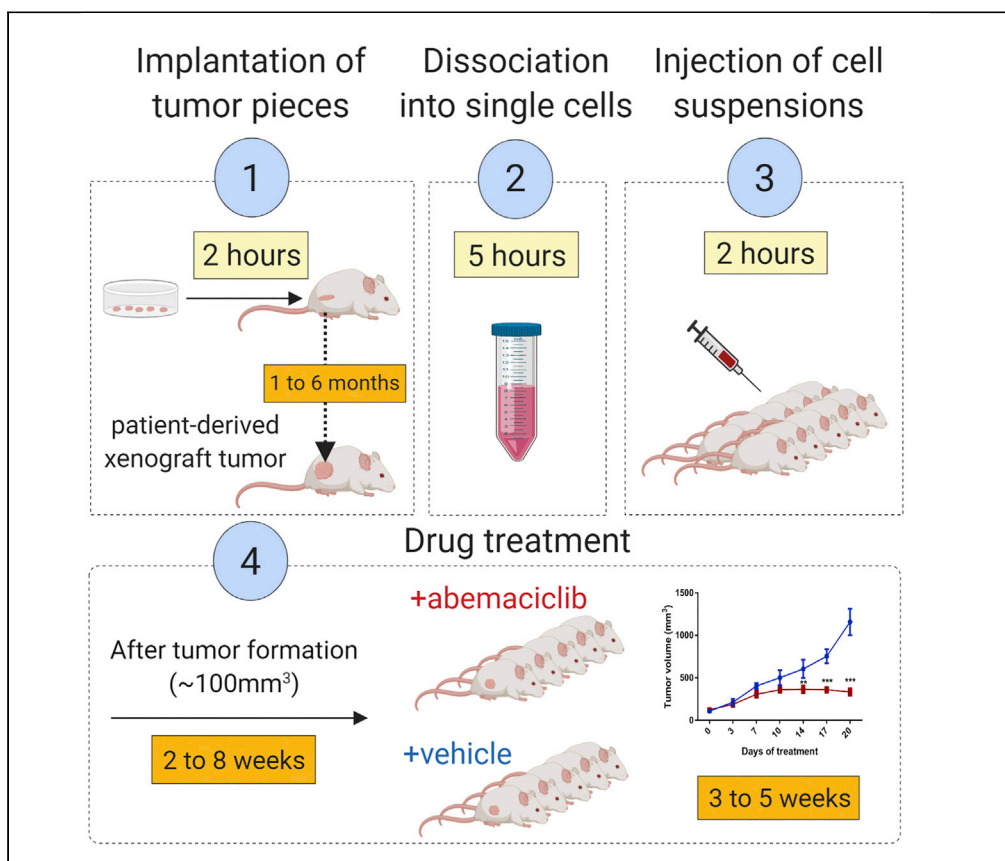


Protocol

Establishment and Use of Patient-Derived Xenograft Models for Drug Testing in Head and Neck Squamous Cell Carcinoma



This protocol provides the steps required for the establishment of patient-derived xenograft (PDX) tumors for head and neck squamous cell carcinomas (HNSCCs) and their utility in examining drug responses. PDXs recapitulate the heterogeneity observed in the corresponding human tumors, which makes them an ideal pre-clinical model system. This protocol outlines detailed steps required for (1) the generation of HNSCC-PDXs, (2) the processing of tumor tissues, and (3) the expansion of PDX models into cohorts for (4) drug testing.

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HIGHLIGHTS

Patient-derived xenograft tumors are successfully generated from HNSCC tissue

Bulk single-cell suspensions of PDX tumors are used to create larger mouse cohorts

Tumor response to candidate drugs can be assessed across several PDX models

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Protocol

Establishment and Use of Patient-Derived Xenograft Models for Drug Testing in Head and Neck Squamous Cell Carcinoma

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SUMMARY

This protocol provides the steps required for the establishment of patient-derived xenograft (PDX) tumors for head and neck squamous cell carcinomas (HNSCCs) and their utility in examining drug responses. PDXs recapitulate the heterogeneity observed in the corresponding human tumors, which makes them an ideal pre-clinical model system. This protocol outlines the detailed steps required for (1) the generation of HNSCC-PDXs, (2) the processing of tumor tissues, and (3) the expansion of PDX models into cohorts for (4) drug testing. For complete details on the use and execution of this protocol please refer to Karamboulas et al. (2018).

BEFORE YOU BEGIN

Note: Figure 1 provides an overview of all four steps.

Note: All steps are to be done in a laminar hood and under sterile conditions.

Implantation of Tumor Pieces

1. Primary tumor samples are obtained from patients undergoing surgery for head and neck squamous cell carcinoma. Samples are collected with informed consent and must be performed with the approval of the Research Ethics Board (REB) or Institutional Review Board (IRB).

△ **CRITICAL:** Be aware of the potential presence of human pathogens. Be sure to adhere to appropriate biosafety protocols when handling human tissues. Also, antibiotics should be added to the media to prevent bacterial contamination during tissue processing.

△ **CRITICAL:** To preserve tissue viability, it is important to obtain and to process the patient sample as soon as possible, within 1 to 4 hours of surgical resection.

2. All mouse experiments must be performed with the approval of an Animal Care Committee that is relevant to your research institution.

3. Immunocompromised mice are used for implantation of patient tumors, with a preference for NOD-SCID-IL2R γ^{null} (NSG) mice (6 to 8 weeks old) since they have a more severely impaired



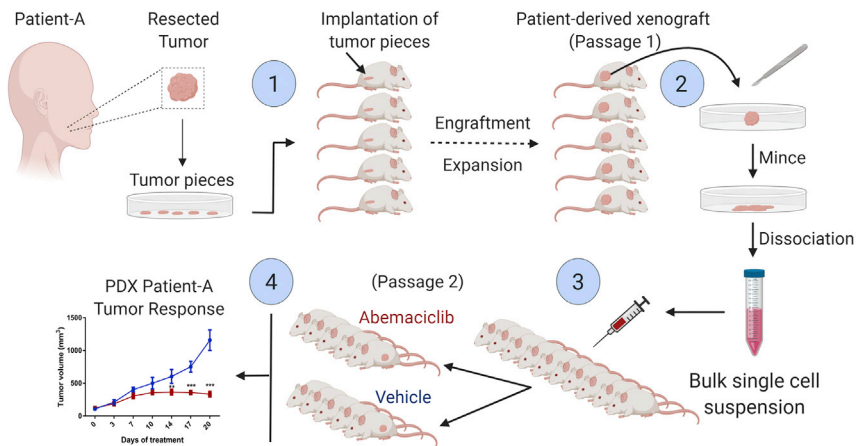


Figure 1. Protocol Overview

Steps required for establishing Head and Neck Squamous Cell Carcinoma (HNSCC) patient-derived xenografts (PDXs) and for preparing the subsequent cohorts for drug testing. (1) The surgically resected patient tumor is cut into 5-10 pieces (2 mm x 2 mm) (number of pieces implanted depends on size of the patient tumor). One tumor piece is implanted per mouse subcutaneously into the left flank of NSG mice. (2) Passage 1 (P1) PDX tumors are extracted, minced and enzymatically dissociated into a single cell suspension that is subcutaneously (3) injected (100,000 to 250,000 cells per mouse) into the left flanks of a cohort of at least 10 mice. (4) The drug is administered daily when PDX tumors are approximately 80 to 120mm³. Tumor volumes are measured every 4 days.

immune system. Supplier of NSG mice is the Jackson Laboratory (<https://www.jax.org>). Alternatively, NOD-SCID or NOD-Rag1^{null}-IL2r^{null} can be used.

4. A strict barrier environment is required to house and maintain immune-compromised mice. All food, water, bedding and cages entering the room should be autoclaved or sterilized. Personal protective equipment (sterile gown, mask, hair net, double gloves) is essential to minimize the spread of bacteria to mice. Mouse cages must be opened in a Type II Biological Safety Cabinet. Sterile surgical instruments and frequent disinfection of gloves and equipment with Clidox (1:5:1) and 70% ethanol must be performed when handling mice. It is good practice to place mice into fresh cages weekly to maintain their health.
5. All the surgical instruments must be autoclaved and opened in the laminar flow hood.
6. An isoflurane chamber with nose cone attachment is required to anesthetize the mice.
7. Prepare a pack of the following surgical instruments and autoclave: Sharp/blunt scissors; 1 curved forceps; 1 Straight forceps

Dissociation into a Single-Cell Suspension

8. Bulk tumor PDX cell suspensions are used to establish cohorts in Step 3. Injecting a bulk cell suspension (rather than implanting pieces) allows for a more even distribution of tumor cells into each mouse and thus a similar growth rate of tumors across all the mice.
9. As in step 1, the tumor is processed under sterile conditions in a laminar flow hood and all materials must be sterile.
10. A 37°C incubator (5% CO₂) is required for incubation during dissociation.

Drug Treatment

11. Prepare drug stocks (e.g. abemaciclib) and store aliquots in freezer.
12. Begin treatments when tumors reach between 80 to 120 mm³.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Biological Samples		
HNSCC tissue samples	University Health Network	N/A
Patient-derived xenograft tissue	Ailles Lab	N/A
Chemicals, Peptides, and Recombinant Proteins		
Surgical Scrub Betadine	Purdue Products LP	6904214-40890
Meloxicam	Boeinger Ingelheim	L20805A-42
Fetal bovine serum	Gibco	N/A
Dimethyl sulfoxide	Fisher	BP231-1
Tear gel	Optixcare Eye Lube	10699
Ethyl Alcohol Anhydrous	Commercial Alcohols	PO16EAAAN
Media 199	Wisent	316-010-CL
Phosphate Buffered Saline (PBS)	Wisent	311-010-CL
Deoxyribonuclease I (DNase), Code D, 20mg	Worthington	LS002004
10X Collagenase/hyaluronidase in DMEM	Stem Cell Technologies	07912
ACK Lysing Buffer	Thermo Fisher Scientific	A1049201
Trypan Blue Solution	Thermo Fisher Scientific	15250061
IMDM	Thermo Fisher Scientific	12440061
Penicillin/Streptomycin (10,000 U/ml)	Thermo Fisher Scientific	15140-122
Matrigel Matrix Basement Matrix	VWR	CACB354234
LY2835219 (abemaciclib)	MedChem Express	Cat# HY-16297; CAS: 1231930-82-7
Betadine Iodine Solution	Purdue Products LP	00158348
Experimental Models: Organisms/Strains		
NOD/SCID/IL2Rg ^{-/-} (NSG) mice	Original source: Jackson Labs. Bred in-house at University Health Network Animal Resource Centre	Cat# 4142; RRID:BCBC_4142
Other		
Electric clippers	Wahl	Model 1854
Sterile Gauze Sponge (2x2")	VWR	95041-740
Sharp/blunt scissors (12cm)	Fine Science Tools	91400-12
Curved Specimen forceps	VWR	82027-442
Straight Specimen forceps (2)	VWR	82027-450
Isoflurane chamber with nose cone	Benson Medical	T3ISO
Autoclip Wound Clip (9mm)	VWR	CA-BD427631
Autoclip System - Applicator and Removal Forceps	Fine Science Tools	12020-00
Ear Notcher	Fine Science Tools	24210-02
1 ml syringe	VWR-BD	53548-001
26g needle	VWR	89219-324
Freezing container	VWR	55710-200
Cryovials, 1.5ml	VWR	66008-710

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
50ml Falcon Tubes	VWR	CA21008-940
Sterile Petri Dish	VWR	25384-342
Heating Pad - Snuggle Safe	Amazon	310
Scalpel with blade no. 10	VWR-Miltex	21909-654
Sterile razor	VWR	55411-050
Centrifuge Allegra 6KR with Safety Caps	Beckman	A37909
Cell Strainer (70micron)	VWR	CA21008-952
25ml Serological Pipette	Thermo Fisher Scientific	170357
10ml Serological Pipette	Thermo Fisher Scientific	170367
Hemocytometer	VWR	15170-170
Hemocytometer coverglass (26 x 20 mm (1 x 7/8"))	VWR	16007-111
Light Microscope	Leica	DMIL
Plastic Feeding Tubes, 20gx30mm	INSTECH	FTP-20-30
Bel-Art Plastic Calipers	Scienceware	134160001

MATERIALS AND EQUIPMENT

DNase stock at (12,500 U/ml) in Media199 = (100X)

Make 100 μ l aliquots of 100X DNase, which can be diluted directly into 10 ml of dissociation solution. Store the aliquots in a -20 °C freezer.

Avoid freeze/thaw cycles.

1X Dissociation Solution

Note: A volume of 10 ml is required per $\sim 1,755\text{mm}^3$ tumor volume:

- 8.9 ml of Media199
- + 1 ml 10X Collagenase/Hyaluronidase (Stem Cell) (Final 1X)
- + 100 μ l of 100X DNase1 (Worthington) (Final 125 U/ml)

Iscove's Modified Dulbecco's Medium (IMDM)

- +2% Fetal Bovine Serum
- + 1% Penicillin/streptomycin

Note: This is used for washing the dissociated cells through the 70 μ m filter

STEP-BY-STEP METHOD DETAILS

Implantation of Tumor Pieces

⌚ TIMING: ~ 2 h

This step describes what is required to implant the primary patient samples into NSG mice to establish the patient-derived xenograft (PDX) tumors (Figure 1, Step 1). Five to ten pieces of the patient tumor are implanted subcutaneously into 5 to 10 mice (1 piece per mouse). Aim for 10 pieces, however, due to small patient samples a smaller number of tumor fragments may have to be implanted.

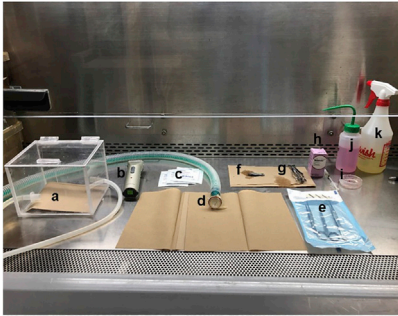


Figure 2. Preparation for Surgical Implantation

After disinfecting the biological safety cabinet prepare the following: (a) Isoflurane chamber, (b) electric clippers, (c) sterile gauze sponges, (d) isoflurane tube with nose cone, (e) sterilized surgical instrument pack, (f) sterile ear notcher, (g) autoclip applicator, (h) meloxicam, (i) human patient tumor pieces, (j) ethanol (70%), (k) clidox (1:5:1). Missing in picture but required: surgical scrub betadine, betadine solution, tear gel.

Note: This first generation of PDXs (or passage 1, P1) is established to expand the initial tissue so that enough cells can be obtained for further experiments (Figure 1, Step 2-4). The growth of even 1 of 10 tumors implanted allows for further experiments to be performed. Of course, non-engrafting tumors cannot be tested further.

1. Collect the patient tumor tissue from surgical pathology. Place the specimen in a 50 ml conical centrifuge tube containing 20 ml sterile PBS and store on ice until ready to process.

△ **CRITICAL:** The sample should be processed as soon as possible (i.e. within 1–4 h) to preserve cell viability.

2. In a laminar flow hood, place the patient tumor in a small petri dish and under sterile conditions cut into 2 x 2 mm pieces (5 to 10). One piece will be implanted per mouse. Add sterile PBS to the dish to prevent the tissues from drying out, seal the dish with paraffin film and place on ice.

Optional: Extra tumor pieces can be cryopreserved. Place 5 pieces of tumor per cryovial and add 900 μ l FBS + 100 μ l DMSO to freeze down. Place the vials into a Mr. Frosty and then store the Mr. Frosty in a - 80 °C freezer for a minimum of 4 h. For long term storage transfer the vials into a liquid nitrogen freezer.

3. Before entering the mouse facility, put on personal protective equipment (sterile gown, mask, hair net, and double gloves).
4. Spray and wipe down the inside of the biological safety cabinet first with Clidox (1:5:1) followed by 70% Ethanol.
5. Place the dish with the tissue pieces and the required materials in the biological safety cabinet to prepare for surgical implantation (Figure 2).
6. Place a warm heating pad under a new clean cage with sterile paper towel on top of the bedding to serve as the recovery cage.
7. Anesthetize the mice in an isoflurane chamber using Isoflurane at 5% and an oxygen flow of 1.5 L/minute until breathing reaches thoraco-abdominal region. Reduce anesthetic to 2.5% for the remaining surgical procedure.
8. Once anesthetized, position the mouse in the nose cone in ventral recumbence.
9. Place tear gel in eyes, ear punch mice for identification and use electric clippers to shave the left (or right) flank for implant.
10. Inject mouse with meloxicam 5 mg/kg subcutaneously for pain management.
11. Place the mouse back into the induction chamber to clean up shaved hair and area before surgical procedure.
12. Replace sterile paper towels under nose cone and lay out sterile paper towels for surgical instruments. Open the sterile pack of instruments described in the “Before you begin” section.
13. Position mouse in the nose cone in ventral recumbence.

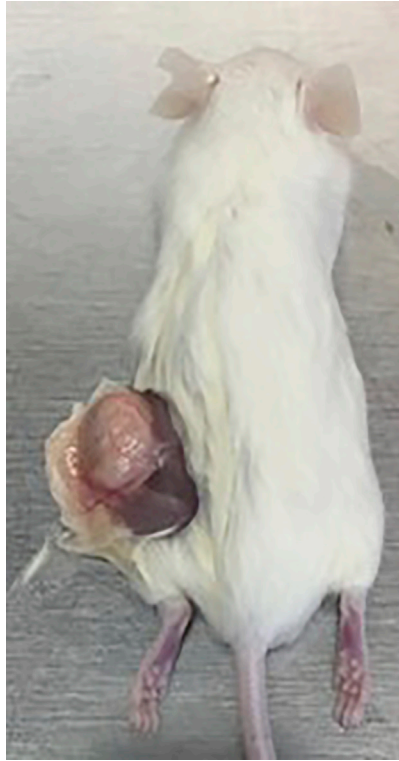


Figure 3. Patient-derived Xenograft Tumor

At endpoint, the mouse is euthanized, and the tumor is extracted under sterile conditions.

14. Scrub the surgical site with Scrub Surgical Betadine then wipe away surgical scrub with 70% Ethanol.
15. Swab surgical site with Betadine Solution and allow it to dry.
16. Test that the mouse is in surgical plane by performing a toe pinch. This is done by taking the toes of the back foot of the mouse and pinching between your thumb and index finger. The mouse should not retract its leg once it has reached surgical plane.

Note: See [Video S1](#) for step 17 to 20

17. At a 45-degree angle to the midline, with the scissors, make a 0.5 cm incision halfway between the knee and spine.
18. Pull up the cut skin using curved specimen forceps with one hand and use straight specimen forceps to blunt dissect the skin away from the abdominal wall towards the leg to form a pocket (See [Video S1](#))
19. Using the same straight forceps place the tumor fragment into the pocket. Make sure the tumor goes into the pocket as it can slip out. Use the curved forceps to bring the cut skin back together.
20. Staple the opposed wound edges using the Autoclip staple applicator.
21. Place the mouse in recovery cage until fully mobile.
22. Check the mice the following day to ensure staples are intact.
23. Remove staples using the Autoclip removal forceps 7-10 days after surgery.
24. Palpate for tumor growth after 2 weeks and up to 6 months.
25. End point is either 6 months if NO tumor is palpable or once the xenograft tumor reaches the maximum size allowed (15 mm in diameter or 1,755 mm³) ([Figure 3](#)). The mouse must be euthanized, and the tumor extracted in a laminar flow hood under sterile conditions. See Step 2 for tumor extraction.

Extraction and Dissociation of PDX Tumor into a Single-Cell Suspension

⌚ TIMING: ~ 5 h

This section describes the steps for extraction, viable tissue banking/cryopreservation and dissociation of the PDX tumor into a single cell suspension (Figure 1, Step 2).

26. Once the tumor reaches the maximum size allowed (15 mm in diameter or 1,755 mm³) (Figure 3), euthanize the mouse and extract the tumor in a laminar flow hood under sterile conditions
27. Place the tumor in a 15 ml conical centrifuge tube containing sterile PBS and place on ice.

⚠ **CRITICAL:** Dissociation of fresh tissue immediately after extraction results in optimal tumor cell viability. Keep tumor on ice or in fridge until ready to process.

Optional: While not recommended, in cases where the tumor cannot be processed immediately, it can be stored at 4°C for up to 24 h and processed the next day.

28. In a laminar flow hood use sterile forceps to transfer the sample into a 10 cm petri dish.

⚠ **CRITICAL:** The sample size should be no bigger than 1,755mm³ per 10 ml of 1X dissociation solution in a 10 cm plate to ensure efficient dissociation.

Optional: Multiple tumors from the same PDX model (i.e. PDX tumors derived from the same patient sample; recall up to 5 to 10 mice were implanted) can be dissociated separately and then pooled together at the end if higher cell numbers are required.

Optional: PDX tumor pieces (Passage 1, P1) can be viablely banked by placing 5 pieces per cryovial (900 µl FBS + 100 µl DMSO). Place the vials into a Mr. Frosty and then store the Mr. Frosty in a -80°C freezer for a minimum of 4 hours. For long term storage transfer the vials into a liquid nitrogen freezer.

Note: For steps 29-38, see Figure 4.

29. Use a sterile scalpel to cut the tumor into smaller pieces and then mince using a sterile razor blade.
30. Add 10 ml of the 1X dissociation solution. Agitate the dish gently by moving it side to side, back and forth to mix the minced tissue with the dissociation solution. Place the plate with the tumor mixture in a tissue culture incubator (at 37°C, 5 % CO₂) for 15 min.

⚠ **CRITICAL:** FBS should NOT be included in the 1X dissociation solution as FBS inhibits dissociation.

31. Gently pipette the mixture up and down several times using a 25 ml and then a 10 ml serological pipette to aid in the dissociation process and place it back into the incubator.
32. Pipette every 15 min for up to 3 hours until a homogenous solution is obtained.

⚠ **CRITICAL:** If the tissue is very fibrous, there may be some small tissue pieces remaining. Do not incubate the tissue longer than 3 h as cell viability decreases with prolonged dissociation.

33. Once the dissociation is complete, place a 70 µm strainer into a 50 ml conical tube and pass the 10 ml of cell suspension through the strainer.
34. Wash the strainer by adding 10 ml of IMDM (+ 10 % FBS).

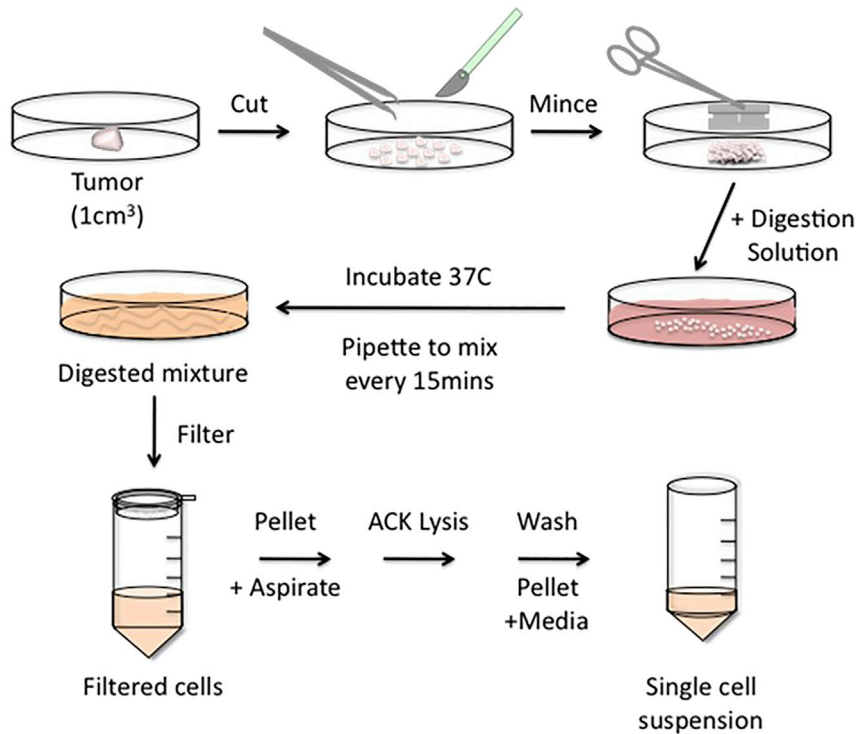


Figure 4. Dissociation Steps for Solid Tumor into a Single Cell Suspension

A tumor piece of $\sim 1 \text{ cm}^3$ or smaller is placed in a sterile 10 cm petri dish and cut into smaller pieces and then minced finely. Digestion solution (10 ml) is added to the minced tissue and incubated in the tissue culture incubator (37°C) for up to 3 hours. Every 15 min pipette the mixture to facilitate the digestion. Once the dissociation is complete transfer the digested solution through the $70 \mu\text{m}$ strainer into a 50 ml conical tube and wash with fresh 10 ml IMDM (+10 % FBS). Pellet, use lysing-buffer (ACK) to degrade red blood cells, wash and pellet. Pellet can be used immediately or cryopreserved.

35. Centrifuge the collected strained suspension of cells $300 \times g$ for 5 min to pellet the cells. Aspirate the media.
36. Add 1 ml of cold Ammonium-Chloride-Potassium (ACK) lysing buffer (to lyse the red blood cells) and incubate no more than 5 min on ice.

△ CRITICAL: Do not incubate cells with ACK lysing buffer for more than 5 min as it will negatively affect the viability of tumor cells.

37. Add 10 ml of PBS to wash and centrifuge again $300 \times g$ for 5 min. Aspirate the PBS.
38. Resuspend the cell pellet in 10 ml of PBS and count the number of viable cells using Trypan Blue.

The cells are now ready for injection into mice (Step 3) OR for cryopreservation for storage.

- a. For cryopreservation, resuspend up to 10×10^6 viably counted cells in $900 \mu\text{l}$ FBS + $100 \mu\text{l}$ DMSO per cryovial and freeze down. Place the vials into a Mr. Frosty and then store the Mr. Frosty in a -80°C freezer for a minimum of 4 hours. For long term storage transfer the vials into a liquid nitrogen freezer.

▮ PAUSE POINT: Bulk suspensions can be cryopreserved, however upon thawing, cell viability can decrease up to 50% and this varies between tumor samples. Be sure to count viable cells

when frozen vials are thawed for recovery. Alternatively, proceed directly to injection of bulk suspensions to avoid freezing/thawing.

Injection of Bulk Tumor Single-Cell Suspension into NSG Mice

⌚ TIMING: ~ 2-3 h

This section describes the steps for preparation and injection of bulk tumor cells into a large cohort of mice (Figure 1). Injecting a bulk cell suspension (rather than implanting pieces) allows for a more even distribution of tumor cells into each mouse and thus allows for a similar growth rate of tumors across all the mice. The following steps were used to establish the cohorts of mice from our recent publication that were treated with abemaciclib (Karamboulas et al, 2018).

39. Prepare a bulk suspension of fresh tumor cells or thaw a vial of previously cryopreserved cells.
40. Count the total number of cells suspended in PBS and determine the number of cells to inject per mouse for a group of at least 10 mice. Typically, between 100, 000 to 250, 000 cells are enough to ensure engraftment across all mice and to produce an even growth rate. (However, some tumors may require higher cell numbers (i.e. 400, 000 to 500, 000).

⚠ **CRITICAL:** Preparation of larger cohorts of mice is suggested when possible depending on the number of cells available to ensure the sample size per treatment group is large enough to distinguish small changes in mean tumor volume between groups.

41. Cold Matrigel matrix will be mixed at a 1:1 ratio with cell suspension in cold PBS.

Note: Matrigel matrix contains extracellular matrix components that allow for the cells to remain localized at the injection site.

⚠ **CRITICAL:** It is essential that the cell/Matrigel mixture be kept cool on ice as Matrigel forms a gel above 10°C.

42. A volume of 100 µl total (50 µl Matrigel + 50 µl cell suspension) is injected per mouse.
43. Prepare the cells plus Matrigel for all 10 injections in one tube in order to reduce variation from mouse to mouse. Be sure to prepare a few extra doses due to loss of sample in the needle hub.
44. Before filling the 1 ml (26-gauge needle) syringe, ensure Matrigel plus cells are mixed well by tapping the tube and/or inverting it a few times. Keep on ice.

⚠ **CRITICAL:** It is essential to thoroughly mix the cold cell/Matrigel mixture immediately before injections to ensure even distribution of cells as they will settle to bottom of tube.

45. Fill the syringe with cell/Matrigel mixture at the time the mice are ready to be injected. If the syringe is prepared with the cell/Matrigel mixture too far in advance, the cells will start to settle in the syringe and can get trapped inside the hub. It is critical to add the cell/Matrigel mixture right before you are ready to inject to ensure that the cells are evenly distributed in the syringe and that each mouse is injected with an equal number of cells.
46. Wet a sterile gauze sponge with 70 % ethanol. Place the sponge on the floor of the laminar hood and gently drag the needle across the sponge to wipe off excess cells on the outside before proceeding with injection.
47. Hold the mouse in an Intraperitoneal (IP) hold and subcutaneously inject 100 µl into the left flank of each mouse.
48. Ear notch each mouse to identify.

Drug Treatment of PDX Cohorts

⌚ TIMING: ~2 h

This section describes the steps that were used to treat HNSCC-PDX mice with abemaciclib (LY2835219) in our recent paper (Figure 1). This protocol can be used for treating with other drugs of interest, using the appropriate vehicle, dose and delivery route that is appropriate.

49. Monitor the tumor growth of each mouse.
50. Using calipers measure and record the measurements for each tumor (length and width, mm).
 - a. $\text{Volume} = (L \times W^2) \times 0.52$
51. Treatment will begin when the volumes of the tumors reach approximately 80 to 120mm³

⚠ **CRITICAL:** It is important that all tumors are approximately of similar volume.

52. Randomize the ten mice into two groups of 5 (Drug (abemaciclib) (5) vs Vehicle Control (sterile milli-q water) (5)).
53. Weigh each mouse before treatment starts to determine the volume of abemaciclib to administer so that each mouse is receiving 100 mg/kg. Use this volume until the end of treatment.

Note: Working concentrations should be determined such that you are administering an appropriate volume based on the method of delivery. Oral gavage is limited to 1% volume of total body weight, e.g., a 20 g mouse is given a maximum of 0.2 ml.

The working concentration of drug must take into account the volume of drug that can be delivered safely. In our case, we used a working stock concentration of 25 mg/ml which gave a range of 70 - 130 μl depending on the body weight of the animal.

Example calculation:

$$0.025 \text{ kg (body weight of mouse)} \times 100 \text{ mg/kg (dose)} \times 1 \text{ ml/25 mg} = 0.1 \text{ ml volume of drug}$$

54. Mice are treated daily with abemaciclib or sterile water by oral gavage/feeding tubes and measurements are recorded twice a week (Figure 5A).
55. Treatment ends once the tumors in the control group reach the humane endpoint size of 1755 mm³ in volume and mice are euthanized.
56. Extract tumors and weigh each for an endpoint weight (Figure 5A).
57. See the “Quantification and Statistical Analysis” section for more detail into the analysis of the results between the two groups (Abemaciclib vs Control).

EXPECTED OUTCOMES

The tumors take 1 to 4 months to engraft where some PDX tumors are palpable by 2 weeks and others after several weeks. By 6 months, if no tumor has formed, it is unlikely that a tumor will form and therefore 6 months is used as an endpoint for tumor growth after implantation and mice are sacrificed.

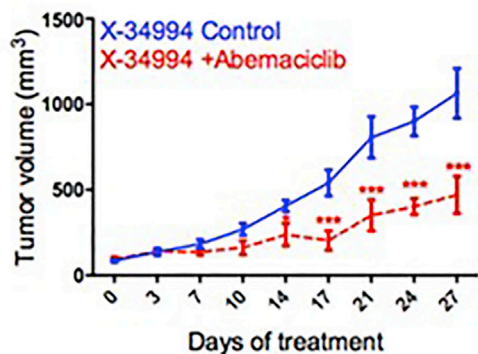
The implanted samples that show no growth in mice are termed non-engrafters. Engraftment efficiency will vary between patient samples. For example, more aggressive patient tumors will engraft at 90 to 100% efficiency (9 to 10 of the pieces implanted grow out tumors) and less aggressive tumors will engraft at 10 to 20% efficiency (*i.e.* 1 to 2 of the 10 pieces).

A

Tx started		28 Feb 17																	
D#	X2-34994																		
Cell dose injected	160,000	27 Jan 17																	
CAGE#	1473	Control (water)																	
MEASUREMENT																			
MOU# ID	1= (29.5 g (120u))			2= (30 g (120u))			3= (30 g (120u))			4 = (28.5 g (120u))			5= (26.5 g 110			#/G	turn	Stdv	
DATE	LENGTH	WIDTH	TOTAL vol.	LENGTH	WIDTH	TOTAL vol.	LENGTH	WIDTH	TOTAL vol.	LENGTH	WIDTH	TOTAL vol.	LENGTH	WIDTH	TOTAL vol.	vol			
2/23/2017	5.0	5.0	65.0	4.4	4.8	52.7	5.9	4.8	70.7	3.0	3.0	14.0	5.1	5.3	74.5	55.4	24.5		
2/28/2017	5.6	5.7	94.6	4.5	5.1	60.9	5.1	5.2	71.7	5.8	5.7	94.6	5.4	6.1	104.5	65.3	18.2		
3/3/2017	6.0	6.6	135.9	5.0	5.4	75.8	5.9	6.4	125.7	6.0	6.8	144.3	7.4	7.3	205.1	137.3	46.3		
3/7/2017	7.8	7.5	228.2	6.0	6.0	112.3	6.6	6.9	163.4	6.6	6.7	154.1	7.5	6.1	255.9	162.6	58.2		
3/10/2017	8.0	8.0	268.2	6.8	6.9	188.3	7.3	8.2	265.2	7.1	8.5	268.7	8.8	9.2	387.3	268.8	78.0		
3/14/2017	10.0	8.8	402.7	8.8	8.2	300.7	8.3	9.3	373.3	9.8	9.7	469.7	9.7	9.8	484.4	408.2	74.8		
3/17/2017	10.1	10.1	535.8	7.1	8.5	286.7	9.9	10.2	535.6	11.5	10.9	710.5	10.8	10.8	665.1	540.7	171.0		
3/21/2017	11.9	11.9	678.3	9.8	9.1	413.4	10.7	11.6	748.7	13.9	12.7	1168.8	12.0	11.5	825.2	805.9	270.3		
3/24/2017	12.6	12.0	843.5	11.1	10.0	571.2	13.0	11.8	941.3	11.5	13.4	1073.8	12.9	12.0	988.0	900.3	188.6		
3/27/2017	12.9	12.6	1066.0	10.9	9.7	520.3	12.8	12.7	1073.5	15.0	13.3	1378.7	13.4	13.5	1288.9	1064.3	325.5		
tumor weights (g)	1.4			0.6			0.7			1.0			1.3						

CAGE#		1474		Abemaciclib (100mg/kg)															
MEASUREMENT																			
MOU# ID	1= (30.5 g (120u))			2= (30.5 g (120u))			3 = (27 g (110u))			4 = (29 g (120u))			5 = (24 g (100u))			#/G	turn	Stdv	
DATE	LENGTH	WIDTH	TOTAL vol.	LENGTH	WIDTH	TOTAL vol.	LENGTH	WIDTH	TOTAL vol.	LENGTH	WIDTH	TOTAL vol.	LENGTH	WIDTH	TOTAL vol.	vol			
2/23/2017	5.8	5.9	105.0	3.0	3.0	14.0	3.0	3.0	14.0	5.8	5.6	94.6	3.0	3.0	14.0	48.3	47.1		
2/28/2017	7.1	6.0	132.9	6.0	5.4	91.0	5.1	5.2	71.7	6.0	5.9	108.6	5.4	5.4	81.9	97.2	24.1		
3/3/2017	7.2	6.6	173.1	7.4	6.4	157.6	5.9	5.8	92.7	7.0	6.6	158.6	6.2	5.9	112.2	138.8	34.5		
3/7/2017	8.9	8.9	170.8	6.6	5.9	119.5	5.6	5.5	88.1	7.2	7.0	183.5	5.9	6.3	121.8	136.7	38.5		
3/10/2017	8.9	7.8	261.6	6.2	6.0	116.1	6.0	6.0	112.3	7.5	7.6	225.3	5.2	5.2	73.1	161.7	67.7		
3/14/2017	10.3	9.1	443.5	7.5	6.9	186.7	6.0	6.3	123.8	8.5	8.6	326.9	5.9	5.9	106.8	237.3	144.2		
3/17/2017	9.5	8.6	365.4	6.0	6.3	128.8	6.3	6.1	121.9	7.9	8.7	310.9	6.2	5.5	97.5	203.9	124.5		
3/21/2017	11.9	10.3	658.5	7.9	8.4	249.9	7.3	6.7	170.4	8.9	9.9	453.6	7.2	7.0	183.5	360.8	205.1		
3/24/2017	10.6	9.3	478.7	8.5	8.7	334.5	8.0	9.1	344.5	9.9	10.3	548.2	8.3	8.4	304.5	401.3	104.5		
3/27/2017	13.0	10.6	759.6	8.6	8.3	306.1	7.3	7.1	191.4	11.2	10.8	679.3	9.4	9.2	413.7	470.4	242.2		
tumor weights (g)	0.6			0.4			0.2			0.5			0.3						

B



C

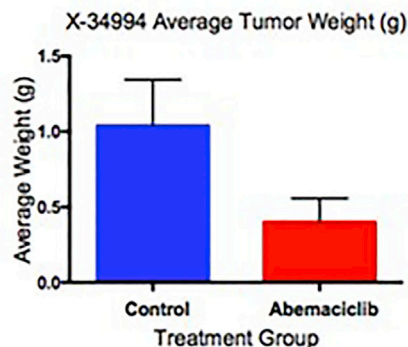


Figure 5. Record of Tumor Measurements during Treatment Study and Analysis

An example of a spreadsheet used to record tumor sizes with a caliper (LXW) during Abemaciclib study (A). At the end of the study a growth curve is generated (B) and average tumor weight is calculated (C).

10 mice were set up for each patient model where 5 mice were treated with abemaciclib and 5 mice were treated with vehicle control. A power calculation indicated that, based on typical tumor volumes and standard deviations, a sample size of 5 mice per group provided 85% power to detect a decrease in tumor volume of at least 50% in the treated group relative to the control group at the end point of the experiment. Note that larger cohorts of mice would be required to detect smaller differences in mean tumor volume between treatment and control groups. Ideally, a power

calculation should be performed to determine the appropriate sample size to detect the desired effect size.

QUANTIFICATION AND STATISTICAL ANALYSIS

1. It is helpful to create a file that can be used to record information and results during the treatment (Figure 5A).
2. The average tumor volume for each time point is calculated and graphs to depict tumor growth are generated (Figure 5B, e.g. we used GraphPad Prism6.)
3. At the endpoint, the weight for each tumor is recorded and the average tumor weight is calculated for each treatment group (Figure 5C).
4. A Student's t-test can be used for statistical analysis between the two groups at individual time points or at end point. For growth curve analysis, which involves longitudinal samples with repeated-measures, a type II ANOVA or (preferably) a mixed model regression analysis should be used.

LIMITATIONS

One limitation of the PDX model system includes the failure for a patient tumor to successfully engraft. This is a common feature across other cancer types as well. Indeed, individual patient samples will vary in their rates of tumor formation (for example, some patient samples engraft at high rates (90 to 100%), some low (10 to 20%), and some in between), however, some will NOT form tumors and cannot be studied further. In addition, due to the nature of NSG mice, some population types in a PDX tumor cannot be examined. For example, *human* tumor stroma cells (ie. immune cells, endothelial cells, fibroblasts) do not survive during xenograft formation. While mouse fibroblasts replace the lost human fibroblasts, NSG mice lack an intact immune system and therefore contribution of immune cells cannot be studied. Moreover, although mouse fibroblasts replace the human fibroblasts in the PDX tumor, cell signaling and protein-protein interactions may be altered due to mouse-human compatibility issues. Lastly, few patient samples produce ulcers and cystic tumors when implanted into mice which affect the viability of tumor cells recovered. These tumors may not be suitable for further studies.

TROUBLESHOOTING

Problem

No tumors formed.

Potential Solution

Ensure that the tumor tissue is fresh and that pieces are cut at 2 x 2 mm. Be sure to cut from the fleshy portion of the tumor. Recognize necrotic areas of the tumor and scrape those areas out.

Problem

Some patient samples will NOT form PDX tumors,

Solution

This is likely due to unknown features of the patient tumor and mouse model system. This is common in other cancer types as well.

Problem

Difficulty dissociating samples.

Solution

Highly keratinized HNSCC samples are more difficult to dissociate and often present with reduced recovery of cell number and quality due to the increased level of debris.

Problem

Low viable tumor cell count after dissociation.

Solution

Be sure to mix the minced tumor and dissociation solution thoroughly and often during the 2 to 3 hours of incubation. This trituration is essential for effective dissociation of the tumor tissue. Also, some tumors will be thoroughly dissociated in 1.5 to 2 hours. Look at the cells under the light microscope to look for single cells. Dissociating tissues too long can decrease overall cell viability.

Problem

Not all mice are engrafting for mouse cohort establishment using injection of bulk cells suspensions.

Solution

For injections of tumor cell suspensions, cell numbers that are below the threshold to initiate a tumor should be taken into account when considering how many mice to inject. Most tumor suspensions will initiate tumors efficiently at cell numbers of 100,000. If not all mice are engrafting in your cohort than try injecting at a higher cell number.

Problem

During injections of tumor cell suspensions, cells can leak out.

Solution

This can happen for a variety of reasons but generally relates to the speed and/or force of injection. If the injection is done too quickly, the Matrigel, which adds structure and keeps cells in close contact, will not have enough time to warm and solidify in the injection site. To avoid this, place needle under the skin, inject the cells and hold for a count of 3 seconds. Turn the needle a quarter of a turn and gently pull the needle out. This should leave a formed bubble under the skin and minimize cells from following the needle out of the injection site. Another precaution to avoid cell leakage is to ensure that the restraint of the mouse during injection is not too tight. Be sure to have enough loose skin on the flank to be able to create a pocket for the cells. When the skin is pulled too tight during injection this will prevent the ability of the cells to form a pocket and the added pressure will cause the cells to leak out around the needle during injection.

Problem

Lymphomas can develop at the engraftment site.

Solution

Characterize the histology of engrafted tumors to rule this out. These are derived from EBV-infected lymphocytes present in the engrafted specimens (Bondarenko et al., 2015; Chen et al., 2012). Lymphomas have a distinct histology, with a small round blue cell appearance. Suspected lymphomas can be confirmed if necessary by pan-cytokeratin (anti-pan Cytokeratin antibody [C11], ab7753, abcam) staining, as these will be cytokeratin-negative, whereas HNSCC tumors will be positive. If necessary, consult a pathologist to confirm engrafted tumors are HNSCC.

Problem

Ulcerations near engraftment site.

Solution

Work with your Animal Care Committee to provide appropriate treatment.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at <https://doi.org/10.1016/j.xpro.2020.100024>.

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AUTHOR CONTRIBUTIONS

C.K. wrote and prepared the manuscript. J.S. contributed to writing Step 1 section, Implantation of Tumor Pieces, performing the implantation in the movie, and editing of the manuscript. L.A. edited and helped in section organization.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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