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An oncogenic hepatocyte-induced orthotopic mouse model of hepatocellular cancer arising in the setting of hepatic inflammation and fibrosis.

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Cover Letter

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November 3, 2018

Dear Dr. Ron Myers

I am pleased to submit an original research article entitled “A clinically relevant mouse model of hepatocellular cancer arising in the setting of inflammation and liver fibrosis”. We have developed a mouse model to study hepatocellular carcinoma that recapitulates the typical immune features seen in human tumors, and this manuscript provides the protocol to replicate the tumor model.

We believe that this manuscript is appropriate for publication by JoVE because it provides an opportunity for other researches to develop an animal model to study hepatocarcinogenesis and various therapeutic strategies for liver cancer.

This manuscript has not been published and is not under consideration for publication elsewhere. We have no conflicts of interest to disclose.

Thank you for your consideration!

Sincerely,

Guangfu Li, DVM & Ph.D

TITLE:

An Oncogenic Hepatocyte-Induced Orthotopic Mouse Model of Hepatocellular Cancer Arising in the Setting of Hepatic Inflammation and Fibrosis

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KEYWORDS:

hepatocellular cancer, tumor model, mouse, murine model, liver fibrosis, cancer

SUMMARY:

Here, we describe the development of a clinically relevant murine model of liver cancer recapitulating the typical immune features of hepatocellular cancer (HCC).

ABSTRACT:

The absence of a clinically relevant animal model addressing the typical immune characteristics of hepatocellular cancer (HCC) has significantly impeded elucidation of the underlying mechanisms and development of innovative immunotherapeutic strategies. To develop an ideal animal model recapitulating human HCC, immunocompetent male C57BL/6J mice first receive a carbon tetrachloride (CCl_4) injection to induce liver fibrosis, then receive histologically-normal oncogenic hepatocytes from young male SV40 T antigen (TAg)-transgenic mice (MTD2) by intra-splenic (ISPL) inoculation. Androgen generated in recipient male mice at puberty initiates TAg expression under control of a liver-specific promoter. As a result, the transferred hepatocytes become cancer cells and form tumor masses in the setting of liver fibrosis/cirrhosis. This novel model mimics human HCC initiation and progression in the context of liver fibrosis/cirrhosis and reflects the most typical features of human HCC including immune dysfunction.

INTRODUCTION:

Hepatocellular cancer (HCC) is the most rapidly increasing type of cancer in the United States (US)[1–3]. Every year, approximately 850,000 new cases are diagnosed[4,5] and 700,000 patients die from this lethal disease[6–10], making it the second-highest cause of cancer-related death worldwide. Management of HCC includes surgical resection, transplantation, ablation, chemoembolization, or systemic therapies, such as sorafenib[11]. Early diagnosis and management with surgical resection or transplantation have the highest overall survival benefit[4]. Unfortunately, the majority of patients present at a later stage and require management with ablation, chemoembolization or sorafenib[12]. Sorafenib, a receptor tyrosine kinase inhibitor (RTKI), was approved by the Food and Drug Administration in 2008 as the only systemic drug therapy available for treating unresectable HCC. Although the drug only provides a modest increase in overall survival, from 7.9 to 10.7 months[13], it provided a new therapeutic strategy that could be utilized to manage HCC.

Manipulating the immune system to eliminate established cancers is a rapidly growing field in cancer research[14]. Immune checkpoint studies have considerably advanced immunotherapeutic drug development in cancer treatment[15,16]. The FDA approved the use of antibodies (Abs) against cytotoxic T-lymphocyte antigen 4 (CTLA-4), programmed cell death protein 1 (PD-1), and its ligand PD-L1 for the treatment of melanoma, lung cancer, head and neck cancer, and bladder cancer[17–20]. Clinical trials of monotherapy or combination therapy using one or multiple antibodies against PD-1, PD-L1, or CTLA-4 for the treatment of advanced HCC are ongoing[21–23], and some trials have shown favorable results. In 2017, the FDA granted accelerated approval for anti-PD-1 antibody to treat HCC patients, who are resistance to sorafenib, but the overall response rate of this therapy is only 14.3%. Other strategies have not been translated into clinical practice at this time[24,25]. Overcoming tumor-induced profound immune tolerance to improve immune checkpoint therapy[26]; predicting efficacy of immune checkpoint therapy; preventing immune-related adverse events; optimizing administration route, dosage, and frequency; and finding effective combinations of therapies[27–29] all remain extremely challenging tasks.

There are several conventional approaches used to induce HCC in mouse models currently and are utilized depending on the investigator's particular research question[30]. Chemically-induced HCC mouse models with genotoxic compounds mimic injury-induced malignancy. Xenograft models through either ectopic or orthotopic implantation of HCC cell lines are suitable for drug screening. A number of genetically modified mice have been engineered to investigate the pathophysiology of HCC. Transgenic mice expressing viral genes, oncogenes and/or growth factors allow the identification of pathways involved in hepatocarcinogenesis. Due to inherent limitations, these models do not recapitulate the typical immune characteristics seen in human HCC, which has significantly impeded elucidation of the underlying mechanisms and development of innovative immunotherapeutic strategies[14, 15]. We recently created a clinically relevant murine model. This novel model not only mimics human HCC initiation and progression but also reflects most typical features of human disease including immune dysfunction. We have characterized its biological and immunological characteristics. Leveraging this novel model, we have explored various immunotherapeutic strategies to treat HCC[31–37]. This unique platform allows us to study mechanisms of tumor-induced immunotolerance and to develop proof-of-

concept therapeutic strategies for HCC toward eventual clinical translation.

PROTOCOL:

NOTE: All the procedure including animal subjects have been approved by the IACUC at the University of Missouri. All mice received humane care according to the criteria outlined in the "Guide for the Care and Use of Laboratory Animals". The following procedure for cell isolation and inoculation should be performed in a hood. All performers should wear the standard personal protective equipment for handling of the mice and tissue.

1. Induction of Liver Fibrosis and Cirrhosis with IP Injection of Carbon Tetrachloride (CCl_4)

NOTE: See **Figure 1.**

1.1. Obtain male C57BL/6J mice that are six to eight weeks old (see **Table of Materials**).

1.2. Prepare 10% CCl_4 (v/v) solution in corn oil in a centrifuge tube. Determine total volume based on number of mice to be injected (see step 1.6).

1.3. Use appropriate mice handling technique to select one mouse for injection.

1.4. Manually restrain the mouse with its dorsal (abdomen) side up.

1.5. Clean the injection site on abdominal wall of the mouse by alternatively scrubbing with 70% alcohol and betadine three times.

1.6. Inject male C57BL/6J mice with 160 µL of 10% CCl_4 solution by intraperitoneal (IP) injection using a 25-gauge disposable needle.

1.7. Ensure that the needle penetrates just through the abdominal wall (approximately 4–5 mm) with bevel-side up and slightly angled at 15–20 degrees.

1.8. Inject mice twice a week for a total of four weeks–each mouse will receive a total of eight injections.

NOTE: Two weeks after the last injection, treated mice are ready for ISPL inoculation of oncogenic hepatocytes from MTD2 mice.

2. Isolating Tag-transgenic Hepatocytes from Line MTD2 mice

NOTE: See **Table 1** for solution recipes.

[Place **Table 1** here]

2.1. Obtain line MTD2 mice[38] to serve as the source of oncogenic hepatocytes.

2.2. Anesthetize 5-week-old MTD2 mice using 2.5% isoflurane.

NOTE: Proper anesthetization will be checked by toe pinch method. In brief, using two fingers, give the mouse toe/foot a good squeeze. If there is no withdrawal reaction, the animal is judged deep enough to commence surgery.

2.3. When adequately sedated, place mice in a supine position and fix the extremities with tape to provide adequate exposure of abdominal surface.

2.4. Perform a midline laparotomy incision using scissors along the length of the linea alba large enough to provide an adequate exposure of the liver.

2.5. Displace the intestines to the left to provide better exposure of the liver and portal triad.

2.6. Dissect above the liver to expose the inferior vena cava (IVC).

2.7. Ligate the IVC above the liver using an artery clamp.

2.8. Returning to the inferior border of the liver, use a butterfly needle (see materials) to gain IV access to the portal vein. Fix catheter by hand.

2.9. Successively perfuse the mouse liver using an injection syringe at 8.9 mL/min with with 15 mL of solution 1, 15 mL of 0.75% collagenase solution 2, and 15 mL of solution 2 via the catheter.

2.10. Harvest the perfused liver by cutting and taking the tumor mass from MTD2 mice in a 50 mL conical tube with 10–15 mL of PBS.

2.11. Remove PBS and wash an additional time with PBS; do not centrifuge at this step.

2.12. Cut the liver into smaller pieces using scissors and then wash again with PBS 2x to remove remaining blood.

2.13. Add 5 mL of complete RPMI medium to the conical tube and continuously mince the liver with scissors to small pieces (<3 mm)–tissue should smoothly go through a 5 mL pipette.

2.14. Add complete RPMI to a final volume of 30 mL and suspend liver using a 5 mL pipette.

2.15. Filter the mixed solution with a 70 µm strainer into a 50 mL conical tube.

2.16. Wash the strainer several times with complete RPMI and adjust the final volume to 50 mL by adding additional RPMI medium.

2.17. Quickly spin the suspension by centrifuge to a maximum of 500 rpm; once the speed is accelerated to 50 x *g*, the centrifuge should be stopped.

2.18. Decant the supernatant and suspend pellets in 20 mL of PBS.

2.19. Count cells using Trypan blue exclusion and a hemocytometer, then adjust the cell concentration to 2.5 x 10^6/mL for the following cell inoculation.

NOTE: The expected yield from 5 grams of tumor tissue is 80 million hepatocytes with viability >95%.

3. Inoculating the hepatocytes from MTD2 mice to the liver of wild type C57BL/6J mice by ISPL injection

3.1. Anesthetize the CCl_4-treated male C57BL/6J mice with 2.5% isoflurane.

3.2. Prepare syringes with 200 µL of hepatocytes for injection.

3.3. When adequately sedated, position mice with left side up.

3.4. Shave the entire left flank of the mice, then scrub the area, alternating between 70% alcohol and betadine three times.

3.5. Administer 5 mg/kg of carprofen subcutaneouly prior to surgical incision.

3.6. Make a 1 cm incision on the left flank parallel to the 13^{th} rib from the dorsal extreme beginning just below the spine muscle.

3.7. Identify the spleen, then exteriorize it using blunt-pointed forceps.

3.8. Clip the spleen with two medium-sized titanium clips. Place both clips between the splenic artery and vein, leave room between the clips to cut later after inoculation.

NOTE: The goal is to isolate the inferior pole of the spleen to reduce risk of seeding.

3.9. Inject 200 µL (0.5 million) of the prepared hepatocytes into the inferior pole of the spleen using a 27 G needle.

3.10. Clip the inferior branch of the pedicle (inferior splenic pole vessels) with one medium-sized clip.

3.11. Cut the spleen between the two initially placed clips.

3.12. Remove the inferior pole of the spleen that was directly injected with tumor cells.

3.13. Use 3-0 polyglactin 910 interrupted suturing to close the inner muscle layer.

3.14. Use sterilized steel wound clips to close the outer skin layer.

NOTE: Steel clips are preferred over sutures to avoid animals chewing out sutures, leaving a gaping wound.

3.15. Place all recovering animals on a temperature-controlled heating pad and monitor closely until fully recovered from anesthesia.

3.16. Give mice free access to water after surgery. If the mouse becomes dehydrated during surgery, administer subcutaneous fluids (<1 mL).

3.17. Remove skin clips at 7-10 days post-operatively.

REPRESENTATIVE RESULTS:

Oncogenic hepatocytes isolated from TAg-transgenic mice (**Figure 2**) were seeded in the liver of wild type mice by intra-splenic injection (**Figure 3**). The transplanted hepatocytes successfully and reliably grew orthotopic HCC tumors (**Figure 4**) with tumor specific antigen SV40 TAg (**Figure 5**) in the setting of hepatic inflammation and fibrosis (**Figure 1**).

FIGURE LEGENDS:

Figure 1: Schematic for preparing mouse model of HCC. The experimental design for establishing an innovative murine model of HCC. C57BL/6J mice are first treated with IP injection of CCl_4 twice weekly for four weeks to induce liver fibrosis. Two weeks following the last IP injection, the CCl_4-treated mice receive hepatocytes isolated from young male MTD2 mice via ISPL inoculation.

Figure 2: Isolation and purification of hepatocytes from line MTD2 mice. (**A**) Hepatocytes isolated from MTD2 mice were prepared according to protocol and stained with trypan blue to detect viability and purity of cell isolation. The left panel shows both hepatocytes (black arrow) and tumor infiltrating lymphocytes (red arrows) that are isolated during the cell extraction. The right panel shows the cell population remaining after low speed centrifugation, including hepatocytes (black arrow) and significantly fewer tumor infiltrating lymphocytes. Magnification = 10x objective, scale bar = 100 µm. (**B**) Hepatocytes isolated from MTD2 mice are stained with trypan blue solution prior to inoculation of wild type C57BL/6J mice. Cell isolation is determined to be successful if viability is >95%. Dead cells will be stained with the trypan blue (red arrows), while viable cells will not be stained (black arrow). Magnification = 10x objective, scale bar = 100 µm. The graph on the right shows the results of cell isolation with >95% of hepatocytes being viable, statistical data come from 5 different observed field under microscope; error bars represent $\pm$SD.

Figure 3: Intrasplenic inoculation of hepatocytes into the liver of wild type C57BL/6J mice. The experimental design for ISPL inoculation of hepatocytes to induce HCC in C57BL/6J mice. (1) The

spleen is identified and exteriorized. (2) The spleen is clipped with two medium-sized titanium clips. (3) Prepared hepatocytes are injected into the inferior pole of the spleen. (4) The inferior branch of the pedicle (inferior splenic pole vessels) is clipped with one medium-sized clip.

Figure 4: Progressive tumor development in C57BL/6J mice after inoculation with MTD2-hepatocytes. (**A**) Hepatocellular carcinoma (HCC) orthotopic tumor model was established according to protocol. Anatomical images were taken before inoculation and at sub-sequential time courses. (a) Shows healthy liver prior to inoculation with MTD2 hepatocytes. (b) Mouse liver 3 months post inoculation with evidence of gross tumor development. (c) 3.5 months after inoculation with increasing tumor burden. (d) 4.5 months after inoculation. (e) 6 months after inoculation with evidence of tumor throughout the liver. (**B**) Tumor nodules were harvested and weighed at the time points indicated after inoculation with MTD2 hepatocytes. Error bars represent $\pm$SD. ***$P < 0.001$, statistical analysis was performed by *t*-tests. (**C**) Representative images of wildtype and MTD2-inoculated liver sections. Hematoxylin and eosin (H&E) staining depicts pseudogland formation (black arrows). There is nuclear crowding, and tumor cells are eosinophilic with high nucleus-to-cytoplasm ratios (magnified image). Magnification = 20x objective, scale bar = 50 µm. The insets on the upper right are further manually amplified. (**D**) Sirius red staining indicating abnormal collagen deposition, consistent with liver fibrosis. Magnification: 40x objective, scale bar = 20 µm.

Figure 5: Specific detection of SV40 T antigen gene in tumor. (**A**) Tumor tissue and additional tissue samples throughout mice were collected from tumor-established mice. Genomic DNA was isolated from tissue and primers for both SV40 T Ag and P53 (control) were synthesized for conventional PCR. SV40 T antigen gene was detected in tumor tissue but is not present in either the normal tissue or other tissue in the mice. (**B**) Tumor tissue was collected from tumor-bearing mice and stained with anti-SV40 T antigen antibody. Left panel is a negative control from healthy liver tissue collected from naïve mice. Right panel shows significant SV40 T antigen staining of tumor tissue collected from tumor-bearing mice (brown color). Magnification = 40x objective, scale bar = 20 µm.

Table 1: Solution recipes.

DISCUSSION:

With this protocol, we have established a reliable and reproducible murine model of HCC that mimics human HCC initiation and progression. Clinically, many risk factors successively induce liver injury, liver fibrosis, cirrhosis and the final stage of HCC. In our protocol, IP injection of CCl_4 is used to first produce liver fibrosis in wild type mice, which allows the subsequent oncogenic hepatocytes to form the tumors in the setting of liver fibrosis. We found that tumor formation occurred most successfully in mice that received the hepatocyte inoculation two weeks after CCl_4 treatment, compared to mice receiving injections at other time points. In addition, we found liver fibrosis can be detected up to four months after CCl_4 injection. This approach results in more than 90% of mice developing HCC tumors compared to the mice without exposure to CCl_4. In our model, hepatocytes transferred from line MTD2 mice into C57BL/6J mice, traffic to the liver where they become incorporated as hepatocytes that express TAg as a tissue

antigen. Isolation of hepatocytes from MTD2 is a critical step during this protocol. MTD2 mouse livers are perfused thoroughly with the solution 1 and 2 to remove circulating red blood cells within the liver completely. The collagenase solution is used to perfuse the liver at the indicated concentration and speed to generate proper liver digestion to release hepatocytes. The use of lower concentrations of collagenase is insufficient for digestion, resulting in clumps of hepatocytes. In contrast, high concentrations are too harsh on the tissue, resulting in a significant reduction of viable hepatocytes. We also find that brief centrifugation as described in our protocol is required to improve the purity of hepatocytes. The lower spin we used for centrifugation can precipitate hepatocytes and leave leukocytes in the supernatant based on the density difference of these two types of cells.

The next critical step is the route for inoculating hepatocytes into wild type mice. In our pilot studies, we explored various ways to conduct hepatocyte inoculation. The successful orthotopic tumor growth in the liver is seen best in mice receiving intrasplenic inoculation of oncogenic hepatocytes at a dose of half-a-million cells per mouse, compared to the mice receiving cells administered via tail vein and intraperitoneal injection at various doses. These findings suggest that the orthotopic HCC growth is route and dose-dependent. Malignant transformation occurs gradually and is limited to the subpopulation of transplanted hepatocytes rather than the whole liver parenchyma. Continued cell proliferation results in tumor nodules developing throughout the liver.

For HCC or other cancers to progress it must evade the immune system; in fact, avoiding immune destruction is now considered a hallmark of cancer[39]. However, the lack of tumor-specific antigen is a critical barrier to elucidating the underlying mechanisms. In our model, TAg is expressed in the tumors, not other organs, which acts as a tumor-specific antigen. Also, TAg has numerous well-defined epitopes that can be recognized by CD8 T cells in C57BL/6J mice. Regarding TAg epitope-I, we have generated line 416 mice which transgenically express T cell receptors for this epitope[34]. Targeting TAg to examine tumor antigen-specific immune response allows us to investigate tumor immune surveillance during tumor initiation and progression, which is not possible using models induced by DEN or genetic manipulation. Elucidating the underlying mechanisms enables us to identify critical cells and molecules mediating tumor-induced immune tolerance. Targeting these key factors can significantly advance our development of innovative immunotherapeutic strategies against HCC. Using this unique HCC model and established tools, we have investigated the mechanisms underlying tumor-induced tolerance[25, 35] and explored various immune-based antitumor immunotherapies[31, 32, 36, 37].

In summary, our established murine model of HCC reflects some typical features of human disease. In our previously published article, we were able to establish this as a clinically relevant tumor model that had typical features of human HCC. We showed that the mice treated with CCl_4 and MTD2 hepatocytes developed tumors that expressed HCC-associated antigens, AFP and GPC3[36]. Pathology determined that the lesions in our murine model are similar both macroscopically and pathologically to human HCC. Leveraging this reliable model and the developed tools, we can study this complex human disease including insight into mechanisms of HCC development and clinically available treatment.

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DISCLOSURES:

There are none to declare.

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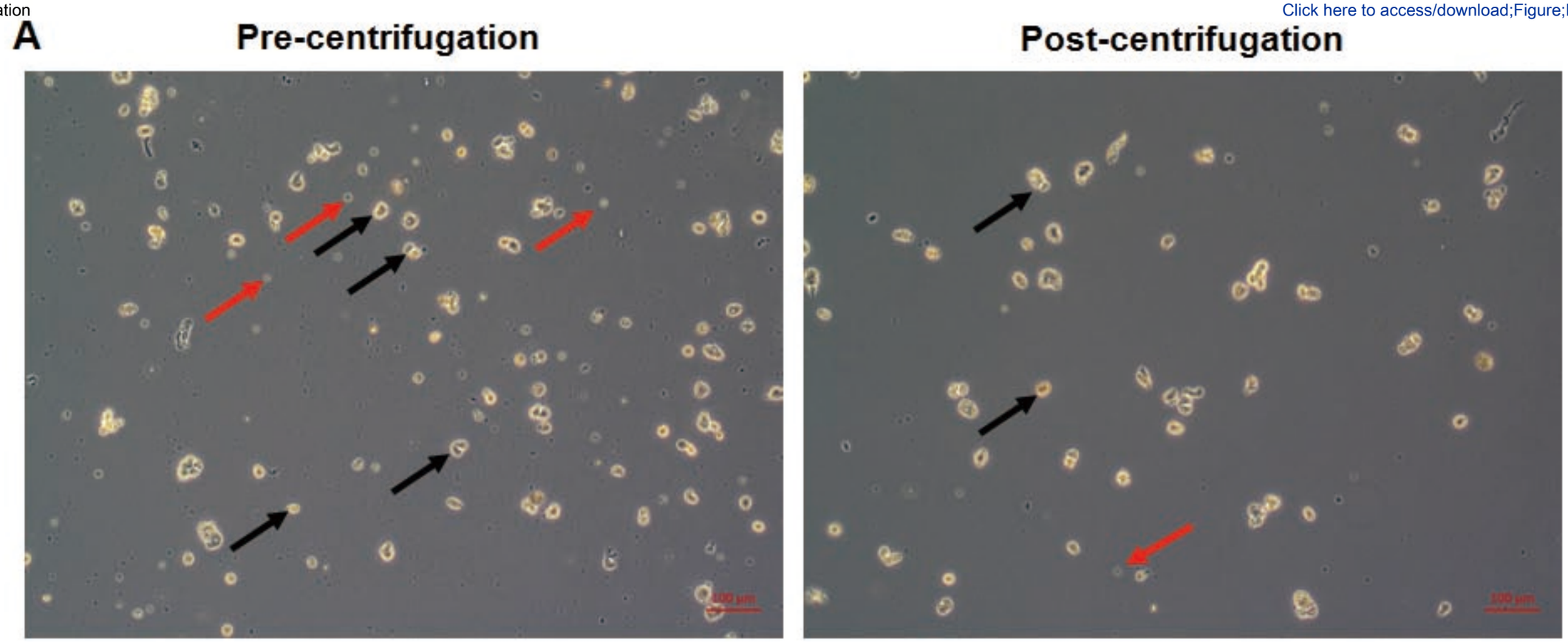
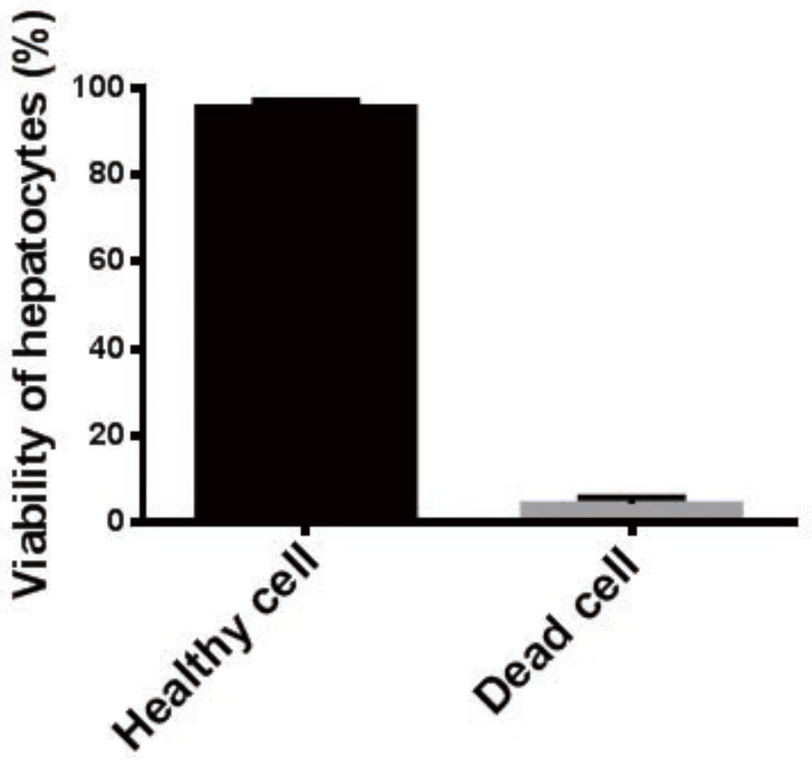
Figure 1 Mouse Schema

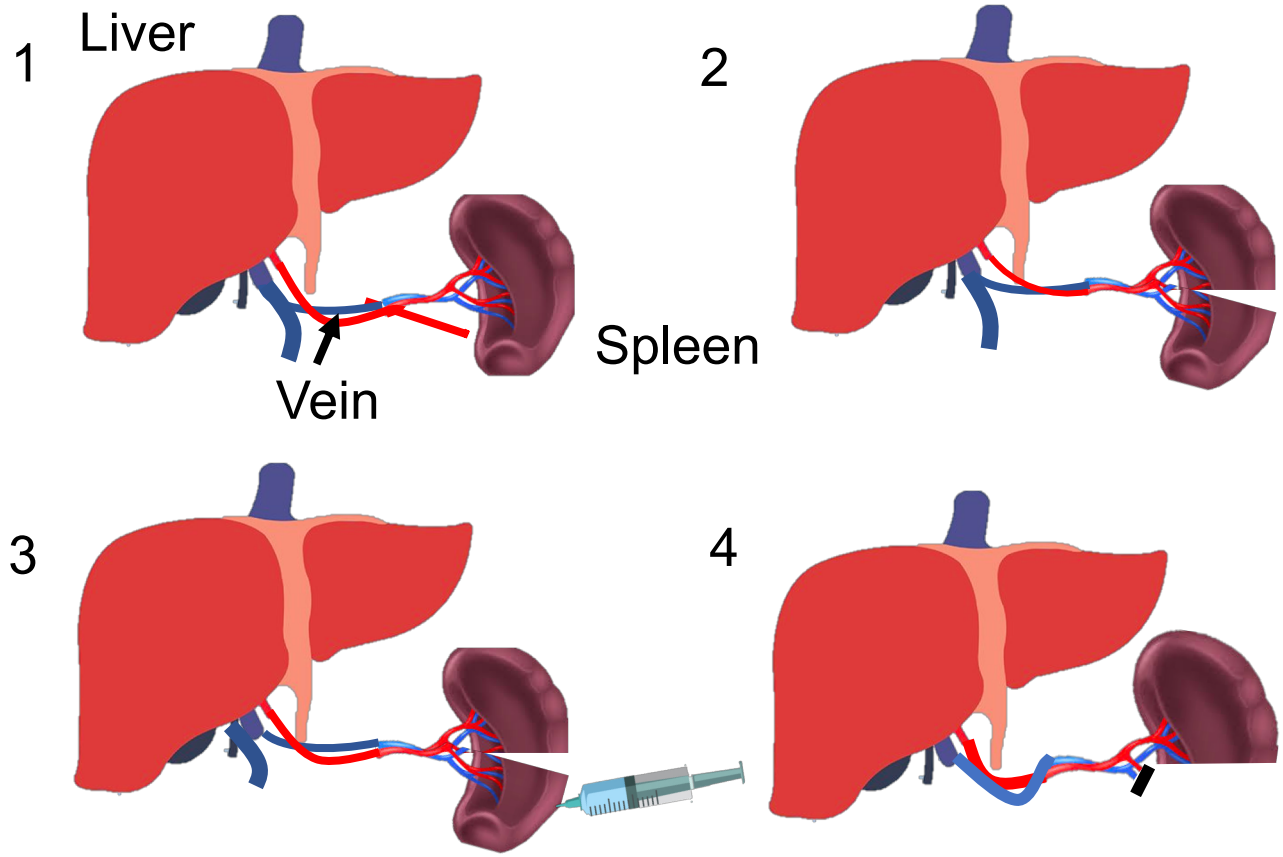
C57BL/6J

Week 1 – Week 4
Twice weekly injections

Week 7
MTD2-line hepatocyte injection







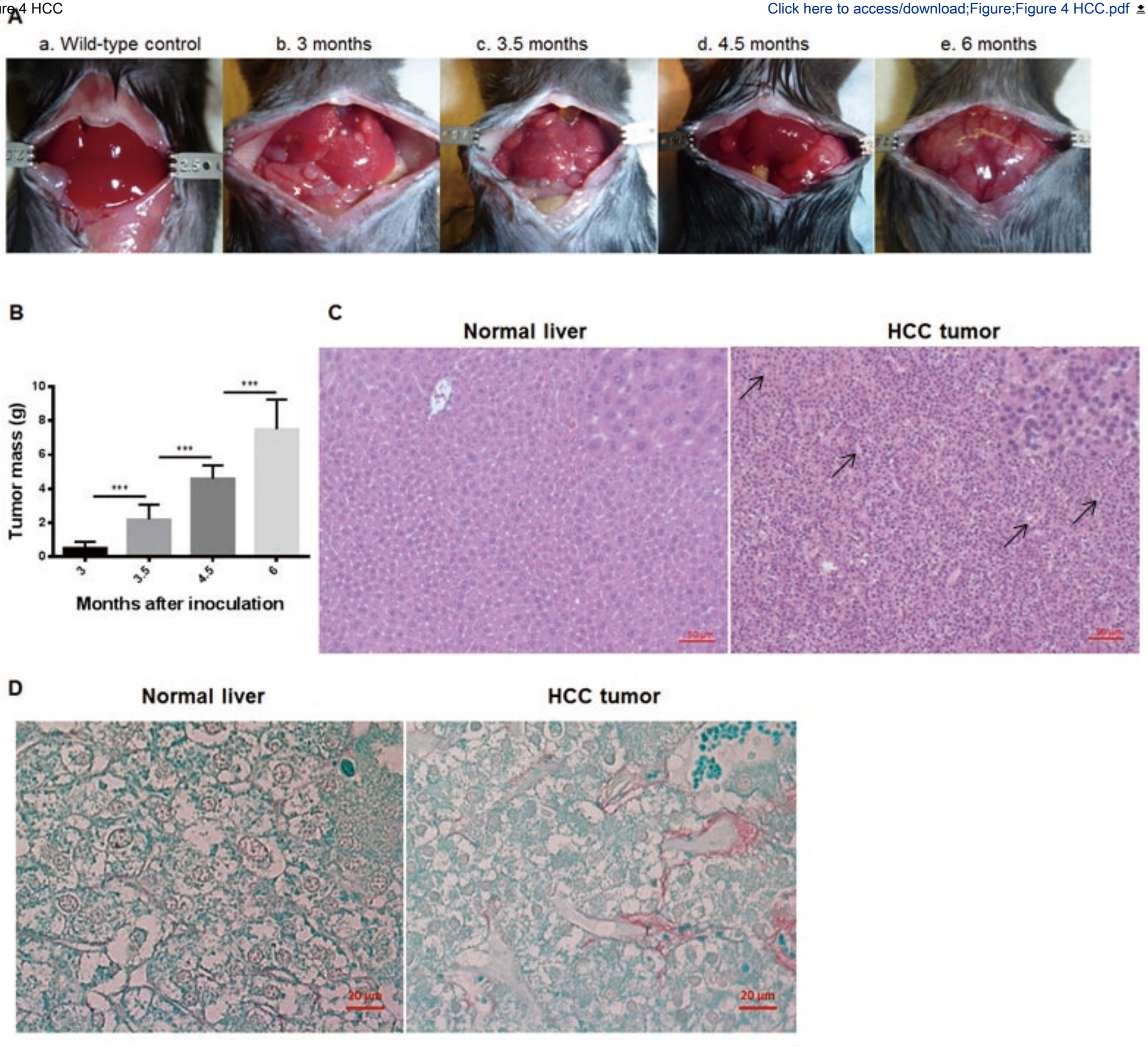
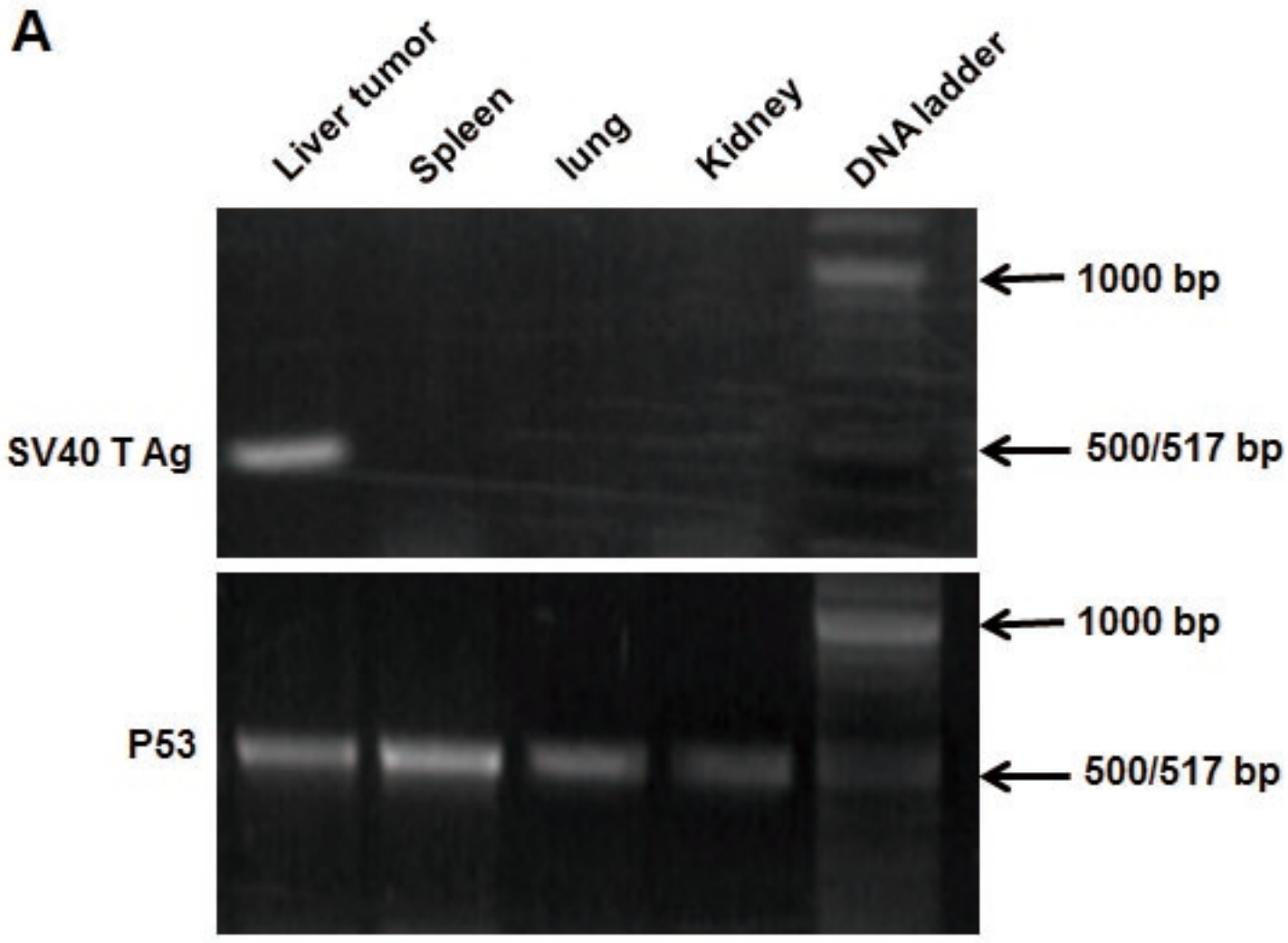
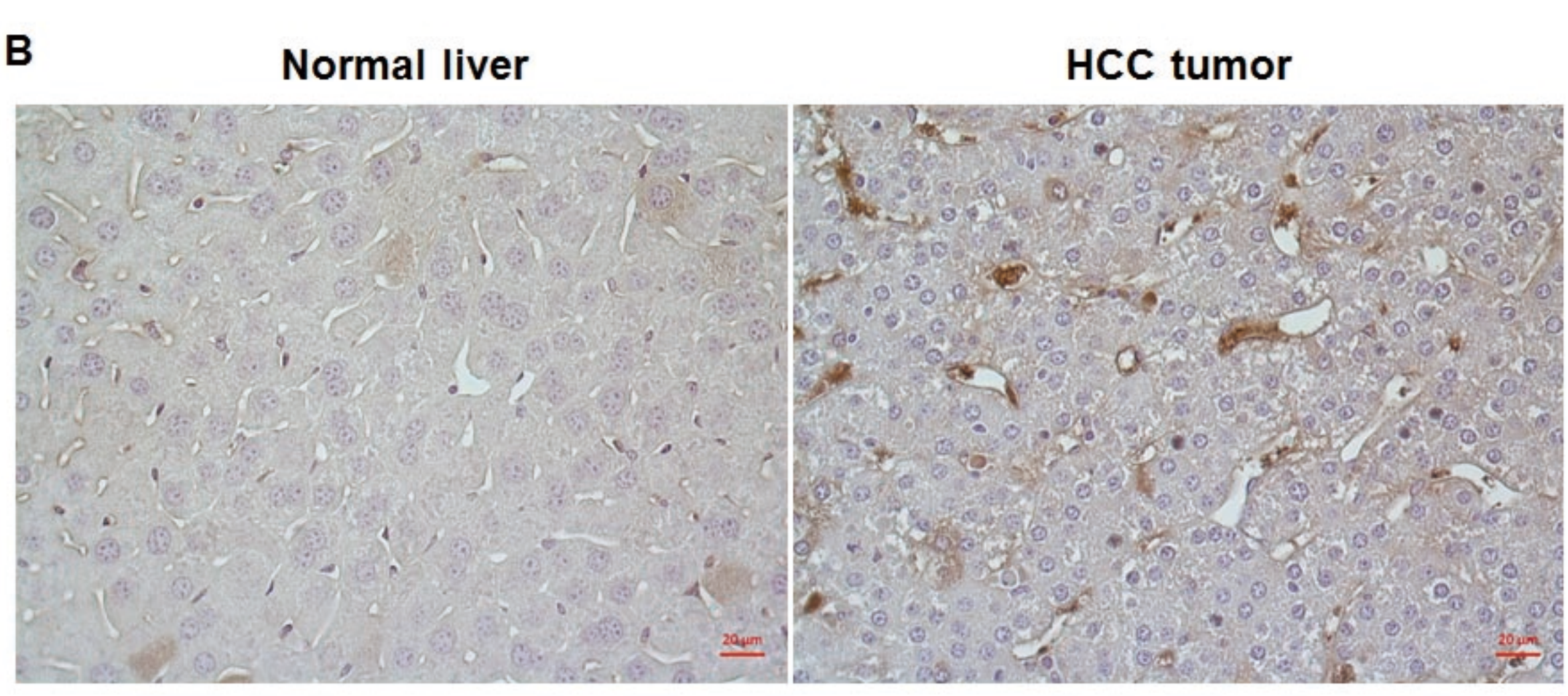


Figure 5 SV40 TAg

Click here to access/download;Figure;Figure 5 SV40 TAg.pdf





10x Earle's Balanced Salt Solution (EBSS)
10x Earle's Balanced Salt Solution without Ca or Mg (EBSS without Ca or Mg)
Solution 1
Solution 2
0.75% collagenase solution
Complete Medium

2 g $CaCl_2$ 4 g KCl 2 g $MgSO_4 \cdot H_2O$ 68 g NaCl 1.4 g $NaH_2PO_4 \cdot H_2O$ 10 g dextrose Add water to 1 liter, pH to 4.32 Pass through filter
4 g KCl 68 g NaCl 1.4 g $NaH_2PO_4 \cdot H_2O$ 10 g dextrose Add water to 1 liter, pH to 4.32 Pass through filter
20 mL 10x EBSS without Ca or Mg 44 g $NaHCO_3$ 1.33 mL 1.5M Hepes 10 mL of 10 mL EGTA Add water to 200 mL
100 mL 10x EBSS 2.2 g $NaHCO_3$ 6.67 mL 1.5 M Hepes Add water to 1 liter
15 mg collagenase type 1 20 mL of solution 2
2 RPMI 50 mL FBS 5 mL 100x Penicillin-Streptomycin

Table of Materials

Click here to access/download;Table of Materials;JoVE_Materials_1.14.xlsx

Name of Material/ Equipment	Company
Anesthesia machine	VETEQUIP
Butterfly needle	BD
C57BL/6 mice	Jackson Lab
Carprofen	CRESCENT CHEMICAL
Cell Strainer	CORNING
Centrifuge	Beckman Coulter
Clips	Teleflex Medical
Microscope	Zeiss
Mtd2 mice	N/A
Needle	BD
Suture	ETHICON
SV40 T Ag antibody	Abcam
Syringe	BD
Trypan blue	SIGMA
Wound clips	Reflex

Catalog Number	Comments/Description
IMPAC6	anesthesia machine for surgery
8122963	Needle used for liver perfusion
000664	mice used in prototol
20402	carprofen for pain release
REF 431751	Cell strainer, 70µm, for hepatocytes isola
Allegra X-30R	centrifuge for cell isolation
REF 523700	Titanium Clips for spleen
Primovert	microscope for cell observation
	Gift from Dr. William A Held at roswell Pa
REF 305109	BD precisionglide needle, 27G x 1/2 (0.4r
J303H	coated VICRYL suture
ab16879	anti-SV40 T-antigen antibody for IHC
REF 309626	1 mL TB syringe for cell injection
T 8154	Trypan blue solution for cell viability test
reflex9, Part. No. 201-1000	stainless steel wound clips for wound clo

ation

ark Cancer Institute in 2002, maintained in our lab
mm x 13mm)

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Title of Article: A clinically relevant mouse model of hepatocellular carcinoma in the setting of inflammation and liver fibrosis

Author(s): Xiangkang Qi, Emily Schaper, Diego Avella, Eric Kimchi, Jussuf Kaifi, Kevin Staveley-O'Carroll, Guangfu Li

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CORRESPONDING AUTHOR

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Department:	Dept. of Surgery
Institution:	University of Miami
Title:	Ph.D.
Signature:	[signature]
Date:	11/05/2018

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RE: JoVE59368; "A clinically relevant mouse model of hepatocellular cancer arising in the setting of inflammation and liver fibrosis."

In reference to the above identified manuscript that you recently reviewed, we would like to thank you and the reviewers for your comments and interest in our study. We greatly appreciate your time, effort, and the insightful and constructive comments about the manuscript. We acted on these comments, and in doing so, have substantially improved the manuscript. The supplementary materials and protocol now provide more detailed descriptions as requested. Please find the point-by-point reply to the reviewer's comments on the following pages (Reviewers comments are in italics, and responses follow).

Regarding the Editorial comments:

1. *Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.*

Answer:

We have proofread the manuscript.

2. *Please revise lines 42-43, 45-50, 72-73 to avoid previously published text.*

Answer:

We have revised the above-mentioned lines.

3. *Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .pdf, .svg, .eps, .psd, or .ai file.*

Answer:

We have uploaded the figures into the account in the appropriate format.

4. *Figure 1: Please use subscripts in chemical formulae to indicate the number of atoms, e.g., CCl4 (4 should be in subscript form).*

Answer:

We have corrected the chemical formulas to include subscripts.

5. *Figure 2B: Please define the error bars in the figure legend.*

Answer:

The error bars represent SD±, it has been added to manuscript.

6. *Figure 4B: Please include a scale bar for all images taken with a microscope to provide context to the magnification used. Define the scale in the appropriate figure Legend.*

Answer:

The corresponding scale bars have been added in the images. The definition has been added in figure legend.

7. *Figure 5A: Please label the last lane as well.*

Answer:

The last lane in figure 5A has been labeled.

8. *Please revise the title to be more concise.*

Answer:

The title has been revised to “An oncogenic hepatocytes-induced orthotopic mouse model of hepatocellular cancer arising in the setting of hepatic inflammation and fibrosis”

9. *Please provide an email address for each author.*

Answer:

We have included all authors email addresses under the authors and affiliations.

10. *Line 77: Please ensure that the reference numbers are correct and make them superscripts.*

Answer:

We have corrected the references and made them superscripts.

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Answer:

We have removed all commercial language.

12. *Please revise the protocol to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible. Please move the discussion about the protocol to the Discussion.*

Answer:

We have reformatted our sentences into the imperative tense. We have added the following, “The following procedure for cell isolation and inoculation should be performed in a hood. All performers should wear the standard personal protective equipment for handling of the mice and tissue.”

13. *Lines 110-148: The Protocol should contain only action items that direct the reader to do something. Please either write the text in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.), or move the solutions, materials and equipment information to the Materials Table.*

Answer:

We have reformatted the protocol sentences to the imperative tense and moved materials and equipment information to the Materials table.

14. *Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.*
Answer:
We have added more detail to the protocol.

15. *1.1: Please list an approximate volume of solution to prepare.*
Answer:
The volume is dependent on the number of mice. We have clarified by stating, "Determine total volume based on number of mice to be injected, refer to step 1.6."

16*. 2.2, 3.1: Please specify the concentration of isoflurane used and mention how proper anesthetization is confirmed.*
Answer:
The revision has been made. The concentration of isoflurane is 2.5%; the proper anesthetization will be checked by toe pinch method. In brief, person uses two fingers giving mouse toe/foot a good squeeze. If there is no withdrawal reaction, the animal is judged deep enough to commence surgery.

17. *2.3, 2.6, 3.5, etc.: Please specify the surgical instrument used throughout the protocol.*
Answer:
2.3 "with a scissor" has been added.
2.6 "using injection syringe" has been added.
3.5 "using scissor" has been added.

18. *2.8: Please describe how to harvest the perfused livers.*
Answer:
"by cutting and taking tumor mass" has been added.

19. *2.10: Please define "smaller pieces".*
Answer:
"The minced tissue is defined by smoothly going through a 5mL pipette" has been added.

20. *2.15: Please convert centrifuge speeds to centrifugal force (x g) instead of revolutions per minute (rpm).*
Answer:
The centrifuge speed has been changed.

21. *2.17: What is used to adjust the cell concentration?*
Answer:
"Using hemocytometer"

22. *At the end of the protocol, please also describe post-surgical treatment of animal, including recovery conditions and treatment for post-surgical pain.*
Answer:
All recovering animals will be placed on the temperature-controlled heating pad and monitored closely post-operatively until they are fully recovered from anesthesia. The mice will be given free access to water after surgery. If the mouse becomes dehydrated during surgery, they will be given subcutaneous fluids (<1 ml). Carprofen is administered at a dosage of 5mg/kg s.c. prior to surgical incision. Skin clips are removed 7-10 days post-op.

23. *Please include single-line spaces between all paragraphs, headings, steps, etc.*
Answer:
We have made the recommended change.

24. *After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.*
Answer:
We have revised the highlighted portions of the protocol.

25. *Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Notes cannot usually be filmed and should be excluded from the highlighting. Please do not highlight any steps describing anesthetization and euthanasia.*
Answer:
We have highlighted steps in the manuscript to be filmed.

26. *Please include at least one paragraph of text to explain the Representative Results in the context of the technique you have described, e.g., how do these results show the technique, suggestions about how to analyze the outcome, etc. The paragraph text should refer to all of the figures. However for figures showing the experimental set-up, please reference them in the Protocol. Data from both successful and sub-optimal experiments can be included*.
Answer:
The new paragraph has been added in manuscript.

27. *References: Please do not abbreviate journal titles.*
Answer:
We have corrected journal titles.

28. *Table of Materials: Please revise the Table of Materials to include the name, company, and catalog number of all relevant supplies, reagents, equipment and software in separate columns in an xls/xlsx file. Please sort the items in alphabetical order according to the name of material/equipment.*

Answer:

The table of materials has been revised according to guide.

Reviewer #1:

This work describes the protocol for a liver tumor model in fibrotic livers.

1. *The title and the description of the model is misleading. What makes the authors believe that this is a "clinically relevant model" of HCC? They do not provide data for this. In fact, the transfer of foreign hepatocytes into the spleen is far from the "normal" hepatocarcinogenesis (in which endogenous hepatocytes transform). These highly misleading statements (title, abstract, text) need to be removed and rephrased.*

Answer:

The title has been revised.

2. *The authors should provide some exemplary data that the model can be helpful in determining the efficacy of immune-directed therapies (e.g., interventions with anti-PD-1 antibodies). This would be helpful to the readers.*

Answer:

We have followed the reviewer's instruction and provided a exemplary data in the part of discussion on page 6.

3. *Figure 4 should contain a statistical summary of the data, e.g., number of tumor nodules, liver-to-body weight ratio, size of nodules etc. This would help the readers to estimate what type of tumor burden can be expected by following the protocol.*

Answer:

A statistical summary data for total tumor mass in each mouse after inoculation has been added. The number of tumor nodules is random.

4. *Figure 4B: What does the circle mean? Why does this picture show HCC?*

Answer:

The circle has been removed. The pictures show typical H&E (hematoxylin and eosin) staining pattern for normal liver and HCC tumor in our murine model.

5. *Text: "We have recently published our results in several journals 29-31 including the Journal of Hepatology (14-16) and Gastroenterology 32." The references do not fit.*

Answer:

We corrected the reference numbers.

Reviewer #2:

Manuscript Summary:

Guangfu et al. describe in their manuscript an animal model that mimics human HCC initiation and progression in the context of liver fibrosis and cirrhosis. This model has a very complex workflow. Therefore, a detailed protocol is highly required. To make all described necessary steps reproducible there are still a number of critical points that need to be addressed by the authors.

Major Concerns:

1. *The table of materials is incomplete and therefore has to be extended to make the protocol reproducible.*

Answer:

The table of materials had been revised.

2. *Step 2: It should be mentioned where the MTD2 mice can be obtained from.*

Answer:

MTD2 mice were a gift from Dr. William A. Held at Roswell Park Cancer Institute in 2002. We have maintained this mouse line.

3. *Point 2.2. and 3.1.: It is not mentioned at which concentration Isoflurane is used for anesthesia.*

Answer:

The revision has been made. The concentration of isoflurane is 2.5%

4. *Point 2.7.: Authors should mention the flowrate for successful perfusion of the liver. In established protocols for the isolation of liver cells, the last perfusion solution contains collagenase. The authors should explain why they end with a perfusion step with solution 2 and not with the collagenase solution if the order was not changed by mistake here.*

Answer:

The flowrate for perfusion is 8.9 mL/min, it has been added in protocol. The last solution used in perfusion step is solution 2 without collagenase, by which the remnant of collagenase will be removed, thus the hepatocyte viability will not be hurt.

5. *Point 2.17.: The expected yield and viability of isolated hepatocytes should be added. This would make it also more obvious how many mice can be injected with the hepatocytes from one MTD2 mouse.*

Answer:

The 80 million of hepatocytes will be expected with viability > 95% from 5 gram of tumor tissue. It has been added.

6. *Point 3.1.: The male mice are not 6 weeks old. They receive CCl4 starting at the age of 6-8 weeks for 4 weeks and then have a recovery period of 2 additional weeks. It should be made clear that these mice were treated with CCl4 before this step.*

Answer:

Removed “six-week-old” and replaced with “CCl4-treated” mice.

7. *Fig. 1: A time schedule should be added. The schematic representation should include the MTD2 mice and their use.*
Answer:
Added timeline to this schematic.

8. *Fig. 3: The experimental design is not explanatory enough. It should be clearly indicated where the clips are positioned. The authors should add photographs of each step in addition to the schematic drawing.*
Answer:
Clips are placed within the vascular pedicle, between the artery and vein leaving space between the adjacent clips to later be cut after inoculation. The position of clips will be shown on video.

9. *Fig. 4: Tumor initiation is not circled as indicated in the figure legend. It is not indicated from which timepoint the H&E stainings are. Moderate nuclear pleomorphism should be indicated in the picture. More than one mitotic cell should be shown to state that the mitotic activity was increased. A representative H&E staining of different time points should be included, similar to 4A.*
Answer:
The new pictures have been used in fig. 4B, which show typical H&E staining pattern for normal liver and HCC tumor in our murine model. The H&E staining pattern for HCC tumor is consistent.

10. *Fig. 5: It is not clear where the specific SV40 TAg staining is since there seems to be unspecific (brown) staining in the negative control. All tumor cells should be SV40 TAg positive, nevertheless there are only three arrows indicating positive cells.*
Answer:
The new images have been taken, and used in manuscript. It is clear specific SV40 T Ag staining on the HCC tumor tissue, but not on normal liver tissue.

11. *The expected amount of fibrosis should be shown (i.e. Sirius Red or similar).*
Answer:
The fibrosis staining has been added.

12. *Since the authors claim that this is a model recapitulating human HCC initiation and progression and also reflects most typical features of human disease including immune dysfunction, they should show what can be expected in regard to these characteristics.*
Answer:
Given this model recapitulates human HCC initiation and progression as well as tumor-induced immune suppression, we consider that this model provides a unique platform for us to explore and develop therapeutic strategies including immunotherapy against HCC. This has been highlighted in the first paragraph of discussion on page 6.

13. *The typical load and size of tumors per mouse should be mentioned in addition to the typical time that it takes to develop neoplasms and tumors. Also variability in this model should be discussed.*

Answer:

The data of typical tumor mass in model has been added. The mice will not die from tumorigenic hepatocytes inoculation before developing large HCC tumor unless surgical accident or other disease.

14. *It should be mentioned which control mice need to be considered (i.e. Oil injection control for CCl4 treatment, sham operation,…).*

Answer:

The mice receiving vehicle solution without oncogenic hepatocytes are used for control.

Minor Concerns:

1*. It should be mentioned if C57BL/6J or 6N mice are used/recommended since it is known that there can be different responses even between these two strains.*

Answer:

Clarified in the protocol to use C57BL/6J.

2. *The authors state that they recently published in several Journals including the Journal of Hepatology. This publication is missing in the list of references.*

Answer:

We have corrected these references.

3. *Point 1.4.: This should be made clearer. Mice receive 8mL/kg of the 10% CCl4 solution? It is not clear what is meant by "mice will receive 10% CCl4".*

Answer:

Changed to volume needed to be injected. “inject male C57BL/6J mice with 160uL of 10% CCl4 solution”

4. *Step 2 Reagents required: It is not clear if all solutions for Step 2 need to be filtered and pH adjusted. For some it is written for some not. For solution 1 the concentration of EGTA is missing. For isolation of viable cells from mouse liver, perfusion solutions have to be warmed to 37-42°C prior to their use. Such a step is not mentioned in the manuscript.*

Answer:

All solutions need to be filtered, only EBSS and EBSS without Ca/Mg need to be pH adjusted. The concentration of EGTA has been added. Based on our experience, the perfusion buffer can be used at room temperature without damaging cells.

5. *Point 2.4.: It is not mentioned how the catheter is fixed in place.*

Answer:

Catheter is fixed by hand which will be shown in video.

6. *Point 2.9.-10.: It is not clear how the washing is performed. Is there centrifugation involved?*
Answer:
The washing is performed by removing dirty PBS and adding clean PBS and do it again, no centrifuge is needed.

7. *Point 3.2.: The total number of injected hepatocytes per mouse and the needle size for injection should be mentioned.*
Answer:
0.5 Million of cell is injected per mouse by 27G needle that is listed in material table. The revising has been made in manuscript.

Reviewer #3:
Manuscript Summary:
the manuscript describe a HCC model based on transplantation of tumor cells in a healing liver.

Major Concerns:
General:
1. *The model is proposed as an ideal model to study tumor-induced immunotolerance. It is however not discussed why this model is more appropriate than others (for ex DEN or genetic) for this specific purpose.*
Answer:
In this model, TAg is only expressed in tumors rather than other organs. Therefore, TAg functions as a tumor-specific antigen enabling us to study tumor antigen-specific immune response which could not be done with other models induced by DEN or genetic. This information was added into the first paragraph of discussion on page 6.

2. *Please give all specifications of mouse strains used (a) C57Bl6 (J? N? Origin?, It is understood that MDT2 mice are not commercially available. Please indicate how people can have access to those mice. This is mandatory if the protocol has to be used by other scientists.*
Answer:
Line MTD2 mice are transgenic C57BL/6J mice expressing SV40 large T antigen under the Major Urinary Protein (MUP) promoter. MTD2 mice were a gift from Dr. William A. Held at Roswell Park Cancer Institute in 2002. We have maintained this mouse line.

3. *In the model, Injection of MTD2 hepatocytes is performed on C57BL6 mice having undergone a CCl4 pretreatment followed by a 2 weeks recovery period.*
Necessary information is lacking: (a) what is the residual liver fibrosis and inflammation at the time of cell injection? (b) why perform injections 2 weeks after the end of CCl4 (why not 1 or 4?) (c) what is the efficiency of tumor development (% of animal with tumors) when %TD2 cells are injected into a "naïve" normal liver, versus previously exposed to CCl4?
Answer:
The following explanation was included in our discussion on page 7: "In developing this model, we performed hepatocyte inoculation at varying time points after completing the CCl_4

injections, including immediately, one week, and two weeks post-CCl_4 administration. Tumor formation was only detected in the mice receiving hepatocyte inoculation two weeks after CCl_4 treatment. In addition, we found liver fibrosis is able to be detected even up to four months after CCl_4 injection. This strategy is reliable and reproducible as more than 90% of mice are able to form HCC tumors compared to the mice without exposure to CCl_4."

4. *It appears (Fig 2) that a mixture of hepatocytes and lymphocytes from MTD2 mouse liver are injected. Please comment on the following: what is the % of purity of hepatocyte orearation (what is the contamination by lymphocytes and other non parenchymal cells). Why not performing an additional step for exclusion of hematopoietic cells? Some death cells contaminate the hepatocyte suspension: what lower percentage of viable cells is acceptable for injection. In other words, when do you discard the cell preparation for injection (<90% cell viability, <95%???).*

Answer:

In the legend of figure 2, we have clarified the purity and viability of hepatocytes. We didn't use additional method to exclude hematopoietic cells because the extent of this purity and viability is sufficient for us to successfully make HCC model.

5. *Figure 4B is unclear. Left normal liver, right HCC bearing liver. Actually, it look like the opposite. Please provide low magnification H&E figures so architecture including HCC can be evaluated, higher magnification to cell atypia. Also provide a sirus red staining (or other fibrosis stain) so ECM deposits and organization can be visualized. The picture provide do not convince that there is tumor tissue!*

Answer:

The new pictures have been used in fig. 4B, which show typical H&E staining pattern for normal liver and HCC tumor in our murine model. The H&E staining pattern for HCC tumor is consistent. The fibrosis staining has been added.

6. *Figure 5B: IHC are unreadable. Left panel is negative control: I see there some hepatocytes (4?) that might be positively stained (nuclear and plasma membrane stain). In right panel (+ve!), I only see non specific staining in sinusoids.*

Answer:

The new images of IHC have been taken, and used in manuscript. It is clear specific SV40 T Ag staining on the HCC tumor tissue, but not on normal liver tissue.

RE: JoVE59368; "A clinically relevant mouse model of hepatocellular cancer arising in the setting of inflammation and liver fibrosis."

1.It is still unclear the extent to which this is a good model of HCC. Do you have something like Western blot data (or equivalent) to further demonstrate this? What data specifically show its reliability? It is fine to publish without these data (or just reference other articles with such data), but claims should be adjusted accordingly.
Answer:
We have added the following to our discussion. "In our article previously published, we were able to establish this as a clinically relevant tumor model. We showed that the mice treated with CCl4 and MTD2 hepatocytes developed tumors that expressed HCC-associated antigens, AFP and GPC3. Pathologist reviewed MTD2-inoculated mice livers and determined that the lesions in murine and human HCC are similar on both macroscopic and pathologic exam."

2. You have removed claims of clinical relevance from the title, but they remain in the manuscript itself. Please do so (or justify the 'clinical relevance' further).
Answer:
We have added the following to our discussion. "In our article previously published, we were able to establish this as a clinically relevant tumor model. We showed that the mice treated with CCl4 and MTD2 hepatocytes developed tumors that expressed HCC-associated antigens, AFP and GPC3. Pathologist reviewed MTD2-inoculated mice livers and determined that the lesions in murine and human HCC are similar on both macroscopic and pathologic exam."

3. Please include a more detailed description of what is being shown in the Figures and how they represent typical outcomes of the protocol. For, example, how exactly does Figure 4 show that transplanted hepatocytes grow orthotopic HCC tumors? What does the staining in 4C indicate? How does the staining in 4D indicate liver fibrosis, exactly?
Answer:
We have changed the Figure legend for Figure 4 to the following: " (C) Representative images of wildtype and MTD2-inoculated liver sections. H&E staining depicts pseudogland formation. There is nuclear crowding, and tumor cells are eosinophilic with high nucleus-to-cytoplasm ratios. H&E, magnification: ×20 objective, the blocks on right-up corner are further manually amplified. (D) Sirius red staining indicating abnormal collagen deposition, consistent with liver fibrosis. Magnification: x40 objective."

4. Please combine separate pages of Figures (Figure 2, Figure 4, Figure 5) into one complete image per Figure. Note that we do not have limits on Figure size apart from the file size.
***Answer**:*
We have combined the separate images of Figures 2, 4, and 5.

5. Please remove titles from the Figures themselves.
***Answer**:*
We have removed the titles from the figures.

6. Please reference Figure 5 in the manuscript (outside of the Figure Legends).
Answer:
We have referenced Figure 5 outside of figure legend.

7. Figure 4: What significance cutoffs do the asterisks indicate?
Answer:
Asterisks indicate P < 0.001

8. Please format the 'Reagants required' section as a table/tables or numbered protocol steps in the imperative.
***Answer**:*
We have reformatted the Reagants required into table format