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Orthotopic model of hepatocellular carcinoma in mice

Flavia Lambertucci^{1,2}, Sijing Li^{1,2,3}, Omar Motiño^{1,2}, Léa Montégut^{1,2,3}, Uxia Nogueira Recalde^{1,2,4}, Hui Chen^{1,2,3}, Gerasimos Anagnostopoulos^{1,2,3}, Maria Chiara Maiuri^{1,2,5}, Guido Kroemer^{1,2,6,#} and Isabelle Martins^{1,2,#}

¹ Centre de Recherche des Cordeliers, Equipe labellisée par la Ligue contre le cancer, Inserm U1138, Université Paris Cité, Sorbonne Université, Paris, France;

² Metabolomics and Cell Biology Platforms, Gustave Roussy, Villejuif, France;

³ Faculté de Médecine, Université de Paris Saclay, Kremlin Bicêtre, France;

⁴ Rheumatology Research Group (GIR), Biomedical Research Institute of A Coruña (INIBIC), Professor Novoa Santos Foundation, A Coruña, Spain;⁵ Department of Molecular Medicine and Medical Biotechnologies, University of Napoli Federico II, Napoli, Italy;

⁶ Institut du Cancer Paris CARPEM, Department of Biology, Hôpital Européen Georges Pompidou, France-HP, Paris, France.

#Correspondence: Guido Kroemer (Kroemer@orange.fr) or Isabelle Martins (isabelle.martins@inserm.fr).

Contact Information:

Centre de Recherche des Cordeliers U1138
Metabolism, Cancer and Immunity laboratory
15 rue de l'École de Médecine
75006 PARIS
FRANCE

Summary/Abstract

Orthotopic models of hepatocellular carcinoma (HCC) consist in the implantation of tumor cells into the liver by direct intrahepatic injection. In this model, tumorigenesis is triggered within the hepatic microenvironment thus mimicking the metastatic behaviour of HCC. Herein, we detail a surgically mediated methodology that allows the reproducible and effective induction of liver-sessile tumors in mice. We enumerate the steps to be followed before and after the surgical procedure, including HCC cell preparation, the quantity of cancer cells to be injected, presurgical preparation of the mice and, finally, postoperative care. The surgical procedure involves laparotomy to expose the liver, injection of cells into the left-lateral hepatic lobe and closure of the incision with sutures followed by wound clips. We also provide information concerning the subsequent tumor growth follow-up, as well as the application of bioluminescence imaging to monitor tumor development.

Key Words

Bioluminescent imaging, hepatocellular carcinoma, intrahepatic injection, orthotopic cancer model, surgical procedure

1. Introduction

Most cases of liver cancer occur in the setting of chronic liver disease, making the design of adequate mouse models challenging **(1)**. For a detailed and effective characterization of hepatocellular carcinoma (HCC) treatment, different models should be employed. Animal models in which development of initially non-malignant liver diseases is followed by hepatic oncogenesis and tumor progression are best suited to study the biology of hepatocarcinogenesis **(2)**. Nevertheless, mimicking the physiological changes of HCC development requires long-lasting treatments. Injection of HCC tumor cells into mice is suitable for establishing a biologically relevant model of the disease in order to evaluate novel treatment strategies. Thanks to their simplistic setup and increased reproducibility, implantation models are often used to test new drugs **(2)**.

Injection of murine or human cancer cell lines as well as implantation of tumor tissue subcutaneously or into the liver of recipient mice, represent the most common methods to establish HCC tumors **(3)**. Injection of a tumor into its tissue of origin is known as an orthotopic model, whereas tumors inoculated into a foreign tissue, for example, subcutaneously, constitute heterotopic models.

The dual hepatic vascularization with its connection to the gut, the tumor–stroma interactions and the metastatic behaviour of HCC require orthotopic models more than in any other cancer field **(4)**. When compared to subcutaneous implantations, orthotopic models are technically more challenging to perform. However, they may reflect more accurately the tumor microenvironment (TME). Orthotopic tumors derived from mouse or human cells can be introduced via direct intrahepatic, intrasplenic or intraportal injection **(5)**.

In syngeneic models, murine cancer cells or murine tumors are implanted in immunocompetent mice. In xenograft models, human cancer cell lines or human tumors are injected in immunocompromised murine recipients to avoid rejection of the foreign tissue. For this reason, xenograft models are not expected to mimic the TME and the immune reactions against the tumor **(6, 7)**. Nowadays, it is of major importance to consider the role of the TME on HCC development, progression and response to therapy **(8, 9)**.

The present protocol describes in detail all the steps required for a syngeneic mouse liver orthotopic implantation model. This HCC model constitutes a powerful tool to uncover the mechanisms regulating tumor initiation and progression **(10)**. As an example, we established a unifocal HCC model developing a single tumor nodule by implantation of Hep55.1C-Luc murine tumor cells in the liver of syngeneic 7 weeks-old female C57BL/6J mice. Tumor evolution can be monitored by bioluminescent imaging (BLI) as well as histologically. The implantation of Hep55.1C cells in the syngeneic mice liver enables the fast growth of a highly differentiated tumor, expressing fibrotic and cancer marker genes **(5)**. This model represents a reproducible HCC model in the C57BL/6J mouse strain, which has a low propensity to develop spontaneous cancers.

Established luciferase-expressing HCC cell lines have been successfully used in an orthotopic immunodeficient xenograft mouse model, which enables easy repeated monitoring of tumor growth by in vivo bioluminescence that correlates well with MRI imaging **(11)**. BLI consists in the detection of luminescence emitted following the expression of luciferase genes in tumor cells and the administration of the substrate luciferin **(12)**. Although BLI has been found to correlate with other

imaging modalities, when tumors reach the end stage of disease, BLI may underestimate tumor growth as the tumor outgrows its blood supply **(13)**. Nevertheless, due to the local injection of tumor cells, mice develop a solid single tumor nodule that it is easily detected with BLI. Additionally, BLI is a useful tool to monitor changes in tumor growth induced by different treatment modalities **(14, 15)**.

Prior to injection, HCC cells are trypsinized, resuspended in culture medium, and maintained in ice until the moment of injection (section 3.1). After the recipient mouse is anesthetized, a parallel incision along the linea alba is made in the abdominal wall to expose the liver. Then, the injection is performed orthotopically into the left-lateral hepatic lobe. The peritoneum is closed with suture, and wound clips are used for closure of the skin. Intrahepatic implantation of mouse HCC cells requires 20-30 min per mouse (section 3.2). Tumor growth is followed by BLI for 2 to 8 weeks after implantation. Mice are sacrificed 6-8 weeks after implantation, and histological analysis of the liver by HES staining is performed (section 3.3). Tumor growth varies according to the tumor cell line and mouse strain used. Additionally, in later stages of the disease, the mice may develop local metastases in adjacent lobes of the liver, as well as lung metastases.

Development of one big tumor nodule, which can be easily distinguished from the rest of the liver tissue, is useful for a precise measurement of tumor size and for immunotherapy studies, as mononuclear cells from the tumor and surrounding liver tissue can be analyzed separately. This distinguishes this model from others (e.g. genetically engineered mice) which may generate several tumors throughout the liver **(16)**. Since the protocol consists in a surgical procedure on living mice, it requires the acquisition of basic surgery skills to perform the

procedure in an ethically accepted, effective, safe, and reproducible manner. Finally, variations of this protocol can be performed in the context of non-alcoholic fatty liver disease (NAFLD), injecting HCC cells orthotopically in preconditioned livers with high-fat-diet feeding or underlying liver cirrhosis **(10, 15)**. Indeed, simultaneous induction of tumor growth and development of liver disease can help to understand the crosstalk between hepatic tumors and the local environment.

2. Materials

2.1. Reagents

1. Culture medium: DMEM high-glucose medium (pH 7.0 to 7.4, with 4.5 g/L D-glucose, L-glutamine, phenol red, sodium pyruvate), 10% fetal bovine serum (FBS), 1% penicillin/streptomycin antibiotics
2. Trypsin suitable for cell culture
3. 70% ethanol
4. 10% povidone iodine solution or Betadine
5. Luciferase-expressing HCC line: Hep 55.1c-Luc
6. Serological pipettes and pipette tips
7. T75 Flask (suitable for cell culture)
8. Eppendorf and centrifuge tubes
9. C57Bl/6J female mice, 7 weeks-old
10. Isoflurane (gas anesthesia system; according to the specific animal protocol at your institution and animal facility)
11. Analgesia (according to the specific animal protocol at your institution)
12. 1X PBS, pH 7.4
13. 4% Formaldehyde solution, buffered, pH 6.9 (approx. 10% formalin solution) for histology
14. IVISbrite D-luciferin potassium salt bioluminescent substrate, XenoLight

2.2. Equipment

1. Centrifuge
2. Bright-field microscope
3. Incubator

4. Laminar flow hood
5. Heating pad
6. Heat lamp
7. Isoflurane vaporizer
8. Electric trimmer
9. Scale (capable of measuring mouse weight)
10. Bioluminescence in vivo imaging system (IVIS Spectrum)
11. Caliper

2.3. Surgical supplies

1. Eye protective gel
2. Tape
3. 1 mL syringe
4. 25-Gauge needle
5. Autoclip applier plus 9-mm autoclips
6. Sterile cotton tips
7. Sterile gauze
8. 5-0 PDS (Polydioxanone Suture), vicryl or silk suture for peritoneum closure
9. Sterile absorbable gelatin sponge
10. Straight forceps
11. Curved fine forceps
12. Straight scissor
13. Hartman Hemostats
14. Wound clip remover

3. Methods

3.1. HCC Cell preparation for injection_ Time: 40-60 min

1. Prewarm culture medium (previously prepared) in a 37 °C water bath before use and prewarm trypsin at room temperature.
2. Remove the medium from the flask containing Hep55.1C-Luc cells at 80% confluence (see **Notes 1, 2 and 3**). Rinse cells twice with 1X PBS.
3. Add 2 mL of trypsin to the cells. Gently shake the flask to ensure full coverage of the surface area by the trypsin solution. Put the flask at 37 °C in the incubator (see **Note 4**).
4. After 3–5 min, check the cells detaching with a bright-field microscope. Gently tap the flask to facilitate cell detachment (see **Note 5**).
5. Once cells are detached, add 6-8 mL of serum containing culture medium to neutralize the trypsin in the flask. Gently resuspend and mix the cell suspension to finish detaching cells. Collect the cells in a 15-mL centrifuge tube.
6. Centrifuge the cells for 5 min at 200–250 *g* at room temperature and resuspend the cells in culture medium.
7. Count cells to determine the number of cells in the suspension. Adjust the cell concentration to 30×10^4 cells per 50 μ L in DMEM-10% FBS (corresponding to the final cell concentration and volume to be injected). Keep the cell suspension in ice (see **Note 6**).

3.2. Implantation of Syngeneic HCC Cells in Liver_ Time: 20-30 min/mouse

3.2.1. Pre-surgical Setup

(see **Notes 7, 8 and 9**) (**Figure 1**)

1. Properly arrange all instrumentations and solutions that are used during the experimentation prior to surgery.
2. For anesthesia of mice, an isoflurane chamber with nose cone attachment is needed. Anesthetize the mouse by inhalation of 1.5-3 vol% isoflurane in 100% oxygen at a flow rate of 1 L/min. The depth of anesthetization is sufficient when the following vital criteria are reached: regular spontaneous breathing, no reflex after setting of pain stimuli between toes, and no response to pain. Apply pre-operative analgesia subcutaneously, either meloxicam (5 mg/kg, subcutaneous injection) or buprenorphine (0,1 mg/kg, subcutaneous injection).
3. During the complete experimentation, keep the animal on a heating pad at a temperature of 37 °C and fix the legs of the animal with stripes of tape.
4. Shave the abdominal fur of the mouse using an electric trimmer and disinfect the skin with 70% ethanol followed by sterile gauzes with a 10% povidone-iodine solution. Protect the eyes from drying out by usage of eye protective gel (see **Note 10**).

3.2.2. Surgical procedure

1. Grab the skin with a pair of straight blunt forceps and perform a midline abdominal incision of the skin (below the thoracic cavity). Incision should be 2.0–2.5 cm long. Do not to penetrate into the peritoneal cavity.
2. Gently free the skin from the peritoneum. Grab the peritoneum lifting straight upward and open the peritoneal cavity with a midline laparotomy of a length of approximately 1.5-2 cm, ensuring to see the tip of the scissors as to not injure underlying organs. Incision in the peritoneum should be performed

- along the linea alba (midlines white fascia). If the incision is correctly made, the liver should be the first organ to be visualized (see **Note 11**) (**Figure 2a**).
3. Gently pull out the left-lateral lobe with a saline-moistened cotton tip and expose the lobe out of the peritoneal cavity (see **Note 12**) (**Figure 2b**).
 4. Immediately before orthotopic implantation, cell suspension should be mixed. Fill a 1 mL syringe attached to a 25-gauge needle with 50 μ L of cell suspension (all 50 μ L should be injected). This will ensure a final cell number of 30×10^4 cells implanted (see **Note 13**).
 5. Keep immobilizing the liver lobe with the help of a cotton tip and visualize the injection site.
 6. Insert the needle into the liver parenchyma traversing several millimetres deep (under the liver capsule) and gently inject the cell suspension (**Figure 2c**). The colour of the liver surface injected should slowly change to a brown-to-white shade during cell implantation (see **Notes 14 and 15**) (**Figure 2d**).
 7. After injecting the cell suspension, keep the needle for a few seconds in the same position to then slowly retract it.
 8. Immediately after removing the needle of the liver parenchyma, place a small piece of sterile sponge (0.5 \times 0.5 cm) on the injected site applying gentle pressure with a cotton tip for a few minutes to prevent possible bleeding or leakage (see **Note 16**) (**Figure 2e**).
 9. Replace the liver to its physiological position, leaving the sterile sponge onto the injection site which will be slowly resorbed (**Figure 2f**). Using a 5-0 suture, close the peritoneal layer by continuous or interrupted sutures (**Figure 2g**). Cut the ends of the sutures and sterilize the operation area with a gauze swab moistened with 10% povidone-iodine solution.

10. Close the skin layer of the abdominal wall by placing two or three 9-mm autoclips using the autoclip applicator. Simple interrupted sutures can also be used (see **Note 17**) (**Figure 2h**).

3.2.3. Postoperative care

1. Allow the mouse to recover in a cage warmed up by a heat lamp until the mouse is fully awake and active. A heating pad may also be used instead of a heat lamp (see **Note 18**).
2. Apply postoperative analgesia according to the specific animal protocol at your institution.
3. Place animals back in their cages with unlimited access to food and water.

3.3. Tumor growth follow-up_ Time: 6-8 weeks

1. Wound clips must be removed in 7-10 days once the incision has healed.
2. Closely monitor the mice for tumor progression and continue to assess their health until the experiment is finished (see **Note 19**).
3. Anesthetize the mice using 2% Isoflurane in an anesthesia chamber.
4. Administer an intraperitoneal injection of 150 mg/kg of D-luciferin in 1X PBS. Wait 5 min.
5. Perform imaging. We utilized the IVIS Spectrum *in vivo* imaging system (**Figure 3**). Images were acquired with the same settings and exposure times for all animals (see **Note 20**).
6. After imaging, let the mice recover from anesthesia in a cage with a heat lamp.
7. After 6-8 weeks sacrifice animals and retrieve liver for histological and biochemical analysis (see **Note 21**) (**Figure 4**).

8. Excised tumors and normal liver can be fixed in 10% formalin solution for later histological analyses like hematoxylin-eosin-safranin (HES) staining (**Figure 5**).

4. Notes

1. Avoid using antibiotics to cultured cells at least 1 week before this step. Cells should be in culture for approximately four passages prior to injection and cell passaging should be avoided at least 2 days before this step.
2. Here we use Hep55.1C-Luc cells as HCC cells since they are syngeneic and can be injected and grown in C57BL6J mice; other HCC cells can also be used. Also, the use of a luciferase expressing cell line allows a follow-up on tumor growth by BLI after implantation of cells.
3. Hep55.1C-Luc cells were maintained in Dulbecco's modified Eagle's medium supplemented with 4.5 g/L glucose (see **Materials** section) and 10% FBS in a 5% CO₂ humidified chamber. Cell lines used should be regularly checked to ensure they are not infected with mycoplasma.
4. Some commercial trypsin reagents don't require a previous wash with PBS before trypsinization.
5. Do not leave the cells in trypsin for more than 5 min.
6. Prepare 20% more volume of the cell suspension than the exact volume needed for injection. Use Eppendorf tubes, to allow easier collection of cells with the syringe in the moment of injection.
7. Use latex gloves, face mask, surgical gown and hair cap as protective equipment both to protect the human operator and to keep the surgical field reasonably aseptic.
8. Experiments must comply with institutional and ethics regulations concerning the use of animals for research purposes.
9. Carry out all procedures under clean but non-sterile conditions. All the surgical instruments must be autoclaved prior to use and opened on a clean bench.

Use small surgical instruments, which should be appropriate for rodent surgery. Clean surgical instruments should be used for each mouse.

10. Surgical area should be completely shaved and disinfected, to avoid contamination of the wound through contact with fur and to keep a sterile surgical field.
11. A proper abdominal incision makes the procedure easier with a good surgical view of the injection site. Improper placement may lead to a struggle delivering the liver out of the wound for injection. A smaller incision allows a better and faster mice recovery from surgery. A Colibri retractor can be used for a better visualization of the surgical field if necessary.
12. We inject the left-lateral lobe of the murine liver as it is generally easily accessible and larger than the right or middle lobes. It is useful to use a moistened cotton tip to pull out the hepatic lobe, if necessary, pulling out the lobe with the fingers can be done, applying no pressure on the tissue. Do not use forceps to manipulate the hepatic lobes, since the liver can be lacerated and this may provoke some undesirable bleeding.
13. We have tested different gauge size needles from 31G to 25G. The larger needle would be preferred to decrease the impact of shear stress on the cells. The 25G needle did not cause any significant hemostatic or leakage problems.
14. Inject the cell suspension continuously and as slowly as possible to avoid leakage and bleeding. A precise location of the needle is crucial in this procedure to prevent backflow of the cell suspension and allow consistent and reproducible injection of the same number of cells. If the cell suspension is not properly injected under the liver capsule, a bubble will be visible. Do not

insert the needle more than 2-3 mm to not cross the hepatic lobe. Do not move the needle from its initial position while injecting to prevent laceration of the liver.

15. This procedure requires surgical skill and practice to obtain reproducible results and perform the implantation in an acceptable amount of time. It is critical to inject viable cells, the tumor cells should not to be in ice for an extended period of time.
16. Possible issues for orthotopic tumor implantation are bleeding, leakage or undesirable peritoneal metastasis. To avoid these problems, we propose (1) to reduce the volume of cell suspension injected (50 μ l per injection or less), (2) to inject cell suspension as slowly as possible, (3) to extend the time applying gentle pressure with the sterile sponge after injection.
17. Clips may be used if BLI imaging is not planned on being performed during the following week, since clips may cause interference with the imaging. We normally use wound clips to close the skin, remove them after 7 days (necessary time for skin healing) and start to perform tumor growth follow-up by BLI imaging weekly.
18. An animal should not be left unattended until it has regained sufficient consciousness. An animal which has undergone a surgery should not be returned to the company of other animals until fully recovered.
19. Additionally, after removing wound clips, BLI can be utilize. Tumor growth is monitored on a weekly basis with BLI. Expression of the luciferase gene synthesizes the enzyme luciferase which allows monitoring tumor progression during the course of treatment.

20. Before starting a large experiment, it is important to determine the proper exposure time required as to not saturate the image but have a good signal. When obtaining images with BLI, several factors should be kept in mind including mouse position, the depth of tumor in the tissue, and imaging settings **(17)**. These are important considerations as imaging settings may be different depending on the tumor cell line, and the location of the tumor.
21. The tumors take 2 to 8 weeks to engraft and grow enough to be analyzed **(18, 19)**. In our experience, 90-100 % of animals have tumors after a few weeks of implantation. The mice sacrifice is performed by cervical dislocation following the institutional and ethics regulations concerning the use of animals for research purposes.

Conflicts of interest

GK has been holding research contracts with Daiichi Sankyo, Eleor, Kaleido, Lytix Pharma, PharmaMar, Osasuna Therapeutics, Samsara Therapeutics, Sanofi, Sotio, Tollys, Vascage and Vasculox/Tioma. GK has been consulting for Reithera. GK is on the Board of Directors of the Bristol Myers Squibb Foundation France. GK is a scientific co-founder of everImmune, Osasuna Therapeutics, Samsara Therapeutics and Therafast Bio. GK is the inventor of patents covering therapeutic targeting of aging, cancer, cystic fibrosis and metabolic disorders.

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Figure legends

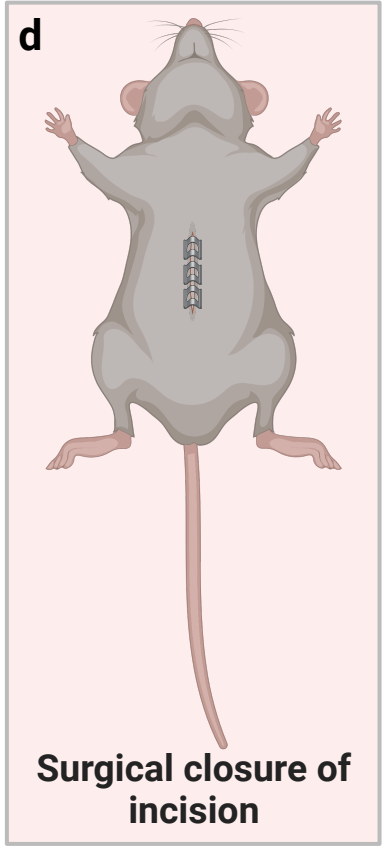
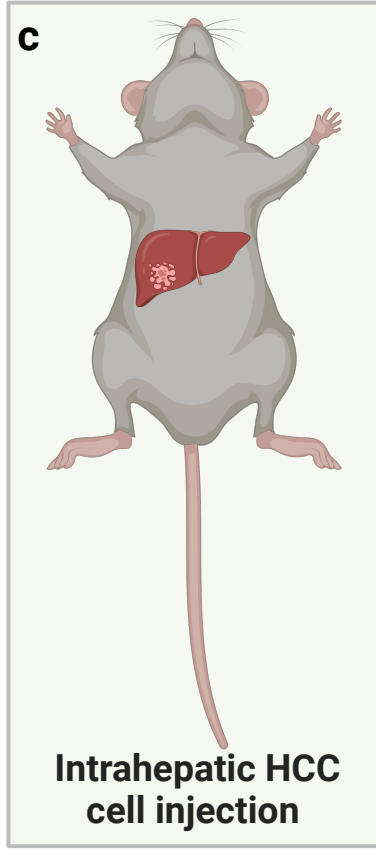
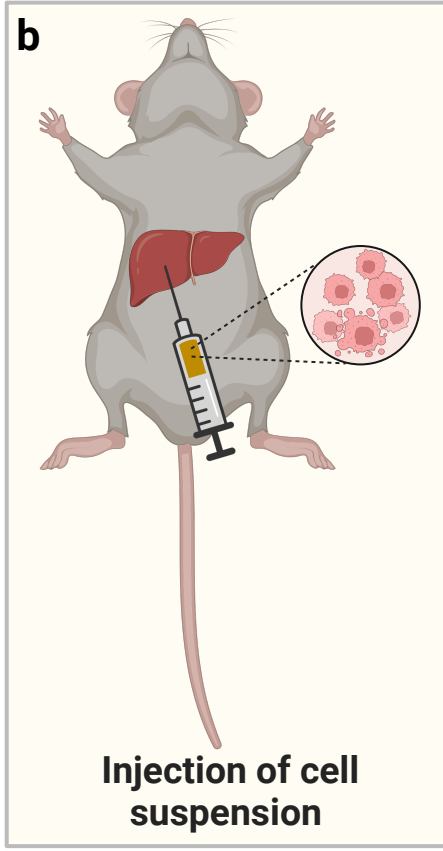
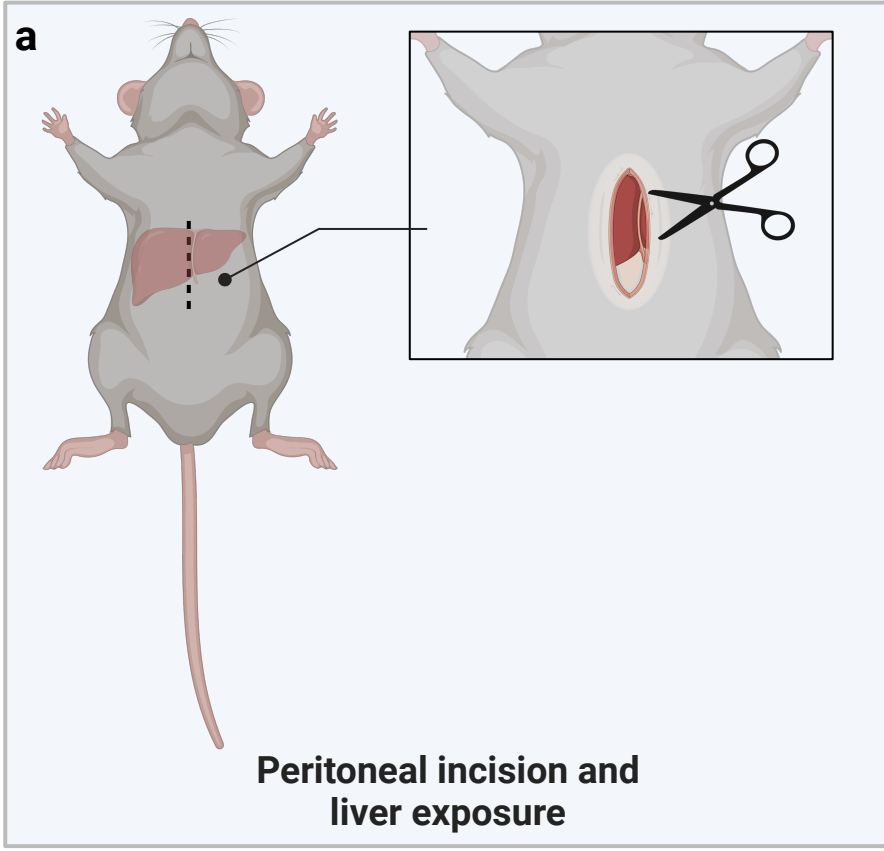
Figure 1. Graphical abstract of the surgical procedure.

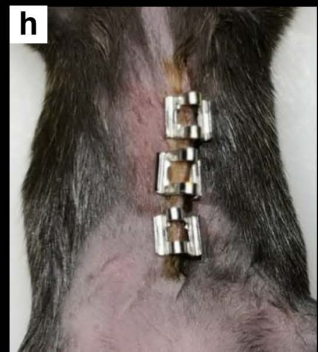
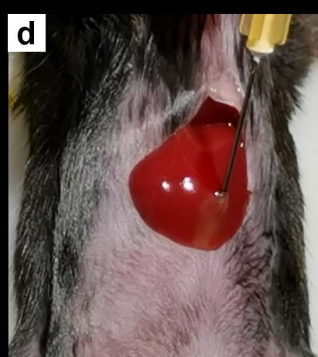
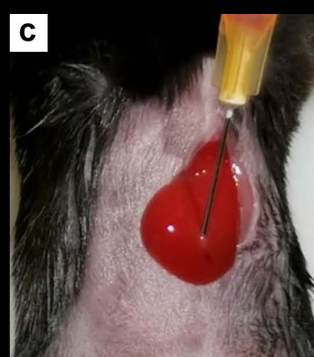
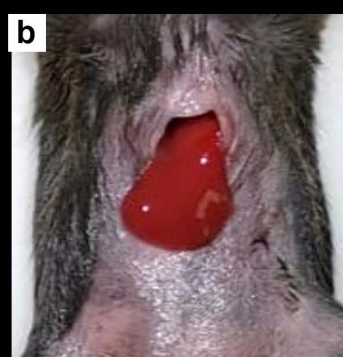
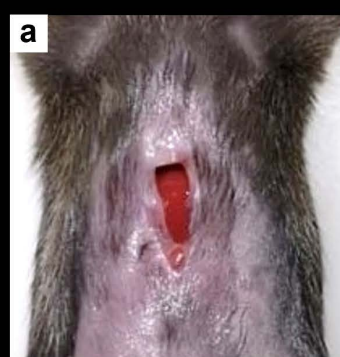
Figure 2. Surgical procedure for intrahepatic HCC cell injection. **(a)** Skin and peritoneal incisions and liver exposure. **(b)** Exteriorization of the left-lateral hepatic lobe. **(c)** Insertion of the needle under the liver capsule. **(d)** Injection of 50 μ l cell suspension and visualization of the change in the liver colour. **(e)** Covering of the left-lateral lobe injection site with a piece of sterile sponge. **(f)** Relocation of the left-lateral lobe in the peritoneal cavity with the piece of sterile sponge. **(g)** Closure of muscle layer by a continuous suture. **(h)** Skin layer closed with 9-mm autoclips.

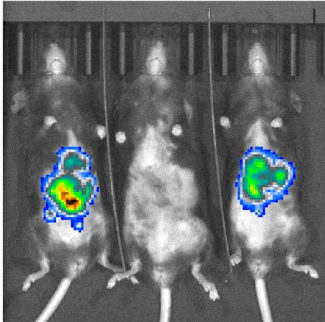
Figure 3. Representative images obtained with IVIS Spectrum *in vivo* imaging system. Bioluminescent signal measured in three anesthetised female mice, two positive controls with tumor and one negative control without intrahepatic tumor injection.

Figure 4. Representative livers after euthanasia of control and tumor implanted mice after 6 weeks.

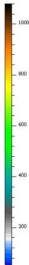
Figure 5. Representative HES staining images of a mice tumor-bearing liver. **(a)** Image of a HES stained liver slide. Digital magnification from **5a**, of the periphery of tumors in the liver **(b)** and the tumor morphology **(c)**.





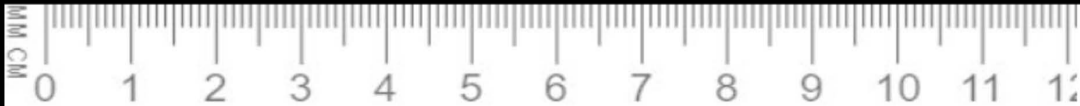


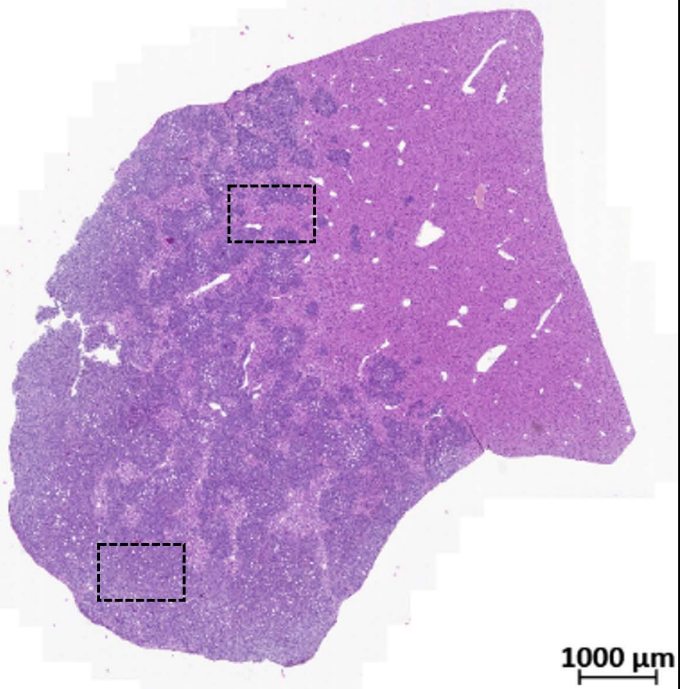
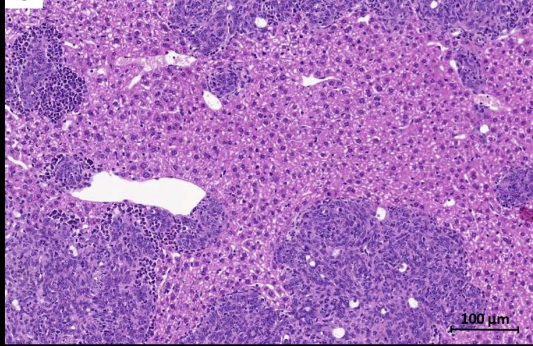
Luminescence



Counts

Color Scale
Min = 0
Max = 1000



a**b****c**