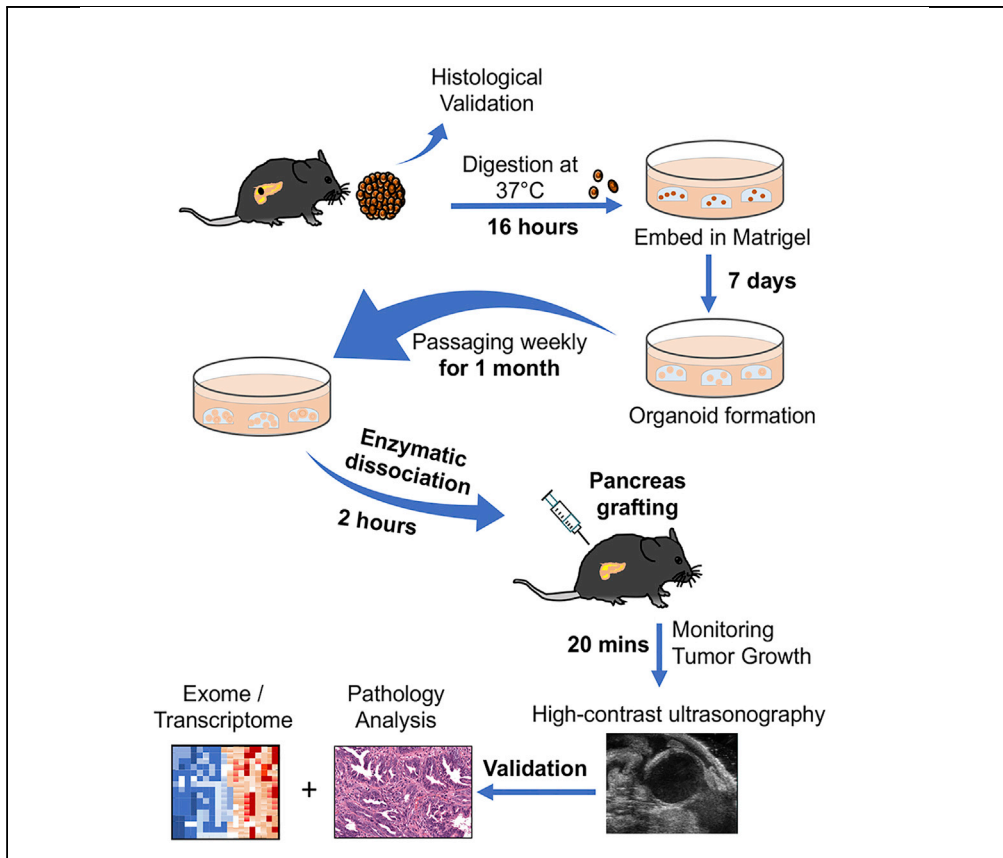


## Protocol

# Generation of Pancreatic Organoid-Derived Isografts



Orthotopic isografts of mouse PDA cell lines have long been used to model tumor progression albeit showing limited similarity to human tumor biology. D'Agosto and colleagues describe a procedure for the generation of organoid-derived isografts that evolve through discrete stages of disease and permit investigation of the biological determinants of progression.

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### HIGHLIGHTS

A procedure for the generation of orthotopic isografts using mouse PDA organoids

Organoid-derived isografts (ODIs) recapitulate progressive stages of PDA

ODI models allow investigation of the biological determinants of human PDA progression

D'Agosto et al., STAR  
Protocols 1, 100047  
September 18, 2020 © 2020  
The Author(s).  
<https://doi.org/10.1016/j.xpro.2020.100047>



## Protocol

## Generation of Pancreatic Organoid-Derived Isografts

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<https://doi.org/10.1016/j.xpro.2020.100047>

## SUMMARY

This protocol is a procedure for generating orthotopic isografts using mouse pancreatic cancer organoids. These isografts can be used to track the evolution of pancreatic ductal adenocarcinoma (PDA) from a preinvasive lesion to a metastatic disease and therefore represent a suitable model for identification of determinants of PDA progression.

For complete details on the use and execution of this protocol, please refer to Boj et al. (2015) and Filippini et al. (2019).

## BEFORE YOU BEGIN

Prepare all solutions before sample processing, following the recipes in the [Materials and Equipment](#) section. Some solutions can be prepared in advance and stored as indicated. Refer to the [Key Resources Table](#) for a complete list of materials and equipment.

## KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, Peptides, and Recombinant Proteins		
Advanced DMEM/F12	Gibco	Cat# 12634-028
DMEM	Gibco	Cat# 41965-062
Glutamax	Gibco	Cat# 35050-061
HEPES	Gibco	Cat# 15630-080
Penicillin/ Streptomycin	Gibco	Cat# 15140-122
Dulbecco's PBS, no calcium, no magnesium (DPBS)	Gibco	Cat# 14190-144
TrypLE Express Phenol Red	Gibco	Cat# 12605-028
DNase I	Sigma-Aldrich	Cat# D5025-150KU
Cultrex HA-R-Spondin I-Fc-293Tcell line	Trevigen	Cat# 3710-001-K
Mouse recombinant EGF (mEGF)	Gibco	Cat# PMG8043
Human Fibroblast Growth Factor 10 (hFGF10)	PeproTech	Cat# 100-26
Murine Noggin (mNoggin)	PeproTech	Cat# 250-38
Rho Kinase Inhibitor (RhoKi) Y-27632	Sigma-Aldrich	Cat# Y0503
Fetal Bovine Albumin (FBS)	Gibco	Cat# 26140079
30% Bovine Serum Albumin Solution (BSA)	Sigma-Aldrich	Cat# A9576

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**Continued**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Collagenase type XI	Sigma-Aldrich	Cat# C9407
Dispase	Gibco	Cat# 17105-041
DMSO	Sigma-Aldrich	Cat# D2650
N-Acetyl Cysteine	Sigma-Aldrich	Cat# A9165
Nicotinamide	Sigma-Aldrich	Cat# N0636
A83-01	Tocris	Cat# 2939
B27 supplement	Gibco	Cat# 17504-044
Human Gastrin I	Tocris	Cat# 3006
Matrigel (growth factor-reduced, phenol-red free)	Corning	Cat# 356231
Trypan Blue	Thermo Scientific	Cat# T10282
10% formalin solution	Mondial	Cat# FM0622
70% alcohol	Diapath	Cat# A0193
<b>Experimental Models: Organisms/Strains</b>		
Mouse: KPC ( $Kras^{+/LSL-G12D}$ ; $Trp53^{+/LSL-R172H}$ ; $Pdx1-Cre$ )	n/a	n/a
Mouse: C57BL/6J (B6J)	Charles river	Cat# B6JSIFE07SZ
<b>Other</b>		
37°C water bath	EuroClone	Cat# ENB-301
Refrigerated 1.5 mL tube centrifuge	Thermo Fisher Scientific	MICROCL 21R
Refrigerated 15 mL tube centrifuge	Thermo Fisher Scientific	IEC CL31R Mutlispeed Centrifuge
Ice bucket	BioCision	BCS-117PL
Digital Timer	Thermo Fisher Scientific	Cat# 1464917
Sterile 10 $\mu$ L pipette tips with filter	Corning	Cat# 4135
Sterile 20 $\mu$ L pipette tips with filter	Corning	Cat# 4136
Sterile 200 $\mu$ L pipette tips with filter	Corning	Cat# 4138
Sterile 1000 $\mu$ L pipette tips with filter	Corning	Cat# 4140
Pipetman (P10, P20, P200, P1000)	Gilson, or others	n/a
1.5 mL Eppendorf tube	Eppendorf	Cat# 286730000
15 mL conical tube	Falcon	Cat# F2097
50 mL conical tube	Falcon	Cat# F2070
250 mL sterile PETG Bottle	Corning	Cat# 431732
24-well culture plate	Greiner	Cat# 662102
6-well culture plate	Sarstedt	Cat# 833920500
37°C tissue culture incubator	Thermo Fisher Scientific	HERA Cell 150
Sterile 5 mL serological pipettes	Falcon	Cat# F7543
Sterile 10 mL serological pipettes	Falcon	Cat# F7551
Sterile 25 mL serological pipettes	Falcon	Cat# F7525
Pipette aid	Corning	Cat# 4099
CryoPure tube	Sarstedt	Cat# 72.377
Hemocytometer	Marienfeld	Cat# 0640011
Ruler	Staedtler	Cat# 562 04-30F
Laboratory Balance	Sartorius	Cat# BCE223-1S
Cell-Gro Tissue Culture Rotator Drums	Thermo Fisher Scientific	Cat# 1647Q
Dish cell culture treated	Corning	Cat# 430167

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### Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Scalpels	Swann Morton	Cat# 0501
Herasafe™ KS (NSF) Class II, Type A2 Biological Safety Cabinets	Thermo Fisher Scientific	Cat# KS12
Inverted Laboratory microscope	Leica	DMIL LED
ActivFlo Routine I Cassettes	Leica	Cat# 39LC-500-1-L
Pencil	STAEDTLER	Cat# 100-HB
Rodent shaver	2biological instruments	Cat# 2BTOSRC
Depilatory cream	Veet	
Dissecting scissors, curved	World precision instruments	Cat# 14394-G
Iris forceps, curved	World precision instruments	Cat# 15915
BD Micro-Fine, 0.5 mL insulin syringes (30G)	Becton Dickinson	Cat# 320927
Applicator Cotton Tip	Dealmed	Cat# 782010
VetSuture FastPGA	Vetsuture	Cat# FPGLA1CN
Reflex 7 mm Clip	2biological instruments	Cat# 59034
Autoclip applier	2biological instruments	Cat# 59036
Clip remover	2biological instruments	Cat# 59037
High-resolution micro-ultrasound imaging system- Vevo 2100	Visual sonics	n/a
Aquasonic gel	Parker laboratories	Cat# 30592037
BD Plastipak syringes 1 mL	Becton Dickinson	Cat# 303174

## RESOURCE AVAILABILITY

### Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Vincenzo Corbo ([vincenzo.corbo@univr.it](mailto:vincenzo.corbo@univr.it)).

### Materials Availability

This study did not generate new unique reagents.

### Data and Code Availability

This study did not generate datasets.

## MATERIALS AND EQUIPMENT

### Wash Medium

Reagent	Volume (mL)
DMEM	490
FBS	5
100× Penicillin/Streptomycin	5

Store at 4°C for up to 1 month.

### Digestion Solution

Reagent	Amount	Final Concentration
Wash Medium	50 mL	n/a
Dispase	6.25 mg	0.125 mg/mL
Collagenase XI	6.25 mg	0.125 mg/mL

**Note:** This solution should be freshly prepared and used immediately.

Make the digestion solution step by step as follows:

1. Take Dispase and Collagenase out of the fridge and allow the container to reach room temperature (20–25°C).
2. Weigh Dispase carefully and pour into a 50 mL conical centrifuge tube.
3. Weigh Collagenase carefully and pour into the same 50 mL conical centrifuge tube.
4. In a biological hood, add 50 mL of Wash Medium to the 50 mL conical tube containing Dispase and Collagenase. Mix by inverting the tube.
5. Place Digestion solution in a 37°C water bath for 20 min to warm up before use.

### Splitting Medium

Reagent	Volume (mL)
Advanced DMEM/F12	485
100× HEPES	5
100× Penicillin/Streptomycin	5
100× GlutaMax	5

Store at 4°C for up to 1 month.

### Dispase Solution

Reagent	Amount	Final Concentration
Splitting medium	50 mL	n/a
Dispase	100 mg	2 mg/mL

**Note:** Used for generation of single cells from organoids.

**Note:** Prepare this fresh before use.

Make the dispase solution step by step as follows:

1. Take Dispase out of the fridge and allow the container to reach room temperature (20–25°C).
2. Weigh Dispase carefully and pour into a 50 mL conical centrifuge tube.

3. In a biological hood, add 50 mL of Splitting medium to the 50 mL conical tube containing Disperse. Mix by inverting the tube.
4. Place Disperse solution in a 37°C water bath for 20 min to warm up before use.

### Mouse Complete Medium

1. Thaw B-27 supplement, N-acetylcysteine, Nicotinamide, mEGF, A83-01, hFGF10 and mNoggin on ice in an ice bucket.
2. Place the bottles containing Splitting medium and R-spondin1 conditioned medium on ice.
3. To make 100 mL Mouse Complete medium, add the following into a 250 mL sterile PETG bottle.

Reagent	Stock Concentration	Final Concentration	Volume (mL)
Splitting medium	NA	NA	86.34
R-spondin1 conditioned medium <sup>a</sup>	10×	1×	10
B27 supplement	50×	1×	2
Nicotinamide	1 M	10 mM	1
N-acetylcysteine	500 mM	1.25 mM	0.25
mEGF	50 µg/mL	50 ng/mL	0.10
hFGF10	100 µg/mL	100 ng/mL	0.10
Gastrin I	100 µM	10 nM	0.01
mNoggin	100 µg/mL	100 ng/mL	0.10
A83-01	25 mM	500 nM	0.002
RhoKi <sup>b</sup>	10.5 mM	10.5 µM	0.10

Store at 4°C for 2 weeks.

<sup>a</sup>Conditioned medium from R-spondin1 expressing 293T cell line.

<sup>b</sup>RhoKi is added to the Mouse complete medium only for initiation of the culture (i.e., initial seeding of the dissociated tissue and first medium refreshing), but not for culture maintenance.

### DNase I Solution

Prepare a 10 mg/mL solution in sterile DPBS, aliquot and store at –20°C.

## STEP-BY-STEP METHOD DETAILS

### Establishment of Mouse Pancreatic Tumor Organoid Culture

⌚ Timing: 24 h

Mouse pancreatic tumor cells are isolated from the tumor bulk of KPC mice ( $Kras^{+/LSL-G12D}$ ;  $Trp53^{+/LSL-R172H}$ ;  $Pdx1-Cre$ ) (Hingorani et al., 2005). The KPC mouse model develops PDA with 100% penetrance, yet the development of tumors is not synchronous, and the presence of overt carcinoma should always be confirmed by histological analysis. As a general rule, pancreas tissue is collected from mice (between 2.5 and 3 months of age), which show a solid lesion by ultrasound imaging (for details of ultrasound imaging see section “Ultrasound imaging”).

1. For tissue collection, euthanize the mice using an approved method, and then dissect out the tumor rather than the entire pancreas. The tumor mass will appear as a white and stiff lesion as opposed to the soft and well vascularized normal pancreatic tissue.
2. In a petri dish, cut the tumor tissue in halves with a scalpel.

3. Place one half of the dissected tumor in a pre-labeled histological cassette and then move the cassette in a container of 10% formalin to fix for histology.
4. Place the other half of the dissected tumor in a 50 mL conical centrifuge tube containing 5 mL of Spitting medium. Keep the tissue on ice until ready for processing.

**Pause Point:** Mouse tumor tissue can be stored at 4°C for up to 48 h in Spitting medium to which FBS has been added to the final concentration of 1%.

5. Warm up 24-well cell culture plate(s) for 12–14 h in the 37°C incubator.
6. Place Digestion solution in a 37°C water bath to warm up; move frozen Matrigel aliquots on ice to thaw.

**Note:** Matrigel should be stored in a –20°C freezer. Allow enough time for your Matrigel aliquot to thaw on ice before use. An 1 mL aliquot of frozen Matrigel takes ~1 h to thaw on ice, and a full vial takes ~8 h.

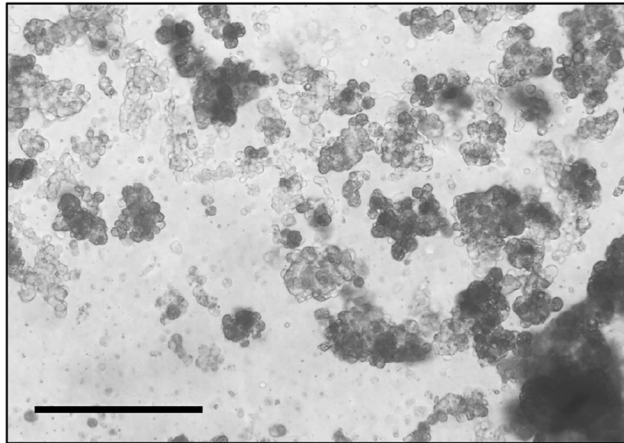
7. Transfer the tissue to a petri dish. Examine the tissue macroscopically and record the shape, size, weight, and morphological characteristics such as the amount of fat and/or necrosis content.
8. Mince tissue into pieces of 1 mm<sup>3</sup> using sterile scalpels.
9. Transfer the minced tissue to a 15 mL conical centrifuge tube and add 10 mL of ice-cold wash medium.
10. Let the tube stand at 20–25°C for 1–2 min. If present, fat will rise to the top and float on the surface, while pancreas tissue material will sink.
11. Remove and discard the medium containing fat and leave ~1 mL of Wash medium with pancreas pieces. Be careful not to aspirate pancreas pieces.
12. Add 9 mL of pre-warmed digestion solution.
13. Place the tube on a rotator at 37°C for an initial digestion of 4 h and for a maximum of 16 h.
14. Check the digestion by sampling 10 µL of the digestion solution and spotting it in a petri dish. Observe the material under an inverted microscope and if several clusters of cells are visible ([Figure 1](#)), stop the reaction by placing the tube on ice and adding 5 mL of ice-cold Wash medium.

**Note:** If clusters of cells are not visible, continue with digestion and check every hour (when possible).

15. Centrifuge for 5 min at 200 × g at 4°C.
16. Discard the supernatant and add 10 mL of ice-cold Wash medium.
17. Centrifuge for 5 min at 200 × g at 4°C.
18. Remove the supernatant leaving the pellet behind, add 1.5 mL TrypLE supplemented with 15 µL DNase I (10 mg/mL) to the pellet, pipette up and down with 1 mL pipette tip to break large clumps, and incubate in a 37°C water bath for 5 min (no more than 10 min).

**Note:** Every 5 min, pipette up and down with 1 mL pipette tip to break large clumps.

19. Pipette the suspension with 1 mL pipette tip for 10 times to ensure that large clumps are broken down.
20. Check the status of the enzymatic digestion by sampling 10 µL of the suspension and spotting it in a petri dish. Observe the material under an inverted microscope and, if several clusters of cells are visible ([Figure 2](#)), stop the reaction adding 10 mL of ice-cold Wash medium.
21. Spin down at 200 × g for 5 min at 4°C. Without disturbing the pellet, carefully remove the supernatant.



**Figure 1. Digestion of Mouse Pancreatic Tumor Tissue with a Mixture of Collagenase/Dispase**  
An ideal digestion of mouse pancreatic tumor tissue. Scale bar, 100  $\mu$ m.

22. Resuspend the pellet in Matrigel. Usually, the pellet from 10 mg mouse tumor tissue is resuspended in about 200  $\mu$ L of Matrigel.
23. Spot 50  $\mu$ L Matrigel dome for each well of a 24-well plate.

**Note:** The volumes described are applicable for 24-well plate. Scale-up accordingly if using different culture conditions, as indicated in the table below.

Culture Dish	No of Matrigel Domes/Well	Matrigel Volume/Dome	Culture Media/Well
24-well plate	1	50 $\mu$ L	0.5 mL
6-well plate	8	50 $\mu$ L	3 mL

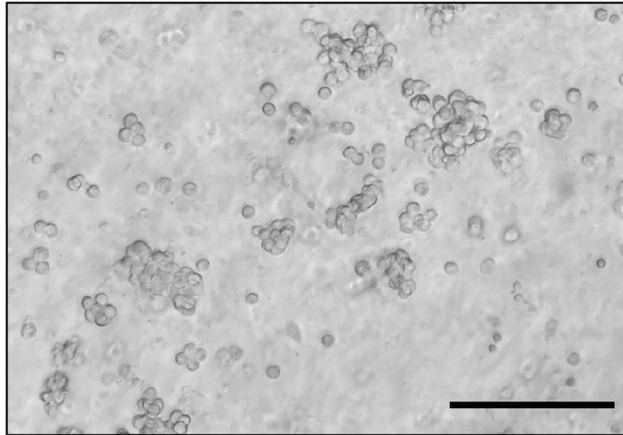
24. Carefully move the plate into a 37°C tissue culture incubator until Matrigel solidifies (~15 min).
25. Add Rho Kinase Inhibitor (RhoKi, 10.5 mM stock solution) to mouse complete medium to a final concentration of 10.5  $\mu$ M.
26. Bring the plate back to the hood and add 500  $\mu$ L of pre-warmed Mouse complete medium supplemented with RhoKi to each well of the 24-well plate containing Matrigel domes. Carefully move the 24-well plate to the 37°C cell culture incubator.

### Post Derivation

⌚ Timing: 24–72 h

27. 24 and 48 h after plating, observe the Matrigel domes under the microscope and take images using 4 $\times$  and/or 10 $\times$  objectives. Organoids should start forming within 1–2 days from isolation.
28. Make sure to complete histological analyses within few days using the tissue that has been fixed for histology. Tumor histology must be confirmed on tissue section.
29. Mouse complete medium is usually refreshed every 3 days after seeding, and cultures are passaged when:
  - a. obtaining a dense culture (for example, see [Figure 3](#))
  - b. obtaining large organoid structures (more than 20% of the diameter of a 50  $\mu$ L Matrigel dome)
  - c. Matrigel softens and the dome tends to detach from the plate





**Figure 2. Example of Ideal Cell Clusters Post Enzymatic Digestion**

Scale bar, 100  $\mu\text{m}$ .

**Note:** RhoKi is added to the mouse complete medium only for culture initiation, i.e. when plating the dissociated tissue and for the first refreshing of the medium. There is no benefit for maintenance of the culture by adding RhoKi to the mouse complete medium.

## Passaging

⌚ Timing: 45 min

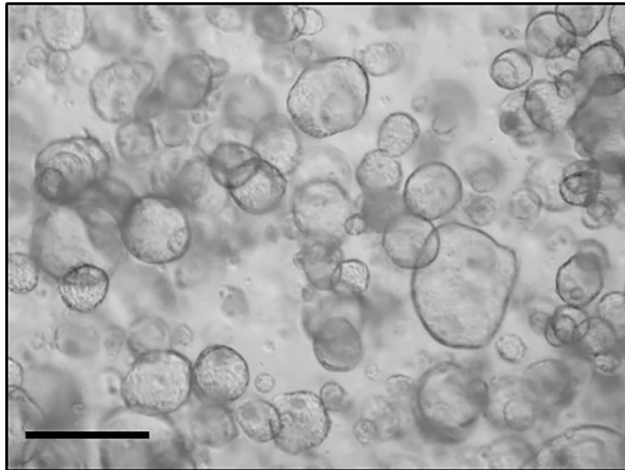
Passaging is performed with a split ratio of 1:4 to 1:8, every week depending how fast the organoids grow.

In order to check for a possible contamination from KRAS wild type cells, standard protocols can be used to assess for the presence of non-cancerous cells (see section “[Troubleshooting](#)”). For culture expansion, we suggest using 6-well plates as collection and passaging of organoids is easier and less time consuming as compared to the use of smaller vessels (e.g., 24-well plates). However, the procedure described below is the same regardless the vessel size.

30. Place Mouse complete medium in a 37°C water bath to warm it up; move frozen Matrigel aliquots on ice to thaw.
31. Aspirate medium from each well and add 3 mL of ice-cold Splitting medium to each well of the 6-well plate.
32. Pipette the liquid up and down until the Matrigel falls apart and the solution is homogeneous.
33. Take the mix of organoids, Matrigel, and Splitting medium and move the solution to a 15 mL conical centrifuge tube previously filled with 6 mL of ice-cold Splitting medium.
34. Rinse each well of a 6-well plate with 1 mL of ice-cold Splitting medium to collect any leftover and transfer it into the 15 mL conical centrifuge tube (total volume at this point should be 10 mL).

**Note:** Repeat this procedure for the remaining wells. Harvest up to 8 Matrigel domes per 15 mL conical centrifuge tube.

35. Centrifuge the tube at 100  $\times$  g for 5 min at 4°C.
36. Aspirate the supernatant until 1.5 mL of volume and add 1 mL of ice-cold Splitting medium.
37. Pipette up and down with a sterile fire-polished pipette and resuspend the pellet  $\sim$ 10 times to shear the organoids.



**Figure 3. Example of a Dense Organoid Culture (~80% Confluency)**

An ideal organoid culture ready to be split. Scale bar, 500  $\mu\text{m}$ .

38. Add Splitting medium to bring the volume to 10 mL and invert the tube a couple of times to resuspend the solution.
39. Spin down at  $100 \times g$  for 5 min at  $4^{\circ}\text{C}$ . Without disturbing the pellet, carefully remove the supernatant.
40. Resuspend the pellet in Matrigel. The volume of Matrigel depends on the density of the organoids and the chosen split ratio. As an example, if starting from two wells of a 6-well plate (16 individual domes) and the required split ratio is 1:4, then add 3.2 mL of Matrigel to the organoid pellet.

**Note:** Each dome requires 50  $\mu\text{L}$  Matrigel, so when splitting with a 1:4 ratio (from 16 domes to 64 domes), the total volume of Matrigel required is  $50 \mu\text{L} \times 64 = 3,200 \mu\text{L}$  (3.2 mL)

41. Spot up to 8 individual Matrigel domes of 50  $\mu\text{L}$  into each well of a pre-warmed 6-well plate.
42. Carefully place the plate into a  $37^{\circ}\text{C}$  tissue culture incubator until Matrigel solidifies (typically 15 min).
43. Bring the plate back to the hood and add 3 mL of pre-warmed Mouse complete medium to each well of the 6-well plate containing Matrigel domes.
44. Carefully move the 6-well plate to the  $37^{\circ}\text{C}$  cell culture incubator.

**Note:** Mouse complete medium is usually refreshed once a week after passaging.

### Mouse Pancreatic Tumor Organoids Dissociation

⌚ Timing: 2 h

For transplantation of tumor organoids into the pancreas of a recipient mouse, a suspension containing 1,000,000 single cells is prepared starting from 8 domes in one well (400  $\mu\text{L}$  of Matrigel, one well of a 6-well plate), each having ~80% confluency (Figure 3).

45. Place Dispase solution in a  $37^{\circ}\text{C}$  water bath to warm up; move frozen Matrigel aliquots on ice to thaw.

**Note:** Dispase solution must be warm when you add it to Matrigel domes.

46. In the cell culture hood, aspirate medium from each well, and add 3 mL of pre-warmed Dispase solution to each well of a 6-well plate.
47. Return the plate to the incubator for 20 min.
48. Place Splitting medium and DPBS on ice.
49. For organoid collection, prepare 15 mL conical centrifuge tubes each filled with 6 mL of Splitting medium and place them on ice.

**Note:** A maximum of 8 Matrigel domes, corresponding to one well of a 6-well plate, are generally collected in one tube.

50. Using a 1 mL pipette tip, pipette up and down the Dispase solution to dislodge the Matrigel and shear the organoids in each well of the 6 well-plate.
51. Transfer the Dispase solution containing the organoids from one well (~3 mL) to an ice-cold 15 mL conical centrifuge tube filled with 6 mL of ice-cold Splitting medium.
52. Rinse the well with 1 mL of ice-cold Splitting medium to collect any leftover and transfer it into the same 15 mL conical centrifuge tube (total volume at this point is 10 mL).

**Note:** Repeat steps 51–52 for the remaining wells using different tubes.

53. Spin down the organoids for 5 min at 200 × g at 4°C.
54. Carefully remove the supernatant and resuspend the pellet in 1 mL of TrypLE.

**Note:** Aspirate carefully, keeping the tip of the aspirator pipet at the top of the liquid.

**Optional:** Before resuspension in TrypLE, perform additional washes with ice-cold Splitting medium until the organoids sediment efficiently as a white pellet at the bottom of the tube. If organoids appear dispersed throughout a layer of Matrigel gel in the tube, then continue with washes.

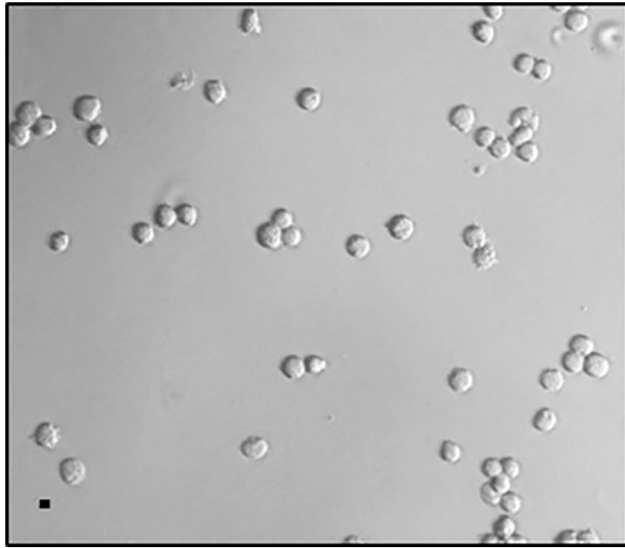
55. Pipette cells up and down through a 1 mL pipette tip, looking for a reduction of visible particle size.
56. Incubate the tube in a 37°C water bath for 10 min, shaking every 3 min.
57. Add 2 mL of Dispase solution to the 15 mL conical centrifuge tube (containing 1 mL of TrypLE) and pipet up and down the solution ~4 times with 1 mL pipette tip to make a single-cell suspension.

**Note:** At this step, the addition of dispase will ensure complete digestion of any leftover proteinaceous materials (from previous Matrigel digestion), which will impair the successful obtainment of a single cell suspension.

58. Add 30 µL of the 10 mg/mL DNase I Solution to digest the DNA released from dying cells.
59. Incubate the tube in a 37°C water bath for an additional 10 min, shaking every 3 min.
60. Using 1 mL pipette tip, pipette up and down the cell suspension ~4 times to make a single-cell suspension.

**Optional:** Increase incubation time to 15 min if dissociation is not complete. [Figure 4](#) shows an example of successful dissociation.

61. Quench with 7 mL of ice-cold Spitting medium (the tube now contains approximately 10 mL of solution).
62. Spin down the cells for 5 min at 200 × g at 4°C.
63. Remove supernatant and re-suspend the cells with 1 mL of ice-cold Splitting medium.



**Figure 4. Dissociation of Mouse Pancreatic Tumor Organoids**

A single cell suspension after enzymatic treatment. Scale bar, 10  $\mu\text{m}$ .

**Note:** Conical centrifuge tubes should be maintained on ice all the time.

64. Count and evaluate the number of live cells using available methods. For example, mix 10  $\mu\text{L}$  of cell suspension with 10  $\mu\text{L}$  of trypan blue solution (0.4%). Load 10  $\mu\text{L}$  of the mixture to the hemocytometer. Count and calculate live cells concentration.
65. Dilute cells in ice-cold DPBS to a final concentration of 1,000,000 live cells/mL and transfer 1 mL of the cell suspension in a 1.5 mL Eppendorf tube.
66. Spin down the cells for 5 min at 200  $\times g$  at 4°C.
67. Remove supernatant and re-suspend cell pellet with 33  $\mu\text{L}$  of Matrigel and 17  $\mu\text{L}$  of ice-cold PBS for a total volume of 50  $\mu\text{L}$ . Store cell suspension on ice.
68. Repeat steps 65–67 in two separate tubes and combine the 50  $\mu\text{L}$  cell suspension from these two tubes into one tube for a total of 100  $\mu\text{L}$  cell suspension.

**△ CRITICAL:** The cell number required for each injection is 1,000,000 live cells/50  $\mu\text{L}$ . It is ideal to prepare 2,000,000 live cells in 100  $\mu\text{L}$  per Eppendorf tube for each injection since a little volume of solution will be retained by the syringe needle after each injection.

### Isograft Generation

⌚ **Timing:** 20 min

69. Anesthetize the transplant host (B6J mice) using methods approved by your Institutional Committee, e.g., 4% isoflurane; when the mouse is asleep (approximately 2–3 min), put it on the right flank on the operative field.

**Note:** Keep the mouse sedated for the entire procedure, e.g., with 2.5%–0.5% isoflurane.

70. Remove the fur from the left flank using the rodent shaver, and then apply a thin layer of depilatory cream using a cotton stick and leave it for approximately 3 min.

**Note:** Do not keep depilatory cream on mouse skin more than 3 min; it may cause skin irritation.

71. Clean the shaved area with a wet tissue and then with 70% alcohol.
72. Using dissecting scissors, practice a small incision (~1 cm) in the flank by first cutting the skin and then the peritoneum.
73. Using iris forceps, gently lift the spleen to expose the pancreas.
74. Keeping the cell/Matrigel mix as cold as possible, resuspend single cell suspension with 200  $\mu$ L pipette tip and slowly draw up 100  $\mu$ L of the cell suspension through the needle of a 0.5 mL insulin syringe.
  - △ CRITICAL:** Carefully resuspend cells to avoid formation of bubbles. At this point it is important to remove air from a 0.5 mL insulin syringe before taking the required volume.
75. Warm the syringe content by holding it for approximately 30 s so that the Matrigel will begin to solidify; then, clean the needle using 70% alcohol before the injection.
  - △ CRITICAL:** Do not warm the syringe for more than 30 s, otherwise the suspension cannot be injected as Matrigel completely solidifies.
76. Using iris forceps, hold the spleen so that the pancreas will be in a vertical position and slowly inject 50  $\mu$ L of the single cell suspension into the pancreatic tail (Figure 5); if the injection is performed correctly, a small bubble will be visible at the injection site.
  - △ CRITICAL:** It is important to avoid any leak, otherwise tumor cells will spread and grow outside the pancreas. To avoid leaks, it is important to inject the solution slowly. Avoid applying too much pressure while injecting.
77. Using a cotton stick, gently clean the injection site.
78. Gently reposition the viscera into the peritoneal cavity using forceps.
79. Using the needle holder and the VetSuture FastPGA, suture the peritoneum.
80. Apply clips with the autoclip applier to close the skin wound.
81. Gently position the mouse in the housing cage and keep it under a red light to warm it up, until it completely recovers from the anesthesia following approved post-surgery care procedures for the animals.

### **Tumor Growth Monitoring**

Prior to proceeding to tumor growth monitoring, remove external clip using the autoclip remover.

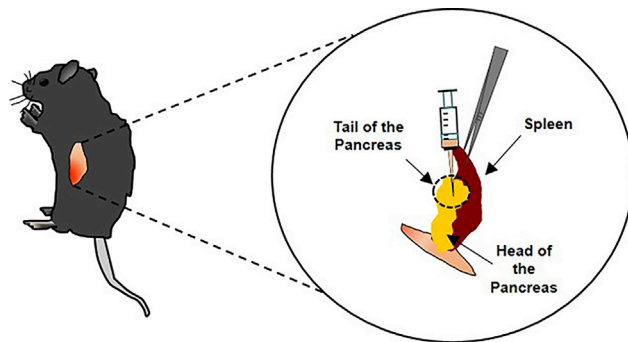
**Note:** Wound closure requires approximately 10–15 days.

#### *Abdominal Palpation*

⌚ **Timing: 3 min**

To check for the presence of a palpable mass, perform weekly abdominal palpation following the described procedure.

81. Hold the mouse from the tail and place it on the grid of the cage.
82. Gently palpate the abdomen with the thumb and the index finger moving backward and forward for a solid mass feeling.



**Figure 5. Schematic Representation of the Injection of a Solution Containing Single Cells from Organoid Culture into the Pancreatic Tail**

- △ **CRITICAL:** Don't confuse mouse feces with tumor mass; this procedure is difficult to reproduce and subjective. Please, use ultrasound imaging to dispel any doubts (see below).

#### Ultrasound Imaging

⌚ **Timing:** 20 min

To effectively measure tumor mass and volume, and monitor tumor growth over time, available methods should be used. For High-resolution micro-ultrasound imaging system - Vevo 2100, follow the protocol described below.

83. Turn on the imaging system following the manufacturer's instructions and switch on the heating pad (~37°C).
84. For abdominal imaging, choose B-mode imaging since it is the most suitable to locate anatomical structures.
85. Apply ultrasound gel on the transducer head.
86. Anesthetize the animal with 4% isoflurane; when the mouse is asleep (approximately 2–3 min), put it on the right flank on the operative field and keep it sedated for the entire procedure with 2.5 % isoflurane.
87. Remove the fur first with the help of the rodent shaver and then apply the depilatory cream with the help of a cotton stick (Figure 6). After 3 min, remove the cream with a wet tissue.

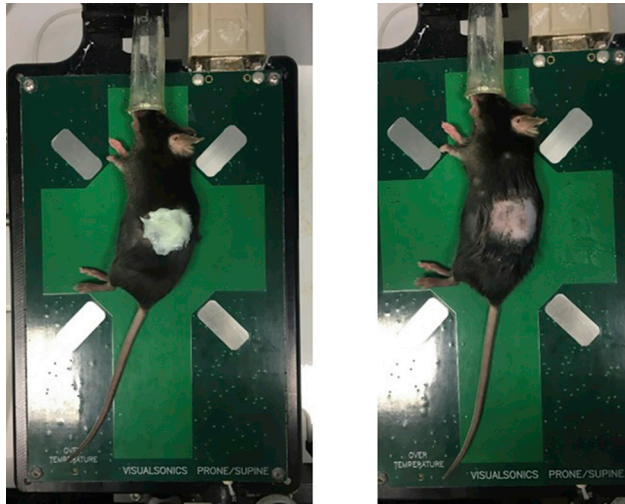
**Note:** Do not keep depilatory cream on mouse skin more than 3 min; it may cause skin irritation.

88. Apply ultrasound gel on the flank of the mouse and get the transducer in position.

**Note:** Do not perform heavy compression on the mouse flank.

89. To localize the tumor, scan backward and forward trying to localize the spleen, and then the pancreas.

**Note:** Pancreatic tumors are usually visualized as hypoechogenic formations. During the progression from pre-invasive lesions to carcinoma, transplants grow as small cystic structures (spherical structure with clear borders), and then they become full solid structures (Figure 7).



**Figure 6. Fur Removal from Mouse Flank with Depilatory Cream**

90. Once the tumor mass is identified, using the ultrasound caliber find the two extremities of the mass. Place the transducer at the same distance from the start/end of the tumor and scan the entire tumor mass.

**Note:** This step is important to measure tumor volume.

91. At the end of the procedure, remove the ultrasound gel from the mouse abdomen and place the animal in a recovery cage.

92. Once the scan is over, track two diagonal lines to measure the mass (Figure 8).

93. To measure volume with the Vevo Imaging Workstation, click on 3D settings and select Volume.

94. Select Parallel from the Volume area and then Start to start creating the first contour; proceed by drawing subsequent contours both inward and outward to measure the total volume of the tumor mass.

95. Once generated the desired number of contours, click on Finish to end the procedure and obtain the volume. Volume measurement should be performed once a week.

**Note:** When tumor volume reaches the size of approximately  $1 \text{ cm}^3$ , mice should be euthanized, and tissues harvested.

### Necropsy

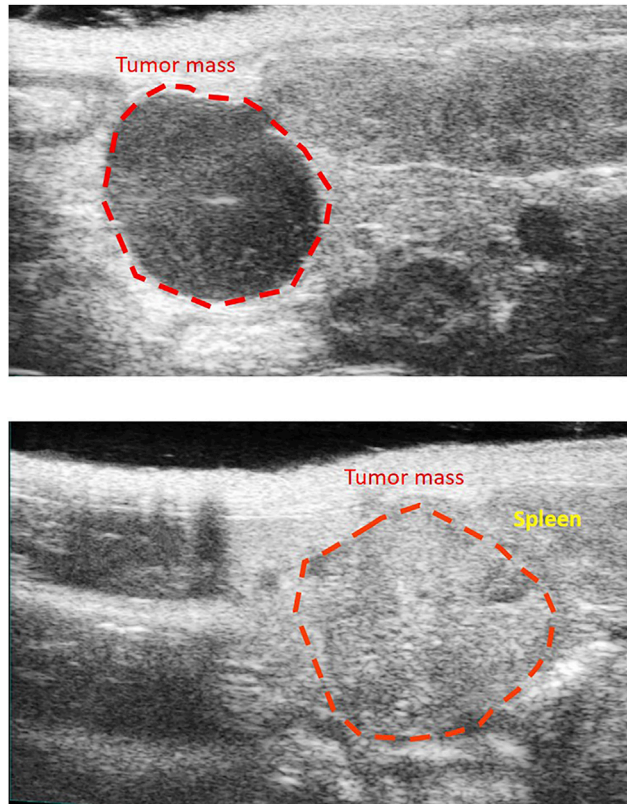
⌚ Timing: 20 min

96. Anesthetize the animal using approved methods (e.g., 4% isoflurane) and, once asleep, euthanize the mouse according to approved methods. For example, put the sedated mouse supine on the operative field and practice cervical dislocation to euthanize the mouse.

97. With the curved forceps, hold the skin in the center of the abdomen and cut it with the curved scissors performing an "upside-down V" (Figure 9); perform the same procedure with the peritoneum.

98. Check for the presence of a tumor mass attached to the pancreas.

99. Using the forceps, pull out the intestine and collect pancreas; if a visible tumor mass is present, gently detach it from the pancreas.



**Figure 7. Ultrasound Image of Tumor Mass**

Upper panel: ultrasound image of a tumor mass with cystic appearance.  
Lower panel: ultrasound image of a full solid tumor mass (red circle).

100. Place the pancreatic tumor tissue in a petri dish, which has been previously tared on a digital scale, and record the weight of the pancreas
101. Cut the pancreas tumor in halves with a scalpel.
102. Place one half of the pancreas in a pre-labeled cryotube and flash-freeze in liquid nitrogen. Then store the samples in  $-80^{\circ}\text{C}$  until needed.
103. Place the other half of the dissected tumor in a pre-labeled histological cassette and fix for 12–14 h with a 10% formalin solution (fixative volume should cover completely the tissue).

**△ CRITICAL:** Make sure to cut the mass carefully. If it is a cystic mass, ensure wearing personal protective equipment to avoid exposure to splashes.

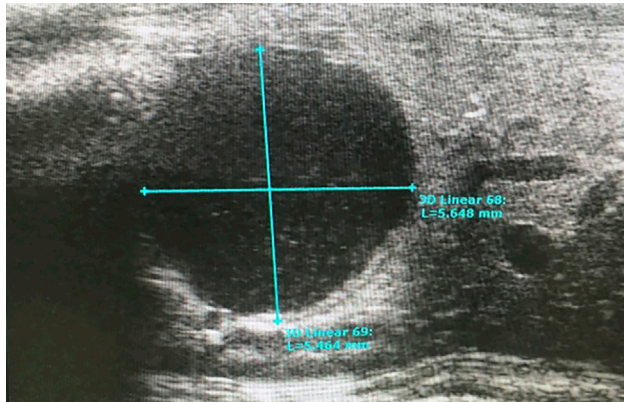
104. Collect organs which are potential site of metastasis, such as liver and lungs.
105. Properly dispose of the carcass according to institutional guidelines.

**Note:** For paraffin processing of Tissue and Haematoxylin/Eosin Staining proceed according standard Protocols.

### EXPECTED OUTCOMES

It has been demonstrated that orthotopic transplants of mouse pancreatic cancer organoids in syngeneic immunocompetent mice slowly progress from preinvasive lesions (PanIN-like lesions) to overt carcinomas. Organoid-derived isografts (ODIs) can be established from pancreatic organoid cultures previously subjected to genetic manipulation (e.g., deletion or overexpression of specific





**Figure 8. Tumor Mass Measuring**

Example of tumor mass measuring using micro-ultrasound imaging.

genes), and this enables to verify whether a given perturbation accelerates or delays tumor progression *in vivo*.

In line with this, the effect of specific perturbation can also be assessed in the context of changes induced in the tumor microenvironment during progression of the disease.

Growth of ODIs can be easily monitored using ultra-sound imaging, which reveals that transplants grow initially as small lesions developing from a cystic structure that tend to close over time. Materials from ODI can be used for different downstream analyses, including histopathological analysis, immunohistochemistry, FACS, and isolation of nucleic acids for DNA-sequencing and mRNA expression analyses as described in our publication (Filippini et al., 2019).

In our experience, it takes 1–2 months for ODIs of tumoral organoids to develop as low- to high-grade preinvasive lesions that resemble mouse PanIN, 3–4 months to progress into well – differentiated/moderately – differentiated tumors, 5–6 months to progress into poorly differentiated carcinoma.

## LIMITATIONS

Successful generation of mouse pancreatic organoid-isografts is dependent upon generation of viable cell suspension from organoid cultures as well as proper injections of cells into the pancreatic tail. Viable cell suspensions are easily obtained from healthy cultures of mouse pancreatic organoids. It is critical to avoid nutrients exhaustion in spent culture medium. Therefore, we strongly recommend refreshing culture medium and passaging organoids at the appropriate time. The generation of viable cell suspension is also heavily influenced by the time required for relieving organoids from Matrigel: for this, we suggest starting from ~80% confluent cultures as cell recovery from Matrigel is usually more efficient. As compared to the generation of isografts starting from monolayer cell cultures of mouse pancreatic cancer cells, the time required for the generation of full-blown carcinoma from organoid cultures is longer (up to 5 months); yet, the ODIs recapitulate relevant pathophysiological features of PDA (e.g., desmoplastic reaction), which are usually absent in 2D derived isografts (Boj et al., 2015).

## TROUBLESHOOTING

### Problem 1

Extensive cell death after digestion of pancreatic tumor tissue.



**Figure 9.** Performing an Upside-Down V Cut during Necropsy

### Potential Solutions

Prolonged digestion of minced pancreatic tumor tissue with a solution containing a Collagenase/Dispase mixture may result in a significant reduction of cell viability, which will ultimately affect the successful establishment of the organoid culture. Therefore, it is crucial to: (i) prepare digestion media appropriately to avoid having a solution with excessive amount of the enzymes; (ii) monitor the digestion every hour looking for clusters of cells, and when those are visible (Figure 1) stop the reaction immediately. Reduced viability of the digestion may also result from prolonged incubation with TrypLE; therefore, we recommend incubating the sample for no longer than 10 min.

### Problem 2

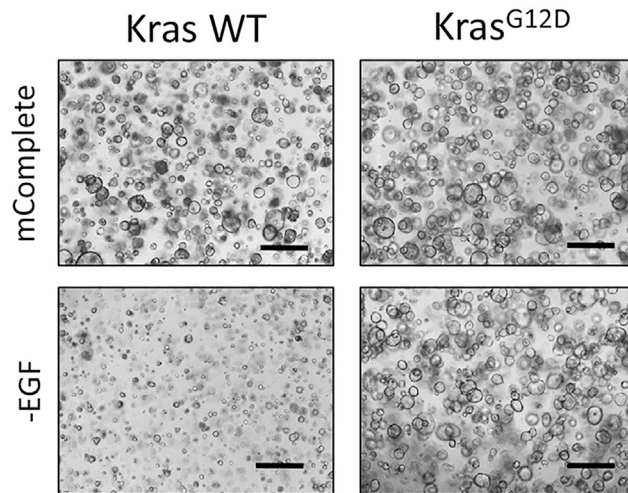
Failure in establishing the organoid culture.

### Potential Solutions

Organoids are usually visible within 24–48 h from the initial plating of the tissue' digestion. A possible explanation for the lack of visible organoids in culture might be the incorrect composition of the medium. Make sure to prepare the mouse complete medium correctly and to include the Rho Kinase Inhibitor (RhoKi) for the first steps of the protocol (i.e., establishment and media refreshing 3 days after).

### Problem 3

Normal contamination in culture.



**Figure 10. Representative Images of a Normal Mouse Organoid Culture (Left) and a Mouse Tumor Organoid Culture (Right Panels) Grown with (mComplete) or without (-EGF) Epidermal Growth Factor**  
Scale bar, 500  $\mu$ m.

#### Potential Solutions

Following establishment of organoid cultures from KPC bulk tumors, it is possible (although extremely rare) to have contamination from normal cells. Upon establishment of KPC tumor-derived organotypic cultures, it is recommended to perform PCR-based confirmation of  $Kras^{LSL-G12D}$  recombination. To check for the presence of KRAS wild-type cells, sequencing should be performed (Boj et al., 2015; Hingorani et al., 2005). In case of contamination from non-neoplastic cells, it is advisable to remove epidermal growth factor (EGF) from the culture medium as the EGF-depleted medium does not support the growth of KRAS wild-type cells (Figure 10).

#### Problem 4

Failure in organoid propagation.

#### Potential Solutions

Mouse pancreatic organoids should be cultured under strict sterile conditions and quality controls should be routinely performed. Check for the presence of any contamination in the culture (e.g., bacteria, fungus, mycoplasma) using available methods.

#### Problem 5

Failure of transplantation.

#### Potential Solutions

Keep conditions standard for a specific study in order to minimize variability. The success of the transplant depends on the injection. It is important during the procedure to keep the syringe needle still and in place. Remove it only when the appropriate volume of cell suspension has been injected. If material spills out during the injection, consider that mouse out of the study.

Make sure that the bubble does not break when you pull out the needle.

Make sure to dab the injection site with a cotton stick by applying gentle pressure to avoid material leaking out.

### ACKNOWLEDGMENTS

V.C. is supported by Associazione Italiana Ricerca sul Cancro (AIRC; grant no. 18178). S.D. is supported by a fellowship award of the AIRC (24043). The funding agency had no role in the writing of the manuscript.

### AUTHOR CONTRIBUTIONS

S.D. and F.L. conducted the experiments, analyzed the data, and wrote the manuscript. V.C. designed the experiments and wrote the manuscript.

### DECLARATION OF INTERESTS

The authors declare no competing interests.

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