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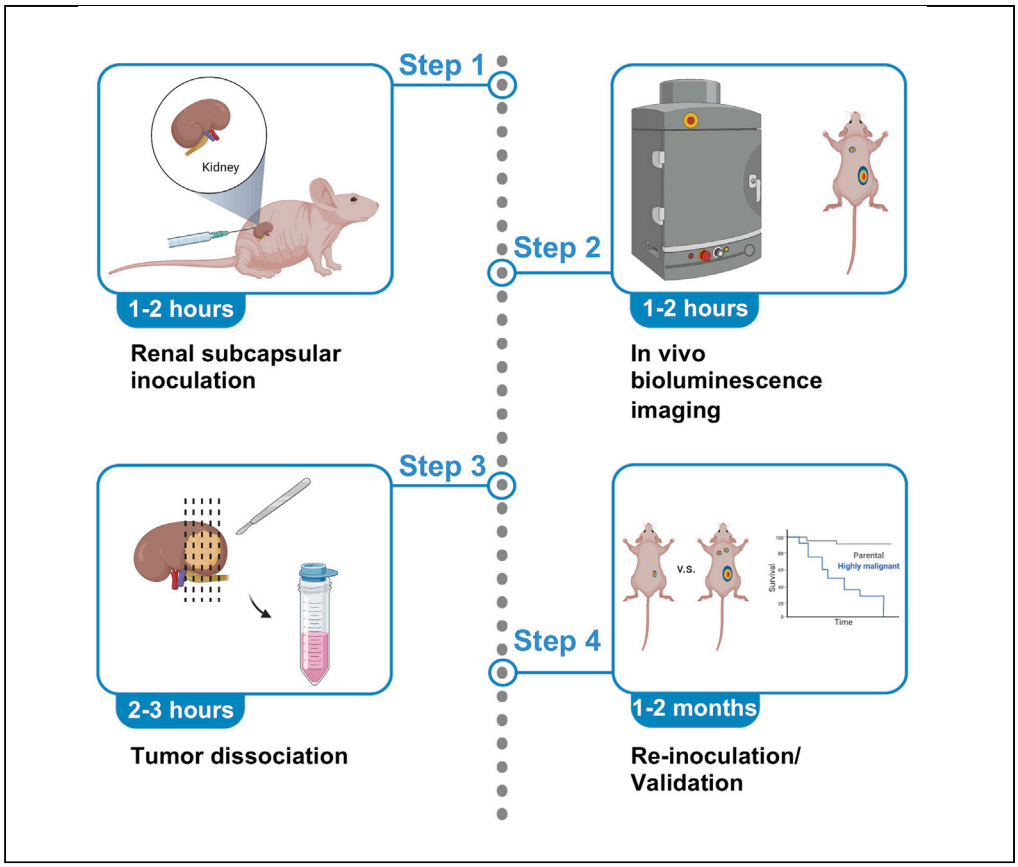
An *in vivo* orthotopic serial passaging model for a metastatic renal cancer study



We developed an *in vivo* serial passaging model for renal cancer with orthotopic renal subcapsular inoculation. We detail the procedures for renal subcapsular inoculation of cancer cells in mice, followed by *in vivo* and *ex vivo* bioluminescence imaging, tumor-bearing kidney dissociation, and *in vivo* passaging. This protocol is capable of reproducing the coevolution between cancer cells and the primary tumor microenvironment. It enables us to unveil the systemic dynamics of metastasis and develop a therapeutic strategy for metastatic renal cancer.

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Highlights

A protocol to obtain highly malignant derivatives of renal cancer cells

Renal cancer is recapitulated by renal subcapsular inoculation in mice

Repeated orthotopic inoculation endows cancer cells with highly metastatic phenotype

This protocol is applicable for the analysis of immune microenvironment in renal cancer

Nishida et al., STAR Protocols *3*, 101306
June 17, 2022
https://doi.org/10.1016/j.xpro.2022.101306

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An *in vivo* orthotopic serial passaging model for a metastatic renal cancer study

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https://doi.org/10.1016/j.xpro.2022.101306

SUMMARY

We developed an *in vivo* serial passaging model for renal cancer with orthotopic renal subcapsular inoculation. We detail the procedures for renal subcapsular inoculation of cancer cells in mice, followed by *in vivo* and *ex vivo* bioluminescence imaging, tumor-bearing kidney dissociation, and *in vivo* passaging. This protocol is capable of reproducing the coevolution between cancer cells and the primary tumor microenvironment. It enables us to unveil the systemic dynamics of metastasis and develop a therapeutic strategy for metastatic renal cancer.
For complete details on the use and execution of this protocol, please refer to Nishida et al. (2020).

BEFORE YOU BEGIN

The protocol described below lists specific procedures for the use of human renal cancer cells. However, a similar protocol is available for mouse renal cancer cells.

Institutional permissions

All of our animal experiments were performed under the approval of the Animal Ethics Committee of The University of Tokyo. Animal experiments described in this manuscript should be recapitulated in line with the permitted protocol of each institution.

Preparation of cancer cells and mice

Timing: 2 weeks to 1 month

1. Preparation of cancer cells labeled with fluorescent protein and luciferase.
   a. Transduce mCherry and Luc2 via lentiviral vectors.
      i. One day before transduction, plate 2.0 × 10^4 cells with 500 μL of culture media in several wells of 24-well plate.
      ii. Infect lentivirus encoding mCherry or Luc2 to cells with several different volumes.

⚠ CRITICAL: Multiplicity of infection (moi) of lentivirus varies in each cell line. It is good to add different volumes of lentivirus to several wells of cells. We have observed that the

volume of lentivirus successfully introduced in ≥ 95% of OS-RC-2 is 10-fold higher than that of 786-O or Caki-1 cells.

iii. One day after adding lentivirus, replace the culture media.
iv. Expand cells by avoiding confluent.

Alternatives: Although we transduced individual lentiviral vectors of mCherry and Luc2 separately in this context, it is a good to engineer two-in-one vector containing mCherry and Luc2 in one vector (e.g., mCherry-T2A-Luc2).

b. Two to three days after transduction, validate expression of mCherry under the microscope.
c. Validate the activity of Luc2.
   i. Suspend an arbitrary number of cells in 100 μL of PBS and plate into the optimal 96 well plate for the luminometer.
   ii. Add 10 μL of 12.5 mg/mL of D-Luciferin potassium salt (VivoGlo™ Luciferin, In Vivo Grade) - PBS solution to each well.
   iii. Measure the intensity of bioluminescence signal by a luminometer.

Note: We verified that 786-O, Caki-1, and OS-RC-2 cells were successfully transduced. We did not experience the loss of expressions of mCherry and Luc2 at least 10 passages after the transduction.

2. Preparation of immunocompromised mice.

Note: The extent of immunodeficiency varies depending on the cancer cells of interest. It may be important to validate the engraftment of cancer cells in advance. We verified that athymic mice (five-week-old male mice) are acceptable for the engraftment of human cancer cell lines, including 786-O, Caki-1, and OS-RC-2.

⚠ CRITICAL: This protocol was optimized for human cancer cell lines inoculated into immunocompromised mice. For application in mouse cancer cell lines, please be aware that fluorescent protein and luciferase can be immunogenic (Gambotto et al., 2000; Limberis et al., 2009).

⚠ CRITICAL: To avoid exceeding clonal selection, prepare 3 or more tumor-bearing mice.

Renal subcapsular inoculation of cancer cells

Timing: 1–2 h

3. Preparation of cell suspension.
   a. Collect cancer cells from culture and wash once with PBS.
   b. Suspend cells into Hanks' Balanced Salt Solution (HBSS) to be 2.0×10^6 cells/mL.
4. Renal subcapsular inoculation (Methods video S1).
   a. Anesthetize mice with an approved protocol.
   b. Make an incision (≤1 cm) on the back of an anesthetized mouse above the right kidney. Sanitize the area with an alcohol swab before the surgery (Figures 1A and 1B).
   c. Lift the kidney upon the body surface (Figure 1C).
   d. Prepare 50 μL of cell suspension into the low-dose syringe.
   e. Insert the needle from the inferior pole of the kidney. Stab one-third to half the length of the 13-mm needle of an insulin syringe into the parenchyma to reach the superior subcapsular space (Figures 1D–1F).
   f. Inoculate 50 μL of cell suspension to be 1.0×10^5 cells per mouse.

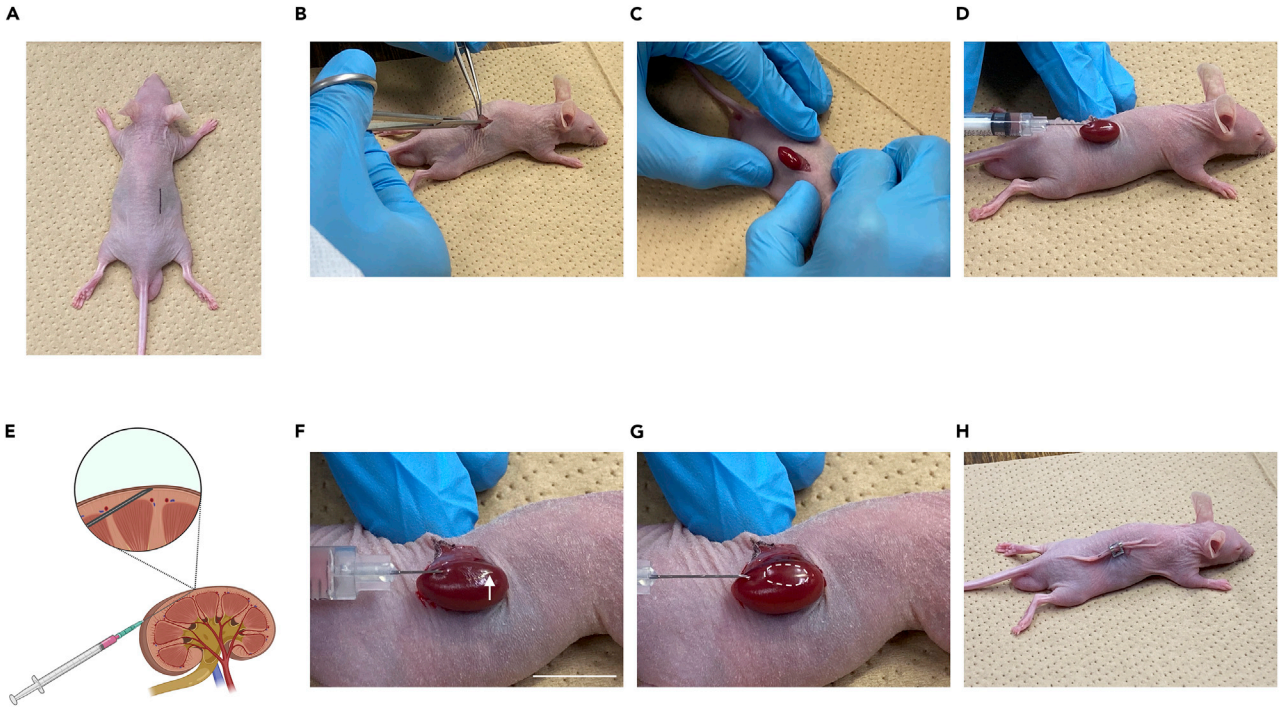

Figure 1. Renal subcapsular inoculation into an athymic mouse

(A) The point of incision to be made (black line; see before you begin; step 4b).

(B) Incision being made on the back of an anesthetized mouse (before you begin; step 4b).

(C) Kidney lifted upon the body surface (before you begin; step 4c).

(D) Insertion of needle (before you begin; step 4e).

(E) Schematic of injection site. Needle will be inserted from the inferior pole of kidney with the cutting edge face-up (i.e., on the capsule; before you begin; step 4e).

(F) Inserted needle. Cutting edge of the needle is visible under the capsule (white arrow; before you begin; step 4e: Scale bar = 1 cm).

(G) After the injection. Make sure that cell suspension is remaining in the subcapsular space (white dotted circle; before you begin; steps 4f and 4g).

(H) Close the wound with clip (before you begin; step 4h).

g. Gently remove the needle (troubleshooting 1; Figure 1G).

h. Close the wound with a clip (troubleshooting 2; Figure 1H).

Note: It is preferable to inject into the right kidney. When injecting into the left kidney, the spleen will also be lifted simultaneously.

⚠ CRITICAL: At the time of inoculation, the cutting edge of the needle should face the kidney capsule (Figure 1E). The cutting edge of the needle should be visible just under the capsule when the needle reaches the subcapsular space. It is important to ensure that the cell suspension does not leak from the insertion site and remains in the subcapsular space (Figure 1G, white dotted circle). If the injected cell suspension does not appear in the subcapsular space and enters the parenchyma, this mouse should be excluded from the experiment.

In vivo bioluminescence imaging

Timing: 1–2 h

5. Anesthetize tumor-bearing mice following an approved protocol.
6. Intraperitoneal injection of D-Luciferin.

a. Inject 200 μL of 12.5 mg/mL of D-Luciferin potassium salt - PBS solution into the intraperitoneal space.
b. Wait for 10 min.

7. Visualize and quantify bioluminescence by in vivo bioluminescence imaging (troubleshooting 3).

Note: The duration of the exposure time for in vivo bioluminescence imaging varies depending on the machine and tumor size. It is important to image the tumor tissues with several different exposure times to avoid saturation at the late time point of imaging.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
FITC Anti-CD11b antibody (Clone M1/70; 1:200 dilution)	BioLegend	Cat# 101206; RRID: AB_312789
BV421 Anti-CD45 antibody (Clone 30-F11; 1:200 dilution)	BioLegend	Cat# 103134; RRID: AB_2562559
PE Anti-F4/80 antibody (Clone BM8; 1:200 dilution)	BioLegend	Cat# 123110; RRID: AB_893486
APC/Cy7 Anti-Ly-6C antibody (Clone HK1.4; 1:200 dilution)	BioLegend	Cat# 128026; RRID: AB_10640120
PE/Cy7 Anti-Ly-6G antibody (Clone 1A8; 1:200 dilution)	BioLegend	Cat# 127618; RRID: AB_1877261
PerCP/Cy5.5 Anti-I-A/I-E (MHC class II) antibody (Clone M5/114. 15.2; 1:200 dilution)	BioLegend	Cat# 107626; RRID: AB_2191071
APC Anti-SiglecF antibody (Clone S17007L; 1:200 dilution)	BioLegend	Cat# 155508; RRID: AB_2750237
Chemicals, peptides, and recombinant proteins		
VivoGlo™ Luciferin, In Vivo Grade (D-Luciferin potassium salt)	Promega	Cat# P1043
PBS	Takara Bio	Cat# T900
HBSS	Thermo Fisher Scientific	Cat# 14170112
Lipofectamine 2000	Thermo Fisher Scientific	Cat# 11668019
Type-I collagenase	Worthington Biochemical Corporation	Cat# LS001652
ACK Lysing Buffer	Thermo Fisher Scientific	Cat# A1049201
Zombie Aqua Fixable Viability Kit	BioLegend	Cat# 423102
FcR Blocking Reagent, mouse	Miltenyi Biotec	Cat# 130-092-575
Experimental models: Cell lines		
Human: OS-RC-2 cells	RIKEN BRC	Cat# RCB0735; RRID:CVCL_1626
Human: Caki-1 cells	ATCC	Cat# HTB-46; RRID:CVCL_0234
Human: 786-O cells	ATCC	Cat# CRL-1932; RRID:CVCL_1051
Experimental models: Organisms/strains		
Mouse: BALB/cSlc-*nu/nu* (Male; 5 weeks old)	Sankyo Labo Service/ Japan SLC	N/A
Recombinant DNA		
Plasmid: pCSII-CMV-Luc2	Nishida et al. (2018).	N/A
Plasmid: pCSII-EF-mCherry	Nishida et al. (2018).	N/A
Plasmid: pCAG-HIVgp	RIKEN BRC	Cat# RDB04394
Plasmid: pCMV-VSV-G-RSV-Rev	RIKEN BRC	Cat# RDB04393
Software and algorithms		
GraphPad Prism 6	GraphPad Software	https://www.graphpad.com/scientific-software/prism/
Other		
In vivo bioluminescence imaging	Berthold Technologies	NightOWL LB981

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
24 well plate	Corning	Cat# 3524
6 well plate	Corning	Cat# 3516
10 cm dish	Corning	Cat# 353003
Insulin syringe with 29G needle	TERUMO	Cat# SS-10M2913
40-μm nylon cell strainer	Corning	Cat# 352340

STEP-BY-STEP METHOD DETAILS

Ex vivo bioluminescence imaging

Timing: 1 h

This section describes how to visualize luciferase-expressing cancer cells in resected organs.

1. Euthanize mice with an approved protocol.
2. Resect the tumor-bearing kidney from three or more mice.
3. Incubation of the kidney with D-Luciferin.
   a. Place all the resected tumor-bearing kidneys on a 10 cm plate or an according size of plate.
   b. Add 12.5 mg/mL of D-Luciferin potassium salt - PBS solution directly onto the kidney on the plate by a pipet. The volume of luciferin - PBS solution needed will be almost 200 μL per kidney.
   c. Incubate for 5 min.
4. Visualize and quantify bioluminescence by in vivo bioluminescence imaging.

Note: This protocol is also applicable to visualizing luciferase-expressing cancer cells in the lungs in cases where lung metastasis is of interest.

Dissociation of tumor-bearing kidney

Timing: 2–3 h

This section describes the preparation of a single cancer cell suspension from the resected kidney (Methods video S2). This protocol is also applicable for metastatic lung tumors in cases where lung metastasis is of interest.

⚠ CRITICAL: Process of dissociation is individually performed for each tumor-bearing kidney.

5. Separate tumor from the kidney.
6. Mince the collected kidney with scalpel to be smaller than 1 mm cubic.

Note: To avoid contamination, samples are sprayed with 70% Ethanol.

7. Dissociate with collagenase.
   a. Collect the minced kidney in 400 unit/mL of type-I collagenase dissolved in native cell culture media (without any additives like FBS) used for the cell lines of interest.
   b. Incubate at 37°C for 30 min.
8. Filter cell suspension through cell strainer.
   a. Pass tumor chunk through 40-μm nylon cell strainer placed on 50 mL tube and transfer cell suspension in a 15 mL tube.
   b. Centrifuge at 400 × *g* at 10 min.

c. Remove the supernatant.

9. Wash with PBS.
   a. Add 5 mL of PBS.
   b. Centrifuge at 400 × *g* at 10 min.
   c. Remove the PBS.
10. Red blood cell lysis.
   a. Add 1 mL of ACK lysing buffer.
   b. Incubate at room temperature (18°C–25°C) for 5 min.
   c. Add 9 mL of PBS.
   d. Centrifuge at 400 × *g* at 10 min.
   e. Remove the supernatant.

⚠ CRITICAL: If this is the last step of repeated in vivo passaging, cells can be suspended in the culture media and returned to normal cell culture conditions. Cancer cells can be purified using a cell sorter based on the fluorescent protein.

11. Myeloid cell characterization by flow cytometry analysis.
   a. Dead cell staining with Zombie Aqua Fixable Viability Kit.
      i. Add 1 mL of 1:500 Zombie Aqua Fixable Viability Kit diluted in PBS.
      ii. Incubate at room temperature (18°C–25°C) for 10 min.
      iii. Add 9 mL of 2% FBS-PBS.
      iv. Centrifuge at 400 × *g* at 10 min.
      v. Remove the supernatant.
   b. Fixation with 4% PFA-PBS.
      i. Add 1 mL of 4% PFA-PBS.
      ii. Incubate at room temperature (18°C–25°C) for 10 min.
      iii. Add 9 mL of 2% FBS-PBS.
      iv. Centrifuge at 400 × *g* at 10 min.
      v. Remove the supernatant.

Pause point: After fixation, samples can be stored in 2% FBS-PBS at 4°C.

   c. Blocking with FcR Blocking Reagent.
      i. Add 100 μL of 1:10 FcR Blocking Reagent diluted in 2% FBS-PBS.
      ii. Transfer samples into a U-bottom 96 well plate.

Note: When preparing samples stained with control isoform antibody, each sample is split into 2 wells with 50 μL each.

Alternatives: A V-bottom plate will be a good alternative if cell pellet will not be packed well at the time of centrifuging a U-bottom plate.

      iii. Incubate at room temperature (18°C–25°C) for 10 min.
      iv. Centrifuge at 110 × *g* at 10 min.
      v. Discard the supernatant.
   d. Antibody staining.
      i. Add 100 μL of antibody cocktail.
      ii. Incubate at room temperature (18°C–25°C) for 30 min.
      iii. Add 100 μL of 2% FBS-PBS.
      iv. Centrifuge at 110 × *g* at 10 min.
      v. Discard supernatant.
   e. Wash with 2% FBS-PBS.
      i. Add 200 μL of 2% FBS-PBS.

ii. Centrifuge at 110 × *g* at 10 min.
iii. Discard the supernatant.
f. Analyze with a flow cytometer.

In vivo passage

Timing: 1–2 h

This section describes the preparation and re-injection of the cancer cells into the next mouse.

12. Wash with HBSS.
    a. Merge dissociated cells from each mouse into 10 mL of HBSS.
    b. Centrifuge at 400 × *g* at 10 min.
    c. Remove the supernatant.
13. Cell preparation for inoculation (troubleshooting 4).
    a. Suspend cells into HBSS.
    b. Count cell number and adjust the concentration of cancer cells to be 2.0 × 10^6 cells/mL.

Note: Both the cancer and stromal cells are included in samples of different cell sizes. In general, cancer cells are larger than stromal cells, with a diameter between 5 and 20 μm. It is good to separately count the cancer cells from the stromal cells based on their size. Repeated renal subcapsular inoculation can be performed with the mixed population of the cancer cells and the stromal cells.

⚠ CRITICAL: To avoid exceeding clonal selection, prepare three or more tumor-bearing mice.

14. Renal subcapsular inoculation. Please follow the protocol shown above. Inoculate 1.0 × 10^5 cells of cancer cells suspended in 50 μL.
15. While considering humanitarian endpoints, the mice were kept. Once the survival time of derivative-bearing mice reaches almost half that of those bearing parental cells, cells can be established as highly malignant derivatives.
16. Evaluate activity of Luc2 in highly malignant derivatives. Please follow the protocol shown in step 1 before you begin (troubleshooting 5).

Highly malignant clear cell renal cell carcinoma (ccRCC) cell derivatives

Derivative	Parental cell line	Passage
OS5K-1, -2, -3	OS-RC-2	5 times
786-3K	786-O	3 times
Caki-1-3K-1, -2, -3	Caki-1	3 times

EXPECTED OUTCOMES

In each passage, the newly established derivatives shortened the survival of tumor-bearing mice. Highly malignant derivatives grew faster in the primary site and in metastatic lungs than in the primary cells (Figure 2). This was independent of the behavior in cell culture (i.e., agar-gel colony formation assay or chamber migration assay; Nishida et al., 2020).

LIMITATIONS

One of the major limitations of this study is the selection of models. Xenograft models of human cancer cells into nude mice are limited in their ability to study adaptive immunity. Although syngeneic

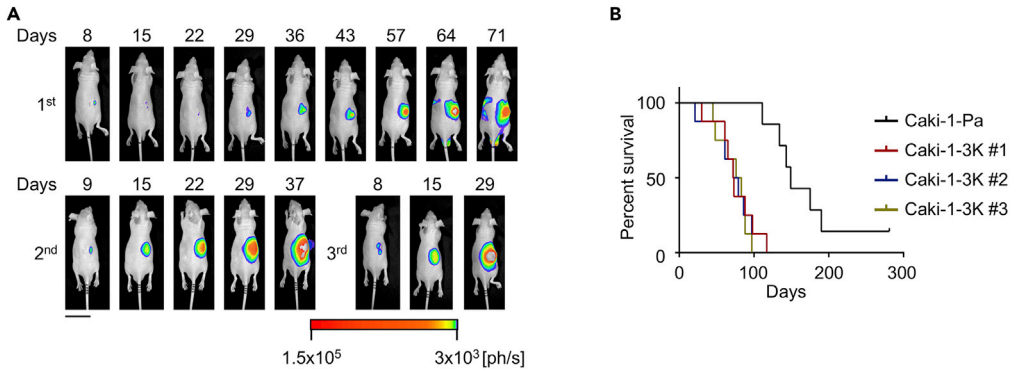

Figure 2. *Invivo* serial passaging of renal cancer cells

(A) In vivo bioluminescence imaging of Caki-1-bearing mice at each process (Scale bar = 3 cm).

(B) Kaplan–Meier plot of mice bearing parental or highly malignant Caki-1 cells.

mouse models can overcome this limitation, exogenous genes including fluorescent protein, luciferase, and Cas9 can be immunogenic (Gambotto et al., 2000; Chew et al., 2016; Limberis et al., 2009). In contrast, our previous study focused on the enhancer remodeling of cancer cells. Since the enhancer landscape is distinct between humans and mice, a xenograft model is suitable for this purpose. Several human cancer cells failed to engraft in nude mice. In this case, it is necessary to consider the use of less immunogenic mice. This limits the experimental set-up in terms of cost, breeding environment, and ethical reasons.

In our orthotopic inoculation model, the lung was the common metastatic site. Macroscopic metastases in other tissues such as the brain and bone cannot be detected in our setting. Therefore, it may be necessary to take the surgical removal of primary tumors into account for the long-term evaluation of metastasis.

TROUBLESHOOTING

Problem 1

Significant variability of the tumor size engrafted or cancer cell dissemination occur because of the suspension backflow (before you begin step 4g).

Potential solution

By inoculating softly, we did not observe the significant backflow of cell suspension inducing significant variability of the tumor size or cancer cell dissemination in our model. In case the cell of interest induces these problems, it is good to hold the insertion site with a cotton swab just after the inoculation to avoid backflow.

Problem 2

The incision made is not closed properly (before you begin step 4h).

Potential solution

Make sure to clip both peritoneum and skin at once. A clip can be usually removed 1 week after the inoculation. Suturing will also be a good option.

Problem 3

Bioluminescent signal from tumor is not visible when performing in vivo bioluminescence imaging (before you begin step 7).

Potential solution

In some cancer cells, the bioluminescent signal may drop off 1–2 weeks after the inoculation. We have also observed the signal drop-off at day 15 or day 22 after the inoculation of parental Caki-1 cells (Figure 2A). If the signal is not visible at early time point, it is good to continue monitoring tumor weekly. In case cancer cells do not form tumor at all, the number of cancer cells inoculated can be increased. We succeeded to form kidney tumor without technical problems when injecting up to 3.0×10^5 cells in our hands. More than 3.0×10^5 cells may not be packed in the inoculation volume (50 μL) in some types of cancer cells like human kidney cancer cell line OS-RC-2 because of their large cell size.

Problem 4

Bacterial contamination of newly established cancer cell derivatives (step 9).

Potential solution

Add penicillin-streptomycin to the culture and consider using an anti-bacterial reagent, amphotericin B.

Problem 5

The intensity of luciferase is occasionally different between parental cells and newly established derivatives (step 16).

Potential solution

Monitor luciferase activity in both parental cells and derivatives (see step 1 in before you begin). When comparing the bioluminescence signal among the different cells, the intensity of in vivo bioluminescence should be normalized by the activity of the luciferase in individual derivatives. The weight of the parental renal tumor can also be evaluated by subtracting the weight of the tumor-bearing kidney from that of the unaffected kidney. Metastasis is also detectable with fixed frozen tissue by observing native fluorescence or FFPE sectioning by immunostaining using anti-luciferase or anti-fluorescent protein antibodies.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Shogo Ehata (ehata@wakayama-med.ac.jp).

Materials availability

For the request of highly malignant ccRCC cells, please contact lead contact.

Data and code availability

This study did not generate any datasets.

SUPPLEMENTAL INFORMATION

Supplemental information can be found online at https://doi.org/10.1016/j.xpro.2022.101306.

ACKNOWLEDGMENTS

We would like to thank K. Ogikubo (The University of Tokyo) for technical support. This work was supported by a KAKENHI Grant-in-Aid for Scientific Research on Innovative Area on Integrated Analysis and Regulation of Cellular Diversity (17H06326) from the Ministry of Education, Culture, Sports, Science and Technology (MEXT) of Japan (to K. Miyazono and S.E.); a Grant-in-Aid for the Japan Society for the Promotion of Science (JSPS) Research Fellow (16J05993; to J.N.); a KAKENHI Grant-in-Aid for Scientific Research (C) (19K07684; to S.E.) from JSPS; and the Princess Takamatsu Cancer Research Fund (to S.E.). This work was also supported in part by a grant from the Endowed

Department (Department of Medical Genomics) from Eisai Co., Ltd. Schematics in the graphical abstract and Figure 1E were created using BioRender.com.

AUTHOR CONTRIBUTIONS

J.N. and K. Miyakuni conducted the experiments. J.N., K. Miyazono, and S.E. designed the experiments and wrote the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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