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Portal Vein Injection of Colorectal Cancer Organoids to Study the Liver Metastasis Stroma

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TITLE:

Portal Vein Injection of Colorectal Cancer Organoids to Study the Liver Metastasis Stroma

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SUMMARY:

Portal vein injection of colorectal cancer (CRC) organoids generates stroma-rich liver metastasis.

This mouse model of CRC hepatic metastasis represents a useful tool to study tumor-stroma interactions and develop novel stroma-directed therapeutics such as adeno-associated virus-mediated gene therapies.

ABSTRACT:

Hepatic metastasis of colorectal cancer (CRC) is a leading cause of cancer-related death. Cancer-associated fibroblasts (CAFs), a major component of the tumor microenvironment, play a crucial role in metastatic CRC progression and predict poor patient prognosis. However, there is a lack of satisfactory mouse models to study the crosstalk between metastatic cancer cells and CAFs. Here, we present a method to investigate how liver metastasis progression is regulated by the metastatic niche and possibly could be restrained by stroma-directed therapy. Portal vein injection of CRC organoids generated a desmoplastic reaction, which faithfully recapitulated the fibroblast-rich histology of human CRC liver metastases. This model was tissue-specific with a higher tumor burden in the liver when compared to an intra-splenic injection model, simplifying mouse survival analyses. By injecting luciferase-expressing tumor organoids, tumor growth kinetics could be monitored by *in vivo* imaging. Moreover, this preclinical model provides a useful platform to assess the efficacy of therapeutics targeting the tumor mesenchyme. We describe methods to examine whether adeno-associated virus-mediated delivery of a tumor-inhibiting stromal gene to hepatocytes could remodel the tumor microenvironment and improve mouse survival. This approach enables the development and assessment of novel therapeutic strategies to inhibit hepatic metastasis of CRC.

INTRODUCTION:

Colorectal cancer (CRC) is a major cause of cancer mortality worldwide[1]. More than half of the CRC patients develop hepatic metastasis that occurs through the portal vein dissemination[1]. Currently, there are no effective therapeutics that can cure advanced liver metastasis, and most patients succumb to metastatic disease.

The metastatic niche or tumor microenvironment plays a key role in engraftment and growth of disseminated CRC cells[2]. Cancer-associated fibroblasts (CAFs), a prominent component of the tumor microenvironment, promote or restrain cancer progression through secreting growth factors, remodeling the extracellular matrix (ECM), and modulating immune landscapes and angiogenesis[3–5]. CAFs also confer resistance to chemotherapies and immunotherapies[3]. Moreover, CAFs regulate initiation and progression of CRC liver metastasis and predict prognosis in patients with CRC[3,6–8]. Thus, CAF-related factors could be exploited for the development of therapeutic strategies to inhibit CRC liver metastasis. However, the lack of satisfactory mouse models to study the metastatic tumor stroma has been a major obstacle to developing stroma-targeted therapies.

Currently, animal models to study CRC liver metastasis include primary CRC models that spontaneously develop hepatic metastasis and cancer cell transplantation models into the liver. Primary CRC mouse models, such as genetically engineered mouse models and colonic injection of cancer cells, rarely show metastasis to the liver[9–12]. Moreover, even if a liver metastasis is observed, these models show long latency from the primary tumor induction to metastasis, and

potentially die of primary tumor burden[12]. To efficiently generate CRC liver metastases, cultured CRC cells are transplanted into the liver using three injection approaches: intra-splenic injection, direct intra-parenchymal injection into the liver, and portal vein injection. Intra-splenically injected cancer cells spread into the splenic vein, the portal vein, and ultimately to the liver[13,14]. However, the intra-splenic injection yields a lower tumor take ratio compared with other transplantation models[15,16]. With intra-splenic injection, surgical removal of the spleen is performed to avoid cancer growth in the spleen, which can potentially compromise immune cell maturation[17]. Furthermore, intra-splenic injection can also result in unintended tumor growth in the spleen and abdominal cavity[18], complicating liver metastasis analyses. Direct intra-parenchymal injection into the liver efficiently induces hepatic metastasis[16,19,20]. Nonetheless, this approach does not fully recapitulate a biological step of liver metastasis that naturally occurs through portal vein dissemination. Using direct injection into the liver, entry of cancer cells into a non-portal, but systemic circulation can also result in multiple large lung metastases[16]. Although a majority of patients with CRC liver metastasis show multiple tumor nodules in the liver[21], direct injection into a specific liver lobe generates a single tumor mass[19,20]. Portal vein injection or mesenteric vein injection, though technically challenging, allows efficient delivery of tumor cells into the liver in a manner that recapitulates the growth patterns seen in patients[17]. This strategy can minimize the possibility of secondary-site metastases and enables rapid growth of cancer cells in the liver, simplifying mouse survival analyses.

Historically, colorectal cancer cell lines such as mouse MC-38, human HT-29, and SW-620 were used to generate mouse models of hepatic metastasis[22,23]. However, these colorectal cancer cell lines do not induce a desmoplastic stromal reaction. Low stromal content in the tumors makes it difficult to investigate the biological roles of cancer-associated fibroblasts. Recent advances in CRC organoids and their transplantation have offered useful platforms to assess vital roles of the stroma in cancer progression[24]. Liver transplantation of CRC organoids generates a fibroblast-rich tumor microenvironment and has provided novel insights into stromal research[6,25]. Currently, portal or mesenteric vein injection of organoids has become a gold standard approach to generate CRC liver metastasis[6,25–28]. Nonetheless, to our knowledge, no previous papers have described detailed methods for the portal vein injection of colorectal tumoroids. Here, we present a methodology for using portal vein injection of CRC organoids to develop novel adeno-associated virus (AAV)-mediated stroma-directed therapy.

Hepatocytes are an important constituent of the metastatic tumor microenvironment in the liver and play a critical role in metastatic cancer progression[29]. Inspired by the success of AAV gene therapy approaches to induce protein expression in hepatocytes in non-neoplastic patients[30,31], we investigated a similar approach but aimed at modifying the liver tumor microenvironment in CRC[25]. As such, we also describe herein the tail vein injection of AAV8 to induce expression of anti-tumorigenic proteins to modify the liver tumor microenvironment. The AAV8 serotype, designated by the choice of viral capsid protein during virus production, leads to high transduction efficiency specifically of hepatocytes (i.e., targeted gene expression in the liver tumor microenvironment)[32]. We have previously shown that *Islr* (immunoglobulin superfamily containing leucine-rich repeat) is a CAF-specific gene that induces bone morphogenetic protein (BMP) signaling, reduces CRC tumoroid growth, and promotes *Lgr5*$^{+}$ intestinal stem cell

differentiation[25]. We tested whether AAV8-mediated overexpression of the cancer-restraining stromal gene, *Islr*, in hepatocytes could attenuate hepatic metastasis progression by performing portal vein injection of CRC tumoroids in AAV8-*Islr*-treated mice.

In this paper, we first describe the tail vein injection procedure of liver tropic AAV. Then, we describe a method for tumoroid cell preparation and portal vein injection into the AAV-treated mice. Finally, we present approaches to monitor metastatic tumor progression to assess the efficacy of stroma-directed therapeutics.

PROTOCOL:

All animal procedures in this article were reviewed and approved by the South Australian Health and Medical Research Institute Animal Ethics Committee (Approval number, SAM322).

1. Tail vein injection of adeno-associated virus

NOTE: Adeno-associated virus (AAV) should be handled as a biohazard under Biosafety Level 1 guidelines. Please refer to the published protocol for AAV preparation, purification, and titration[33]. Hepatocyte-tropic AAV, AAV8[34], encoding the cytomegalovirus (CMV) promoter-*Islr* gene, was used in this study[25]. To induce AAV-mediated overexpression, AAV dosing might require optimization depending on the promoter activity, gene, and mouse weight.

1.1. Dilute AAV vector into 150 µL aliquots containing 1.0 x 10^{11} viral genomes, using sterile phosphate-buffered saline (PBS) and keep it on ice. Personal protective equipment should be worn to handle the AAV.

NOTE: Repeated freeze and thaw cycle decreases virus titer and should be avoided. The stock viral solution should be stored in a -80 °C freezer.

1.2. Turn on a heat box (animal warming chamber) to preheat to 35 °C.

1.3. Hold a mouse and apply a topical anesthesia cream to the whole length of the tail at least 15 min prior to the tail vein injection.

NOTE: This is an optional step and might not be necessary if it is not required by the animal ethics committee of the institute. Follow the protocol approved by the local animal ethics committee. In this experiment[25], a Rosa26-Cas9 mouse was used for AAV injection and subsequent portal vein injection of CRC organoids. Given that tumoroids used in this study were derived from a Rosa26-Cas9 mouse (C57BL/6 x 129 genetic background), this mouse strain was also used as immunocompetent, syngeneic recipients of the tumor transplant. Male and female Rosa26-Cas9 mice (6- to 24-week-old) were used.

1.4. Place the mouse into the heat box. Leave the mouse for up to 15 min to warm and dilate the tail veins.

1.5. Gently secure the mouse in a rodent restrainer. Place the tail under a heat lamp to ensure full dilation of tail veins.

1.6. Draw up 150 µL of diluted AAV (prepared in step 1.1) into a low dead space sterile syringe with a 27–30 G needle.

1.7. Move the heat lamp and identify the lateral tail vein located on the sides of the tail. Put slight tension on the tail with the fingers so that the tail becomes straight.

1.8. Slowly insert 2–3 mm of the needle, bevel up, into the vein. The needle should be almost parallel to the tail (up to 15˚ from the tail).

NOTE: Blood influx into the syringe might be observed if the needle is successfully located into the vein.

1.9. Inject slowly. If a resistance is felt or skin swelling is observed, remove the needle and re-insert above the first site or to the other lateral vein.

1.10. Wait for about 5 s after the completion of the injection, and then slowly remove the needle. Immediately apply gentle pressure to the injection site with a clean gauze or paper towel until bleeding stops.

1.11. Gently release the mouse into its cage. Monitor the animal to ensure bleeding has stopped.

NOTE: Gene overexpression in the liver can be assessed 1 to 2 weeks after AAV tail vein injection. This can be confirmed, for example, by RNA *in situ* hybridization (ISH), immunohistochemistry (IHC), western blotting, or quantitative real-time polymerase chain reaction (qRT-PCR; **Figure 1A,B**). In a previously published study[25], AAV8-*Islr* was used to overexpress mouse *Islr* gene in hepatocytes and the overexpression was detected by RNA ISH (**Figure 1A,B**).

2. Cell preparation for colorectal cancer organoids

NOTE: CRC organoids used for this experiment solely contain epithelial cells. Culture and generation of CRC organoids has been previously described[25,35]. In short, normal colonic epithelial cells were isolated from the colon of a Rosa26-Cas9 mouse using a crypt isolation buffer (5 mM EDTA (ethylenediaminetetraacetate) in ice-cold PBS), and then embedded in basement membrane matrix medium, and cultured in organoid growth medium as described in reference[35]. Then, *Apc* and *Trp53* mutations were introduced to the colonic epithelial cells by overexpressing single-guide RNAs that target *Apc* and *Trp53* using lentivirus expression protocol. Single organoid clones were handpicked[25]. *Apc*$^{\Delta/\Delta}$ and *Trp53*$^{\Delta/\Delta}$ colon cancer organoids (AP tumoroids), were injected as 5.0 x 10^5 single cells in 100 µL of PBS with 10 µM Y-27632 into the portal vein per mouse, with organoid culture and single-cell preparation described below.

2.1. Culture the CRC organoids in the basement membrane matrix medium domes in a 24-well plate or 10 cm dish 3–5 days before the portal vein injection to obtain 50–400 µm diameter organoids.

2.2. Prepare sufficient amount of cell detachment solution by adding Y-27632 to 10 µM concentration, enough to digest the number of organoids being cultured for the injection. Pre-warm in a 37 °C water bath.

NOTE: The cell detachment solution used in this protocol is a recombinant cell-dissociation enzyme mix and is used as a substitute for trypsin in organoid culture (see **Table of Materials**). It reduces cellular damage caused by cell dissociation compared to trypsin[36]. Y-27632 is a Rho kinase inhibitor and inhibits dissociation-induced cell death, thereby increasing single-cell survival[37].

2.2.1. For injection into up to five mice, prepare two tubes containing 40 mL of the cell detachment solution to digest 10–24 x 50 µL of basement membrane matrix domes with each dome containing approximately 300 organoids (50–400 µm diameter), i.e., each mouse is injected with 2–4.8 domes of organoids (equivalent to about 600–1440 organoids). The number of organoids necessary to obtain sufficient cell number is dependent on the organoid line, and thus should be optimized.

NOTE: The second 40 mL tube enables a repeat digestion with fresh cell detachment solution to obtain dissociated single cells.

2.3. Carefully aspirate the organoid medium from each well.

2.4. Add 1 mL of ice-cold PBS to each well in a 24-well plate. If a 10 cm dish is used, add 10 mL of ice-cold PBS to the dish.

2.5. Scratch off the basement membrane matrix medium using a P1000 pipette tip. Transfer the PBS/medium slurry to a 15 mL centrifuge tube.

2.6. Rinse each well with the same amount of PBS to collect basement membrane matrix fragments and add to the 15 mL tube(s).

2.7. Incubate for 5 min on ice. This incubation helps dissolve the basement membrane matrix medium.

2.8. Centrifuge the tube at 400 x *g* for 5 min at 4 °C.

2.9. Aspirate the supernatant ensuring not to disturb the pelleted medium and cells.

2.10. Add 5 mL of pre-warmed cell detachment solution prepared in step 2.2 to the pellet, resuspend the pellet 10 times, and transfer it back into a 50 mL tube containing fresh, pre-warmed cell detachment solution.

2.11. Place the tube in the 37 °C water bath. Incubate for 5 min.

2.12. Centrifuge the tube at 400 x *g* for 3 min at 4 °C.

2.13. Repeat steps 2.9–2.11.

NOTE: Repeated enzymatic digestion allows for the efficient cell dissociation into single cells.

2.14. Check whether the organoids are dissociated into single cells by pipetting 100 µL into a 96-well plate and observing under a microscope. If many organoids show cell clumps consisting of more than four cells, longer incubation with the cell detachment solution and physical dissociation by trituration with a 10 mL pipette may be necessary.

2.15. Once most cells are single cells, add 4 mL of fetal bovine serum (FBS) into the 40 mL cell suspension to stop the digestion. Rinse a 40 µm cell strainer with 5 mL PBS.

2.16. Pass the cell suspension through the cell strainer into a 50 mL collection tube to remove any cell clumps.

2.17. Centrifuge the tube at 400 x *g* for 5 min at 4 °C.

2.18. Aspirate the supernatant. Add 10 mL of cold PBS to the cell pellet and transfer it to a 15 mL centrifuge tube.

2.19. Centrifuge the tube at 400 x *g* for 5 min at 4 °C.

2.20. Aspirate the supernatant. Resuspend the pellet in 500 µL cold PBS with 10 µM Y-27632. Count the cells.

2.21. Adjust the cell concentration to 5.0 x 10^5 single cells/100 µL, using PBS with 10 µM Y-27632. Place the tube on ice until portal vein injection is performed.

NOTE: It is recommended to inject dissociated cells within 4 h of dissociation.

3. Portal vein injection of CRC organoids

NOTE: All surgical instruments and surgical gauzes must be autoclaved or sterilized before surgery. This protocol is modified from a previous protocol[17]. In this experiment[25], portal vein injection was performed using Rosa26-Cas9 mice treated with AAV-*mRuby2* or AAV-*Islr* in step 1.

3.1. Prepare an aseptic surgical area using sterile drapes on a heating pad.

3.2. Prepare surgical instruments (scissors and forceps), surgical and hemostatic sponge, 4-0 polyglactin suture, cotton buds, skin staplers, stapler applicator, saline, buprenorphine, and 33 G needle attached to a Hamilton syringe. Cut the hemostatic sponge into 1.0 cm x 1.0 cm pieces.

3.3. Adjust the position of the light source to illuminate the surgical area.

3.4. Inject 0.1 mg/kg body weight of buprenorphine subcutaneously to a mouse for surgical pain management.

3.5. Anesthetize the mouse with isoflurane in an anesthesia chamber. Isoflurane concentration for induction and maintenance are usually 5% and 2.5%, respectively. Check for the absence of a reaction to toe pinch before commencing the next procedure.

3.6. Shave the middle to the upper abdomen of the mouse with an electric shaver. Shaving should be performed in an area distant from the aseptic surgical area to avoid hair contaminating the site.

3.7. Wipe the shaved area with 70% Isopropyl alcohol wipes to remove hair from the site.

3.8. Place the mouse in the surgical area on a heating pad in a supine position with maintenance isoflurane anesthesia. Place a surgical drape with a hole over the abdomen of the mouse.

3.9. Lift the abdominal skin with forceps and make a 2–3 cm skin incision at the midline with scissors, cutting the skin only (not underlying peritoneum). The incision should range from the mid-abdomen to the xiphoid process of the sternum. The incision should not go above the lower end of the xiphoid process.

3.10. Fully lift the peritoneal wall with forceps and make a similar 2–3 cm incision to the peritoneum with scissors. Avoid cutting the intestine and the diaphragm.

3.11. Soak the surgical gauze with warm saline and place it on the left side of the incision (on the left side of the mouse's body; to the surgeon's right).

3.12. Gently pull the internal organs (small and large intestines) out using a cotton bud soaked with saline. Place the intestines on the gauze soaked with saline.

NOTE: The mouse's left-side intestines (i.e., intestines on the surgeon's right) should be pulled out first, and then the mouse's right-side intestines (i.e., intestines on the surgeon's left) can be pulled out.

3.13. Adjust the position of the intestines to visualize the portal vein. Cover the intestines with

additional wet gauze to keep the intestines moist.

3.14. Gently pull the intestine to the left side with the wet gauze and apply gentle tension to the left. This facilitates visualization of the portal vein (**Figure 2A**).

NOTE: If visualization of the portal vein is difficult, gently adjusting the position of the stomach with a wet cotton bud might help the visualization.

3.15. Gently pipette tumoroid suspension several times to obtain a homogenous cell suspension. Slowly draw up 100 µL of the cell suspension into a Hamilton syringe attached to a 33 G needle. Avoid air bubbles.

3.16. Slowly insert the needle, bevel up, into the portal vein. The insertion depth along the needle should be 3–4 mm, with the needle angle almost parallel to the portal vein.

NOTE: Injection should be performed into the well-visualized part of the portal vein (usually up to 2 cm away from the hepatic hilum). Avoid movement of the needle after it is fully inserted into the portal vein.

3.17. Inject tumor cells for 30 s. The injection should be slowly performed to prevent occlusion of the portal vein. If the injection is successful, the color of the liver temporarily changes from red to white.

3.18. Remove the needle slowly. Immediately apply gentle pressure to the injection site with a dry cotton bud and wait for 5 min.

3.19. Remove the cotton bud and simultaneously apply a hemostatic sponge to the injection site. Hold hemostatic sponge with a cotton bud or forceps and apply gentle pressure for 5 more min.

3.20. Remove the pressure to the hemostatic sponge and confirm that there is no bleeding from the injection site.

NOTE: The bio-absorbable hemostatic sponge does not need to be removed. Trying to remove the gauze could cause re-bleeding from the injection site.

3.21. If bleeding occurs, immediately perform pressure hemostasis with a cotton bud for about 10 min. Then, apply an additional hemostatic sponge for a further 5 min.

NOTE: If uncontrollable blood loss is observed, the mouse should be euthanized according to the protocol approved by the animal ethics committee of the institute.

3.22. Remove the surgical gauzes on the intestines. Using a syringe filled with 5 mL saline, squirt saline to the intestines to prevent organ adhesion.

NOTE: Do not apply saline to the portal vein injection site. This could cause re-bleeding.

3.23. Gently place the intestines back inside the abdominal cavity.

3.24. Suture the peritoneum using 4-0 polyglactin sutures.

3.25. Lift up both sides of the skin with forceps. Apply skin staplers to close the skin incision. Be careful not to staple the intestine.

3.26. Turn off the isoflurane but keep the oxygen flow running. Carefully monitor the mouse. When the mouse awakens, place the mouse in an empty cage on a heating pad. The mouse typically awakens within 5 min.

3.27. Carefully monitor the mouse recovery for more than 10 min.

3.28. Subcutaneously inject 0.1 mg/kg buprenorphine to the mouse 4 h after the surgery.

3.29. Inject 0.1 mg/kg buprenorphine to the mouse at the end of the day for the subsequent 2 days.

3.30. Carefully monitor the mice daily for a week after surgery. Check sutures and wound healing.

3.31. 7–10 days after the surgery, remove skin staplers using a stapler remover.

4. Assessment of tumor growth kinetics by *in vivo* bioluminescent imaging

NOTE: If Firefly-expressing tumoroids are used for injection, metastatic tumor progression can be monitored weekly by *in vivo* imaging as described[38,39]. Luciferase expressed by cancer cells could elicit immune responses against the cancer cells and limit tumor growth[40]. Thus, caution is warranted in analyzing immune phenotypes and cancer progression in a mouse model using luciferase-expressing tumor cells.

4.1. Prepare a 30 mg/mL solution of D-luciferin using sterile PBS. Protect it from light. D-luciferin should be stored in aliquots at -20 °C until use.

4.2. Shave the abdomen and thorax with an electronic shaver. This can be done up to 1 day before *in vivo* imaging.

4.3. Inject 150 mg/kg body weight of D-luciferin intraperitoneally into mice (i.e., if the mouse body weight is 30 g, inject 150 µL of the D-luciferin solution).

4.4. Place the mice in an anesthesia chamber and anesthetize them. Use 5% of isoflurane for

induction and 2%–3% for maintenance.

4.5. 10 min later, place the mice in a lateral position (right side up). Acquire bioluminescence images using an *in vivo* imaging system (IVIS) as described previously[38,39].

NOTE: Mice are more stable in a lateral position compared to the supine position. Therefore, a lateral position is preferable to obtain a luminescence image from a consistent focal plane.

4.6. Place the mice in an empty cage and monitor their recovery.

4.7. Define regions of interest on the upper abdomen using Living Image Software as described[38]. Quantify total flux as a surrogate for tumor cell number.

5. Survival analysis and tissue collection

5.1. Monitor mice carefully for clinical symptoms of metastases such as a distended abdomen.

5.2. Euthanize a mouse by CO_2 inhalation once it reaches humane endpoints.

NOTE: Use a study endpoint approved by the animal ethics committee of the institute. To determine a humane endpoint, a clinical record sheet was used; scores were calculated by one point being given for the presence of each of the following observations: weight loss > 15%, hunched posture, ruffled coat, dehydration, decreased movement, distended abdomen, or facial grimace. Once a score of 3 was reached, the mice were humanely euthanized.

5.3. Immediately following euthanasia, perform transcardial perfusion fixation with 30–50 mL of 10% formalin in a fume hood, as described[41]. Make several small incisions (up to 1 cm each) with scissors into the normal part of the liver before perfusion to generate outlets for blood and formalin. Upon perfusion, the liver color will change from red to brown.

NOTE: If micrometastases in macroscopically normal liver areas are a subject of study, we recommend researchers to not cut the liver, but to snip the superior vena cava instead. However, when we have used this method, fixation of the liver seems poorer when compared with cutting the liver. Especially if RNA *in situ* hybridization (ISH) is planned to be performed on liver sections, we recommend making incisions into the liver before intra-cardiac injection of formalin, as described above. This enables sufficient fixation of the liver tissue, resulting in preservation of RNA integrity for ISH.

5.4. Place the liver and lung tissues into 10% formalin and fixate overnight. Replace the formalin with 70% ethanol, followed by paraffin-embedding.

5.5. Perform hematoxylin and eosin staining to histologically evaluate tumor area. Perform immunohistochemistry for stromal markers of interest. Perform Picro-Sirus Red staining to evaluate collagen-positive areas.

NOTE: The ImageJ software[42] can be used to quantify immunohistochemistry and Picro-Sirius Res staining data. A color deconvolution function and the MRI fibrosis tool can be used to evaluate 3,3'-Diaminobenzidine (DAB)-positive areas and Picro-Sirius red-positive areas, respectively.

REPRESENTATIVE RESULTS:

To induce AAV-mediated overexpression of a tumor-restraining stromal gene, *Islr*[4,25,43,44], in hepatocytes, we intravenously injected *Islr*-encoding AAV8. 1.0×10^{11} viral genomes (vg) of AAV8-*Islr*, or as a control, AAV8-*mRuby2*, was injected into the adult mouse tail vein (**Figure 1A**). Two weeks after the tail vein injection, livers were harvested to validate the overexpression of *Islr* in hepatocytes. We performed RNAscope *in situ* hybridization[45] and confirmed that 3,3'-Diaminobenzidine $(DAB)^+$ signals were observed throughout the liver (**Figure 1B**). No DAB^+ signals were detected in the liver from an AAV8-*mRuby2*-treated mouse.

We practiced the portal vein injection procedure by injecting India ink (1:1000 dilution in PBS). After making an incision into the upper abdomen, the intestines were gently taken out from the abdominal space to allow visualization of the portal vein (**Figure 2A**). Portal vein injection of India ink delivered the ink throughout the liver, but not to the lung (**Figure 2B**). If the ink is mistakenly injected into other vessels such as the inferior vena cava (IVC) or abdominal aorta, systemic circulation of the ink alters the lung color to black. India ink also helps identify the amount of leakage from the portal vein, which indicates pancreatic or peritoneal dissemination.

Two weeks after the tail vein injection of AAV-*mRuby2*, $Apc^{\Delta/\Delta}$ and $Trp53^{\Delta/\Delta}$ colon cancer organoids (AP tumoroids) were dissociated to single cells and injected into the mouse portal vein (**Figure 3A**). To confirm metastatic tumor growth and evaluate histology, we collected the livers 3–4 weeks after the portal vein injection (i.e., at a timed point rather than at a humane endpoint), before prominent necrosis confounds histopathological analyses. Macroscopically, the portal vein injection of tumoroids resulted in multiple white tumor nodules in the liver (**Figure 3B**). We found that intra-splenic injection of the same cell number of tumoroids did not generate a large metastatic tumor mass. This suggests that the portal vein injection approach more efficiently induced liver metastases compared to the intra-splenic injection model. Hematoxylin and eosin staining of the CRC hepatic metastases induced by the portal vein injection demonstrated histopathology of moderately differentiated tubular adenocarcinoma accompanied with a desmoplastic stromal reaction and necrosis (**Figure 3C**). This stroma-rich histology faithfully recapitulated that of human CRC liver metastases (**Figure 3C**), making this model suitable for translational research investigating the metastatic tumor stroma. Moreover, immunohistochemistry for alpha-smooth muscle actin (αSMA), a well-established marker for CAFs, showed that about 7% of tumor areas were αSMA-positive, confirming the presence of CAFs in this mouse model (**Figure 3D,E**). Picro-Sirius red staining that stains collagen[46] demonstrated abundant ECM in the tumor mesenchyme with approximately 13% of tumor areas positive for collagen (**Figure 3D,E**). Immunohistochemistry for EPCAM, an epithelial lineage marker, revealed that the metastatic CRC showed tumor budding (a single tumor cell or a cell cluster of up to four tumor cells) that is a characteristic of poor prognosis colorectal cancer[47] (**Figure 3F**). Ki67 labeling index in the metastatic CRC was approximately 80%, indicating that most tumor cells are mitotically active (**Figure 3G,H**).

We performed mouse survival and tumor growth kinetics analyses in this preclinical model. The mice in our first pilot cohort showed a median survival of 57 days after tumoroid injection into the portal vein (N = 4 mice; **Figure 4A**). This was later replicated in larger control AAV8-*mRuby2*-injected groups[25]. At humane endpoint (as shown in NOTE, step 5.2), 3 out of 4 mice in the pilot group demonstrated ascites, which is also observed in patients with advanced liver metastasis[48]. To assess the tumor growth, *in vivo* imaging system (IVIS) was used to measure tumoroid-derived luminescence (**Figure 4B,C**). The bioluminescence signals were observed within the upper abdomen, suggesting liver-specific tumor growth (**Figure 4B**). If IVIS signals are observed in the lower abdomen, this indicates peritoneal dissemination or secondary metastases to abdominal organs. The weekly *in vivo* imaging allowed for a longitudinal assessment of tumor growth in each mouse (**Figure 4C**), making it easier to monitor a therapeutic effect in our subsequent larger study. The tumor take rate was 100% (4/4 mice) as assessed by IVIS signals. In this experiment, no macroscopically apparent lung metastasis was observed at the time of tissue collection (0/4 mice).

Finally, we investigated whether AAV-mediated hepatocyte-directed delivery of a cancer-restraining CAF gene, *Islr*[4,25], could inhibit CRC hepatic metastasis growth in this preclinical mouse model. Notably, AAV8-*Islr*-treated mice showed improved mouse survival and decreased IVIS signals from tumors (**Figure 5A–C**)[25]. Immunohistochemistry for phosphorylated Smad1/5/8 demonstrated that hepatocyte overexpression of ISLR, a BMP signaling potentiator, augmented BMP signaling in metastatic tumors (**Figure 5D**). Treatment with AAV8-*Islr* decreased the number of $Ki67^{+}$ proliferating cells in the CRC hepatic metastasis (**Figure 5E**). For full description of the results, refer to Kobayashi et al., Gastroenterology, 2021[25]. Our collective data indicate that AAV8-mediated delivery of a tumor-inhibitory gene to hepatocytes, a vital constituent of the liver metastatic tumor stroma[29], could be an effective preventive/therapeutic approach for CRC liver metastases (**Figure 5F**).

FIGURE AND TABLE LEGENDS:

Figure 1: Tail vein-administered AAV8-*Islr* generates *Islr* overexpression in the liver. (**A**) Experimental scheme showing the tail vein injection of AAV8, followed by portal vein injection of colorectal cancer (CRC) organoids. ISH, *in situ* hybridization; IHC, immunohistochemistry; qRT-PCR, quantitative real-time polymerase chain reaction. (**B**) Representative pictures. RNA *in situ* hybridization for *Islr* was performed using livers from AAV8-*Islr* or AAV8-*mRuby2*-treated mice. The livers were collected 2 weeks after tail vein injection. Scale bars, 50 µm.

Figure 2: Portal vein injection of India ink results in its delivery to the liver, but not the lung. (**A**) Representative pictures showing anatomy of the upper abdomen after laparotomy. Note that, for visualization of the portal vein, the intestines are taken outside the abdominal cavity. The right-hand side picture shows an anatomical annotation of each organ and vessel. The yellow arrow denotes the injection site. IVC, Inferior vena cava. (**B**) Representative pictures of the liver and lung following portal vein injection of India ink. The yellow arrowheads indicate vessels stained with the India ink. Scale bars, 1 cm.

Figure 3: Portal vein injection of colorectal cancer organoids generates stroma-rich liver metastases. (**A**) Experimental scheme showing portal vein injection of *Apc*$^{\Delta/\Delta}$ and *Trp53*$^{\Delta/\Delta}$ colon cancer organoids (AP tumoroids). Scale bar, 200 μm. T, tumoroids. (**B**) Representative macroscopic pictures of the livers that were collected 3–4 weeks after portal vein injection (left) or intra-splenic injection (right). 5.0 x 10^5 single cells from AP tumoroids were injected into the portal vein or spleen. Intra-splenic injection was performed as described[13]. White nodules indicate tumors. T, Tumor; N, Normal liver. (**C**) Representative hematoxylin and eosin (H&E) staining pictures of the CRC liver metastases from the portal vein injection mouse model (left and middle) and human (right). T, Tumor; N, Normal liver; S, Stroma; Nec, Necrosis. The yellow dotted line indicates a border between the tumor and normal liver (left). (**D and E**) Immunohistochemistry (IHC) for alpha-smooth muscle actin (αSMA; left) and Picro-Sirius red staining (right). (**D**) Representative pictures. (**E**) Quantification of αSMA-positive areas (left) and Picro-Sirius red-positive areas (right). N = 4 mice, 5 HPFs (High Power Fields; 400x)/mouse. (**F**) Representative picture showing immunohistochemistry for EPCAM, an epithelial cell marker. The green dotted lines denote tumor budding. (**G and H**) Immunohistochemistry for Ki67, a cell proliferation marker. (**G**) Representative picture. (**H**) Percentage of Ki67$^+$ cells in total epithelial cells. Epithelial cells were visualized by hematoxylin counterstaining. N = 4 mice, 5 HPFs/mouse. In (B)-(H), all mouse liver tissues were collected 3–4 weeks after injection of AP tumoroids. Mean ± S.E.M. Each dot represents an average value of 5 HPFs from a mouse (E and H). Scale bars represent 1 cm (B), 1 mm (C; left), 100 μm (C; middle and right), and 50 μm (D, F, and G). Note that the study with human tissues was approved by the Ethics Committee of Nagoya University Graduate School of Medicine (2017-0127).

Figure 4: Survival analysis and tumor growth kinetic analysis by *in vivo* imaging. (**A**) Kaplan-Meier survival curves. N = 4 mice. (**B,C**) *In vivo* imaging system (IVIS) was used to evaluate tumor growth kinetics. (**B**) A representative picture. The area in the red box was used for quantification. (**C**) Growth kinetics. Luciferase signals from each mouse are shown. N = 4 mice.

Figure 5: AAV8-mediated gene delivery of *Islr* to hepatocytes increases BMP signaling, reduces tumor proliferation, and improves mouse survival in a portal vein injection model of CRC liver metastasis. (**A**) Kaplan-Meier survival curve. Two weeks after tail vein injection of AAV-*Islr* or AAV-*mRuby2*, portal vein injection of CRC tumoroids was performed. (**B and C**) Tumoroid-derived luciferase signals were evaluated using IVIS. (**B**) Representative picture. (**C**) Quantification of IVIS signals. N = 5 (AAV-mRuby2) and 8 (AAV-*Islr*) mice. Mean ± S.E.M. (**D**) Representative pictures. Immunohistochemistry (IHC) for phosphorylated Smad1/5/8 (pSmad1/5/8). Smad1/5/8 is a downstream molecule of bone morphogenetic protein (BMP) signaling that is phosphorylated upon BMP signaling activation[49]. N = 4 mice each. (**E**) Representative pictures. Immunohistochemistry for Ki67. N = 4 mice each. (**F**) Graphical summary. AAV-mediated hepatocyte-directed gene delivery of a cancer-restraining stromal gene might serve as a therapeutic strategy to inhibit CRC metastagenesis. AAV8-mediated overexpression of *Islr* in hepatocytes remodeled the metastatic niche by augmenting BMP signaling and limited CRC metastasis progression. For detailed information, refer to Kobayashi et al., Gastroenterology, 2021[25]. Log-rank test (A), and two-way repeated-measures ANOVA (analysis of variance) with post-hoc Sidak's multiple comparison test at Week 3 (C). Scale bars, 50 μm. **Figure 5A–C** has been

DISCUSSION:

In this study, we have shown that portal vein injection of mouse CRC organoids reproducibly generates fibroblast-rich liver metastases that mimic histological features of human CRC hepatic metastases. Furthermore, when combined with stroma-directed therapeutics such as AAV8-mediated gene therapy, this preclinical model serves as a useful tool to assess therapeutic effects on mouse survival and tumor growth.

There are, at least, two critical steps in the protocol. Firstly, it is important to prepare a single-cell suspension of tumoroids by fully trypsinizing the organoids and using a mesh filter to remove cell clumps. Incomplete dissociation of tumoroids results in large cell aggregations, injection of which can occlude the portal vein. This causes infarction of the liver and animal death[22]. Secondly, during portal vein injection, it is imperative to minimize bleeding from the portal vein. The needle should be properly inserted into the portal vein. To avoid puncture of the other side of the portal vein, the angle of the inserted needle should be kept almost parallel to the portal vein. To prevent tearing of the portal vein during injection, the needle should not be moved once it is fully inserted into the portal vein. Immediately after removing the needle from the portal vein, it is vital to apply pressure to the injection site with a cotton bud for at least 5 min.

The cell number for injection needs to be modified according to the recipient mouse (e.g., immunocompetent vs. immunodeficient mouse) and tumorigenicity of the organoid line. In our experiments, injection of 5.0 x 10^5 single cells in 100 µL suspension was sufficient to generate liver metastases in immunocompetent mice. Increasing the cell number could enhance the risk of embolism in the portal vein and death of animals[14], and thus should be carefully considered. Given that previous papers used 5 x 10^4 to 5 x 10^5 cells in 100 µL for tumoroid injection into the portal or mesenteric vein[25–28], we consider that this cell number range would be a good starting point for optimization.

One limitation of the portal vein injection approach is that it does not fully recapitulate the entire cascade of CRC metastagenesis. Hepatic metastasis of cancer requires five major steps: (1) cancer cell invasion at the primary site, (2) intravasation into the vessel, (3) cell survival in the portal circulation, (4) extravasation from the portal vein to the liver parenchyma, and (5) colonization in the metastatic niche[50]. The portal vein injection model only permits investigation of steps (3)–(5). To gain insights into the other metastatic processes, it is necessary to utilize other models such as *in vitro* models and/or a *Notch1*-mutant primary CRC mouse model that frequently metastasizes to the liver[51].

The significance of the portal vein injection method with respect to the existing methods includes liver-specific growth and higher tumor burden. Peritoneal dissemination in other injection models and primary tumor growth in primary CRC models can confound analysis of liver metastasis progression. In contrast, our portal vein approach rapidly generates liver-specific

tumors, simplifying survival and tumor growth analyses.

One major advantage of organoid transplantation over cell line injection is that portal vein injection of tumoroids generated the fibroblast-rich tumor microenvironment that phenocopies a desmoplastic feature of human metastatic CRC. Organoid culture conditions recapitulate features of the tumor microenvironment and support intestinal stem cell populations, including embedding cells in a rich and complex 3D ECM with a defined combination of growth factors[24,52]. This results in tumor cell propagation in a manner that preserves the genetic landscape of the source tumor[52,53]. In contrast, traditional 2D culture of tumor cell lines is associated with clonal selection and aberrant genomic/transcriptomic alterations that do not faithfully reflect characteristics of the original cancers[54]. We speculate that the relatively harsh culture conditions for 2D culture of cancer cell lines force cells to adapt to lack of ECM/growth factor signals that may then result in stroma-poor tumors *in vivo*, as the cells no longer require stroma-derived signals for survival.

By combining the organoid portal vein injection model with AAV8-mediated gene therapy, we found that delivery of a cancer-inhibitory payload to hepatocytes before cancer cell colonization in the liver could modify the (pre-)metastatic niche to inhibit CRC metastasis growth[25]. Encouragingly, clinical trials have demonstrated that AAV-mediated *in vivo* gene transfer to the liver induces long-term expression of a transgene and could be an effective and safe modality to treat non-neoplastic genetic diseases[30,31,55]. In the future, harnessing hepatocytes to prevent liver metastases through the AAV approach might have potential clinical value in cancer patients at high risk of metastases.

In summary, our paper shows that CRC organoid transplantation via the portal vein generates fibroblast-rich liver metastases, which can be exploited for the development of therapeutic strategies targeting the stroma. By coupling the portal vein injection with stroma-directed therapy such as AAV-mediated gene delivery, one may identify novel stromal targets to restrain CRC metastasis progression.

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DISCLOSURES:

The authors declare no conflicts of interest.

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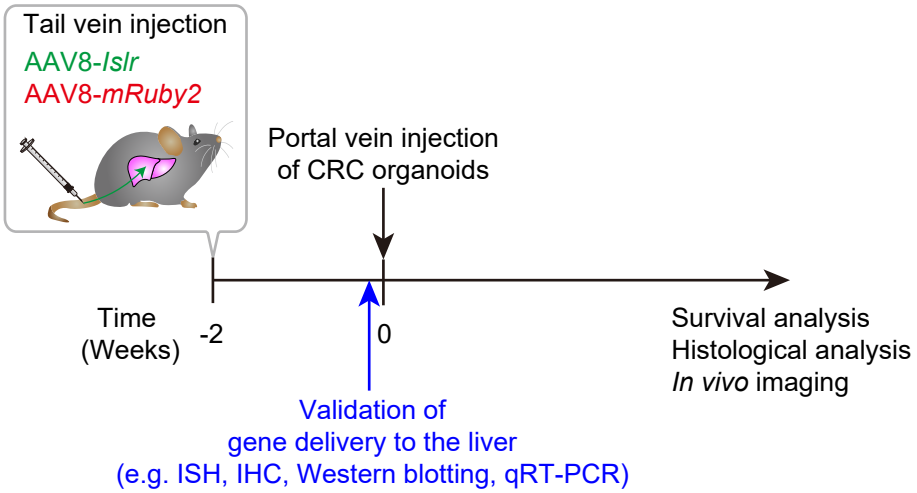
A



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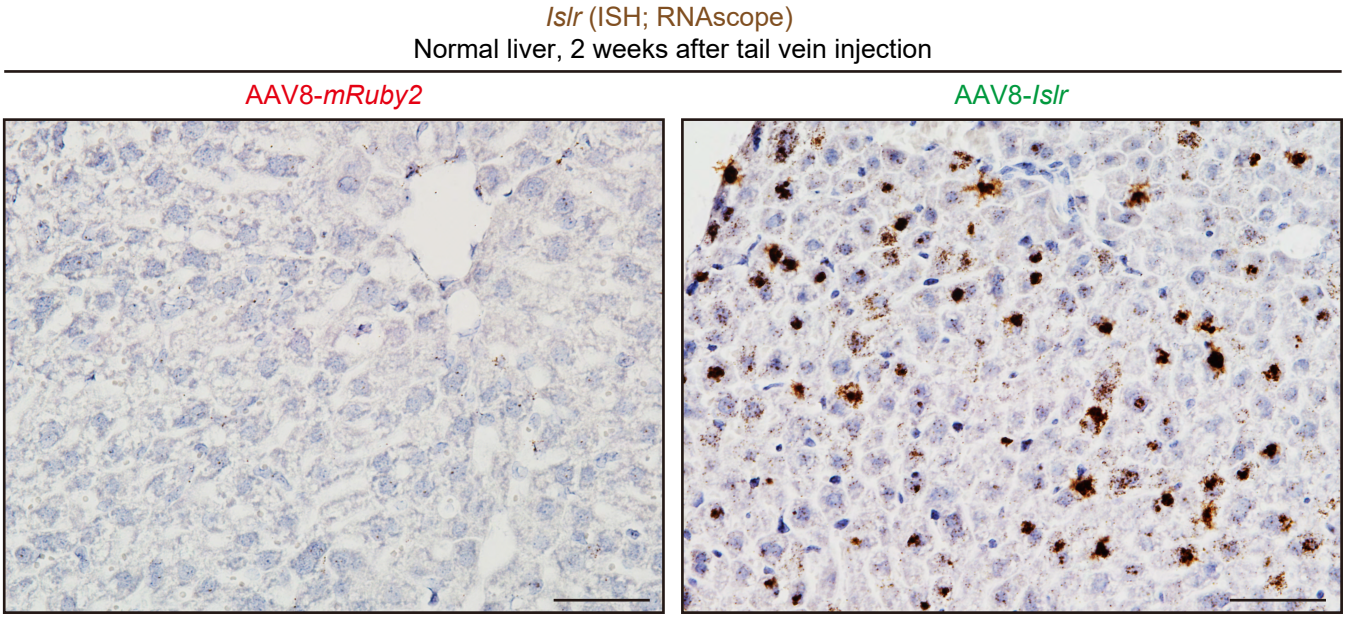

Kobayashi *et al.*, Figure 1.

A

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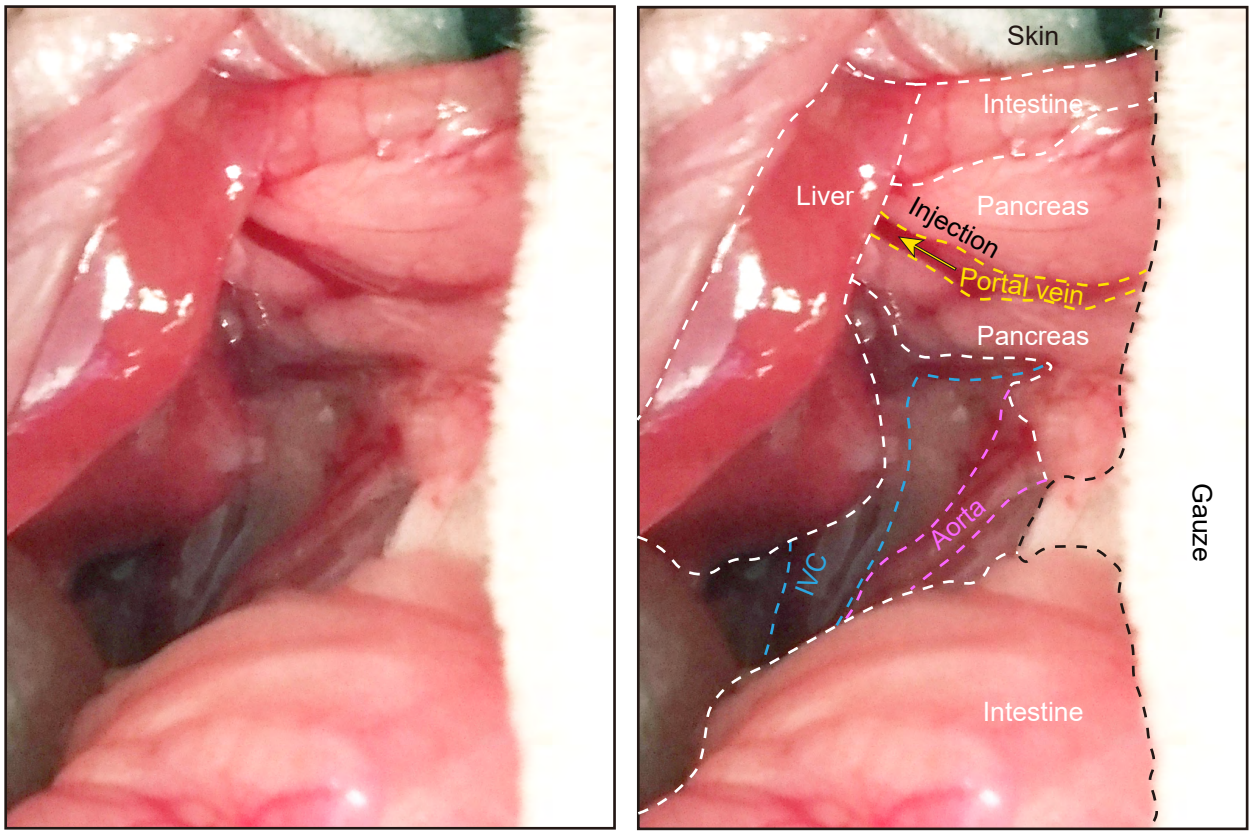
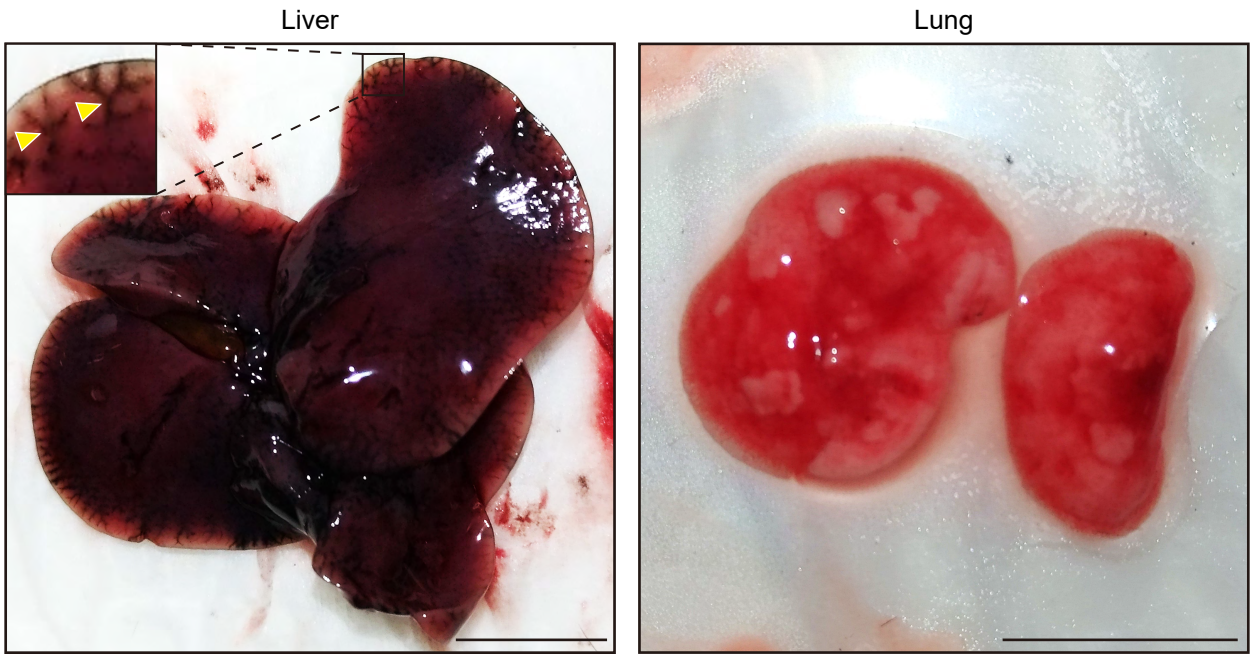


B

Portal vein injection of India ink



Kobayashi *et al.*, Figure 2.

Figure 3

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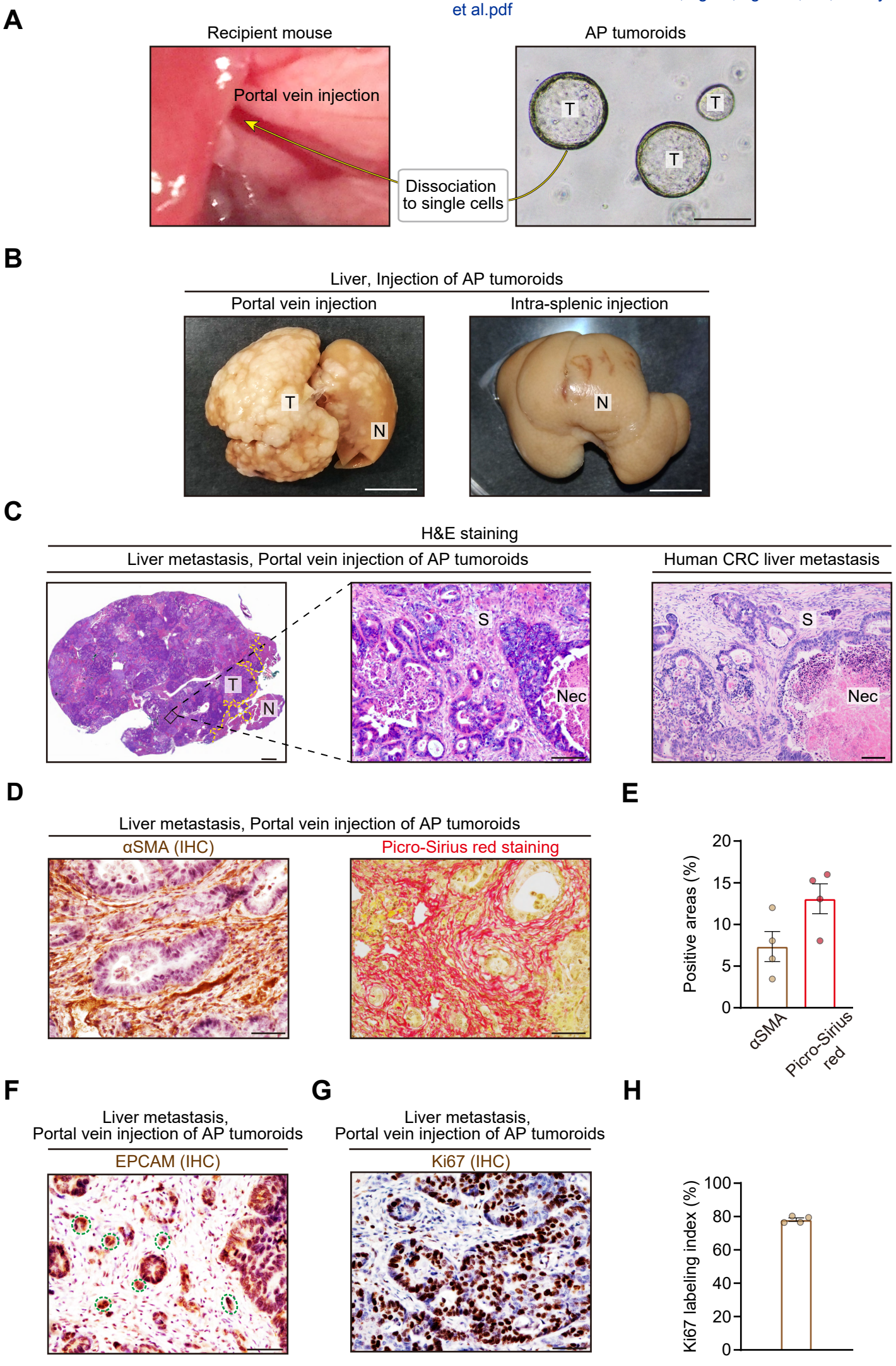
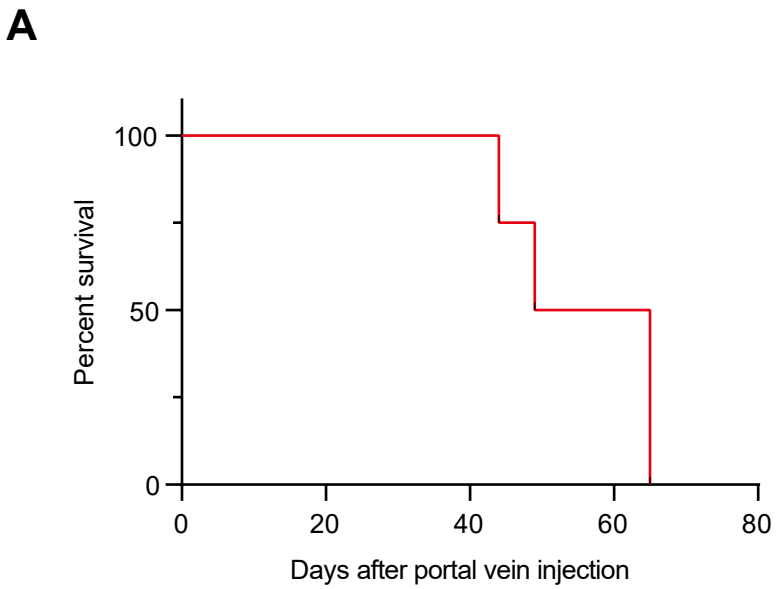
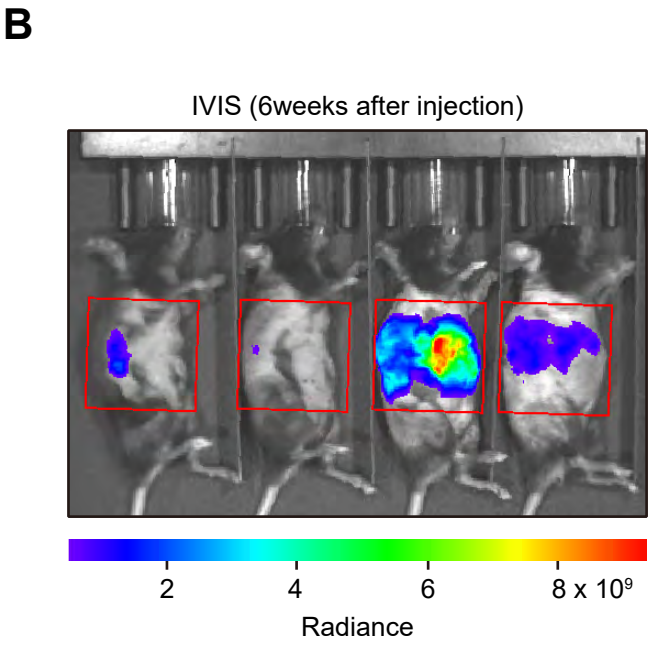
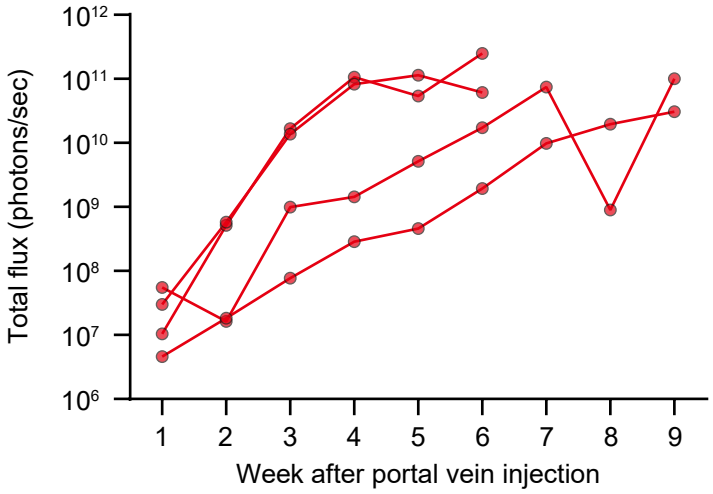


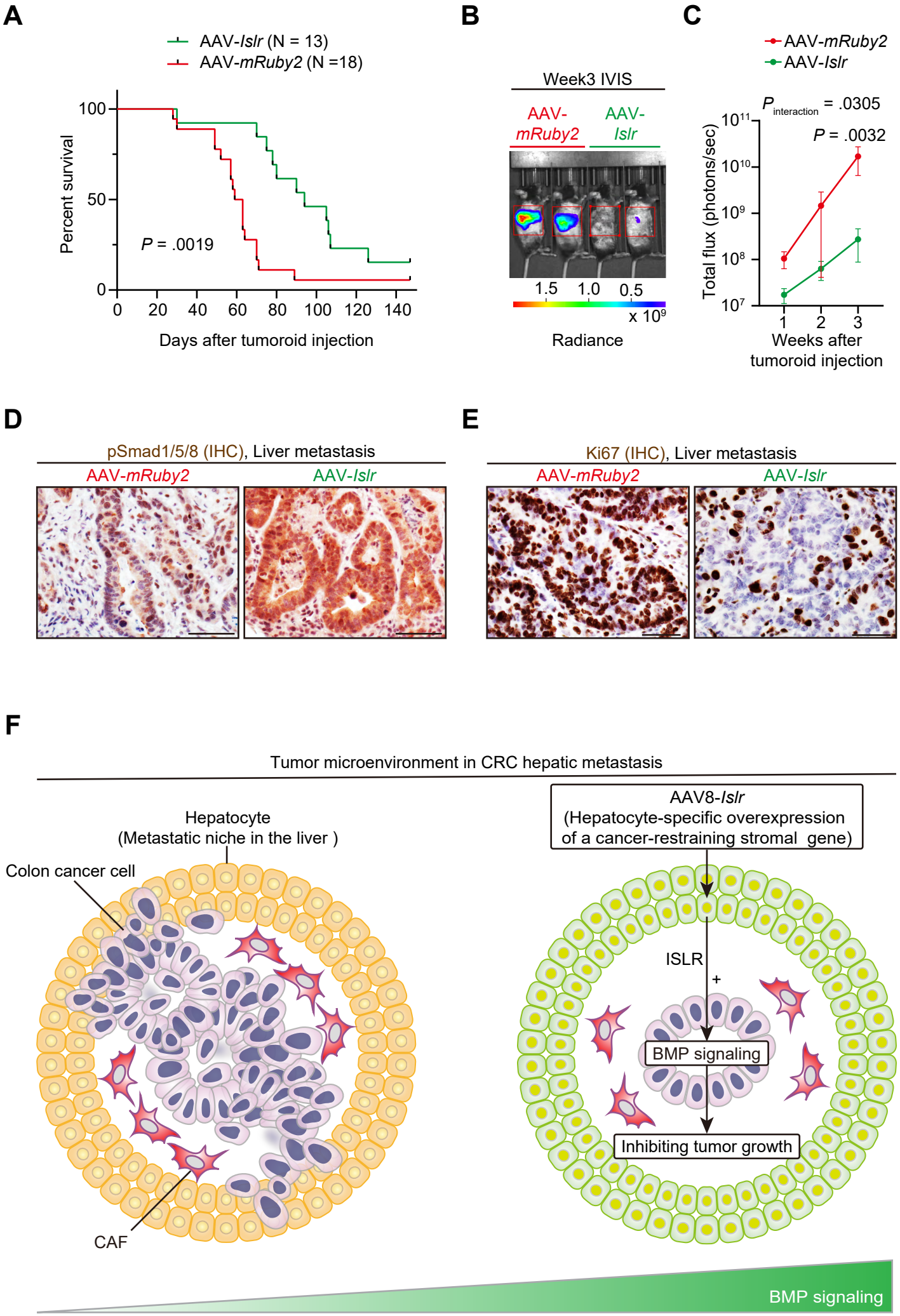
Kobayashi *et al.*, Figure 3.







Kobayashi *et al.*, Figure 4.



Kobayashi *et al.*, Figure 5.

Note to Editors

We are grateful to the editors for their comments. As indicated in our responses below, we have taken all of these into account in the revised version of our manuscript.

We would like to adopt a hybrid filming/production option where, after the manuscript acceptance, we will film ourselves based on a script that the JOVE team will provide. (We are outside the JOVE videographer network.)

Editorial comments:

1. The editor has formatted the manuscript to match the JoVE style. Please retain and use the attached file for revision.

We have retained and used the file for revision. We have used the “Track Changes” function, Microsoft Word software, to denote changes made from the previous version.

2. Please address specific comments marked in the manuscript.

We have now addressed all comments marked in the manuscript. Please see our revised Word document.

3. You are allowed to reuse the figures/tables as long as you obtain the reprint permission from the original publication.

We have now obtained the reprint permission from Elsevier, the publisher for the original figure, and incorporated the data in New Figure 5A-C. The permission document has been uploaded to the Editorial Manager. As written below, we have appropriately acknowledged the source information in the format specified by Elsevier (General Terms 3. Acknowledgement; Page 2, Reprint permission document uploaded to the Editorial Manager).

"Figure 5A-C has been reprinted from *Gastroenterology*, Vol 160(4), Kobayashi *et al.*, The Balance of Stromal BMP Signaling Mediated by *GREM1* and *ISLR* Drives Colorectal Carcinogenesis, Pages 1224-1239.e30, Copyright (2021), with permission from Elsevier." (Lines 635-637)

4. Please include results as asked by the reviewers to support the claims or reduce the scope of the manuscript.

Reviewers 1 and 2 asked for the results showing that our stroma-directed therapy could decrease colorectal cancer metastasis progression (Comment 2, Reviewer 1; Comments 1 and 2, Reviewer 2). In the first round of the revision, we had added representative results demonstrating that AAV8-*Islr* treatment augmented bone morphogenetic protein (BMP) signaling and decreased Ki67 labeling index in our portal vein injection model. However, we had not added mouse survival data and tumor growth kinetics analyzed by *in vivo* imaging system, assuming that we were not allowed to reuse the data from our previous publication (Kobayashi *et al., Gastroenterology*, 2021). In this revision, we have now added these data to reinforce our finding that AAV-mediated stroma-directed therapy could attenuate metastatic tumor growth and improve mouse survival **(New Figure 5A-C)**. We have added the following sentence to Representative Results, the revised manuscript (Lines 558 and 559).

"Notably, AAV8-*Islr*-treated mice showed improved mouse survival and decreased IVIS signals from tumors **(Figure 5A-C)."**

As we have incorporated mouse survival data in New Figure 5A, we have now modified the Abstract sentence as follows. This addresses Comment 2, Reviewer 2, where he/she pointed out that mouse survival data had not been shown in our original manuscript.

"We describe methods to examine whether adeno-associated virus-mediated delivery of a tumor-inhibiting stromal gene to hepatocytes could remodel the tumor microenvironment and improve mouse survival ~~attenuate tumor cell proliferation~~."

5. The title should directly reflect the protocol to be shown in the video.

As AAV injection will not be included in the video, we have removed the phrase “Adeno-Associated Virus” from the title. The title has now been changed from **“Combining an Organoid Transplant Mouse Model of Liver Metastasis and Adeno-Associated Virus to Investigate Stroma-Directed Therapy for Colon Cancer”** to **“Portal Vein Injection of Colorectal Cancer Organoids to Study the Liver Metastasis Stroma.”**

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