumor organoids?

This is an important and valid question. In this specific case, mouse fibroblasts did not grow in this culture condition, which is confirmed by our assay measuring both human and mouse content and found that the mouse content was less than 0.4 %.

-Histopathology: any colorectal marker tested in the organoids, corresponding PDX and matched original tumor to ensure that same markers are preserved?

We confirmed all our CRC models using specific biomarker, which is our standard QC practice of all our models (data not shown), in addition to molecular pathology method (Guo et el, 2015, in the reference list).

-Same question as above for RNA-seq, can you show that colorectal cancer markers are preserved within the models and that correspond to the original patient tumor? This is the only way to know that these are clinically relevant models to use.

See last comments.

-Genomic profiles of PDXO-CR2110 vs. PDX-CR2110, WES and RNAseq: there is just a table presented as figure, but at least global correlation graphs should be shown.

Global correlation graphs is provided as suggested for mRNA expression, as new Figure 2 and the old table 1 is part of Figure 2 in the revised version.

-Can you use different colors (that do not look similar as light blue and blue) for the in vivo graph? Also, it would be visually effective if the two graphs (in vitro and in vivo) show the same color for the line representing each drug response. Do you know if the corresponding patient was treated with Cisplatin and resulted to be insensitive at the time of collection? And in the same way patient was sensitive to irinotecan at the time of collection? Knowing more about patient tumor will help strengthen the correlation observed and therefore the correctness of the protocol presented.

Now, we have redrawn the graphs. Agree with the reviewer on that additional correlated clinical data could be very meaningful, however, we do not have such information, and that this paper is about the method converting PDX to PDXO.

Minor Concerns:

The introduction should have more updated citations since a lot of studies have been out in the last few years:

Line 56 the appropriate citation to consider is Vlachogiannis et al., 2018

Line 58 not sure the reference is correct for the concept expressed.

Line 70 other more recent studies could be added for comparison between organoids and PDX as Pauli et al., Puca et al., Gao et al., 2014 and many more

Appreciate reviewer's suggestions, and we have reexamined the citations to ensure their accuracy

Reviewer #2:

Manuscript Summary:

Manuscript was well written to help scientists facilitate their research with these methods. The methods were excellent to demonstrate pharmacological experiments using your models.

Major Concerns:

Nothing to describe major concerns.

Minor Concerns:

Author's affiliations might be private company. So authors had better to declare any COIs in the manuscript.

Yes, agree. We have disclosure at the end of text that all authors are current the employees of Crown Bioscience.

Reviewer #3:

Manuscript Summary:

Libraries of well-characterized patient derived xenografts have been long viewed as the golden standard of translational in vivo system for testing of chemotherapeutics and/or targeted therapies. Due the limitations such as large scale screening as well as potential application in IO research and drug discovery, patient-derived organoid systems are currently seen as the next potential golden standard for a translation drug discovery platform. The creation of a PDX-derived organoid library based on CrownBio's large xenograft library reflects a very high potential for an enormous library of patient-matched organoid and xenograft model systems. The authors describe in their manuscript the technology behind creation of a PDX-derived organoid (PDXO) model based on one colorectal cancer sample termed (PDXO-CR2110).

Major Concerns:

The authors mentioned in the "long abstract" section as well as in the introduction that patient-derived tumor xenografts are driven by cancer stem cells (line 29, line 57, and line 76). This is a very generalized statement and should certainly be clarified and differentiated for various tumor types, as research is still ongoing whether or not cancer stem cells drive growth of all tumor indications, specifically in the xenograft setting. Correspondingly, while certain tumor types have been shown to contain cancer stem cells in the organoid setting, the lack of well-defined cancer stem cell markers for all tumor types have so far prevented a general statement that organoids are cancer stem cell driven models (line 33).

We have edited the text by toning down the statements.

The statement that high-throughput screens are possible with patient-derived organoids should be exemplified by including a reference (line 72).

Now, cited.

The authors should clarify the exact composition of the organoid suspension which is seeded into the 384-well plates (line 249).

Now we have clarified per suggestion

The IC50 values are calculated based on a dose-response curve (line 267). The authors should explain what kind of positive (untreated?) controls and negative (organoids treated with a toxic

compound?) controls were used to create the signal window (0% and 100% of cell growth inhibition).

Now we have added according to the suggestions.

Apart from the histological characterization by H&E staining (Figure 1B, C, line 281), can the authors provide any data on tumor or epithelial marker assessment by IHC?

In this case, we have not use epithelial marker, although it could be performed in the future.

The RNAseq analysis could become a very critical technology to characterize the nature of the PDX-derived organoid sample (line 287). Apart from the mRNA expression correlation and mutation concordance shown in Table 1, the authors should include an analysis of the mouse RNA content in their PDXO system. This is of specific importance to exclude any contamination of mouse material in the ex vivo established organoid model system. I would suggest to include this into their final discussion statements as a criteria for successful PDXO establishment (line 350).

Totally agreed with the reviewer, we actually did perform mouse content assay, and in this case, we have ensured that there is minimal mouse contamination in this particular organoid model, although data not shown. We added additional comments into the discussion section as well to further clarify this.

The authors should clarify the rationale for choosing cisplatin and irinotecan as two chemotheray drugs. Could the authors demonstrate any data on a target therapy?

Both cisplatin and irinotecan are commonly used chemotherapy drugs for colorectal cancer patients and we have paired data on the model pairs. For this particular model there is no actionable driver mutation for target therapy.

With the regards to irinotecan, the authors should be more careful with the interpretation of their result. It is very important to clarify the exact source of Irinotecan. In the in vivo setting, irinotecan undergoes metabolization. For in vitro experiments, the active metabolite SN-38 should be utilized instead of irinotecan. Could the authors show any data on SN-38 on their PDXO model?

The reviewer is absolutely right. We actually used SN-38, instead of irinotecan. Now we clarified it in the text.

Minor Concerns:

- Line 40: "as solid a dome" wording should be selected differently

Re-worded

- Figure 2: the authors should include a term like "cell viability" on the y axis of the dose response curve

We edited

- Figure 2: the font size of the graph of the tumor growth curve should be increased to the font size of the left plot

Re-graphed

- Figure 2: The title of both plots (currently CR2110B on the left and CR2110 on the right) should have the actual model name (CR2110PDXO and CR2110PDX)

Re-graphed accordingly.