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TITLE:

Patient-Derived Heterogeneous Xenograft Model of Pancreatic Cancer Using Zebrafish Larvae as Hosts for Comparative Drug Assessment

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KEYWORDS:

cancer research, tumor xenograft, zebrafish, pancreatic carcinoma, in vivo drug assessment, BCL2L1

SUMMARY:

This protocol describes optimization procedures in a virus-based dual fluorescence-labeled tumor xenograft model using larval zebrafish as hosts. This heterogeneous xenograft model mimics the tissue composition of pancreatic cancer microenvironment in vivo and serves as a more precise tool for assessing drug responses in personalized zPDX (zebrafish patient-derived xenograft) models.

ABSTRACT:

Patient-derived tumor xenograft (PDX) and cell-derived tumor xenograft (CDX) are important techniques for preclinical assessment, medication guidance and basic cancer researches. Generations of PDX models in traditional host mice are time-consuming and only working for a small proportion of samples. Recently, zebrafish PDX (zPDX) has emerged as a unique host system, with the characteristics of small-scale and high efficiency. Here, we describe an optimized methodology for generating a dual fluorescence-labeled tumor xenograft model for comparative chemotherapy assessment in zPDX models. Tumor cells and fibroblasts were enriched from freshly-harvested or frozen pancreatic cancer tissue at different culture conditions. Both cell groups were labeled by lentivirus expressing green or red fluorescent proteins, as well as an anti-apoptosis gene *BCL2L1*. The transfected cells were pre-mixed and co-injected into the 2 dpf larval zebrafish that were then bred in modified E3 medium at 32 °C. The xenograft models were treated by chemotherapy drugs and/or BCL2L1 inhibitor, and the viabilities of both tumor cells and fibroblasts were investigated simultaneously. In summary, this protocol allows researchers to quickly generate a large amount of zPDX models with a heterogeneous tumor microenvironment and provides a longer observation window and a more precise quantitation in assessing the efficiency of drug candidates.

INTRODUCTION:

Precision oncology aims to find the most beneficial therapeutic strategies for individual patient[1]. Currently, numerous preclinical models such as in vitro primary culture, in vitro organoid culture[2], and patient-derived xenografts (PDX) in mice before or after organoid culture are proposed for diagnosis and to screen/assess the potential therapeutic choices[3]. PDX model generated by the injection of human primary cancer cells into immune-compromised mice, is one of the most promising tools for personalized drug screening in clinical oncology[3,4]. Unlike the cultured cell line in vitro, PDX models usually preserve the integrity and heterogeneity of the in vivo tumor environment, better mimicking the diversity and idiosyncratic characteristics of different tumor patients, and therefore, may predict the potential medical outcome of patients[4]. However, the generation of PDX models in mice requires high quality patient samples and months of time to gather sufficient cells and models for multi group experiments, and the cellular/genetic compositions of the xenograft may drift from those of the original patient's biopsy. The success rate for establishing mice PDX model is also low, making it difficult to be broadly implemented in clinical practice. For the patients carrying rapidly progressed cancers like pancreatic cancer, they may not be able to obtain valuable information from the PDX experiments in time.

In the past few years, zebrafish has been reported to be potential hosts for not only CDX (cell-derived tumor xenograft) models, but also PDX models[5-10]. As a vertebrate model animal, zebrafish harbors sufficient similarities with mammals in both genetics and physiology, with two significant advantages: transparency and small in size[11]. Zebrafish is also highly fecundity, and hundreds of inbred larvae can be obtained within a few days from a single pair of adults[12]. Several studies have employed zebrafish to generate both transgenic and xenograft models of cancer diseases[13,14]. Compared to mice xenografts, zebrafish xenografts allow tracking at single cell resolution. A certain amount of human tissues is capable of generating hundreds of zebrafish PDX models (zPDXs), while may only be sufficient to generate a couple of mice PDX

models[15,16]. Besides, the zebrafish larvae at 2-5 dpf already develop complete circulatory systems and metabolic organs such as liver and kidney, but not the immune system[17], while the remaining yolk sac is a natural 3D medium, ideal for drug screening, drug resistance tests and tumor migration observations[6,18-21].

With an ultimate attempt to use zPDX as a screening/testing platform for clinical use, here, we describe an optimized proposal for zPDX model of pancreatic cancer, which allows the in vivo candidate drug assessment within a short time using fewer cells at lower costs. Compared to the previous references about zPDX[6,9,10], we introduced several optimizations to make the system more feasible and reliable for clinical personalized diagnosis: 1) pre-sorting different cell groups in the primary tumor tissues and stabilizing primary cells for one week before further experiments; 2) labeling the human cells and enhancing the cell viability in xenograft via lentivirus-based genetic modification; 3) optimizing the zebrafish culture condition in both nutriment supplements (glucose and glutamine) and temperature; 4) quantifying the drug responses of different cell types in a comparative manner. We also made changes to the injection solution by adding several supplementary materials. Altogether, those improvements provide the possibility to quickly generate a more patient-like xenograft in zebrafish hosts that can be used as a reliable tool to assess the response of candidate drugs.

PROTOCOL:

All animal procedures were approved and followed the guidelines of the Animal Ethics Committee at Fudan University and all pancreatic cancer specimens were obtained from Fudan University Shanghai Cancer Center. Ethical approval was obtained from the FUSCC Ethics Committee, and written informed consent was obtained from each patient.

1. Preparing the equipment for the microinjection

1.1. Preparing the injection plate.

1.1.1. Prepare a 50 mL solution of 1% agarose dissolved in E3 solution (0.6 g/L aquarium salt in double distilled water + 0.01 mg/L methylene blue). Boil the solution until the agarose dissolves.

1.1.2. Pour 50 mL of the agarose solution into a 10 cm Petri dish and then place the zebrafish embryo fixation mold on the surface. Remove the mold when the agarose solution becomes solidified.

1.1.3. Add 20 mL of E3 solution to the injection plate and maintain it at 4 °C for long-term storage.

1.2. Preparing the injection needles.

1.2.1. Pull a 10 cm glass capillary with an inner dimension of 0.9 mm into two needles on a needle puller.

1.2.2. Use forceps to cut the end of the needle to create an opening under the microscope.

2. Preparing embryos for transplantation

2.1. Place 1 to 2 pairs of adult zebrafish in a mating tank at 7-9 pm and collect the fertilized eggs at around 8 am of the next morning.

2.2. Transfer the fertilized eggs from the mating tank to a Petri dish containing 40 mL of fresh E3 solution and incubate at 28.5 °C.

2.3. After 8 h of incubation in E3 solution, add 0.03% 1-phenyl-2-thiourea (PTU) into E3 solution to inhibit pigmentation. Incubate the embryos in E3 solution with 0.03% PTU at 28.5 °C until 48 hpf. This step can be omitted if using in-bred Casper mutant zebrafish.

3. Isolation and culture of primary human cells from fresh surgical pancreatic cancer specimen or frozen tissue.

3.1. Obtain specimens of human pancreatic cancer tissue of the size around 1 cm^3 during an abdominal surgery, and immediately transfer the tissue into growth media (DMEM with 10% fetal bovine serum (FBS), 10 µM Y-27632, 100 µg/mL primocin, 10 µg/mL putrescine dihydrochloride, 10 mM nicotinamide and 1% penicillin streptomycin).

3.2. Transfer the pancreatic cancer sample into a Petri dish and remove the surrounding necrotic tissue, adipose tissue and connective tissue.

3.3. Rinse the cancer tissue for 5-6 times with phosphate buffer (PBS) and cut the tissue into 1 mm^3 pieces using scalpels.

3.4. Transfer the shredded tissues into 5 mL of HBSS in a 50 mL tube and add collagenase type IV, hyaluronidase and DNase I at final concentrations of 200 units/mL, 100 mg/L and 20 mg/L, respectively. Pipette the mixture up and down to mix well.

3.5. Incubate the mixture at 37 °C in a 5% carbon dioxide incubator for 15-20 min. Pipette the mixture up and down a few times every 5 min.

3.6. Add 7 mL of DMEM to the tube and centrifuge at 110 x *g* for 5 min at 4 °C when digestion is complete.

3.7. Decant the supernatant and re-suspend the tumor mixture in DMEM.

3.8. Plate the mixture into a 6 cm Petri dish in 3 mL of full growth media (DMEM with 10% FBS, 20 µg/mL insulin, 100 ng/mL bFGF, 10 ng/mL EGF, 10 µM Y-27632, 100 µg/mL primocin, 10 µg/mL putrescine dihydrochloride, 10 mM nicotinamide, 1% penicillin streptomycin). Separate

the cells into two groups.

3.9. In group I, add 100x inhibitor of pancreatic cancer fibroblasts into the medium after 48 h to remove the overgrown fibroblasts, leaving the cancer cells as the major cell types;. In group II, the fibroblasts will outgrow the cancer cells within a week.

3.10. Culture both cell groups for 1-2 week depending on the cell densities/purities and change the media every three days. The expected cell types in both group I & II can occupy over 98% in proportion in a typic successful experiment.

4. Labeling the cells with lentivirus expressing anti-apoptosis gene *BCL2L1 (BCL-XL)* and different fluorescent proteins separately

4.1. Lentivirus production

4.1.1. Plate 3 × 10^6 HEK 293T cells with complete DMEM medium (DMEM supplied with 10% FBS) in 10 cm dishes and culture overnight at 37 °C in a 5% carbon dioxide incubator. Replace the medium with 6 mL of serum-free media before transfection.

4.1.2. Prepare solution A: 8 μg of *BCL2L1*-containing lentiviral vectors (pCDH-EF1α-mKate2-E2A-BCL2L1-WPRE or pCDH-EF1α-eGFP-E2A-BCL2L1-WPRE), 2.4 μg of pVSVG, 4 μg pMDL(Gag/Pol), 1.6 μg of pREV and serum-free DMEM in a total volume of 500 μL. Gently pipet the mixture several times, and place it at room temperature for 5 min.

4.1.3. Prepare solution B: 40 μL of PEI (polyethyleneimine) in 460 μL serum-free DMEM. Place it at room temperature for 5 min.

4.1.4. Slowly add solution B into solution A and leave the tube at room temperature for 30 min.

4.1.5. Add the final mixture into the HEK 293T cell culture dish prepared in step 4.1.1 and incubate at 37 °C in a 5% carbon dioxide incubator. After 12 h, add an additional 5 mL of the complete DMEM medium. After 48 h, harvest the medium containing the lentivirus.

4.1.6. Filter the supernatant using a 0.45 μm sterile filter, add the supernatant into a concentration column, centrifuge at 6000 x *g* for 25-30 min at 4 °C. The lentivirus aliquots of 100 μL per tube are made and stored at -80 °C.

4.2. Infection of the primary cells

4.2.1. Seed the cells (group I & II) to be infected in a 12-well plate with 30-40% density and culture the cells overnight at 37 °C in a 5% carbon dioxide incubator.

4.2.2. Replace the medium with 500 μL of serum-free medium containing 8 μg/mL of

polybrene for 4 h. Then, add an additional 100 µL of the lentivirus into the medium (eGFP-E2A-BCL2L1 for Group I or mKate2-E2A-BCL2L1 for Group II). After 12 h, replace the medium with 10 mL of complete medium.

4.2.3. Check the fluorescence markers after 48 h.

4.2.4. Harvest the infected cells and mix them at 1:1 ratio with a final concentration of 10^6/mL.

4.2.5. Centrifuge the cells at 110 x *g* for 5 min and re-suspend the cell mixture in 50 µL of injection solution (1640 medium with 10% FBS, 0.05% hyaluronic acid sodium salt, 0.05% methylcellulose).

5. Injecting mixed cell suspension into the zebrafish

5.1. Add 10x tricaine solution into E3 water to anesthetize zebrafish larvae and transfer the larvae (from step 2.3) to the injection plate filled by modified E3 (E3 with 1 g/L glucose and 5 mmol/L L-glutamine).

5.2. Fill 25 µL of mixed cell suspension into micro capillaries needle and insert the needle into the micro-injection manipulator.

5.3. Set injection pressure and time. Inject 50-80 cells (~8 nL) into the yolk sac of 48 hpf zebrafish.

6. Culture of the xenografted Zebrafish (zPDX model)

6.1. Transfer the post-xenografted zebrafish larvae into 40 mL of mix solution (E3 solution with 1 g/L glucose and 5 mmol/L L-glutamine) at 32 °C.

7. Drug administration on the xenografted zebrafish and the assessment of tumor cells/fibroblasts viabilities

7.1. Determining the optimal concentration of gemcitabine/navitoclax.

7.1.1. Place 10 wildtype zebrafish embryos at 48 hpf into each well of a 12-well plate.

7.1.2. Add different concentrations of gemcitabine or navitoclax in each well and incubate at 32 °C for two days.

7.1.3. After 2 days, calculate the maximal tolerance dosage (MTD) of gemcitabine and navitoclax at which the zebrafish larvae do not shown significant malformation and abnormal behavior, and the working concentrations are set below the MTD.

7.2. Treatment of zPDX models with gemcitabine/navitoclax.

7.2.1. Place 10 xenografted larvae into each well of a 12 well plate.

7.2.2. Divide the larvae into four groups, treat the control group in E3 containing 0.1% DMSO, and treat the other groups with 5 µg/mL gemcitabine and/or 50 µM navitoclax, and incubate at 32 °C for two days.

7.3. Assessment of the cell viabilities and cellular composition in zPDX models.

7.3.1. Anesthetize the xenografted larvae post-treatment and place them in 3% methylcellulose.

7.3.2 Image the larvae from the lateral view using a fluorescence microscope or confocal microscope.

7.3.3 Quantify the intensity of red and green fluorescence signals using ImageJ and GraphPad software.

REPRESENTATIVE RESULTS:

A schematized outline of the procedure is represented in **Figure 1**. In short, the primary cancer tissue cells were seeded into the complete medium after digestion with or without the addition of pancreatic cancer fibroblast inhibitors. Cancer cells and fibroblasts were enriched as two distinct populations that fibroblasts dominated without inhibitors, and cancer cell growth prevailed after the addition of inhibitors (**Figure 2**). Two lentiviral packaging vectors were constructed, which expressed green or red fluorescent proteins and BCL2L1 as shown in **Figure 3**. The virus-based fluorescent labeling also represented the survival status of the cells. The mixed cancer cells and fibroblasts were injected into the zebrafish yolk sac at 48 hpf and were treated by gemcitabine and/or navitoclax for two days. The cell viabilities in different populations and cellular composition of the xenograft were changed as the responses to the drug treatment (**Figure4**).

FIGURE AND TABLE LEGENDS:

Figure 1: Schematic diagram of the generation and drug assessment of the zebrafish xenograft model derived from pancreatic cancer. Surgically removed tissues from patients with pancreatic cancer are sheared and digested, followed by culture at two different conditions, one of which is added by a fibroblast inhibitor, and the other group is not. After 1-2 weeks, the primary cells were genetically modified to express an anti-apoptotic protein *BCL2L1* and different fluorescent proteins. After that, the two populations were mixed and co-injected into 48 hpf zebrafish larvae to assess the effects of chemotherapeutic drugs.

Figure 2: Culture and enrichment of pancreatic cancer cells and fibroblasts. Images of cancer cells and fibroblasts enriched from three different pancreatic cancer patients after 10-days culture. Scale bar, 100 µm.

Figure 3: Structure of lentiviral vector expressing BCL2L1 and different fluorescent proteins. Schematic diagram of two lentiviral vectors constantly expressing mKate2-E2A-BCL2L1 or eGFP-E2A-BCL2L1, respectively.

Figure 4: Effects on xenograft by gemcitabine. (A) Lateral view of the xenograft in the yolk sac of zebrafish larvae at 3 h post transplantation (hpt). **(B)** Representative images of the xenografts in zebrafish larvae at 60 hpt, treated by DMSO or gemcitabine. **(C)** Representative 3D rendering of the xenografts at 60 hpt. **(D)** Statistics of the fluorescence intensity of the xenograft before and after gemcitabine and/or BCL2L1 inhibitor treatment. N = 10 per group for each assay. Scale bar, 100 μm; NS, not significant; *$P < 0.05$; ***$P < 0.0001$; mean ± SD, with statistical differences determined by one-way ANOVA.

DISCUSSION:

Both PDX and CDX models are vital platforms in the field of tumor biology[22], and the critical step of a successful inter-species transplantation is to improve the survival of the xenograft. Recently, some studies have shown that transient expression of *BCL2L1 (BCL-XL)* or *BCL2* may significantly improve the viability of human embryonic stem cells in mice hosts without affecting the cell identities and fates[23-25]. In our manuscript, we labeled the cells with lentivirus constantly expressing anti-apoptosis gene *BCL2L1 (BCL-XL)*, as well as fluorescent proteins. The introduction of *BCL2L1* greatly enhanced the survival time of xenograft human cells in zebrafish to prolong the observation window for the studies, while the fluorescent signals not only label the cells, but also report the living condition of the cells, as we found that the fluorescence fades quickly with the cell death. Meanwhile, we are currently modifying the zebrafish as a better host for human xenografts. On the one hand, the *rag2* and *prkdc* are being knocked out via CRISPR/Cas9 technology to prepare a combined immune-deficient zebrafish, which was similar to combined immune-deficient mice, to make older zebrafish larvae also compatible with human cells and tissues[26]. On the other hand, the zebrafish was also modified to express human proteins such as IGF1 and INS to better support the survival and proliferation of implanted human cells by using the Tol2 transposon-mediated transgenic technology. Moreover, zPDX also lacks a human-like functional immune system, and the interactions between human stromal cells and the immune system, and in future, we may try co-injecting human immune cells to rebuild a short-term humanized immune environment in zebrafish.

In the mice PDX models, the xenograft conditions were traditionally monitored by direct observation on nodes of visible size, which require a long time to develop. In the transparent zPDX models as comparison, the xenograft can be investigated under microscopy in real time. However, it was difficult to assess the cell number alternations in a single-color population without a proper control, and the idea to introduce two cell population of different fluorescence significantly improves the quantitation of cell viability. By mixing and injecting different cells at fixed ratios, we not only mimic the tumor microenvironment, but also are able to accurately quantify the drug response by comparing the two cell groups. Although the original ratio of cancer cells to fibroblasts in primary tissues is highly different, here we started with 1:1 ratio, and the effects of drug treatment on the same cell composition of a different ratio will be investigated in the future. Besides, the effects of 32 °C instead of 37 °C incubations

on the behaviors of human cells also require detailed comparative studies. Lastly, the strategy of the patient-derived heterogeneous xenograft model for pancreatic cancer for comparative drug assessment can also be applied to other types of solid cancers. This approach is particularly useful for PDX using zebrafish larvae as hosts, which are crystal-clear and feasible for analysis at single-cell resolution.

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DISCLOSURES:

No potential conflicts of interest were disclosed.

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Figure 1

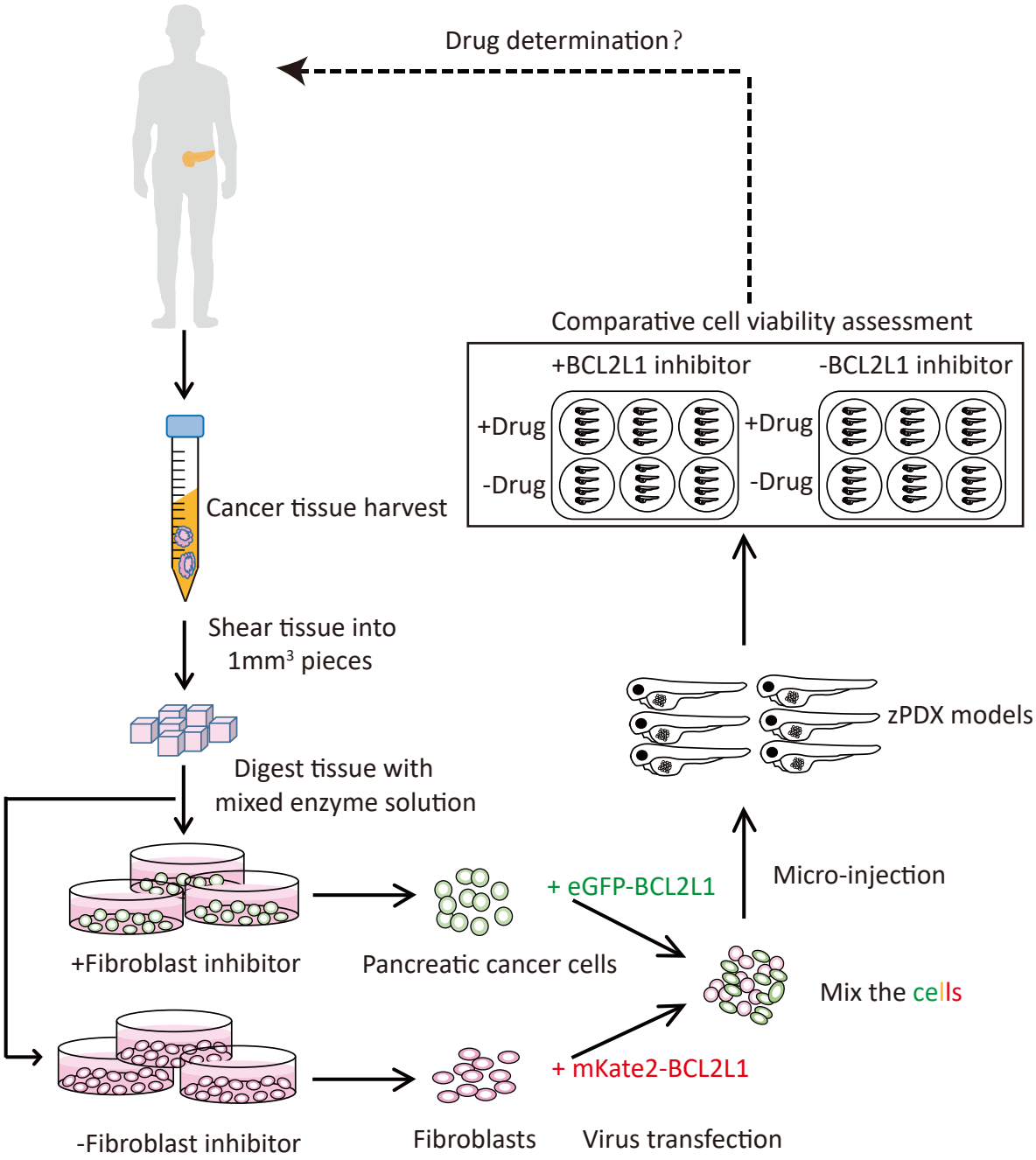

Figure 2

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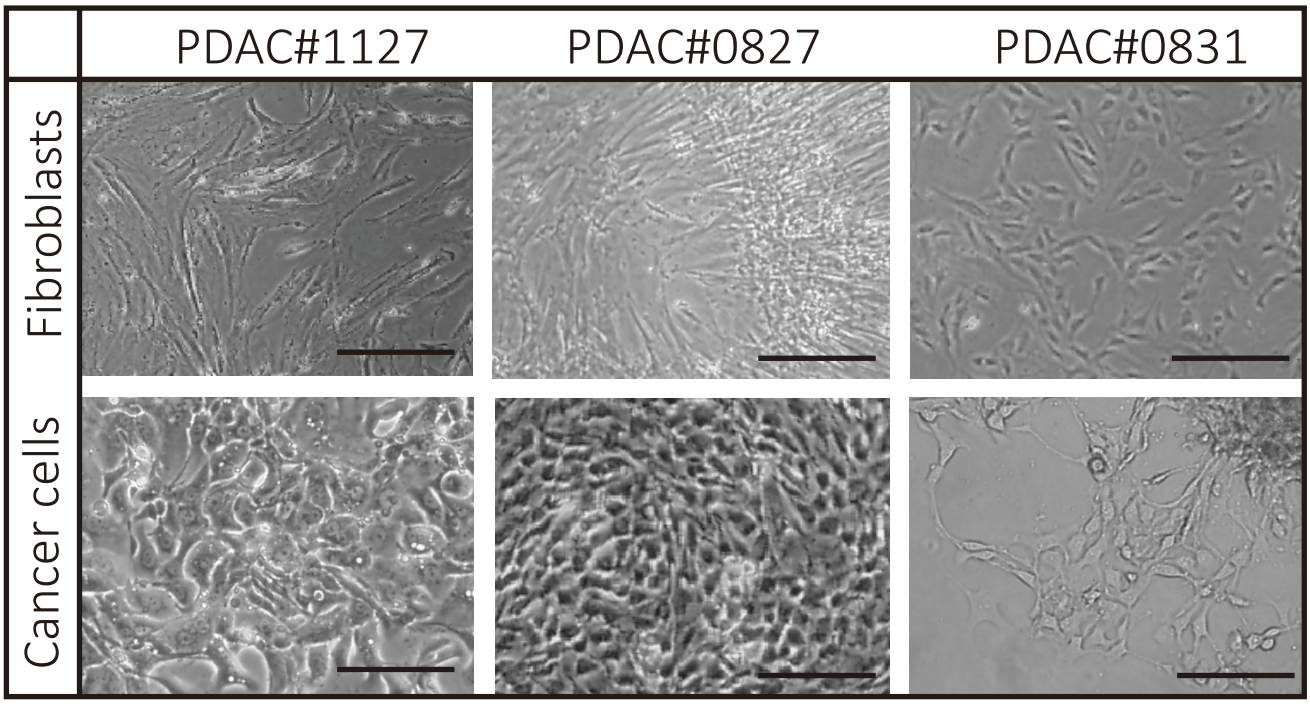

Figure 3

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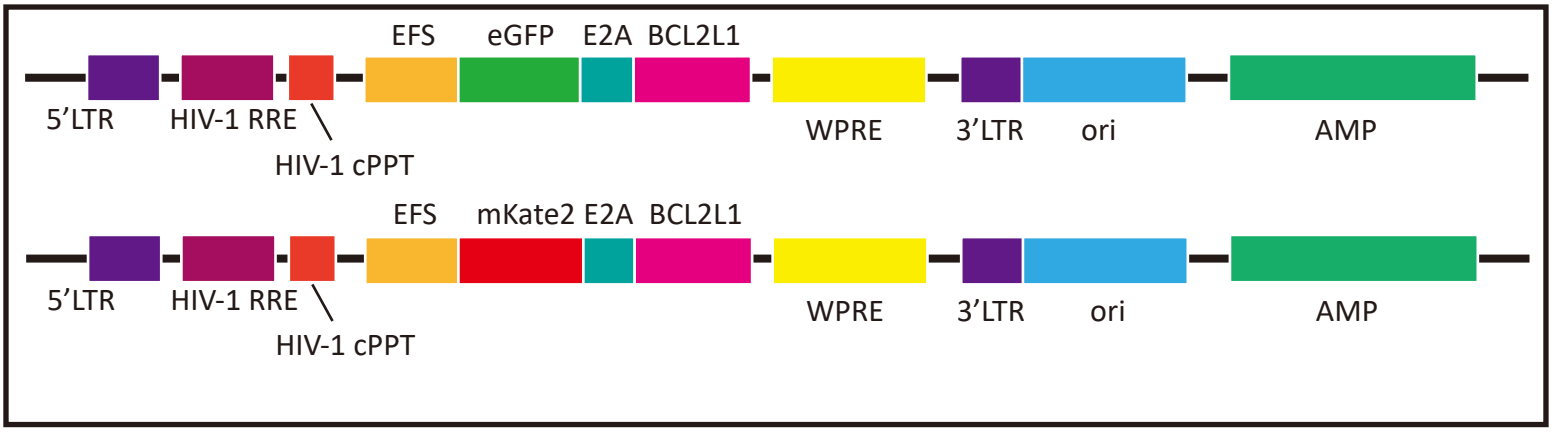

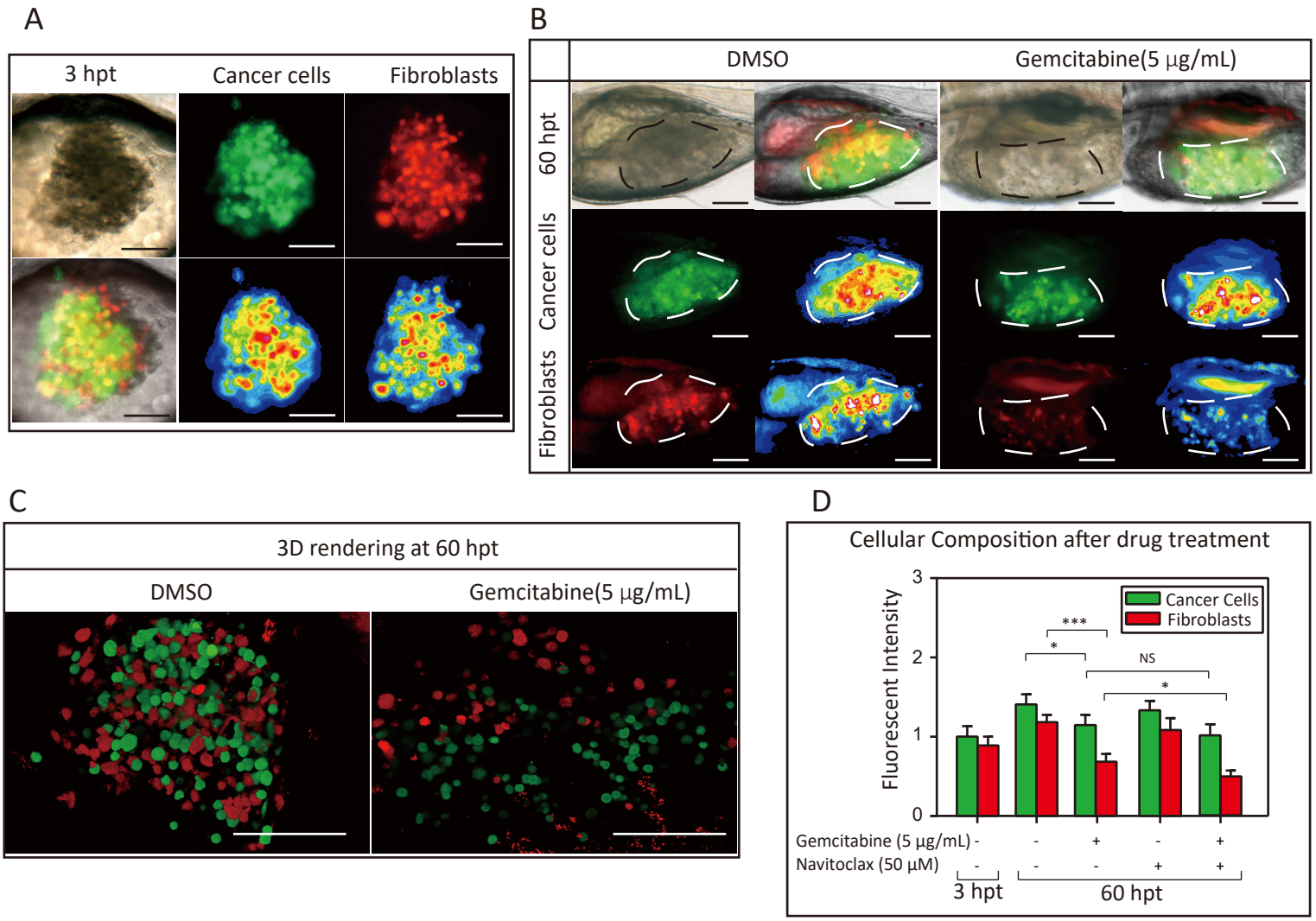

Name of Material	Company
DMEM	GIBCO
FBS	Hyclone
Y-27632	Cliniscience
Primocin	invivogen
Putrescine dihydrochloride	Sigma
Nicotinamide	Sigma
penicillin streptomycin	GIBCO
phosphate buffer (PBS)	GIBCO
HBSS	GIBCO
collagenase type IV	GIBCO
hyaluronidase	Sigma
Dnase Ⅰ	Sigma
insulin	Sigma
b-FGF	GIBCO
EGF	GIBCO
pancreatic cancer fibroblasts ii	CHI Scientific
0.45 μm sterile filter	Millipore
concentration column	Millipore
polybrene	Sigma
Hyaluronic Acid Sodium Salt	Sigma
L-glutamine	GIBCO

gemcitabine	Gemzan
methylcellulose	Sigma
Navitoclax(ABT-263)	Selleck
Equipment	**Company**
Microinjector	NARISHIGE
stereomicroscope	OLYMPUS
Confocal Microscope	LEICA

Catalog Number

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PHG0314

FibrOUT

SLHV033RB

Millipore UFC910008

H9268

H7630

21051024.00

M0262

S1001

Catalog Number

MVX10

SP8

Comments/Description

Rho kinase inhibitor

an antibiotic for primary cell cultures

Concentrate the virus

Bcl-xL inhibitor

Comments/Description

0.00

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Author(s):	Lei Wang1, #, Huan Chen2,#, Fei Fei1,#, Xianfeng He3, Shaoyang Sun1, kunpeng Lv1, Bo Yu2, Jiang Long 2 ,4,5,6 *, Xu Wang1,*

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Date:	12/06/2018

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Dear Editor

Thank you for your comments, and we have fixed those issues and point-to-point responses are listed as below:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

A1: We further checked the manuscript and fixed a few errors.

2. The Summary is over the 50 word limit.

A2: We have reduced the number of words in the summary to 50.

3. JoVE cannot publish manuscripts containing commercial language. This includes company names before an instrument or reagent. 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. Examples of commercial language in your manuscript include CHI Scientific, NARISHIGE, etc.

A3: We have removed CHI Scientific and NARISHIGE, and we did not find other commercial term in the manuscript.

4. Step 7.1.2: What's the temperature for incubation?

A2: We have added the temperature as 32 degrees.

5. 7.2.2: Please write this step in the imperative tense.

A2: We have rephrased the sentence in imperative tense.

6. Please do not abbreviate journal titles for all references.

A2: We have fixed the problem.

Best regards!

Xu

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Jan 07th, 2019

Dear Editors and Reviewers,

Happy New Year!

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

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

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

Sincerely,

Xu

Editorial comments:

The manuscript has been modified and the updated manuscript, **59507_R0.docx**, is attached and located in your Editorial Manager account. **Please use the updated version to make your revisions.**

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For more than 6 authors, list only the first author then et al.

5. Please add a one-line space between each of your protocol steps.

6. There is a 2.75 page limit for filmable content. Please highlight 2.75 pages or less of the Protocol steps in yellow (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

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8. Please do not abbreviate journal titles for all references.

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10. Please use standard SI unit symbols and prefixes such as µL, mL, L, g, m, etc.

11. Please use greek characters for SI unit prefixed, e.g. use 'µL' instead of 'uL'.

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

Reviewers' comments:

Reviewer #1 (Remarks to the Author):

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

Major Concerns:

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

compromising the validity of this tool for clinical purposes. Could the authors address this matter?

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

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

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

Minor comments:

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

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

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

A2. Thank you very much for your comments. To optimize our model to screen for real therapeutic strategies benefitting patients is our final goal, and we have not reached it yet. Currently, we have tested this

model in around 20 cases, but the results were not used to affect clinical decisions made for the patients. However, we will combine information from both bench and bedside, and perform retrospective study.

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

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

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

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

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

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

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

Reviewer #2 (Remarks to the Author):

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

Minor Concerns:

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

a professional editing service.

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

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

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

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

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

.

Reviewer #3 (Remarks to the Author):

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

Major Concerns:

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

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

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

A2：Thank you very much for your comments. We now modify the protocol by adding an additional control experiment (Fig. 1): Co-culture the zebrafish PDX models with Navitoclax (ABT-263) (an oral BCL-XL inhibitor) during the drug test procedures, and Compare the results with the drug test results without Navitoclax treatment in four groups (DMSO, gemcitabine, navitoclax (a BCL2-L1 inhibitor), and gemcitabine & navitoclax). Such comparison will provide an assessment of drug resistances particularly caused by BCL2-L1, and help identify the potential “false negative" outcomes (Fig. 1). In the representative test, the inhibition of BCL2-L1 does not significantly affect the viability of the infected tumor cell population (Fig. 4D). We also added the temperature question into the discussion as a future work:” Besides, the effects of 32 °C instead of 37 °C incubation on the behaviors of human cells also require detailed comparative studies.”

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

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

Minor Concerns:

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

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

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

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

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

A3. Thank you very much for your comments.

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

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

Q4. 5.3 please describe volume? fL?

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

.

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

Sincerely,

Xu Wang, Ph.D.
Young Investigator
Department of Biochemistry and Molecular Biology
School of Basic Medical Sciences
Fudan University
01/07/2019