

# Journal of Visualized Experiments

## Orthotopic Transplantation of Breast Tumors as Preclinical Models for Breast Cancer

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE61173R1
Full Title:	Orthotopic Transplantation of Breast Tumors as Preclinical Models for Breast Cancer
Section/Category:	JoVE Cancer Research
Keywords:	Breast cancer, preclinical model, mammary fat pad transplantation, tumor biology, drug response, biomarker
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Additional Information:	
Question	Response
Please indicate whether this article will be Standard Access or Open Access.	Standard Access (US\$2,400)
Please indicate the <b>city, state/province, and country</b> where this article will be <b>filmed</b> . Please do not use abbreviations.	Houston, TX 77030, U.S.

**TITLE:****Orthotopic Transplantation of Breast Tumors as Preclinical Models for Breast Cancer****AUTHORS AND AFFILIATIONS:**

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**KEYWORDS:**

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**SUMMARY:**

Patient-derived xenograft (PDX) models and transplantable genetically engineered mouse models faithfully recapitulate human disease and are preferred models for basic and translational breast cancer research. Here, a method is described to orthotopically transplant breast tumor fragments into the mammary fat pad to study tumor biology and evaluate drug response.

**ABSTRACT:**

Preclinical models that faithfully recapitulate tumor heterogeneity and therapeutic response are critical for translational breast cancer research. Immortalized cell lines are easy to grow and genetically modify to study molecular mechanisms, yet the selective pressure from cell culture often leads to genetic and epigenetic alterations over time. Patient-derived xenograft (PDX) models faithfully recapitulate the heterogeneity and drug response of human breast tumors. PDX models exhibit a relatively short latency after orthotopic transplantation that facilitates the investigation of breast tumor biology and drug response. The transplantable genetically engineered mouse models allow the study of breast tumor immunity. The current protocol describes the method to orthotopically transplant breast tumor fragments into the mammary fat pad followed by drug treatments. These preclinical models provide valuable approaches to



investigate breast tumor biology, drug response, biomarker discovery and mechanisms of drug resistance.

## **INTRODUCTION:**

Most breast cancer deaths can be ascribed to recurrent disease that is resistant to conventional therapies<sup>1,2</sup>. The inter- and intra-tumor heterogeneity of breast cancers contribute to therapy resistance. Moreover, tumor heterogeneity can impinge on accurate prognosis and challenge disease management<sup>3,4</sup>. Identification of predictive biomarkers of response will significantly improve clinical outcomes of patients with breast cancer. Even though most breast cancer types are immunologically 'cold' tumors that are likely unresponsive to immunotherapy, immune checkpoint inhibitors have shown promise in clinical trials<sup>2,5</sup>. For example, a phase III trial showed improved disease-free survival (DFS) and preliminary evidence that atezolizumab (monoclonal antibody against PD-L1) combined with nab-paclitaxel may provide an overall survival benefit as compared with nab-paclitaxel alone in tumors with  $\geq 1\%$  PD-L1 staining<sup>6</sup>. Development of therapies that sensitize breast tumors to immunotherapy will revolutionize treatment regimens.

Preclinical models that faithfully recapitulate human breast cancer heterogeneity and drug response are critical to study tumor biology and identify potential biomarkers for targeted therapy. Immortalized cell lines are widely used for breast cancer research since these cell lines are easy to grow and genetically modify to study molecular mechanisms. However, due to the selective pressure from long term cell culture in vitro, genetic drift may occur over time and breast cancer cell lines may carry cell line-specific genomic alterations that are distinct from aberrations in primary breast tumors<sup>7-9</sup>.

Patient-derived xenograft (PDX) tumor chunks are able to recapitulate the heterogeneity of human disease, and are histologically and immunohistochemically similar to the tumor of origin<sup>10-29</sup>. Importantly, PDX models are phenotypically stable across multiple transplantations as evidenced by histology, transcriptome, proteome and genomic analysis<sup>10-29</sup>. PDX models show treatment responses comparable to those observed clinically<sup>10-29</sup>. PDX models for estrogen receptor positive (ER<sup>+</sup>), progesterone receptor positive (PR<sup>+</sup>), epidermal growth factor 2 positive (ERBB2<sup>+</sup>, HER2<sup>+</sup>) and triple negative breast cancer (TNBC) PDX models have been established, and provide an excellent platform to test endocrine-, chemo- and targeted therapies. However, one main caveat of PDX models at present is the lack of a functional immune system in the mouse.

The genetically engineered mouse models (GEMM), such as Trp53 homozygous null, cMyc, Wnt1, PyMT, or Her2 overexpression models, allow the study of spontaneous tumor initiation, progression and metastasis in the context of an intact immune system. However, the tumor latency is long, which makes it difficult to conduct preclinical trials with multiple arms<sup>30,31</sup>. However, GEMM can be transplanted to syngeneic hosts to generate sufficient numbers of tumors that closely recapitulate human tumors<sup>32-55</sup>. For example, the mammary epithelium from a p53-null BALB/c mouse was transplanted into the cleared fat pads of syngeneic wild-type recipient mice to form primary tumors, which can be further transplanted into syngeneic hosts<sup>56,57</sup>. The p53-null tumors recapitulated different subtypes of human tumors.

The combination of PDX models and transplantable GEMM provides valuable preclinical tools to investigate breast tumor biology, drug response and anti-tumor immunity. In the current protocol, a method of orthotopic transplantation of PDX and GEMM tumor fragments into the mouse mammary fat pad is described. These models are amenable for serial passages and usually retain a stable phenotype. However, as a precaution to prevent genetic and biological drift we try to avoid multiple serial passages by maintaining the primary tumor bank through replacement of every chunk transplanted with two from the primary outgrowths<sup>29,58</sup>.

## **PROTOCOL:**

All protocols using animals have been reviewed and approved by the Institutional Animal Care and Use Committee (IACUC). The tumor fragments, around 1–2 mm<sup>3</sup> in size, are from viably frozen stock obtained from the Patient-Derived Xenograft and Advanced In Vivo Models Core at Baylor College of Medicine.

### **1. Preparation of cryopreserved mammary tumor fragments for transplantation**

1.1. Transfer the cryovial with tumor fragments from liquid nitrogen to a 37 °C water bath.

1.2. Agitate the cryovial with an occasional gentle flick during thawing.

1.3. After the tissue is thawed, take the cryovial out of water bath and mix by gentle inversion.

1.4. Dry off the outside of the cryovial and spray with 70% ethanol. Transfer it to a biosafety hood.

1.5. Transfer the thawed tumor tissues into a 15 mL conical tube filled with 10 mL of cold Dulbecco's modified Eagle medium (DMEM). Mix well by inverting the tube. Allow the tissue fragments to settle to the bottom of the tube.

1.6. Aspirate the supernatant and resuspend in 10 mL of cold DMEM. Mix well by inverting the tube. Allow the tissue fragments to settle to the bottom of the tube.

1.7. Aspirate the supernatant and resuspend in 10 mL of cold DMEM. Place the tube on ice.

NOTE: The tissue is ready for transplantation.

### **2. Collection and preparation of fresh mammary tumor for transplantation**

2.1. Euthanize the breast tumor-bearing mouse.

NOTE: PDX host may be SCID/Beige, NSG or NRG female mice while in the current study female Balb/c mice aged 3–5 weeks are used.

2.2. Spray the tumor region of the euthanized mouse with 70% ethanol.

NOTE: Try to avoid hair with the tumor sample which might cause contamination of tumor fragments for cryostorage or transplantation.

2.3. With serrated forceps to pinch and lift up the skin surrounding the tumor, use scissors to make a short incision.

2.4. Separate the tumor from the skin with the scissors to dissect the whole tumor from the host mouse. Trim off any remaining mouse fat pad tissue from the outer surface of the tumor. Place the tumor in a 15 mL conical tube filled with 5 mL of cold DMEM.

NOTE: Use tumors at a maximum size of 1 cm diameter since larger tumors are likely to contain necrotic cores.

2.5. In a biosafety hood, transfer the dissected tumor to a sterile 10 cm Petri dish containing enough DMEM to prevent drying.

2.6. Cut the tumor into 1 mm<sup>3</sup> fragments with scalpel or blade under aseptic conditions.

NOTE: The scalpel or blade should be exposed under UV in the biosafety hood for at least 20 min prior to use.

2.7. Transfer the tumor fragments to a 15 mL conical tube filled with cold DMEM. Place the tube on ice.

NOTE: The tissue is ready for transplantation. The dissected tumor fragments from step 2.4 can be, 1) snap frozen in liquid nitrogen for protein and RNA/DNA extraction, 2) fixed with 4% paraformaldehyde (PFA) or 10% neutral buffered formalin (NBF) for hematoxylin and eosin (H&E) and immunohistochemistry (IHC) analysis, or 3) cryopreserved in 1.25 mL freezing medium (10% dimethyl sulfoxide [DMSO], 40% DMEM and 50% fetal bovine serum [FBS]) by slow freezing in a -80 °C freezer overnight and then transferring to liquid nitrogen for long-term storage.

### 3. Prepare animal for surgery

3.1. For animal pain management, subcutaneously inject buprenorphine sustained release 60 min prior to the surgery at a dose of 1 mg/kg or follow institution's guide for 72 h of analgesic coverage.

3.2. Set up the surgical suite according to institutional guidelines for aseptic surgeries.

3.3. Anesthetize a 4-week old female in an induction chamber of the isoflurane anesthesia machine at the rate of ~11.25 mL/h. Transfer the mouse to procedure area, onto the sterile (silicone rubber) surgery board, where it will receive anesthesia through a small facemask. Put

the mouse on its back and tape the legs down in their natural positions.

NOTE: SCID/Beige, NSG or NRG are used for PDX and Balb/c is used for GEMM transplant models.

3.4. Apply ophthalmic ointment to the eyes to prevent dryness.

3.5. Confirm the appropriate level of sedation by toe pinch.

NOTE: No response/movement of the animal indicates that the animal is sufficiently anesthetized and ready for surgery.

3.6. Shave the mouse on the lower abdomen, especially the region around the fourth nipple where the surgery will take place.

3.7. Using a circular motion and starting in the center of the surgery site, work toward the outer edges with povidone-iodine surgical scrub, followed by removal of povidone-iodine with a 70% isopropyl ethanol pad. Repeat two additional times.

#### **4. Transplantation of tumor fragments into the fourth (inguinal) mammary fat pad**

4.1. Use a sterile surgical drape to cover the body of the animal except the incision site.

NOTE: Confirm the appropriate level of sedation by toe pinch before making the incision.

4.2. Using serrated forceps pinch and lift up the skin at the #4 nipple.

4.3. With the blunt side down, use scissors to make a short (about 1 cm), parasagittal incision, from the #4 nipple towards the head.

4.4. Using a cotton tipped applicator, separate the skin from the peritoneum on the medial side of the incision.

4.5. While holding the lateral side of the incision, gently peel the #4 mammary fat pad from the skin using the same swab.

4.6. Once the fat pad is separated, pin down the skin with a 27 G needle close to the animal's body.

4.7. If it is experimentally necessary to clear the fat pad of endogenous mouse mammary epithelium perform the following steps.

218 4.7.1. Using the serrated forceps, gently extend the fat pad away from the body and locate the  
219 inguinal lymph node, which is beneath the intersection points of the major vessels in the gland  
220 (close to where the forceps will be holding the gland).

222 4.7.2. Carefully cauterize the vessels medial to the lymph node and the fat joining the fourth and  
223 fifth fat pads which forms a triangular area.

224  
225 NOTE: Temporarily turn off the oxygen source for this step.

227 4.7.3. Using micro dissecting scissors, cut each cauterized vessel one at a time (to ensure there is  
228 no bleeding after each cut) until the section of the fat pad that contains the lymph node is  
229 removed.

231 4.7.4. Discard the "cleared" fat pad tissue.

233 4.8. Hold the fourth mammary fat pad using blunt separation forceps. With the other hand, insert  
234 the closed tip of the angled fine forceps into the middle of the fat pad above the remaining vessel  
235 and close to the wall of the peritoneum. Slowly open the forceps to make a small pocket. Remove  
236 the angled fine forceps from the fat pad.

238 4.9. Using the angled fine forceps, take a piece of tumor fragment and insert it into the pocket.

240 4.10. Slowly open the forceps tips to release the tumor fragment into the fat pad pocket.

242 4.11. Withdraw the angled fine forceps carefully.

244 NOTE: Look at the pocket to confirm that the tumor fragment remains in the pocket of the  
245 mammary fat pad when withdrawing the forceps. Tumors may be implanted into both  
246 contralateral fat pads when excess tumor tissue is needed for experiments or banking. Animals  
247 with double-sided transplants are not recommended for treatment studies because of known  
248 interactions between contralateral tumors. Alternatively, a trocar device might be used for the  
249 implantation process.

251 4.12. Starting from the base of the incision, collect the skin on each side using the claw and  
252 serrated forceps. Bring the two sides together and lift slightly to prepare the skin for wound clip  
253 application. Uncurl the edges of the incision with the claw forceps and pinch the edges together  
254 to form a continuous surface at the top.

256 4.13. Holding the two sides together with serrated forceps, use the wound clip applicator to place  
257 a wound clip in the center of the incision. If necessary, apply tissue adhesive to the ends of the  
258 incision in order to keep them closed and secured.

260 4.14. Place the animal into a clean cage that is on a warming surface. Monitor for bleeding, signs  
261 of dehiscence, and pain during the postsurgical period. The animal should be up and moving

within minutes postsurgery.

4.15. Pay close attention to the incision site and overall health of the animals for at least 3 days following surgery. Follow institutional guidelines for pain management.

4.16. Clean the surgical tools for 10 s in a glass beads sterilizer before continuing with the next transplant. Wait for the tools to cool down prior to use.

4.17. Repeat steps 4.1–4.15 until all mice are transplanted.

NOTE: Estrogen supplementation is required for ER<sup>+</sup> tumors, which can be supplied through water or slow release pellets<sup>59</sup>.

## 5. Monitoring tumor growth in response to drug treatment

NOTE: Palpable tumors of established transplantable PDXs and p53-null tumors takes between 2 weeks and 8 weeks to develop after surgery, depending on intrinsic tumor growth rates.

5.1. Measure tumors in two dimensions using calipers twice a week. Calculate the volume using the formula:

$$\text{Tumor volume (mm}^3\text{)} = W^2 \times L/2$$

where W is width and L is length of the tumor.

5.2. Start drug treatment when the tumor volume reaches 150–300 mm<sup>3</sup>.

NOTE: Depending on the property of the drugs, oral gavage or intraperitoneal injection may be used to deliver the drugs.

5.3. Collect tumor samples and perform IHC staining<sup>60</sup>, protein and RNA/DNA isolation and immune phenotyping<sup>61</sup> for various purposes. Collect blood and perform immune phenotyping or use it for pharmacokinetic/pharmacodynamic studies.

## REPRESENTATIVE RESULTS:

**Figure 1** shows the equipment (**Figure 1A**) and key procedures (**Figure 1B**) of orthotopic transplantation. **Figure 2** shows characterization of a transplanted PDX tumor (MC1). Tumor fragments (1 mm<sup>3</sup>) of MC1 model were transplanted into the #4 fat pad of SCID/Beige mice. One month later, the average tumor size reached around 350 mm<sup>3</sup>. Tumor volume was monitored twice a week for one month. Normally we obtain palpable tumors for various PDX or GEMM in around 2 weeks to 8 weeks with 1 mm<sup>3</sup> tumor fragments transplanted (**Figure 2A**). Tumor samples can be collected for morphology and signaling analysis (**Figure 2B–D**). H&E staining was performed to analyze the pathology (**Figure 2B**). IHC was used to monitor markers for specific cell lineage (keratin 19 (K19), epithelial cell, **Figure 2C**), cell status (Ki67, proliferation, **Figure 2D**)

or signaling molecule of interest.

#### FIGURE LEGENDS:

**Figure 1: Schematic showing the surgery technique.** (A) Surgical equipment required for the orthotopic transplantation. (B) Representative image showing the exposure of mammary fat pad for tumor trunk transplantation.

**Figure 2: Characterization of the transplanted tumors.** (A) Representative kinetics of tumor growth measured by a caliper. Tumor volume ( $\text{mm}^3$ ) =  $W^2 \times L/2$  ( $W$  = width and  $L$  = length). (B) H&E staining showing pathology of MC1 PDX. IHC showing epithelial marker keratin 19 (C) and proliferation marker Ki67 (D) in MC1 PDX. Scale bar = 20  $\mu\text{m}$ , magnification = 40x.

#### DISCUSSION:

To reduce variations in tumor growth across animals, it is critical to cut the tumor tissue into 1  $\text{mm}^3$  fragments for transplantation. Models that grow soft tissue are harder to work with and the tumor fragments need to be cut slightly larger (1–2  $\text{mm}^3$ ). When placing the tissue into the mammary fat pad pocket take care not to split the tissue into multiple pieces as this will result in multiple small tumors or oddly shaped tumors.

In addition, use fresh tumor for transplanting animals that will be used for drug treatment studies. Implanting tissue from cryopreservation will yield a more variable take rate and slightly slower growth kinetics. Once tumors grow from the cryopreserved material, the second transplant generation will yield the typical take rate and growth kinetics for that model. Moreover, try to use tumors with no or mild necrosis for transplantation. For most models this will be a size range of 400–600  $\text{mm}^3$ . If an obvious necrotic core is observed, use tissue from the outer layer of the tumor for transplantation and do not use the necrotic areas. It is important to keep the tumor tissue on ice and to protect from drying.

To reduce variability among tumor chunks from GEMM that may have been derived from the periphery or tumor core with different microenvironments, an alternative method is to prepare a primary cell suspension and transplant approximately 5,000–30,000 cells depending upon the tumor model into the mammary fat pad. The limited collagenase digestion is carried out with 1  $\text{mg/mL}$  type I collagenase in DMEM/F12 without any additives for 2 h at 37 °C rotating at 125 rpm. Mammary tumor cells can be enriched by 3 short centrifugation steps. Briefly, transfer the cell suspension to a 15 mL conical tube and centrifuge at 450  $\times g$  for 7 s. Aspirate the supernatant and resuspend the pellet in 10 mL of 1x Dulbecco's phosphate buffered saline (DPBS). Repeat the pulse centrifugation for two more times. This will help randomize differences between chunks.

Transplantation of normal mammary epithelium will not regenerate a morphologically normal and functional ductal tree in the presence of endogenous mouse epithelium. It is necessary to remove the endogenous mouse epithelium (clearing) for the normal epithelial transplant to grow<sup>62</sup>. However, neoplastic tissue is able to grow even in the presence of intact endogenous mouse epithelium. Yet this does not necessarily mean no such inhibitory signals exist. Mammary fat pad clearing is necessary for certain experimental protocols to prohibit the interaction of

endogenous mouse mammary epithelium with the engrafted material. In addition, the endogenous epithelium may complicate some downstream analysis such as genome, transcriptome and proteome analysis.

The PDX model and transplantable GEMM can faithfully recapitulate the heterogeneity of clinical subtypes and the response to drug therapy of human breast cancer. Importantly, these models are easy to transplant and maintain a stable phenotype during a limited number of serial passages. Tumor growth can be easily measured with calipers. One caveat of the PDX model and transplantable GEMM is that these models do not recapitulate early steps of tumor initiation. Also, PDX models lack the interaction of the tumor with a functional immune system. These preclinical models provide a valuable system to study breast cancer biology and evaluate drug response. Combining drug response with the genomic and proteomic information for each tumor model will facilitate the identification of biomarkers for response prediction and treatment resistance mechanisms. These types of data may lead to novel targeted therapies that could be used alone and in combination with chemotherapy or immunotherapy to improve patient outcomes.

#### **ACKNOWLEDGMENTS:**

This work was supported by the National Institutes of Health (R37CA228304 and R01HL146642 to Xi Chen, CA148761 to Jeffrey M. Rosen), DOD/CDMRP (CA180759 to Xi Chen, W81XWH-19-1-0035 to Xiangdong Lv) and Cancer Prevention and Research Institute of Texas (RR150009 CPRIT scholar award to Xi Chen), the Patient-Derived Xenograft and Advanced In Vivo Models Core at Baylor College of Medicine (funding from RP170691 CPRIT Core Facility Award and NCI-CA125123 P30 Cancer Center Support Grant).

#### **DISCLOSURES:**

MTL is the founder of a limited partner in StemMed, Ltd. and founder and manager in StemMed Holdings, its general partner. MTL is a founder of and an equity holder in Tvardi Therapeutics, Inc. LED is a compensated employee of StemMed, Ltd.

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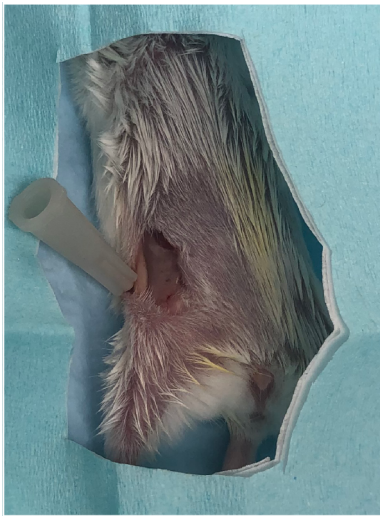
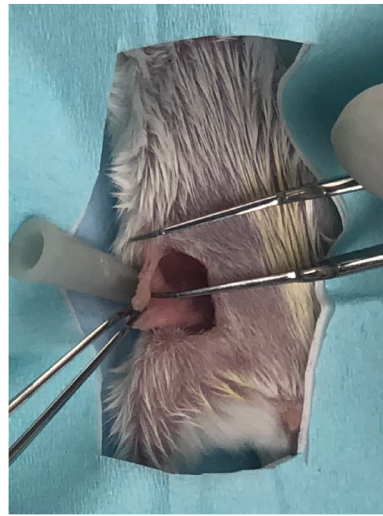
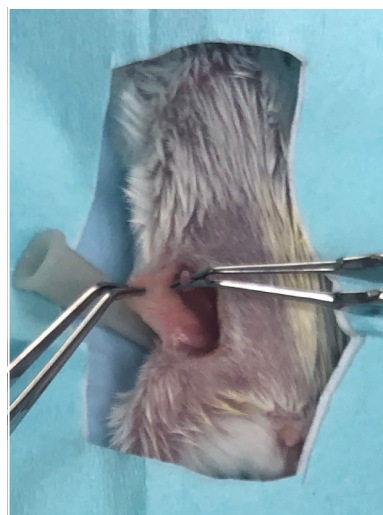
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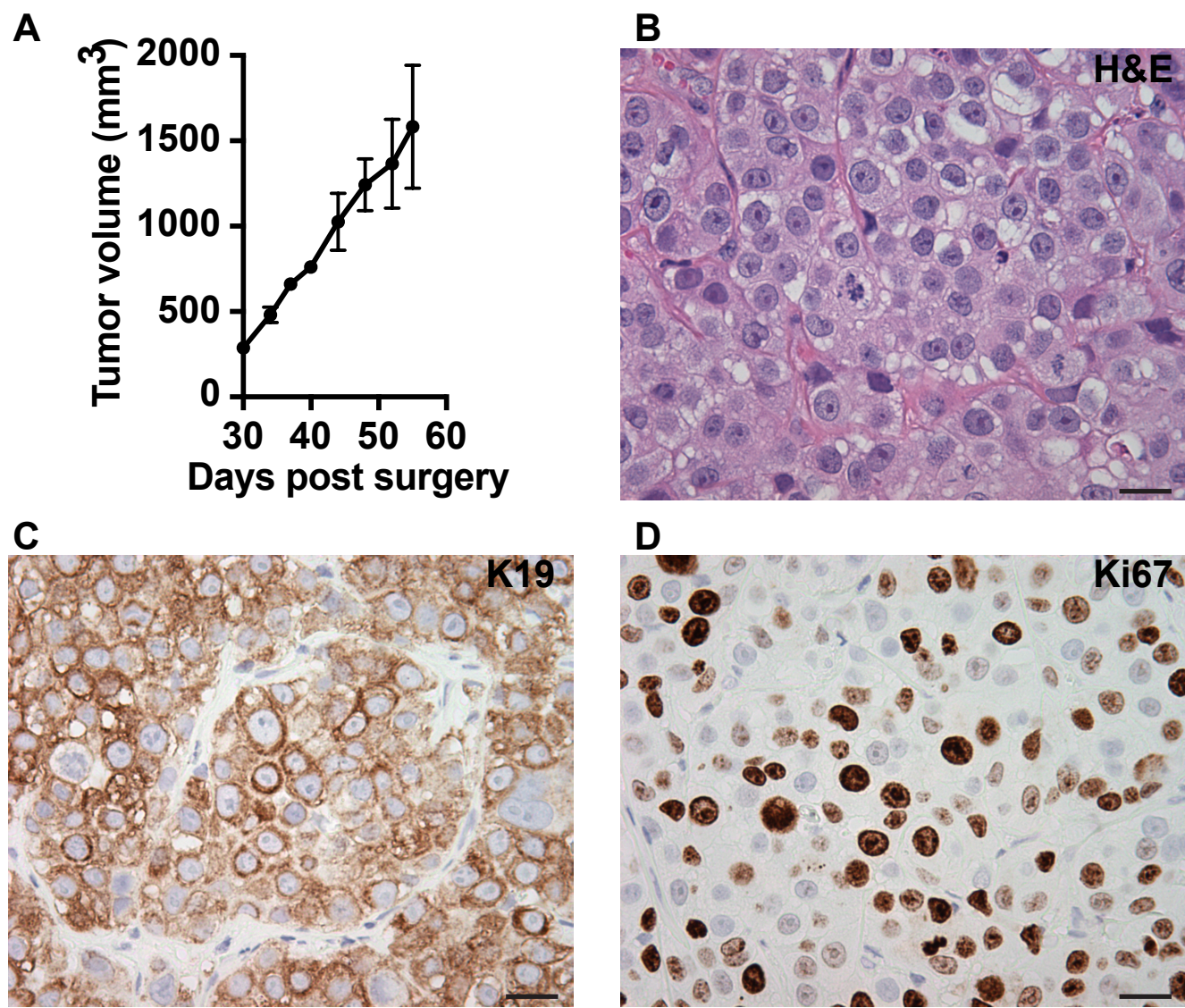
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530 mice. *Cancer Research*. **19** (5), 515 (1959).

**A****B****Expose the fat pad****Make a pocket****Close the incision****Insert tumor fragment**





[illegible]

## Comments/Description

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## Editorial comments:

Changes to be made by the author(s) regarding the manuscript:

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.

**Response:** Thanks. We have thoroughly proofread the manuscript before submission. The changes made during the revision are marked in red color.

2. Please revise lines 167-169, 249-251, to avoid previously published text.

**Response:** We have revised this part as suggested. Please find the revisions below:

For lines 167-169: With the other hand, insert the closed tip of the angled fine forceps into the middle of the fat pad. Slowly open the forceps slightly to stretch a pocket. Remove the angled fine forceps from the fat pad.

For lines 249-251: It is necessary to remove the endogenous mouse epithelium (clearing) for the normal epithelial transplant to grow<sup>58</sup>. However, neoplastic tissue is able to grow even in the presence of intact endogenous mouse epithelium. Yet this does not necessarily mean no such inhibitory signals exist.

3. Please revise the Protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

**Response:** We have revised our manuscript as suggested.

4. 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, dashes, or indentations.

**Response:** We have adjusted the numbering.

5. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.

6. Line 90: Please specify the source and size of tumor fragments.

**Response:** We have added the source and size of tumor fragments in the manuscript. 'The tumor fragments, around 1-2 mm<sup>3</sup> in size, are from viably frozen

stock obtained from the Patient-derived Xenograft and Advanced In Vivo Models Core at Baylor College of Medicine.'

7. Line 104: Please specify the tumor type as well as the age, gender and type of mouse. Please describe how to dissect the tumor from the host mouse.

**Response:** We have added the information into our revised manuscript as described below:

2.1 Euthanize the breast tumor-bearing mouse.

NOTE: PDX host mouse may be SCID/Beige, NSG or NRG female mice while GEMM host mouse strain is Balb/c in the current study. Female mice age 3-5 weeks are used.

2.2 Use 70% ethanol to spray tumor region of the euthanized mouse.

NOTE: Try to avoid hair with the tumor sample which might cause contamination of tumor fragments for cryostorage or transplantation.

2.3 With serrated forceps to pinch and lift up the skin surrounding the tumor, use scissors to make a short incision.

2.4 Separate the tumor from the skin with the scissors to dissect the whole tumor from the host mouse. Trim off any remaining mouse fat pad tissue from the outer surface of the tumor. Place the tumor in 15 mL conical tube filled with 5 mL cold DMEM.

NOTE: Use tumors at a maximum size of 1 cm diameter since larger tumors are likely to contain necrotic cores.

8. Line 126: Please specify the strain of mouse used and concentration of isoflurane.

**Response:** The strain of mouse used was described in our original submission. We have added the concentration of isoflurane into our revised manuscript.

'Anesthetize a 4-week old female in an induction chamber of the isoflurane anesthesia machine at the rate of approximate 11.25mL/hour'.

9. Line 200: Please specify the drug treatment given in this study.

**Response:** We did not show specific drug treatment in this study. Thanks.

10. Lines 203-205: Please describe how to perform IHC staining, immune phenotyping, etc. or provide relevant references here.

**Response:** We add relevant references for IHC staining and immune phenotyping in our revision. Thanks.

11. After you have made all the recommended changes to your protocol (listed above), 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.

**Response:** We have highlighted essential steps of the protocol in green for the video.

12. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Notes cannot usually be filmed and should be excluded from the highlighting. Please do not highlight any steps describing anesthetization and euthanasia.

**Response:** We have highlighted essential steps of the protocol in green for the video.

13. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

**Response:** We have highlighted essential steps of the protocol in green for the video.

14. Figure 2: Please include a scale bar at the lower right corner for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate figure Legend. Please define error bars in the figure legend.

**Response:** We have added a scale bar at the lower right corner. The magnification is 40X, which has been added into the figure legend.

15. In-text citations: Please remove the square brackets enclosing the reference numbers. The corresponding number from the reference list must appear superscripted without a space after the word/group of words it applies to but before any punctuation.

16. 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.

Please do not abbreviate journal titles. See the example below:

Bedford, C.D., Harris, R.N., Howd, R.A., Goff, D.A., Koolpe, G.A. Quaternary salts of 2-[(hydroxyimino)methyl]imidazole. Journal of Medicinal Chemistry. 32 (2), 493-503 (1998).

**Response:** We have updated the reference as requested. Thanks.

### Reviewers' comments:

#### Reviewer #1:

##### Manuscript Summary:

This article describes the detailed transplantation of cryofrozen or orthotopic fragments of breast tumor tissue (human or mouse) into the mammary fat pads of recipient mice. PDX and GEMM models are important tools for drug testing and discovery that continue to increase in the share of in vivo breast cancer experiments. Although a few protocols exist, these are >5 years old, and many labs are still inexperienced in the transplant techniques and updates are needed. The article/protocol is well written with easy to follow steps. Below are a few comments/suggestions that may help clarify a few points for the readers/users.

**Response:** We thank the reviewer #1 for the positive feedback to our manuscript.

##### Minor Concerns:

1. Introduction paragraph lines 62-67. A statement on the particular importance of PDX for ER+ breast cancer and endocrine drug testing would be useful - particularly since it is pointed out in the next paragraph that only the p53-null tumors contain a subset of ER+ tumors adequate for antiendocrine testing.

**Response:** We thank the reviewer for the suggestions. We have added such statement in the introduction part. As detailed below: PDX models for estrogen receptor positive (ER<sup>+</sup>), progesterone receptor positive (PR<sup>+</sup>), epidermal growth factor 2 positive (ERBB2<sup>+</sup>, HER2<sup>+</sup>) and triple negative breast cancer (TNBC) PDX models have been established, which will offer a good platform to test endocrine therapy, chemotherapy and targeted therapy.

2. Protocol part 2 step 1. It might be useful to mention avoiding hair with the tumor sample - this has been a cause of contamination of tumor fragments for cryostorage or transplantation. This can be done by shaving prior to tumor removal and/or dipping the euthanized animal in an antiseptic solution.

**Response:** Thanks. We have added this as a NOTE to step 2.2. As detailed below:

2.2 Use 70% ethanol to spray tumor region of the euthanized mouse.

NOTE: Try to avoid hair with the tumor sample which might cause contamination of tumor fragments for cryostorage or transplantation.

3. Part 4 transplantation, it could be mentioned that an alternative method to implantation is the use of a trochar device (unsure if this is mentioned in other protocols).

**Response:** Thanks for the alternative method for implantation. We have added this as a NOTE to step 4.11. As detailed below:

NOTE: Alternatively, a trochar device might be used for the implantation process.

4. Part 4 transplantation. It should be mentioned that if these are ER+ tumors, most of them will require estrogen supplementation, through water, slow release pellets, etc (references could be given in lieu of describing the methods).

**Response:** We have add this critical information as a NOTE to step 4.17 with reference cited. As detailed below:

NOTE: Estrogen supplementation is required for ER<sup>+</sup> tumors, which can be supplied through water or slow release pellets<sup>59</sup>.

5. Table of materials, since cryostorage is mentioned several times I would add the preferred storage solution by this group (it varies group to group and there are some commercial agents that are quite good i.e. Cryostor).

**Response:** We have add the freezing medium as a NOTE to step 2.10. In brief, the freezing medium contains 10% DMSO, 40% DMEM and 50% FBS.

## **Reviewer #2:**

Manuscript Summary:  
well presented

Major Concerns:  
none

Minor Concerns:

only one concern : I am not sure that serial passages are feasible with this methodology. This is important with regard to the stability and reproducibility of the models. Please expand this issue in the manuscript.

**Response:** Thanks. This is an important question. This methodology is good for serial passages. Importantly, the PDX and transplantable GEMM maintain a stable

phenotype after ten to fifteen passages, as evidence by histology, transcriptome, proteome and genomic analysis. We included this information in the introduction and discussion part of the revision.