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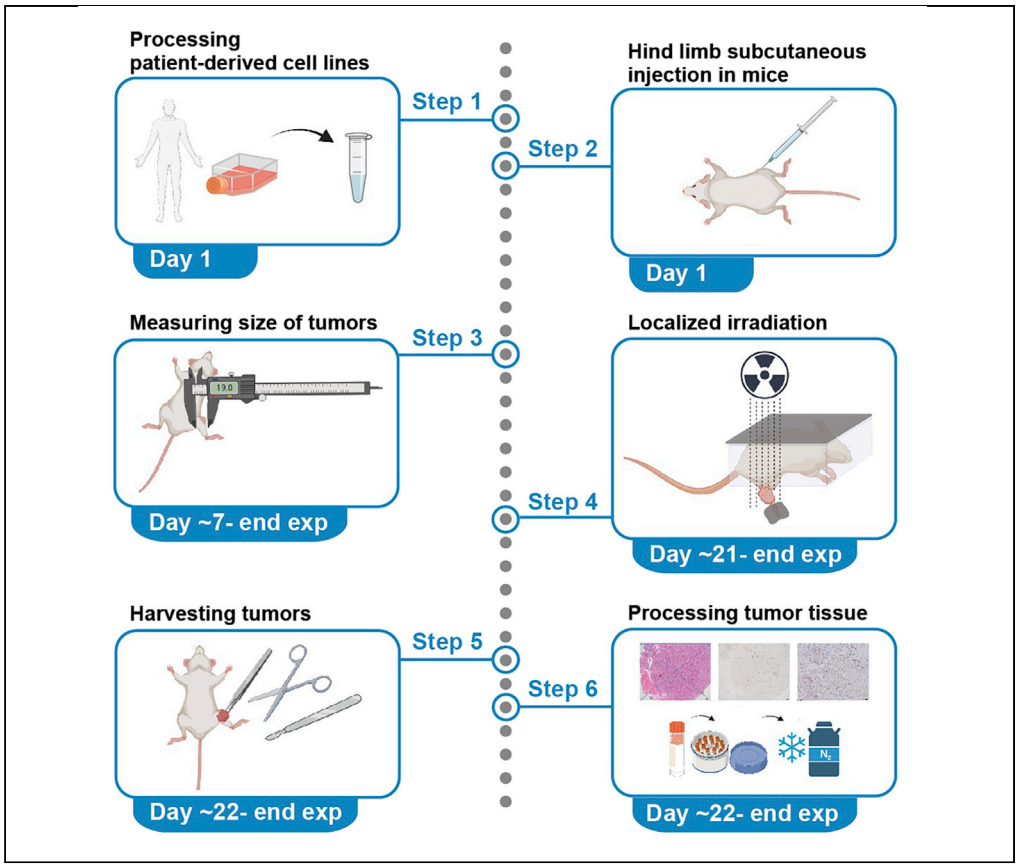
Local irradiation of patient-derived tumors in immunodeficient mice



Severe combined immunodeficient mice are typically used for xenografting experiments and show reliable tumor engraftment; however, their Prkd^{scid} mutation renders them highly sensitive to irradiation. Here, we describe a protocol that allows safe local irradiation of tumor xenografts in immunodeficient mice. We detail the steps for the establishment and handling of patient-derived cancer cultures, subcutaneous injection of cancer cells on the mouse hind limb, localized irradiation in mice, tumor monitoring, and tumor characterization via histological and immunohistochemical assessment.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol for local irradiation of tumor xenografts in SCID mice

Steps for dissociation of patient-derived tissues and establishment of cancer cultures

Subcutaneous injection of patient-derived cells on mouse hind limb and tumor monitoring

Can be widely used for safe irradiation of tumors in immunodeficient mice

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Local irradiation of patient-derived tumors in immunodeficient mice

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SUMMARY

Severe combined immunodeficient mice are typically used for xenografting experiments and show reliable tumor engraftment; however, their Prkd^{scid} mutation renders them highly sensitive to irradiation. Here, we describe a protocol that allows safe local irradiation of tumor xenografts in immunodeficient mice. We detail the steps for the establishment and handling of patient-derived cancer cultures, subcutaneous injection of cancer cells on the mouse hind limb, localized irradiation in mice, tumor monitoring, and tumor characterization via histological and immunohistochemical assessment.

For complete details on the use and execution of this protocol, please refer to Dings et al. (2022).[1]

BEFORE YOU BEGIN

This protocol describes the specific steps to perform localized irradiation of tumor xenografts in immunodeficient mice.

Before starting, make sure the *in vivo* experiments are approved by the local animal test and welfare committee. Mice should be purchased in advance with an acclimatization period of at least 7 days before the start of the experiment. NSG immunodeficient mice should be kept in Specific-Pathogen Free (SPF) facilities to avoid infections. Determine the required group sizes by a power calculation analysis using statistics from previous, similar, experiments.

For this protocol a mouse radiation setup is essential, for which 3D technical drawings as NWD and STP files are available on Mendeley Data: https://doi.org/10.17632/vfjjtwstw9.1.

This protocol is optimized for NOD.Cg-Prkdc^{scid} Il2rgtm1Wjl/SzJ (NSG) immunodeficient mice but can be used for all mice with high radiosensitivity. NSG mice, like all SCID mice, are homozygous mutant for the Prkdc^{scid} gene mutation, involved in DNA double strand brake repair through non-homologous end joining.[2] As a consequence, all mouse strains carrying the Prkdc^{scid} mutation are highly radiosensitive.[3] We advise to use mice between 8 weeks and 6 months of age, as tumor growth and sensitivity to radiation have been shown to be reproducible in this age range.[4]

Additionally, the establishment of patient-derived cell cultures from fresh patient material is described in this primary section. For this, approval from the appropriate Ethics Committee is required as well as informed consent from the patient. When using already established cultures, commercially available cultures or grafting tumor tissue, this step can be skipped. Multiple cell culture lines of two tumor types have been tested with the irradiation setup, both esophageal and cervical cancer. In this protocol we focus on the optimal conditions for esophageal patient-derived 081R cells.

Before starting the experiment, prepare all solutions specified in the "materials and equipment" paragraph.

Institutional permissions

Prior to start, animal experiments should be approved by the animal test committee (Dierexperimentencommissie, DEC) and animal welfare committee (Instantie voor Dierenwelzijn, IvD) according to the local law and performed in accordance with ethical and procedural guidelines.

Dissociation of patient-derived esophageal cancer tissue

Timing: 1 h (for steps 1 to 13)

In this section we describe how to establish patient-derived esophageal cultures from fresh patient resection material. When using already established cultures, publicly available cultures or when grafting tumor tissue, please continue to "step-by-step method details".

1. Collect fresh tumor tissue (biopsy, resection specimen) from the clinic after written informed consent explicitly allowing culturing of the material.

   Note: It is crucial to have effective logistics between the clinic and researchers, to get tissue samples on ice and to the laboratory for processing within a maximum of 2 h.

2. Take tumor tissue on ice into a biosafety cabinet (BSC).

   Note: All steps in this section will be performed on ice.

3. Wash tissue once with 10 mL PBS+++ (see materials and equipment) in a 50 mL conical tube.
4. Mince into small pieces <0.5 mm^2 with a sterile scalpel in a 100 mm petri dish.
5. Add 2 mL Dissociation buffer (see materials and equipment) to the petri dish and transfer suspension into a new 50 mL tube.
6. Rinse remaining tissue from the dish with 1 mL Dissociation buffer and add to 50 mL tube as well.
7. Incubate at 37°C in a water bath for 45 min.
   a. Vortex vigorously for 10 s every 15 min.
8. Resuspend cells in 12 mL of DMEM+++ (see materials and equipment) to block dissociation.
9. Pass suspension through cell strainer (70 μm) on a new 50 mL tube by pouring.

   Note: Do not resuspend by pipetting, as tissue pieces will stick inside the pipette tip.

10. Rinse the cell strainer with 1 mL DMEM+++ to obtain all dissociated cells <70 μm.
11. Spin down at 300 × *g* for 3 min and discard supernatant.
12. Resuspend in 1 mL of desired medium and plate in 1 well of a 12-well plate.

    Note: As each patient tissue sample contains different types of cells, the optimal medium can differ. When obtaining >1 × 10^5 cells, it is advised to resuspend cells in >1 mL medium and plate in multiple wells with different types of medium, e.g., to obtain epithelial tumor cells.

Cancer Stem Cell (CSC) medium (see in materials and equipment) could be considered instead of DMEM+++ or other FBS-supplemented media.

13. Cells will attach within 24–72 h after seeding.

Establishment of patient-derived esophageal cancer cultures

Timing: 3–4 weeks (for steps 14 to 17)

Timing: 1 day (for steps 18 to 24)

14. Monitor growth of cell lines with a phase contrast microscope and replace medium twice a week.
15. When the well(s) of a 12-well plate reach 80%–90% confluence, split cells carefully with trypsin and transfer to a 6-well plate.

Note: when using CSC medium spin down at 300 × *g* after trypsin or use trypsin inhibitor.

16. When the well(s) of a 6-well plate reach 80%–90% confluence, transfer to a T25 flask.
17. Grow cells until a minimum of 80% confluence is achieved in a T25 flask (or 3×10^6 cells), which can take up to 4 weeks.

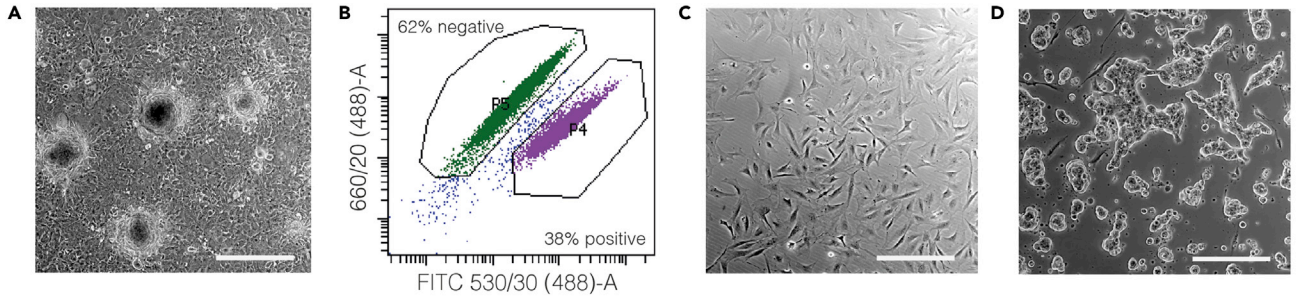
Note: A heterogeneous range of morphologies will appear, with fibroblasts covering the flask and tumor cells growing on top in small spheres (Figure 1A).

18. Place desired media and trypsin at 37°C.
19. Wash cells using PBS and harvest by trypsinization at 37°C.
20. Stain all cells for EpCAM positive and negative cells.
    a. Divide cells into two 15 mL tubes; use approximately 3/4 for EpCAM-stained sample and 1/4 as unstained control.
    b. Spin down at 300 × *g* for 3 min and resuspend in 100 μL of FACS buffer (see materials and equipment).
    c. Centrifuge at 300 × *g* for 3 min and discard supernatant.
    d. Add 100 μL per sample anti-EpCAM antibody at a 1/300 dilution in FACS buffer, incubate for 30 min on ice.
    e. Wash cells 2× with FACS buffer by centrifuging 300 × *g* for 3 min and discarding supernatant.
    f. Stain with FITC secondary antibody at a 1/300 dilution for 30 min on ice, protected from light.
    g. Wash cells 2× with FACS buffer by centrifuging 300 × *g* for 3 min and discarding supernatant.
    h. Add 300 μL per sample appropriate viability marker (end concentration 200 pg/mL) in FACS buffer.

Alternatives: Different viability markers can be used, such as PI or Zombie Aqua.

21. Transfer cells protected from light to FACS-sorting device.
22. Sort cells for EpCAM+ *vs* EpCAM- cells (Figures 1A and 1B).
    a. Select viable cells using a negative gate for the viability marker.
    b. Set gates for the negative population using the unstained control and determine the gate of the positive population in the stained sample.
    c. Sort for both negative and positive cells, ideally at least 1×10^5 cells are sorted in each group.

Note: If more than 1×10^5 positive cells are available, gates can be set strictly with more space between the gates. If few positive cells are available, it is advised to set gates less strict to prevent losing cells and rather sort multiple times.



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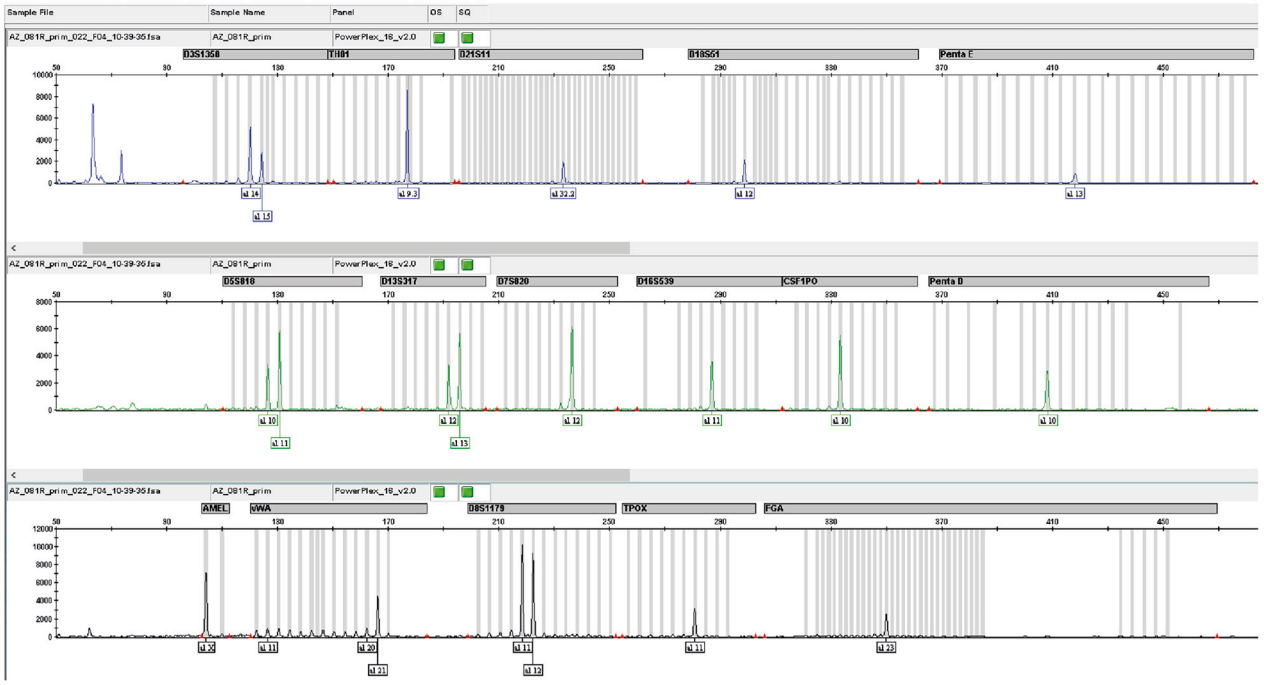

STRsample	source	line	AMEL	CSF1PO	D3S1358	D5S818	D7S820	D8S1179	D13S317	D16S539	D18S51	D21S11	FGA	Penta D	Penta E	TH01	TPOX	vWA
reference	gDNA pt	081R	X	10	14.15	10.11	12	11.12	12.13	11	12	32.2	23	11	(11),13	10	11	21
	P9	081R	X	10	14, 15	10, 11	12	11, 12	12, 13	11	12	32.2	23	10	13	9.3	11	1, 20, 21
	P10	081R	X	10	14	10	12	11	12	11	12	32.2	23	10	13	9.3	11	1, 20, 21

Figure 1. Establishing patient-derived esophageal cancer cell lines

(A) Patient 023 resection (023R) cells before FACS-sorting. Scale bar: 200 μm.

(B) Gating strategy for FACS-sort of FITC (488 nm) with FITC 530/30 *vs* APC 660/20.

(C) 023R EpCAM-negative cancer associated fibroblasts after one sort. Scale bar 70 μm.

(D) 023R EpCAM-positive cancer cells after sorting. Scale bar 70 μm.

(E) STR profiles of 081R cells at different passages (P9, P10), and patient gDNA as reference.

23. Obtain a clean homogeneous cell line by allowing cells to reach 80% confluence in a T25 flask, check morphology carefully and repeat FACS-sort if necessary.[5,6]

Note: Populations after one Epcam+/- FACS-sort yielded esophageal cancer associated fibroblasts (Figure 1C) and esophageal tumor cells (Figure 1D).

24. Profile the first clean passage by performing Short Tandem Repeat (STR) profiling (Figure 1E).

⚠ CRITICAL: As a reference for primary cell lines, patient tumor or gDNA from blood is used. Profile cells regularly to detect cell line contaminations during culturing. Before injection in

mice, cells should be tested for known mouse pathogens such as *C. bovis*. This can be performed by various certified companies, such as IDEXX Bioanalytics.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-EpCAM	Abcam	ab223582
Goat anti-rabbit IgG (FITC) 1:300	Abcam	ab6717
Phospho-Histone H2AX (Ser139) (20E3) Rabbit mAb 1:300	Cell Signaling	9718S
Biological samples		
Patient-derived esophageal cancer tissue (male, 38y)	OR/grossing room	N/A
Chemicals, peptides, and recombinant proteins		
Advanced DMEM F/12	Gibco	12634010
Liberase	Sigma	05401020001
N2 supplement	Invitrogen	17502048
D-Glucose 45%	Sigma	50-99-7
Trace elements B	Fisher Scientific	25-022 CI
Trace elements C	Fisher Scientific	25-023-CI
HEPES 1 M	Thermo Fisher	15630080
Heparin	Sigma	H3149
Insulin	Sigma	I9278-5ML
β-Mercaptoethanol	Sigma	60-24-2
L-Glutamine	Gibco	15410314
Penicillin-Streptomycin	Thermo Fisher	15140122
Fetal bovine serum (FBS)	Gibco	10270106
Phosphate-buffered saline (PBS) tablets	Sigma	524650
DNase	Sigma	10104159001
Fixation/Permeabilization Kit: Cytoperm and Permwash	BD Bioscience	554714
Propium-iodide	Sigma-Aldrich	81845
Isoflurane	Ecuphar	ISOFLO
Virkon	Fisher Scientific	70693-62-8
Matrigel	Corning	356235
Recovery cell culture freezing medium	Thermo Fisher	12648010
Eosin	Klinipath	3871-2500
Hematoxylin	Klinipath	4085-9002
Experimental models: Cell lines		
Esophageal adenocarcinoma 081R primary line, passage number <10	AMC	AMC_EAC_081R
Cervical carcinoma SiHa cell line, passage number <10	ATCC	HTB-35
Experimental models: Organisms/strains		
NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) mice 8 weeks–6 months, males and females	Own breeding	N/A
Nude Hsd:Athymic Nude-Foxn1^{nu} mice 8 weeks–6 months, males and females	Envigo	069
Software and algorithms		
BD FACSDiva software	BD Biosciences	N/A
FlowJo	Tree Star	version 10.2 https://www.flowjo.com/solutions/flowjo/downloads
ImageJ	Schneider et al.[7]	imagej.nih.gov

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Other		
5 mL round bottom polystyrene tube	Corning	352052
Cell culture flask T25	Thermo Fisher	169900
Microscope DM IL LED	Leica	11521266-B
FACS cell sorter	Sony	SH800
CoolCell™ LX Cell Freezing Container	Corning	CLS432001
Radiation source	XStrahl	RS320
Syringe Luer-Lok™ 1 mL	BD	309628
Needle Orange 25G	BD	1712024
Digital caliper	Thermo Fisher Scientific	14-648-17
Deposited data		
Mouse restraining and shielding setup diagrams	University of Amsterdam / Trilobes, Joure, the Netherlands	Mendeley data: https://doi.org/10.17632/vfjjtwstw9.1
3D viewer of diagrams	CAD Soft Tools	https://3d-viewers.com/step-viewer.html

MATERIALS AND EQUIPMENT

- Solutions are provided as a specified final volume, but investigators may choose to prepare different volumes depending on the number of samples processed.

DMEM+++ medium			
Reagent	Stock concentration	Final concentration	Add to 500 mL
Advanced DMEM/F12	1×	1×	440 mL
Fetal Bovine Serum (FBS)	N/A	10%	50 mL
L-Glutamine	200 mM	2 mM	5 mL
Penicillin-Streptomycin	100×	1×	5 mL

Note: Store at 4°C for 3 months.

Cancer Stem Cell (CSC) medium			
Reagent	Stock concentration	Final concentration	Add to 500 mL
Advanced DMEM/F12	1×	1×	478 mL
N2 supplement	100×	1×	5 mL
Glucose	2.5 M	8.5 mM	1.7 mL
Trace Elements B	1000×	1×	500 µL
Trace Elements C	1000×	1×	500 µL
HEPES pH 7.5	1 M	5 mM	2.5 mL
Heparin	2 g/mL	2 mg/mL	500 µL
Insulin	10 g/mL	10 mg/mL	500 µL
β-mercaptoethanol	7.5 M	15 mM	1 mL
L-Glutamine	200 mM	2 mM	5 mL
Penicillin-Streptomycin	100×	1×	5 mL

Note: Store at 4°C for 3 months.

PBS+++			
Reagent	Stock concentration	Final concentration	Add to 500 mL
PBS	Tablets dry powder	1×	1 tablet
H_2O	N/A	1×	440 mL
FBS	1×	10%	50 mL
L-Glutamine	200 mM	2 mM	5 mL
Penicillin-Streptomycin	100×	1×	5 mL

Note: Store at 4°C for 1 month.

Dissociation buffer			
Reagent	Stock concentration	Final concentration	Add to 2 mL
DMEM+++	1×	1×	2 mL
Liberase	2.5 mg/mL	0.1 mg/mL	77 µL
DNase	1 unit/µL	1.5%	30 µL

Note: Use freshly prepared.

FACS buffer			
Reagent	Stock concentration	Final concentration	Add to 50 mL
PBS	1×	1×	50 mL
FBS	100×	1×	0.5 mL

Note: Use freshly prepared.

⚠ CRITICAL: It is important to avoid inhaling isoflurane, as long-term exposure could cause hypotension, tachycardia, respiratory depression, and elevated blood glucose.

Alternatives: Regular laboratory reagents such as PBS or BSA can be obtained from other sources than specified in the key resources table.

STEP-BY-STEP METHOD DETAILS

Harvesting of patient-derived cell lines for injection

Timing: 1 h

This step describes the harvesting, counting, and resuspension of patient-derived cultures prior to subcutaneous injection in the hind limb of NSG mice. These steps are done outside the animal facility. It generally takes 3–5 days since last passage until 081R or SiHa cultures reach 80% confluence. Regarding patient-derived cell lines, it is advised to take a low (<10) passage number to ensure highest possible similarity to the patient tissue. Group sizes should be determined by power calculations prior to starting the experiment.

With SiHa cells, 1×10^6 cells were injected per mouse in a volume of 50 µL. In this step we take 081R cells as an example, injecting 1×10^5 cells per mouse in a total volume of 50 µL.

1. Calculate the volume and cells needed for the number of available mice, taking the total number of mice × 50 µL + minimal 300 µL for loss in syringe needle and spill.

Note: Example with 24 mice: 24 × 50 μL + 300 μL = 1500 μL total.

2. Calculate the amount of Matrigel needed (50% of total volume). Example; 1500/2 = 750 μL.

Alternatives: Basement Membrane Extract (BME) can be used instead of Matrigel.

3. Thaw Matrigel on ice, allowing it to become liquid.
4. Harvest patient-derived cells.
   a. Remove medium from cells.
   b. Wash flask with 2 mL (T25) or 5 mL (T75) PBS.
      i. For cell lines that are hard to detach add 5 mM EDTA in PBS for 2 min at 37°C and remove.
   c. Add 1 mL (T25) or 3 mL (T75) 1× trypsin for 3–10 min at 37°C.
   d. Carefully resuspend the cells with long reach filter tip.
      i. Check under microscope to make sure a single-cell suspension is obtained.

⚠ CRITICAL: CSC medium contains no serum to inactivate trypsin, and if using CSC medium, spin down cells at 300 × *g* for 3 min, remove all medium and resuspend in 1–3 mL CSC medium.

5. Count the number of cells per mL, e.g., in a hemocytometer.
6. Take 1 × 10^5 cells in a total of 50 μL solution for injection.
   a. Volume cell suspension = Total volume needed / (cells per mL / 1 × 10^5 cells).

Example with 24 mice: 24 × 50 μL + 300 μL = 1,500 μL total.

Volume of cell suspension = 1,500 μL / 2 (50% Matrigel) = 750 μL.

750 / (1.16 × 10^6 counted cells / 1 × 10^5) = 64.7 μL of cell suspension in 685.4 μL medium.

7. Resuspend 50% of cell suspension with 50% Matrigel on ice in a 5 mL round-bottom polystyrene tube.
   a. After counting, make sure to freeze down a subset of cells. Document vials including passage number, so future mice experiments can be done with similar passage cell lines.
8. Transport cell-Matrigel suspension on ice to the animal facility.

⚠ CRITICAL: Keep cells in Matrigel on ice to avoid solidification. To avoid cell aggregation and clumps, resuspend or flick the cell suspension tube right before mixing with Matrigel. Resuspension of the cells in Matrigel solution is essential as cells resuspended only in medium may fail to graft.

Hind limb subcutaneous injection in mice

Timing: 2 min per mouse

This step describes the subcutaneous injection of patient-derived cells into the hind limb of mice. This procedure can be performed on one to six mice in a row, depending on personal skills, assistance and equipment available.

9. Work inside a biosafety cabinet (BSC).
10. Randomize mice between different treatment groups.
    a. Distribute mice of one litter across different treatment groups and ensure a similar number of males and females per treatment group.

Note: Group allocations may be corrected for randomization at the start of treatment (step 28).

11. Anesthetize mice with 2% Isoflurane in 100% oxygen for 5 min.
12. Mark animals for identification e.g., using earmarks or color marking using a permanent marker.
13. Carefully remove the hair on the right hind limb of all the mice by shaving (Methods video S1).

Alternatives: Depilatory cream can be used.

14. Disinfect the skin with Virkon.
15. Resuspend pre-made cell suspension from previous steps 1–8.
16. Immediately fill the 1 mL syringe to ensure a homogeneous suspension and attach 25G needle.
17. Inject 50 μL cell suspension into the subcutis in the right hind limb.
    a. Inject below the kneecap, just under the skin to ensure subcutaneous and avoid intramuscular or intracutaneous tumor growth.

Measuring size of tumors

Timing: 3 min per mouse

This step describes measuring and calculating the volume of a tumor on the hind limb of immunodeficient mice. Prior to measuring the tumor sizes, body weights should be measured on a scale.

18. Work inside a BSC.
19. Disinfect the digital caliper.

Note: Check if caliper is zeroed correctly: 0.00 should be indicated when the jaws are closed.

20. Fixate a mouse in your left hand in such a way that the mouse right hind limb is freely accessible.
21. Measure the length, width, and depth of the right (grafted) hind limb (Methods video S2).
22. When measuring for the first time, also measure the left non-tumor injected hind limb of each mouse.

Note: As the tumor volume on the hind limb may be hard to assess, the other hind limb is used as internal control per mouse. In our experiments, most tumors grow subcutaneously as a distinct nodule, though some tumors may grow flat and into the underlying muscle making them difficult to measure. In most cases, the subtraction method to determine tumor graft is required, so we advise to apply this method for all mice.

23. Calculate the average limb *length* for all female and the same for all male mice.

Note: As the length of the limb does not alter during tumor growth, this is a fixed number from this point.

24. Calculate the volume of the tumor by measuring the tumor injected hind limb using the formula: (length × width × depth)/2). *Example: (18 (average length)* × *8.77* × *8.33)/2 = 657 mm*^{3}.

Alternatives: Multiple measuring approaches can be used to measure tumor size. Another option is the formula π/6*(length × width × depth).

25. Subtract the volume of the non-tumor injected control limb. In our case this was on average 107 mm^3; 657–107 = 550 mm^3.

26. Repeat measurements for all mice.
27. Document tumor measurements of each mouse over time.

Localized irradiation of tumors on hind limb

Timing: 20 min per radiation fraction

This step describes a radiation setup to locally radiate on the tumor-injected hind limb, while shielding the rest of the mouse. This provides a method to safely treat a broad range of tumor xenografts in immunodeficient mice that are unable to tolerate (scattering of) whole body radiation. Mice radiation-shielding setup drawings for replication are shown in Figure S1 and 3D technical files are available on Mendeley Data: https://doi.org/10.17632/vfjjtwstw9.1.

28. Begin treatment of mice when tumors reach approximately 50–100 mm^2.

Note: With 081R or SiHa cells, this was the case after two to three weeks after injection. It is advised to start treatment on a Monday, so 5 times daily radiation can be applied.

29. Assess whether randomization should be adjusted based on the size of the tumors, to ensure a similar mean tumor volume in each group at the start of treatment.
30. Select the mice to receive irradiation.

Note: In the described setup there is room for 4 mice at one time.

31. Prepare 3 surgical tape sections of approximately 2–3 cm in length per mouse.
32. Work inside a BSC. Anesthetize mice with 2% Isoflurane in 100% oxygen for 5 min.
33. Assemble the main part of the transparent mouse restrainer; (Figure 2A, 1). Insert the front part in the main part mouse restrainer.
34. Position one mouse into the restrainer with the head positioned to the front (Figure 2A, 2).
    a. Slide the roof onto the restrainer (Figure 2A, 3).
    b. Slide the bottom into the restrainer (Figure 2A, 4), making sure the mouse's tail is not stuck and the right hind limb is completely outside of the restrainer (Figure 2A, 5).
    c. Pull the right hind limb gently outward and forward (Figure 2A, 6).
    d. Place surgical tape behind the limb to secure its position (Figure 2A, 7).
    e. Affix tail to the restrainer roof using surgical tape to secure the position of the mouse (Figure 2A, 8).
35. Position the mice in the radiation setup (Figure 2B).
    a. Place the restrainer containing one mouse and place it on the bottom lead plate with the right limb facing the center of the setup (Figure 2B, 2).
    b. While gently pulling the hind limb outward, secure the limb to the center area with surgical tape on the ankle/paw.
    c. Place the upper protective lead shielding on top of the restrainer (Figure 2B, 3).
    d. Repeat these steps for all 4 mice (Figure 2B, 4).
    e. Place protective lead on top of the feet (ankle and toes) of the mice (Figure 2B, 4).
    f. Place the entire setup inside a transport box (Figure 2B, 5).

Note: Make sure the mice have access to oxygen during all procedures.

36. Take the mouse containing transport box outside of the BSC and bring it to the radiation source. We use the radiation source XStrahl RS320, using a 0.5 Cu filter.
37. Place the radiation setup with the mice on to the setup in the radiation source (Figure 2B, 6).
38. Adjust the X-ray/radiation so that the beam irradiates the non-shielded center area of 110 mm × 110 mm.

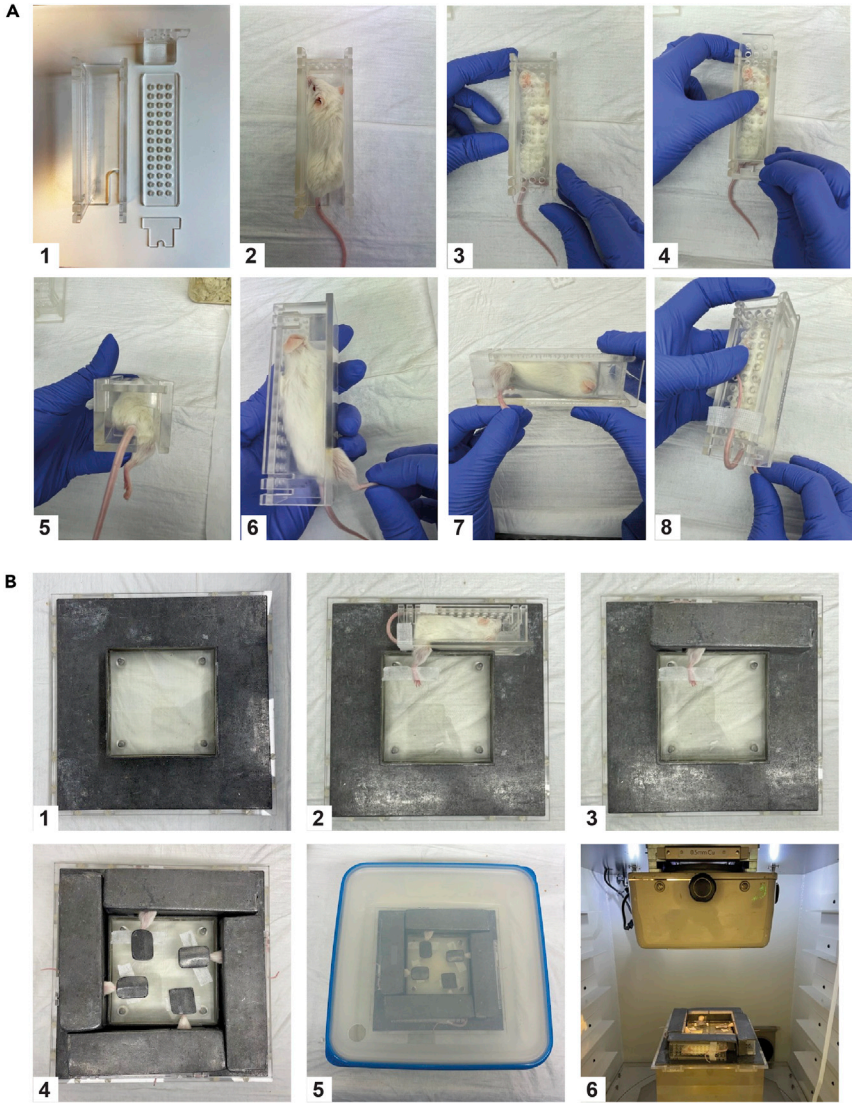

Figure 2. Restraining and local irradiation of hind limb of mice

(A) Assembly of mouse restrainer and positioning of the mouse. Numbers are explained in the main text.

(B) Assembly of shielding material. Numbers explained in the text.

39. Irradiate with a dose of 2 Gy at 220 kVp, 13 mA applied.
40. Repeat steps 30–39 daily.

Note: In our experiments, mice receive 10 fractions in two weeks to reach a total fractionated dose of 20 Gy. The required dose and fractionation may differ for other cancer types, models, and primary outcomes, and will need optimization.

41. Document tumor growth of each mouse three times a week during treatment and after treatment until tumors reach maximum tumor size.

Harvesting subcutaneous tumors from hind limb

Timing: 30 min per mouse

Depending on the experimental setup, repeat "Measuring size of tumors" until a defined timepoint, tumors reach maximum tumor growth or mice reach a humane endpoint.

This step describes how to harvest a subcutaneous tumor from the hind limb and process it for histology analysis, snap freezing tissue for transcriptomic analysis and vital freezing tissue for regrafting experiments.

42. Bring to animal facility: Bucket with ice, 4% PFA in container, labels, tissue cassette, microcentrifuge tubes, cryovial tubes, scissors, tweezer, scalpels, petri dishes.
43. When tumors exceed maximum tumor growth as measured in previous steps or when reaching a humane endpoint, anesthetize mice with 2% isoflurane in 100% oxygen for 5 min and sacrifice mice according to local legislation and ethical guidelines (in our case cervical dislocation).

Note: We use a maximum tumor growth of 500 mm^3, as we observed a necrotic core can occur in larger tumors. It is not advised to let tumors exceed 1,000 mm^3, as tumors on the hind limb can cause more discomfort than on the flank (Figures 3A and 3B).

44. Disinfect the right hind limb area with Virkon thoroughly. This functions to keep the viable frozen specimen sterile and also prevents loose hairs from contaminating the samples.
45. Label tissue cassette, screwcap microcentrifuge tube, and viable freezing vial.
46. Carefully harvest the tumor from the hind leg of the mice (Methods video S3, Figure 3C) and place the tumor on a 100 mm^2 petri dish.

47. Cut the tumor with a scalpel in to three sections. Cut from edge to center, so edge and center are equally represented in each stored tissue section (Figure 3D).
48. Divide the tumor sections (Figure 3E).
    a. Place one tissue section inside a labeled tissue cassette.
    b. Place one tissue section into a 0.5 mL screw cap microcentrifuge tube and put on ice.
    c. Place one tissue section into a viable freezing vial and put on ice.
49. Take samples from mice facility to Biosafety level 1 laboratory.

Processing and characterization of xenograft tissue

Timing: 1 week

This section describes processing of the harvested xenograft tissue in the previous steps. This includes long term storage for transcriptomic analyses or other characterization, vital freezing and preparation for histological and immunohistochemical assessment.

50. Snap freeze tissue.
    a. Add liquid nitrogen in a Dewar carrying flask.
    b. Take samples from ice and submerge screw cap vials in liquid nitrogen.

Note: Snap freezing increases RNA preservation for e.g., transcriptomic analyses.

    c. Store labeled vials at −80°C until further analyses.
51. Vital freeze tissue.
    a. Get freezing container ("Mr. Frosty") out of −80°C freezer in advance.
    b. Take the cryovial from ice, clean it with 70% EtOH and place into a BSC.
    c. Add 0.5 mL freezing medium to cryovial containing the tissue.
    d. Place the vials in a freezing container and freeze the tissue overnight (16 h).
    e. Place vials following day in the liquid nitrogen freezer.

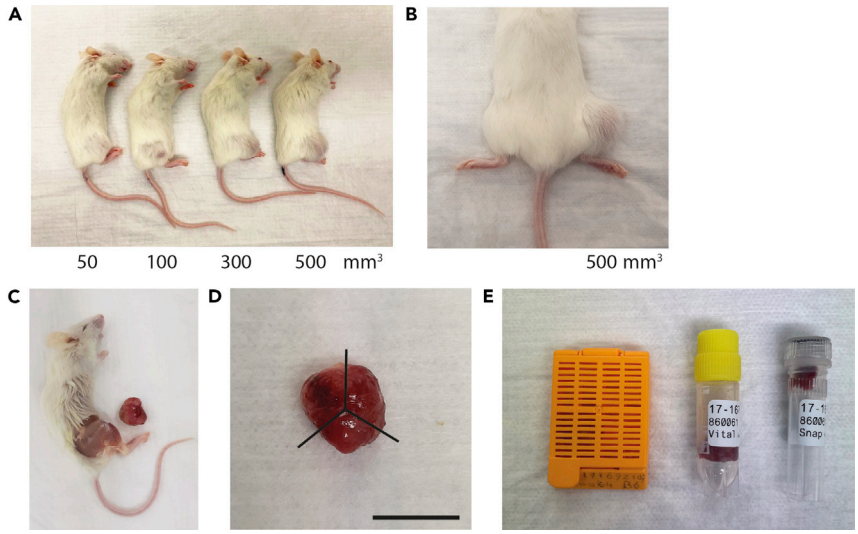

Figure 3. Harvesting tumors from mice

(A) Examples of indicated tumor sizes in hind limbs.

(B) Mice with ~500 mm^3 size tumor on right hind limb, compared to no tumor containing left limb.

(C) Dissected hind limb and harvested tumor.

(D) Diagram explaining dividing the tumor for further processing. Scale bar 10 mm.

(E) Tumor chunk in embedding cassette, in viable freezing vial, and snap-freeze screwcap vial.

f. Years after long term storage, tissue can be regrafted in mice, for instance to assess effects of applied treatments on long-term clonogenicity.[8]

52. Process tissue for histology assessment.
    a. Submerge the cassette in 4% buffered PFA, in a closed container.
    b. Next day remove the 4% PFA and replace by 70% ethanol for 1–7 days.
    c. Process tissue and stain for hematoxylin and eosin (H&E) according to Aziz et al.[9]
53. Assess histology of the tissue by H&E staining and determine the boundaries of the tumor tissue (Figure 4A).
54. Immunohistochemically stain tissue for γ-H2AX (phosphorylated H2A histone family member X, a marker indicative of DNA damage) with a subtle (30 s) Hematoxylin background according to Nagelkerke et al.[10]
55. Specifically in the tumor tissue established with H&E staining, validate radiation efficacy by induction of γ-H2AX immunohistochemistry (Figure 4B).

EXPECTED OUTCOMES

Two to three weeks after tumor cell injection tumors reach ~100 mm^3. This is based on data of esophageal 081R cells and cervical SiHa cells, but can depend per cell line and number of cells grafted. After two weeks of 2 Gy fractionated radiation, tumors are expected to grow out to maximum tumor volume of 500-100 mm^3 in approximately 9 weeks after last irradiation dose (Figure 5). A difference of >4 weeks of maximum tumor size outgrowth between the control group and the irradiated group can be expected. If other treatments combined with radiation are desired, we observed good tolerance of daily oral gavage simultaneously with the irradiation treatment schedule (Figure 5).[1]

LIMITATIONS

In this protocol tumors are irradiated in a setup that radiates from above. As the tumor is situated on top of the hind limb and is target more than any other tissue, this ensures effective targeting of the tumor. However, this setup does not allow radiation from all angles, as often used in a patient setting, resulting in one side of the tumor that received a slightly higher radiation dose than the other side. When processing and analyzing the tumors, this should be considered.

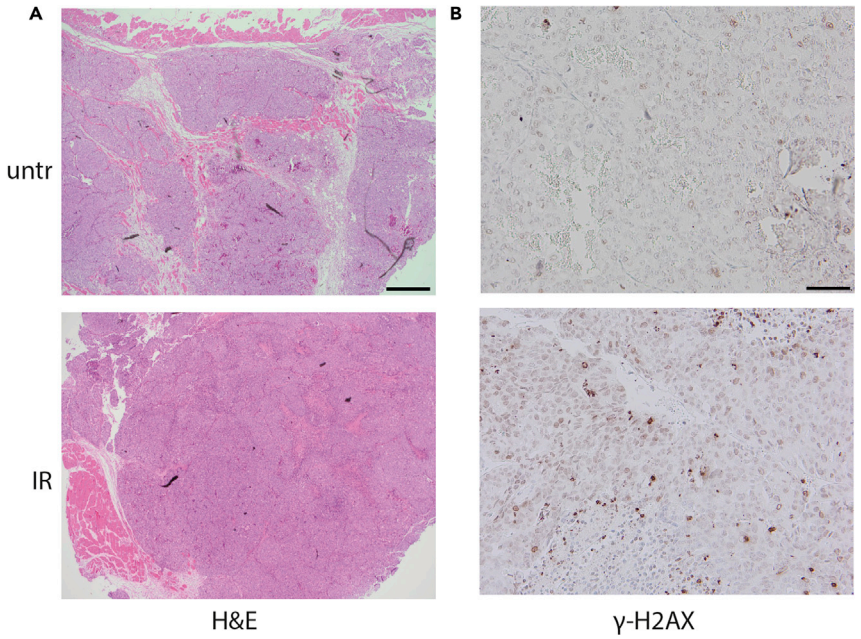

Figure 4. Histopathological analysis of treated tumors

(A) Histopathology by H&E of control and radiation treated tumor harvested as shown in Figure 3. Scale bar is 500 μm. Pink edges: myocyte tissue. Purple: tumor tissue.

(B) Tumor sections as shown in Figure panel A, processed for γ-H2AX immunohistochemistry, using DAB (brown) to visualize target protein. Scale bar 200 μm.

TROUBLESHOOTING

Problem 1

Lack of tumor growth. Even after successful injection, it can happen that tumors grow very slow (>3 months before palpable tumor), or do not grow out at all (related to step 41).

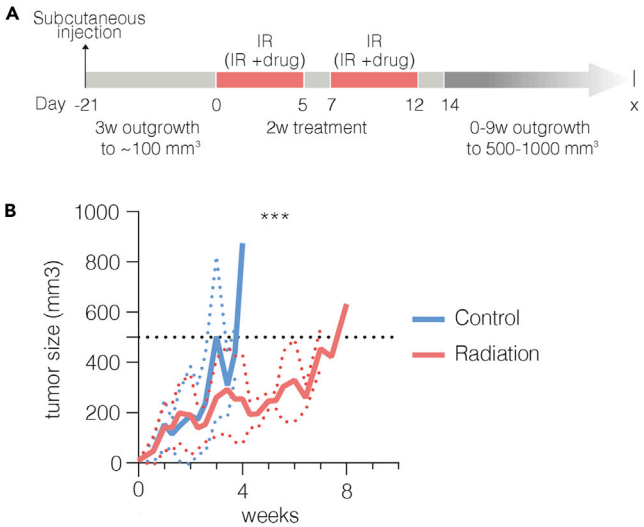

Figure 5. Expected timelines of tumor growth prior to and following irradiation

(A) Timeline of expected tumor growth during experiment.

(B) Average tumor growth in NSG mice of control group versus radiated group two-way ANOVA ***$p < 0.001$. Data from Dings et al.[1]

Potential solution

Patient-derived cell lines can differ tremendously in proliferation tumor take. We recommend performing a pilot experiment first, testing multiple cell lines with two number of injected cells (e.g., 1×10^5 and 1×10^6). Based on *in vitro* proliferation rates, one would likely choose cultures that show a reliable growth, not too fast or too slow. Too fast-growing tumors could cause ulceration, a humane endpoint for mice without reading maximum tumor size. Prior to injection, resuspend the prepared cell suspension extensively just before injection, to avoid cellular aggregates and clumps, as well as preventing to inject many versus no cells in other mice that could cause no tumor outgrowth.

Problem 2

No effect of irradiation on tumor growth (related to step 41).

Potential solution

Radiation sources could radiate either X-rays or gamma radiation. The relative biological effect (RBE) is a function of the beam's characteristics and the tissues with which it interacts. Different tissues within the body respond differently to the same radiation dose. In general, the RBE for X-rays is higher than for that of gamma radiation (30%–40%), so a lower dose (Gy) may be required for X-ray than for gamma radiation. Alternatively, certain cell lines could be more resistant to radiation than others. Therefore, dose adjustment may be necessary.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Arlene L. Oei; a.l.oei@amsterdamumc.nl.

Materials availability

Reagents, resources, and materials employed in this study are detailed in the key resources table. Cell lines used in this study and generated at our laboratory are available upon request. All files to create the mice radiation setup are provided in this article.

Data and code availability

Original data have been deposited to Mendeley Data: https://doi.org/10.17632/vfjjtwstw9.1.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2023.102098.

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AUTHOR CONTRIBUTIONS

Conceptualization, A.Z.; Investigation & methodology, A.Z., S.B., H.R.; Writing, A.Z., M.F.B., A.L.O.; Supervision, M.F.B., A.L.O.

DECLARATION OF INTERESTS

M.F.B. has received research funding from Celgene, Frame Therapeutics, and Lead Pharma and has acted as a consultant to Servier.

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