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Editorial summary: This protocol describes a syngeneic orthotopic hepatocellular carcinoma (HCC) model in immunocompetent mice with liver cirrhosis induced by carbon tetrachloride. This model recapitulates the key features of the necroinflammatory, fibrotic and angiogenic microenvironment of human HCC.

ABSTRACT

Subcutaneous xenografts have been used for decades to study hepatocellular carcinoma (HCC). These models do not reproduce the specific pathophysiological features of HCCs, which occur in cirrhotic livers showing pronounced necroinflammation, abnormal angiogenesis, and extensive fibrosis. As these features are critical for studying the role of the pathologic host microenvironment in tumor initiation, progression, and treatment response, alternative HCC models are desirable. Here, we describe a syngeneic orthotopic HCC model in immunocompetent mice with liver cirrhosis induced by carbon tetrachloride (CCl4) that recapitulates key features of human HCC. Induction of significant hepatic fibrosis requires 12 weeks of CCl4 administration. Intrahepatic implantation of murine HCC cell lines requires 30 minutes per mouse. Tumor growth varies by tumor cell line and mouse strain used. Alternatively, tumors can be induced in a genetically engineered mouse model. In this setting, CCl4 is administered for 12 weeks after tail-vein injection of Cre-expressing adenovirus in *Mst1*$^{-/-}$*Mst2*$^{F/-}$ mice and results in development of HCC tumors (hepatocarcinogenesis) concomitantly to liver cirrhosis.

INTRODUCTION

Hepatocellular carcinoma (HCC) represents the second most common cause of cancer-related death worldwide[1]. HCC occurs almost exclusively in patients with liver cirrhosis[2], underlying the important roles of the pro-inflammatory, angiogenic and pro-fibrotic microenvironment in the cirrhotic liver for hepatocarcinogenesis.[3,4] The degree of liver fibrosis also affects the feasibility and efficacy of systemic therapies, due to impaired drug delivery, altered metabolism, liver dysfunction, and changes in hepatic perfusion.[5,6] In addition, fibrosis and cirrhosis modulate the interactions between cancer cells and extracellular matrix in HCC. Finally, the response of cancer and stromal cells – e.g., tumor-associated macrophages or cancer-associated fibroblasts – to pharmacologic agents is likely to be different in cirrhotic livers compared to that in healthy livers or subcutaneous space due to different local cues and cell origins[7]. Since most cases of HCC (~80%) develop in a cirrhotic liver, current staging algorithms for HCC[8] acknowledge the important prognostic role of the severity of liver cirrhosis and the impact of the underlying liver disease on the efficacy and safety of current treatments. Thus, the severity of underlying fibrosis influence treatment decisions in patients with HCC and this is reflected by current practice guidelines.[9]

Nevertheless, the mechanisms by which the pro-inflammatory and pro-fibrotic liver microenvironment regulate HCC growth and progression or affect HCC response to systemic therapy remain poorly understood.[4] In part, this is due to a paucity of *in vivo* models to study HCC occurring with underlying liver cirrhosis. Thus, clinically relevant animal models of HCC recapitulating the pathologic microenvironment of the cirrhotic liver are required to promote development of novel systemic therapies.[10] We have developed a syngeneic, orthotopic model of HCC in immunocompetent mice with underlying liver cirrhosis.[7,11] This model is potentially useful for studying various biological aspects of HCC, including cancer cell proliferation, apoptosis, epigenetics, metabolism, treatment resistance, angiogenesis, metastasis, tumor microenvironment, and efficacy of novel therapeutic approaches.

Comparison with other murine and human HCC xenograft models

Due to its size, breeding capacity, physiologic and molecular similarities to human biology, and available technology of gene targeting, the laboratory mouse (*Mus musculus*) often represents the model of choice in cancer biology. Mouse models of cancer include syngeneic grafts, xenografts, chemically-induced, and

transgenic/genetically-engineered models (GEMs).

Xenograft models. For experimental HCC studies, a widely used model is subcutaneous injection of human HCC cell lines in immunodeficient mice, usually athymic nude or severe immunodeficient (SCID) mice[12]. Such xenograft models may be useful for certain studies of cancer cell behavior during tumor progression. However, this approach has some key drawbacks: it does not reproduce critical tumor-stroma interactions in HCC, and does not allow studies of spontaneous development of distant or local (intrahepatic) metastases. To overcome these limitations, a strategy developed by several groups has been to use orthotopic (intrahepatic) patient-derived xenografts (PDX) from HCC patients in immunodeficient mice.[13] In addition to growing in the orthotopic location, the PDX tumor occasionally shows distant metastases.[13] However, the utility of PDX HCC models is greatly limited by the dysfunctional or absent immune responses.

Carcinogen-induced models. In other models, HCC is induced in immunocompetent mice using carcinogens – such as diethylnitrosamine (DEN) – to overcome the limitation of immunodeficiency and lack of heterogeneity. However, the time to tumor formation and tumor sizes can significantly vary depending on mouse strain and often multiple tumor lesions may develop. Thus, chemically induced models are difficult to standardize, which results in difficulties in designing and performing experiments, particularly for testing the therapeutic efficacy of new agents. Most importantly, none of these models recapitulates the pathological host environment of a cirrhotic liver that is present in most cases of human HCCs.

Transgenic models. Transgenic models/GEMs of HCC are widely considered the state-of-the-art. They include hepatocyte-specific models (using the albumin promoter) of *c-Myc*[14] and astrocyte elevated gene-1 (*AEG-1*)[15]. In GEMs with dual (albumin-driven) *AEG-1* and *c-Myc* overexpression, mice developed aggressive HCCs as well as lung metastases. Moreover, DEN treatment significantly accelerated hepatocarcinogenesis in albumin promoter-driven *AEG-1/c-Myc* overexpressing mice.[16]

CRISPR-Cas models. Another novel technique to induce HCC tumors in wild-type mice uses hydrodynamic injections of clustered regularly interspaced short palindromic repeats (CRISPR) and CRISPR-associated proteins (Cas). This CRISPR/Cas technology delivers single guide RNAs (sgRNAs)

to target tumor suppressor genes (such as *Pten* or *p53*) or the *CTNNB1* oncogene. This technique enables direct mutations of tumor suppressors or oncogenes to study functional genomics in HCC. Stauffer et al. showed that co-activation of Akt and β-Catenin in mice rapidly induces formation of lipogenic liver tumors[17]. These authors used hydrodynamic injections to deliver activated AKT and β-catenin for initiation of hepatocarcinogenesis. This model resulted in hepatic adenomas, and after *in vivo* passage, these adenoma cells formed alpha-fetoprotein (AFP)- and CD133-expressing HCC tumors. This murine HCC model developed from steatotic-neoplastic transformation of hepatocytes, which mimics the microenvironment in human diet-induced or metabolic-induced HCC in patients with fatty liver disease. This model of HCC in steatotic liver is a reproducible, rapid transgenic model that provides insights in intrinsic molecular mechanism involved in early tumorigenesis. However, it lacks pronounced fibrosis that is found in human NASH cirrhosis, where HCC tumors usually follow the sequential development of hepatic steatosis, inflammatory fatty liver disease, fibrosis and cirrhosis (NASH cirrhosis), and HCC in NASH cirrhosis. Thus, this model recapitulates human HCC in a non-cirrhotic fatty liver disease background.

Adenovirus models. Another hepatocyte-specific HCC model induces oncogenic SV40 large T antigen via adenovirus transfer, and this model has been used to investigate antigen-specific tolerance[18].

Metabolic models. Finally, Wolf et al.[19] showed that metabolic activation of intrahepatic CD8+ T-cells and NKT cells causes NASH and eventually HCC. HCC was established after 12 months of long-term exposure to a choline-deficient high fat diet.

While all these elegant models recapitulate spontaneous carcinogenesis, the mice show only mild fibrosis and do not display underlying liver cirrhosis. While concomitant administration of CCl4 or TAA might be feasible in these models to create a cirrhotic background, this remains to be demonstrated.

Experimental design

Here, we describe a protocol for developing preclinical mouse models of orthotopic HCC in mice with underlying liver cirrhosis. This model can be established using implantation of syngeneic murine HCC cell lines or by induction of hepatocarcinogenesis in GEMs, such as $Mst1^{-/-}Mst2^{F/-}$ mice.[7,20] This GEM is

a model of YAP1 oncogene-driven carcinogenesis, which is a relevant driver of HCC[21,22]. Both strains of mice used in this protocol – C3H mice and *Mst1*$^{-/-}$*Mst2*$^{F/-}$ mice – have intact immune systems, which allows studies of immune responses in HCC.[11,23] The setting of carcinogenesis within a cirrhotic liver tissue provides a clinically relevant model, which recapitulates key features of human HCC progression [7,11]. Thus, these models provide a unique platform to study HCC-stroma interactions and to perform treatment efficacy studies in an inducible and reproducible model of HCC.[7,117,11]

The rationale for developing and using this orthotopic murine HCC model in fibrotic liver is based on previous studies from our lab and others demonstrating an important role of tumor stroma in HCC and other tumors.[3,7,10,11] Previous studies have revealed that inflammatory cells, integrin signaling, growth factor secretion and interactions, and communication between activated hepatic stellate cells and tumor cells in a fibrotic tumor microenvironment all contribute to tumor progression in HCC.[25,26] As discussed in the following section, the protocol can be used to establish clinically relevant murine HCC models for various experimental studies. The protocol includes three main sections: induction of liver fibrosis/cirrhosis in the liver of C3H mice (Steps 1-10); HCC tumor cell implantation in the fibrotic/cirrhotic liver of C3H mice (Steps 11-42); and simultaneous fibrosis and tumor induction in the *Mst1*$^{-/-}$*Mst2*$^{F/-}$ GEM (Box 1). **Figure 1** summarizes these experimental procedures.

Strains of mice. C3H mice and *Mst1*$^{-/-}$*Mst2*$^{F/-}$ mice should be kept under pathogen-free conditions to maintain overall health of mice, control diseases, and prevent infection outbreak. Our group developed and described *Mst1*$^{-/-}$*Mst2*$^{F/-}$ mice; see Zhou et al.[20]. Alternatively, mice with specific deletion of both *mst1* and *mst2* in hepatocytes have been developed and are available from Jackson Labs (stock 017635)[27]. During fibrosis induction (CCl4 administration), after Cre-adenovirus injection, and after orthotopic tumor implantation, mice should be monitored daily for signs of illness by measuring bodyweight and checking general condition and vital signs. Small animal ultrasound can be used to noninvasively monitor HCC tumor uptake and growth in the liver longitudinally over time. All animals must receive humane care according to the criteria outlined in the NIH guide for the care and use of laboratory animals.[28] Tumors derived from murine HCC cell injections into livers are established as a syngeneic mouse model 7~10 days after implantation. The HCC tumor uptake rate can vary by tumor cell

line and mouse strain used. The murine HCA-1 cell line consistently establishes hepatic tumors in >95% of implanted C3H mice.

Considerations about sample calculation and control groups. It is important to plan your experiment in order to obtain a sufficient number of mice per group, especially if your expected difference in the respective readout (e.g., tumor size) is small.

A sample calculation for a typical treatment testing in the cirrhotic HCA-1 model is as follows:

- An experimental group size that usually allows to detect potential statistically differences in tumor size between different treatments is n=8 mice per group. For example, for 3 treatment groups: (1) control group treated with vehicle/placebo, (2) standard therapy (e.g., sorafenib), (3) experimental therapy.
- Consider potential dropout rate during fibrosis induction (usually <2% in male C3H mice, but better calculate it at a conservative rate of 5%)
- Consider HCA-1 tumor uptake rate of 90% in orthotopic graft
- In this case: (8 mice x 3 groups x 1.05 (5% dropout during fibrosis induction) x 1.10 (90% tumor uptake rate) = 28 mice

A sample calculation for a typical experiment for the cirrhotic *Mst1/Mst2*-mutant model is as follows:

- An experimental group size that usually allows to detect potential statistically differences in tumor size between different treatments is n=8 mice per group. For example, for 3 treatment groups: (1) control group treated with vehicle/placebo, (2) standard therapy (e.g., sorafenib), (3) experimental therapy.
- Consider potential dropout rate during fibrosis induction (up to 5% in *Mst1/Mst2*-mutant mice)
- *Mst1/Mst2*-mutant HCC tumor incidence rate after successful Adeno-Cre tail vein injection is 100%
- In this case: (8 mice x 3 groups x 1.05 (5% dropout during fibrosis induction) = 26 mice

An adequate control group during fibrosis induction is administration of a similar amount (volume) of olive oil (which we use as a vehicle for CCl4). The control group of mice for tumor studies should receive orthotopic liver injections of a similar volume (20µL) of matrigel to exclude a potential effect of the surgical procedure and to assess potential toxicity of experimental drugs in cirrhotic mice (without

tumors). A group of *Mst1*$^{-/-}$*Mst2*$^{F/-}$ mice should be injected with PBS instead of Adeno-Cre.

Considerations about costs and time effort. The models described in this protocol have a high cost for reagents, surgical expertise, and long induction times – CCl4 administration to induce fibrosis (12 weeks) and Adeno-Cre injections for HCC formation (8 weeks). Together with a typical treatment-testing period of 3-4 weeks, the timeline for an experiment could extend to up to 5-6 months. Cost calculations for this models must thus also consider animal housing during this timeframe. Additionally, the time effort and expertise needed for animal handling and surgery must be considered..

Murine HCC cell lines. Various human HCC cancer cell lines have been established for development of xenograft models in immunodeficient mice. However, few cell lines are available for syngeneic mice. The illustrative data we show here uses the C3H mouse-derived HCC cell line HCA-1 to establish the orthotopic HCC model.[7,24] HCA-1 cells were cultured using DMEM culture media supplemented with 10% Fetal Bovine Serum (FBS) and were regularly checked for mycoplasma contamination to avoid artificial results due to animal infection. In general, a single intrahepatic implantation of 1×10^5 HCA-1 cells is sufficient to result in tumor formation in C3H mice.

Induction of liver fibrosis or cirrhosis in mice (steps 1-10). Liver cirrhosis results from repeated liver damage and chronic inflammation, which leads to the massive deposition of extracellular matrix (mainly collagen) and formation of scar tissue (septa) in the liver. To model this clinical feature of HCC, we use repetitive CCl4 exposure to induce liver fibrosis in mice. CCl4 is orally (p.o.) administered to 5-6 weeks old male C3H or *Mst1*$^{-/-}$*Mst2*$^{F/-}$ mice (CCl4, 20%v/v in olive oil, 150µL gavage, 3 times per week) prior to orthotopic tumor implantation or after Cre-adenovirus injection. We found formation of excess fibrous connective tissue and collagen deposition after 8 weeks of CCl4 administration (recapitulating human F2 fibrosis stage according to METAVIR). Established cirrhosis (thick bridging septa, recapitulation human METAVIR F4 stage cirrhosis) was observed after 12 weeks of repetitive CCl4 administration (**Figure 2**). These tumor stromal features are not seen in HCCs developing in *Mst1*$^{-/-}$*Mst2*$^{F/-}$ mice without concomitant CCl4 administration. Thus, CCl4 administration after Cre-adenovirus injection is required to induce cirrhosis concomitantly with carcinogenesis in *Mst1*$^{-/-}$*Mst2*$^{F/-}$ mice.

Orthotopic tumor implantation in cirrhotic livers (steps 32-35). HCA-1 cells are directly implanted in C3H mice with liver cirrhosis – induced by 12 weeks of CCl4 treatment (**Figures 1 & 3**). Small volume suspensions containing HCA-1 cells and Matrigel are injected in the subcapsular region of the liver parenchyma in the median lobe. Typically, we suspend 10,000,000 cells in 200µl of serum-free media. Directly before injection we mix the cell suspension 1:1 (vol/vol) with Matrigel in a small tube to increase the viscosity of the injected cell suspension. Small 0.5mL syringes with 28.5G needles are used to inject the cell/Matrigel suspension. To avoid leakage of tumor cells from the injection site – that might lead to local spread and "seeding" metastasis in the peritoneal cavity – we limit the injected volume to 20-30µl. A steady and slow injection should be performed to prevent leakage of the injected cell suspension and minimize the damage of the surrounding liver tissue. After removal of the needle, the liver surface at the site of needle tract should be covered with gelfoam for 5 minutes to minimize bleeding and potential backflow/leakage.

Alternative approaches to intrahepatic cancer cell implantation Lee et al.[29] have described a percutaneous ultrasound (US)-guided approach to inoculate Vx-2 carcinoma cells in the liver of rabbits. This technique resulted in a high success rate (100%) and was less invasive than an open laparotomy method. However, tumor seeding occurred more often (20.8%) in the US-guided approach than in their laparotomy controls (8.3%). In addition, there was thoracoabdominal cancer cell seeding in (20.8% of cases) and also in the peritoneal cavity (8.3% of case)[29]. A percutaneous technique has been previously described for inoculation of human hepatocytes in immunodeficient mice[30]. However, while an US-guided (less invasive) approach might also be possible for cancer cell inoculation in mice, it carries the important risk of peritoneal seeding at the time of inoculation, which could compromise the experiment.

Induction of cirrhosis induction in the $Mst1^{-/-}Mst2^{F/-}$ GEM (Box 1). To induce the homozygous Mst1/2 knockout, we inject Cre-adenovirus into tail veins of 4-5 week old male or female $Mst1^{-/-}Mst2^{F/-}$ mice to trigger liver-selective deletion of *Mst2*.[20] Because female $Mst1^{-/-}Mst2^{F/-}$ mice are smaller and are more susceptible to fibrosis induction, we recommend only one gender is used within one experiment or a balanced male/female ratio between the experimental groups. Next, cirrhosis is induced in male $Mst1^{-/-}$ $Mst2^{F/-}$ mice using iterative CCl4 gavaging (3 times a week) (**Figure 1**). For comparison, $Mst1^{-/-}Mst2^{F/-}$

mice were only injected with Adeno-Cre without fibrosis induction or only administered with CCl4 without Adeno-Cre virus injection.

Alternative approaches to induce liver fibrosis or cirrhosis in mice. Common alternative methods to induce liver fibrosis and cirrhosis in mice include 1) common bile duct ligation (CBDL), a surgical model of secondary biliary cirrhosis in which the common bile duct is double ligated and resected thus causing secondary biliary cirrhosis after 5-8 weeks; and 2) chronic thioacetamide (TAA) administration, usually injected intraperitoneally twice or thrice a week. Of note, the CBDL cirrhosis model is associated with high morbidity, and thus it might be difficult to use in conjunction with additional interventions such as orthotopic tumor cell implantation surgery and long-term drug treatments in mice.

Monitoring orthotopic liver tumor growth in mice. The orthotopic nature of this model does not allow direct monitoring of tumor growth or size. Besides ultrasound, described in this protocol, there are other imaging options such as microCT or MRI that could be used for this purpose if expertise and resources are available. An inexpensive but invasive alternative is to surgically open the abdomen of mice at predefined time-points to directly measure tumor size. However, repetitive surgeries could confound the experimental outcomes. Another method for monitoring tumor growth for grafted tumors is to use prior transfection of cancer cells with luciferase (Gaussia luciferase or Firefly luciferase). While feasible, this method could also confound the results by introducing changes in cancer cells' phenotype[31] and is restricted to implanted tumor models. In summary, for longitudinal evaluation of tumor growth in this model, we recommend an ultrasound device – equipped with specific probes for small animal imaging.

Below we provide a detailed step-by-step protocol that can serve as a research tool to examine the interaction between HCC cancer cells and the fibrotic microenvironment in a cirrhotic liver. This protocol produces clinical relevant animal models for HCC. Furthermore, these models can also be used for preclinical drug testing in HCC.

MATERIALS

Animals and Reagents

- Mice. C3H mice are used for orthotopic implantation of HCA-1 cells after fibrosis induction (main procedure). *Mst1*$^{-/-}$*Mst2*$^{F/-}$ GEMM is used for concomitant fibrosis induction (Box 1). **!CAUTION** All animal studies must be reviewed and approved by the institutional animal care and use committees and conform to all relevant ethics regulations. **CRITICAL** It is important to note that C3H mice are more susceptible to fibrosis induction by CCl4 or other hepatotoxic agents, such as thioacetamide (TAA). Mice of other strains may require optimization of the duration or dose of exposure to CCl4 to induce a similar amount of hepatic fibrosis without increasing CCl4-associated toxicity. **CRITICAL** For experiments, we recommend using male mice 4-5 week of age (at the time when Cre-adenovirus is injected and CCl4 treatment is started). Use of younger (or lighter) mice will result in increased toxicity. Female mice could also be used, but the susceptibility to CCl4 and hepatocarcinogenesis after Cre-adenovirus injection is higher in male mice. Within one experiment, we recommend to use only one gender of mice.
- Isoflurane (Gas anesthesia system)
- Anesthesia: Ketamine (MGH pharmacy) and Xylazine (Webster, cat. no. 200204.00) – add to sterile saline to obtain a concentration of 100mg/mL for ketamine and 10mg/mL for xylazine
- Sterile DMEM High Glucose Medium (Life technologies, Gibco@, cat. no. 11965-092)
- PBS (1x, Cellgro, cat. no. 20-031-CV)
- Antibodies - αSMA (DAKO, cat. no. M0851), and CD31 (DAKO, cat. no. M0823), collagen-I (Abcam, cat. no. 34710)
- Fetal Bovine Serum (Life technologies, Gibco@, cat. no. 10082147)
- Carbon tetrachloride (CCl4) (Sigma, cat. no. 319961) **!CAUTION** Do not inhale or expose your skin and eyes to CCl4. Handle CCl4 in a chemical fume hood while wearing appropriate protective gloves and clothing.
- Olive oil (sigma, cat. no. O1514)
- Ethanol (70% (vol/vol); Pharmco, cat. no. 111000190)
- HCA-1 cell line (established from a C3H mouse tumor in the Steele Lab, MGH) **!CAUTION** Check the authenticity of any cell line prior to use (e.g., genotyping of microsatellite markers to determine mouse origin and exclude mammalian interspecies contamination, such as provided by IDEXX

BioResearch, MO, USA). Regularly test cell lines for mycoplasma infection.

- Adeno-Cre virus (Gene Transfer Vector Core at the University of Iowa, cat. no. VVC-U of Iowa-5) **!CAUTION** Follow the NIH guidelines for handling material containing BSL-2 organisms. Always wear gloves and goggles, use filtered tips, and work under a biosafety (level-2, biohazard signs) hood.
- Paraformaldehyde (P6148; Sigma, cat. no. 6148) **!CAUTION** Do not inhale or expose it to the skin and eyes.
- 10x Trypsin–EDTA, 0.5% (wt/vol) trypsin–EDTA (Gibco, cat. no. 15400)
- BD Matrigel Growth Factor Reduced, Basement Membrane Matrix (BD Biosciences, cat. no. 356230)

EQUIPMENT

- Serological pipettes and pipette aid
- 1-mL insulin syringe (BD Micro-Fine, cat. no. 329412)
- 0.5-mL insulin syringe with 26G x 5/7 inch needle (BD, no. 309597)
- 1mL syringe (BD, cat. no. 309659)
- 50mL centrifuge tubes (Corning, cat. no.430828)
- Heat lamp (for tail vein injections)
- Sterile Petri dishes (Falcon)
- Countertop vortex (Fisher)
- Centrifuge capable of accepting 15- and 50-ml centrifuge tubes.
- Bright-field microscope
- Caliper (Roboz, cat. no. RS-6466)
- Cryomolds (Cardinal Health, cat. no. M7144-13)
- Cryostat (Microm, cat. no. HM-560)
- Confocal microscope
- Incubator

Gavage supplies

- Scale (capable of measuring mouse weight)

- 1mL syringe (BD, no. 309659)
- Gavage needle with ball tip. **CRITICAL** Gauge size and needle length should be determined based on the size and weight of animals. For gavaging of male C3H or *Mst1*$^{-/-}$*Mst2*$^{F/-}$ mice starting at the age of 4-5 weeks and total exposure period of 12 weeks thereafter, we recommend using a 20G (1-1.5 inches) gavage needle.

Surgical supplies (orthotopic HCC cell implantation)

- 1-mL syringe with 26G needle for anesthesia (Fisher Scientific, cat. no. 14-823-2E)
- Eye protective gel
- Electric clipper (Webster, cat. no. 78997-010)
- Sterile gauze
- Heating pad (Shore Line, cat. no. 712.0000.04)
- Curved forceps (Roboz Surgical, cat. no. RS-5135)
- Straight forceps with teeth (Roboz Surgical, cat. no. RS-5132)
- Straight scissors
- Small needle holder
- Autoclip applier (Roboz, cat. no. RS-9260) plus 9mm autoclips (Roboz, cat. no. RS-9262)
- Sterile cotton swabs
- Ethibond suture 5-0 (Ethicon)
- Surgical microscope
- Sterile gelfoam
- Insulin Syringe (BD 1/2 cc U-100, 28 ½ gauge).

REAGENT SETUP

Antibiotic-free media **-** Sterile DMEM High Glucose Medium supplemented with 10% FBS. Store at 4 °C for continued use of up to 4 weeks. ⏏CRITICAL Cells should be cultured in antibiotic-free medium for at least 2 weeks before use in this procedure.

Paraformaldehyde (PFA) 4% (wt/vol) **-** Dissolve 4g PFA into 100ml 1×PBS to prepare a concentration

of 4% (wt/vol). **!CAUTION** Preparation of 4% (wt/vol) PFA should be performed in a chemical fume hood under appropriate protection. Do not inhale or expose it to the skin and eyes. Store the 4% PFA solution at 4°C (cold room, fridge) in closed glass containers.

CCl4 20% (vol/vol) gavage solution **-** To prepare 10mL of final concentration (20% CCl4), add 2mL of CCl4 to 8mL of olive oil. Gently mix the solution. Administer to mice by gavage of 150µL 20% CCl4 solution three times per weeks. Store 20% CCl4 solution at 4°C for 2 weeks or up to 6 months at -20°C.

HCC cells – HCC cells should be counted and a suspension of 10^6 cells per 10 µL Matrigel should be prepared.

Matrigel solution – is suited for *in vivo* implantation (as typically used for subcutaneous injections). We recommend using a high Matrigel concentration to allow the matrix plug maintain its integrity (Matrigel matrix high concentration, Phenol Red-free, BD 354262). The stock solution should aliquoted in Eppendorf centrifuge tubes (e.g. in 500µL aliquots) and stored at –20°C. On the day of the procedure, the aliquots of Matrigel solution should be kept on wet ice (in a liquid state).

Ethanol, 70% (vol/vol) Mix 1.7 liters of water and 4.73 liters of 95% ethanol. Store at room temperature (20°C) in a closed container.

PROCEDURE

Induction of liver fibrosis ● TIMING 12 weeks

1. Prepare CCl4 20% gavage solution (Reagent Setup) before starting the gavaging procedure. **CRITICAL** For longer periods of administration (i.e., 12 weeks), we recommend oral gavage of CCl4/olive-oil because it is better tolerated by mice, and leads to reproducible effects on liver histology. In addition, as corn oil and/or olive oil are associated with peritoneal irritation and potential adhesions – which might complicate surgical implantation of HCC cells.
2. Draw 150µL of CCl4 20% gavage solution (or olive oil as vehicle for control groups) into a 1-mL syringe with the gavage needle. **!CAUTION** Avoid formation of air bubbles while drawing the gavage solution to ensure administration of the right volume. **CRITICAL** The gavage procedure should be performed by well-trained personnel. **CRITICAL** Gavaging needles that are adequate for the size and weight of the mice must be used.

3. Tightly hold the mouse in a vertical position. Place the body of the mouse straight and extend the head. **CRITICAL** Ensure that the position of the mouse is secured before inserting the gavage needle.
4. Insert the gavage needle gently into the mouth of the mouse and search for a position that can allow the needle to bypass the tongue.
5. Smoothly push the gavage needle via the upper palate into the esophagus and introduce the gavage needle further until the tip of the needle reaches the right position in the distal esophagus. **CRITICAL** Never push the needle too hard. Introduce the needle gently to avoid perforation of the esophagus or intubation of the trachea. **?TROUBLESHOOTING**
6. Inject the CCl4 20% gavaging solution (or olive oil as vehicle) while ensuring the needle remains at the right position in the distal esophagus. **CRITICAL** Administer the CCl4 gavaging solution slowly and only with the gavage needle in the right position to avoid aspiration of fluid into the lungs. **?TROUBLESHOOTING**
7. After injection smoothly remove the gavage needle.
8. Put the mice back to the original cage with the regular diets and monitor the recovery of the mice. If any sign of labored breathing or respiratory distress is observed, use CO_2 inhalation to euthanize the mice.
9. Continue the administration of the CCl4 20% gavage solution (or olive oil as vehicle for control groups) 3 times per week for 12 weeks. Closely monitor the health condition of the mice. **?TROUBLESHOOTING**
10. Twelve weeks after the first gavage, proceed to the next step to orthotopically implant HCC cells in the cirrhotic liver. **CRITICAL** When you establish the cirrhotic HCC model for the first time, include an additional subgroup of mice in order to confirm the cirrhotic state of the liver. Sacrifice these mice using CO_2 inhalation, collect the livers, and fix them in 4% PFA solution. After embedding in paraffin, slice the paraffin block, mount the slices on glass slides, and perform using hematoxylin/eosin staining to assess necroinflammation and fibrosis, Masson's Trichrome staining to assess extracellular matrix fraction, or Sirius Red staining to assess fibrillar collagen content.

Generation of orthotopic syngeneic HCC tumors using murine HCC cell lines in mice with CCl4-induced liver fibrosis ● TIMING 3 hours

11. Twelve weeks after the first gavage, stop the treatment of CCl4 20% gavage solution (or olive oil).
12. Two to three days after the last CCl4 treatment, weigh and prepare the mice for the following procedure.
13. Pre-warm PBS and DMEM culture media at 37°C water bath before use. Pre-warm trypsin at room temperature (20°C).
14. Remove the media from the dish containing HCA-1 cells at 80% confluence. **CRITICAL** Avoid using antibiotics to culture cells at least 2 weeks before this step. **CRITICAL** Avoid cell passaging for least 2 days before this step.
15. Gently rinse the dish with pre-warmed PBS (10mL per $10cm^2$ dish) and aspirate the PBS afterwards.
16. Add 2mL of 1x trypsin–EDTA (0.05% (vol/vol) to the dish. Gently shake the dish to ensure full covering of the surface area by the trypsin solution. Put the plates in the 37 °C incubator.
17. After 3~5 min, observe the attached cells on the plate by using a bright-field microscope. Gently tap the dish to facilitate cell detachment. **CRITICAL** Do not leave the cells in the trypsin solution for more than 5 minutes **?TROUBLESHOOTING**
18. When the majority of HCA-1 cells are detached, add 8mL of DMEM media to neutralize trypsin in the dish. Gently resuspend and mix the HCC cell suspension. Collect the cells in a 15mL centrifuge tube.
19. Centrifuge the HCC cell suspension in the 15mL centrifuge tube for 5min at 200-250*g* at room temperature (20ºC). Wash the cells with 10mL PBS. Centrifuge the cells for 5min at 200-250*g* and resuspend the cells in serum-free DMEM media.
20. Use a cell counter to determine the cell number. Adjust the cell concentration to 10^6 cells/10μL in Matrigel (corresponding to the final tumor cell inoculum/injected volume) with serum-free media. Keep the cell suspension on wet ice.
21. Place the Matrigel solution on wet ice.
22. Anesthetize the mice by using ketamine (100mg/mL) / xylazine (10mg/mL) mixture. Use 10-

12µL/g body weight (corresponding to a dose of 100-120mg/kg of ketamine, and 10-12mg/kg of xylazine).

23. After anesthesia place and keep the mice on a heating pad to avoid hypothermia.
24. Shave the abdominal region of the mouse and place the mouse in a supine position.
25. Use sterile gauzes soaked in 70% ethanol to disinfect and clean the shaved abdominal region from any leftover hair.
26. Gently grab the skin with a forceps and perform a midline abdominal incision of the skin (starting about 1cm under the xyphoid process)
27. Identify the linea alba in the midline of the abdominal muscle layer and perform a similar incision of 1cm along the linea alba.
28. Expose the median lobe of the liver by using a sterile cotton swab soaked in sterile saline solution. **?TROUBLESHOOTING**
29. Put a small piece of sterile gelfoam (0.5 x 0.5 cm) on the exposed liver to maintain the liver surface moisture.
30. Immediately before orthotopic implantation (when the liver is already exposed), the HCC cell suspension should be mixed with Matrigel (1:1). Withdraw an equal volume of the cell suspension and Matrigel (i.e. 50µL each) into a sterile 0.5-mL insulin syringe with a 28G needle. Gently mix the cell suspension with Matrigel and keep on wet ice. This will ensure a final cell number of 10^6 cells per 20µL injected volume of HCC cell/Matrigel suspension. **CRITICAL** Maintain the Matrigel solution and the final HCC cell/Matrigel mixture in the insulin syringe on wet ice to prevent gel formation. ● **TIMING** The cell solution in Matrigel is good for two hours on wet ice. Afterwards, a fresh HCC cell/Matrigel suspension should be prepared.
31. Transfer the mouse on the heating pad under a surgical microscope.
32. Place the mouse under the microscope in a position with the mouse head pointing towards the person performing the injection.
33. Gently insert the 28G needle of the 0.5mL insulin syringe with the HCC cell/Matrigel solution under the liver capsule.
34. Slowly inject 20µL of the HCC cell/Matrigel solution (containing $5x10^5$ HCA-1 cells) in the

subcapsular region of the median lobe. Successful inoculation will form a small bubble on the liver surface. **CRITICAL** Inject the HCC cell/Matrigel solution very slowly to avoid leakage and prevent disruption of the liver surface. **?TROUBLESHOOTING**

35. After injecting the HCC cell/Matrigel solution, slowly retract the needle.
36. Prevent leakage by placing a sterile gelfoam for 5min on the needle track. **?TROUBLESHOOTING**
37. Remove the sterile gelfoam and assess the liver surface at the needle track for leakage and bleeding.
38. Gently wash the injection site with sterile saline solution using a 1mL syringe with a 25G needle.
39. Close the muscle layer of the abdominal wall by a continuous suture using 5-0 Ethibond suture 5-0
40. Close the skin layer of the abdominal wall by placing 2-3 9-mm autoclips using the Autoclip applier.
41. Leave the mouse on the heating pad and allow the mouse to recover from anesthesia.
42. Apply postoperative analgesia according to the animal protocol
43. Closely monitor the mice for tumor progression and health condition. **TIMING 4-8 weeks**
44. At appropriate time points (e.g. 7-10 days after implantation, and then every 7 days afterwards), an ultrasound device equipped with high-frequency ultrasound probes can be used to longitudinally assess tumor growth non-invasively under isoflurane anesthesia.

BOX 1: Liver cirrhosis induction in *Mst1*$^{-/-}$*Mst2*$^{F/-}$ GEMM HCC mice. ● TIMING 12 weeks

1. Weigh *Mst1*$^{-/-}$*Mst2*$^{F/-}$ mice to record bodyweight at baseline.
2. Intravenously inject 200µL of phosphate-buffered Cre-adenovirus (1x10^{8} pfu) into the tail vein of *Mst1*$^{-/-}$*Mst2*$^{F/-}$ mice of 4-5 week of age . **?TROUBLESHOOTING**
3. Start fibrosis induction (or olive oil/vehicle administration) 1 week after Cre-adenovirus injection, as described in steps 1-8.
4. Continue fibrosis induction by administration of CCl4 20% gavage solution (or olive oil/vehicle) 3 times per week for 12 weeks, as described in steps 9-10.
5. Closely monitor the mice for tumor progression and general condition.

END BOX

? TROUBLESHOOTING

See table 1 for troubleshooting guidance.

Table 1: Troubleshooting.

Step	Problem	Possible reason	Possible solution
5	Difficulty passing the gavage needle into the esophagus	The mouse is not positioned at the right angle or the ball tip of the gavage needle is too large.	Ensure that the main axis of the mouse is straight and the neck is extended. Use the round tip of the gavage needle to move the tongue forward. Change the type of the gavage needle to a smaller ball tip.
6	Gavage solution leaks back into the oral cavity	Incorrect position of the gavage needle	The injection should only be performed while the gavage needle remains in the correct position and the tip is placed in the distal esophagus.
9	Mice become weak or show signs of compromised health status	The administered CCl4 dose causes unacceptable toxicity to mice.	Reduce the dose of CCl4.
17	Difficulty in detaching HCA-1 from cell culture dish	Over-confluent cell culture.	Avoid over-confluence of HCA-1 cells in culture dishes. Increase the amount of trypsin/EDTA after washing with PBS.
28	Difficulty in forming bubble at injection site	Needle is inserted too deep into the liver tissue.	Insert the needle at an angle more parallel to liver surface and/or decrease insertion depth of the needle.
35	Tumor cell/Matrigel solution leaks from the injection site	Injected volume of cell solution is too large. Cell solution is not condensed enough.	Reduce the injection volume to 15μL. Increase the amount of Matrigel in the cell/Matrigel mixture or use Matrigel of a higher concentration.
Box 1 step 2	Difficulty injecting the Cre-adenovirus into tail vein	Perforation of the tail vein The diameter of the vein is small The mice are too small	Keep the angle of the injection needle to the skin of the tail very flat and go in very slowly. Apply heat (infrared lamp, heating pad) and apply 70% ethanol by a soaked gauze to dilate the tail veins Wait for another week to re-evaluate mouse weight and tail vein diameter

ANTICIPATED RESULTS

Orthotopic (intrahepatic) tumor implantations in the liver have been successfully performed in mice, and the model of CCl4-induced liver fibrosis has been used for decades to study fibrogenesis in various

murine and rodent models of liver cirrhosis. Our protocol provides an orthotopic model of HCC in which tumor growth is modulated by the cirrhotic liver (see **Figure 2**). The amount of fibrosis in the non-malignant (non-malignant liver tissue) can be quantified by Masson's Trichrome or Sirius red staining of fixed liver sections. An experienced liver pathologist should assess the staining and apply a semiquantitative score to determine the presence/absence of fibrosis and cirrhosis. In addition, staining for the hepatic stellate cell (HSCs) activation markers αSMA, desmin, or GFAP and collagen I will provide quantitative measures of fibrosis [7,19]. Once the administration of CCl4 is stopped, a small degree of spontaneous fibrosis regression may occur. However, complete reversal of cirrhosis is usually not observed after a full course of 12 weeks of CCl4 administration in mice. Regression of fibrosis would take up to 16 weeks. Thus, the liver microenvironment retains the pathological features of inflammation, fibrogenesis, and angiogenesis within the time frame of 5 to 8 weeks in which HCCs are induced and treatment is administered. The HCA-1 model also results in abundant angiogenesis and reproducible spontaneous lung metastases (**Figure 4**).[24] Thus, this model can be used to study HCC angiogenesis and metastasis. Evaluation of lung metastases should be based on both morphology and immunohistochemistry to confirm the expression of typical HCC markers (i.e., for alpha fetoprotein (AFP). Since there are multiple primary tumors in Mst-mutant mice and multiple nodules in lung metastasis from HCA-1 primary tumor grafts, both the number of nodules and their size should be used for quantification of tumor burden.

In addition, these models show adaptive resistance to sorafenib – a standard therapy for advanced HCC – thus, allowing studies of treatment resistance mechanisms. For example, we found upregulated fibrogenesis (collagen I depositions and ECM accumulation), higher levels of proinflammatory cytokines (such as CXCL12/SDF1α), and hypoxia (as indicated by CA9 expression) in cirrhotic (CCl4-induced) livers compared to healthy control livers[7]. Furthermore, we observed that HCA-1 tumor growth was facilitated when the cells were implanted into a cirrhotic liver as compared to a healthy liver (**Figure 5**).[24] Moreover, the tumor growth delay after sorafenib was not as pronounced in syngeneic C3H mice with underlying liver cirrhosis.[3] Based on our experience, essentially all $Mst1^{-/-}Mst2^{F/-}$ mice developed HCC after injection with Cre-adenovirus, with or without combination of CCl4 treatment. However, we observed that induction and growth of HCCs in $Mst1^{-/-}Mst2^{F/-}$ mice was accelerated by CCl4 treatment

as compared to *Mst1*$^{-/-}$*Mst2*$^{F/-}$ mice without CCl4 treatment.

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Competing interests statement

The authors declare that they have no competing financial interests.

Author Contributions

Concept and design of the study: TR, YC, AXZ, RKJ, DGD.

Acquisition of data: TR, YC, RRR, TH, PH, SR, CF, RS, PH, GYL.

Analysis and interpretation of data: TR, YC, RRR, TH, RS, PH, GYL, AXZ, NB, RKJ, DGD.

Drafting of the article and revising for important intellectual content: TR, YC, RRR, TH, CF, RS, SR, PH, GYL, AXZ, NB, RKJ, DGD.

FIGURE LEGENDS

Figure 1: Experimental Design. (A, B) Timeline of HCC development and cirrhosis induction by carbon tetrachloride (CCl4) administration: At least 12 weeks are required to set up the model. Tumors can be concomitantly induced either by orthotopic implantation – for example of HCA-1 cells in C3H mice after cirrhosis induction (see **A**, main procedure) – or by using a genetic model, e.g., using *Mst1*$^{-/-}$ *Mst2*$^{F/-}$ mice injected with Cre-adenovirus to induce hepatocarcinogenesis (**B**, Box 1). Institutional regulatory board permission was obtained for all procedures performed within this protocol.

Figure 2: Microscopic appearance of hepatocellular carcinoma in mice with cirrhotic liver. (**A)** Immunofluorescence staining of collagen I (anti-collagen I antibody, ab34710, Abcam, Cambridge, MA) in mice with liver cirrhosis (Laennec 4 score) after 12 weeks of CCl4 treatment. In blue, DAPI cell nuclear counterstaining (VECTASHIELD HardSet Antifade Mounting Medium with DAP, H-1500, Vector Laboratories, Burlingame, CA). Scale bar: 1mm. (**B, C)** Masson's Trichrome staining of liver tissue from C3H (**B**) or *Mst*-mutant (**C**) mice with cirrhosis. Scale bar: 50µm (**D, E**) Representative images of CD8+ T lymphocyte infiltration (green, anti-CD8 antibody, Ab22378, Abcam) in HCA-1 (D) and *Mst*-mutant tumors (E) with DAPI (VECTASHIELD HardSet Antifade Mounting Medium with DAP, H-1500, Vector Laboratories) for nuclear counterstaining, in blue. Scale bars: 50µm. Institutional regulatory board permission was obtained for all procedures performed within this protocol.

Figure 3: Surgical procedure for intrahepatic HCC graft implantation. (A) Median skin incision. (**B**) Median muscle layer incision, exposure of the xyphoid process. (**C**) Exposure of the median liver lobe, resection of the xyphoid process. (**D**) Exteriorization of the median liver lobe using a moisture steril cotton swab. (**E**) Covering of the median liver lobe (injection site with gelfoam). (**F**) Insertion of the needle and injection of 20µL of HCC cell/Matrigel solution in the subcapsular region. (**G**) Inspection of the injection site and re-location of the median liver lobe in the peritoneal cavity by removing the sterile cotton swab. (**H**) Closure of the muscle layer by a continuous suture. Institutional regulatory board permission was obtained for all procedures performed within this protocol.

Figure 4: Macroscopic appearance of primary HCC and lung metastases. (**A)** HCA-1 grafted in cirrhotic liver (after 12 weeks of CCl4 treatment). Scale bar: 10mm. (**B)** HCCs forming after Cre-adenovirus injection in *Mst1*$^{-/-}$*Mst2*$^{F/-}$ mice with liver cirrhosis (after 12 weeks of CCl4 treatment) Scale bar: 10mm. (**C)** Primary HCC and lung metastases in healthy (top), fibrotic (middle), and cirrhotic (bottom) livers in the orthotopic HCA-1 graft model. Scale bar: 10mm.

Figure 5: Differential growth kinetics of orthotopic versus ectopic (subcutaneous) HCC grafts. (**A**) This growth curve shows the differential progression of HCA-1 tumors according to their location in the mouse (orthotopic versus subcutaneous) and according to the microenvironment state of the liver (healthy versus cirrhotic). Each datapoint represents the mean tumor size in the respective tumor group (n=8 mice per group), the error bars represent the standard error of mean (SEM). The (*) asterisks mark the timepoints when there was a significant difference between tumor size in healthy versus cirrhotic liver. ($p<0.05$). (**B**) This panel shows the growth (mean tumor size) of HCA tumors implanted orthotopically in mice with cirrhotic livers that were treated with vehicle (brown, n=8) or sorafenib (pink, n=8). The error bars represent the standard error of the mean (SEM). *, timepoints when there was a significant difference between tumor size in vehicle– versus sorafenib-treated HCA-1 tumors ($p<0.05$).

Supplementary Information

Supplementary Figure S1: Ultrasound evaluation of orthotopic hepatocellular carcinoma by using abdominal ultrasound. (**A**) B-mode image of a HCA-1 Tumor 10 days after orthotopic implantation in a cirrhotic C3H mouse. Scale bar: 2mm. (**B**) B-mode image of a HCC tumor developed 10 weeks after Adeno-Cre injection and CCl4 administration in a *Mst1*$^{-/-}$*Mst2*$^{F/-}$ mouse. Scale bar: 2mm.

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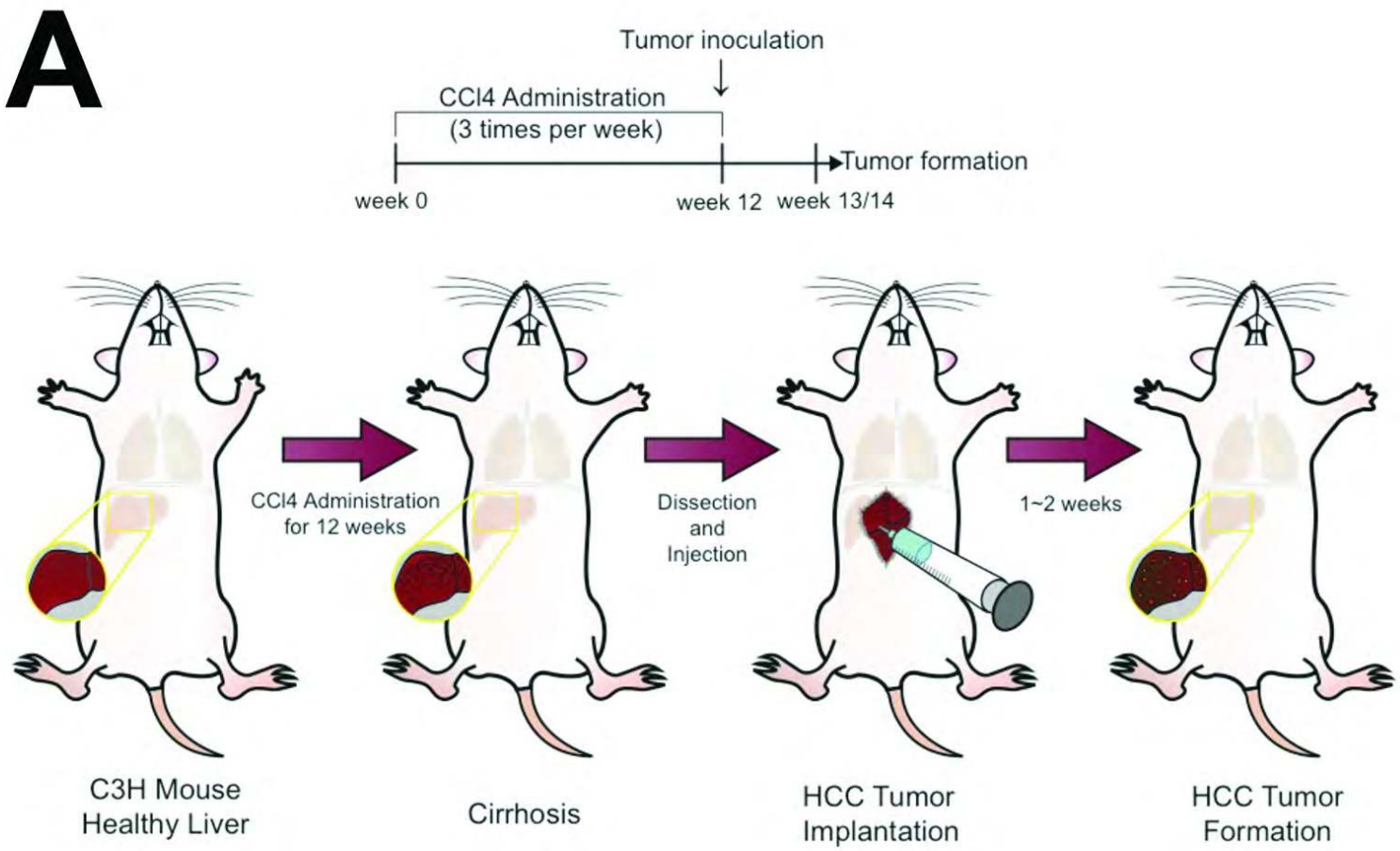
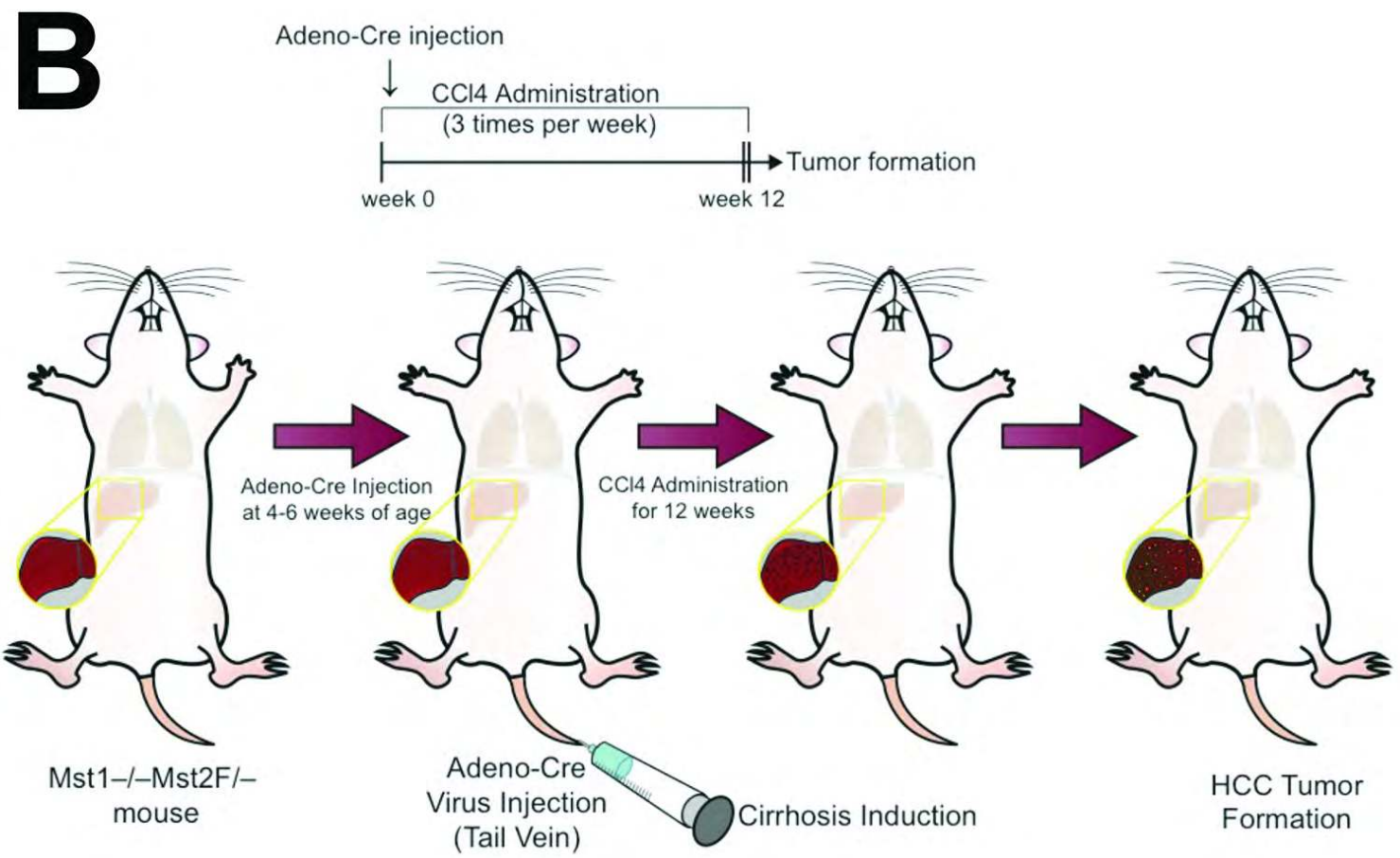
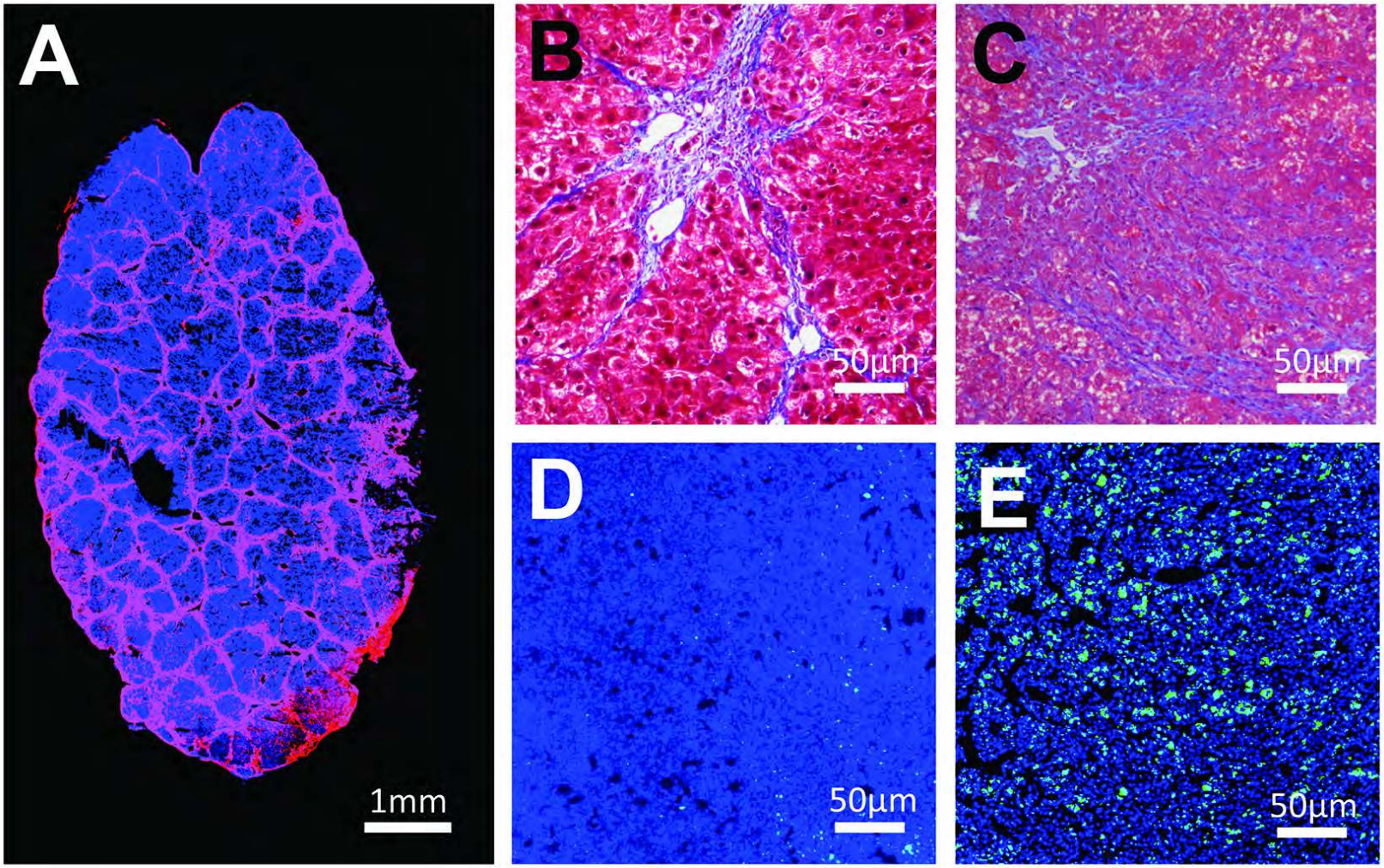

A
Tumor inoculation
CCl4 Administration
(3 times per week)
Tumor formation
week 0
week 12
week 13/14
CCl4 Administration
for 12 weeks
Dissection
and
Injection
1~2 weeks
C3H Mouse
Healthy Liver
Cirrhosis
HCC Tumor
Implantation
HCC Tumor
Formation


B
Adeno-Cre injection
CCl4 Administration
(3 times per week)
Tumor formation
week 0
week 12
Adeno-Cre Injection
at 4-6 weeks of age
CCl4 Administration
for 12 weeks
Mst1–/–Mst2F/–
mouse
Adeno-Cre
Virus Injection
(Tail Vein)
Cirrhosis Induction
HCC Tumor
Formation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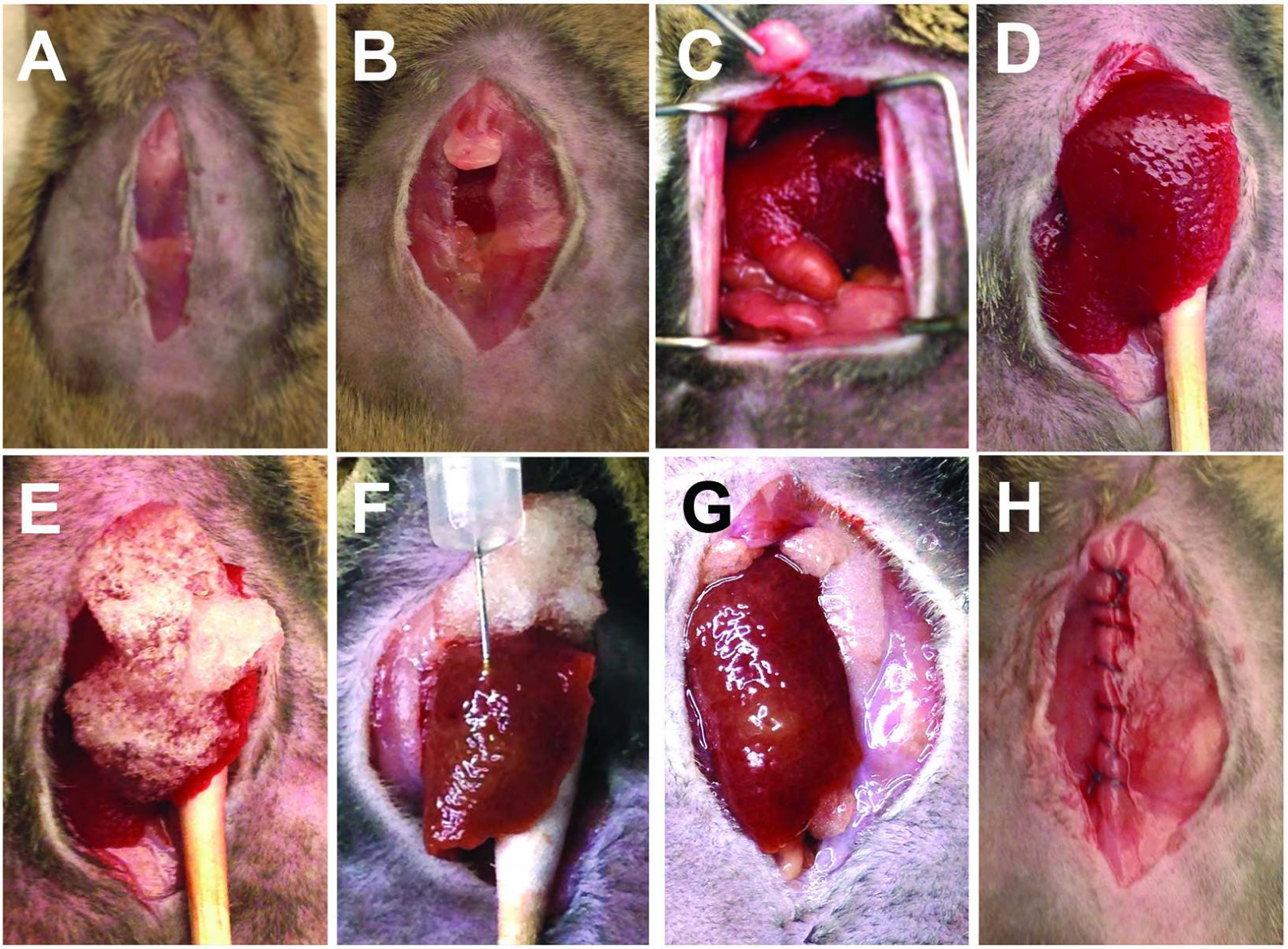
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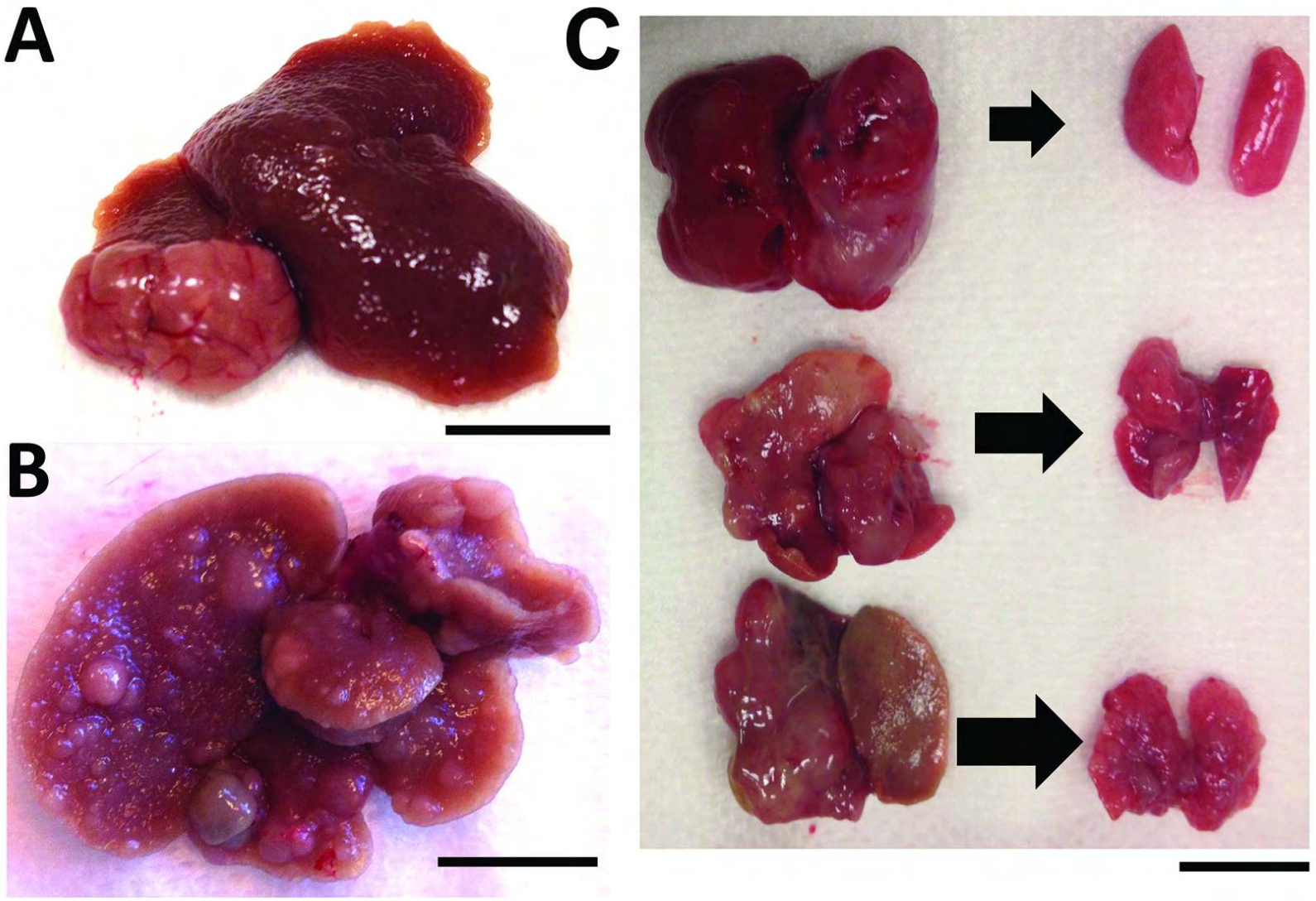
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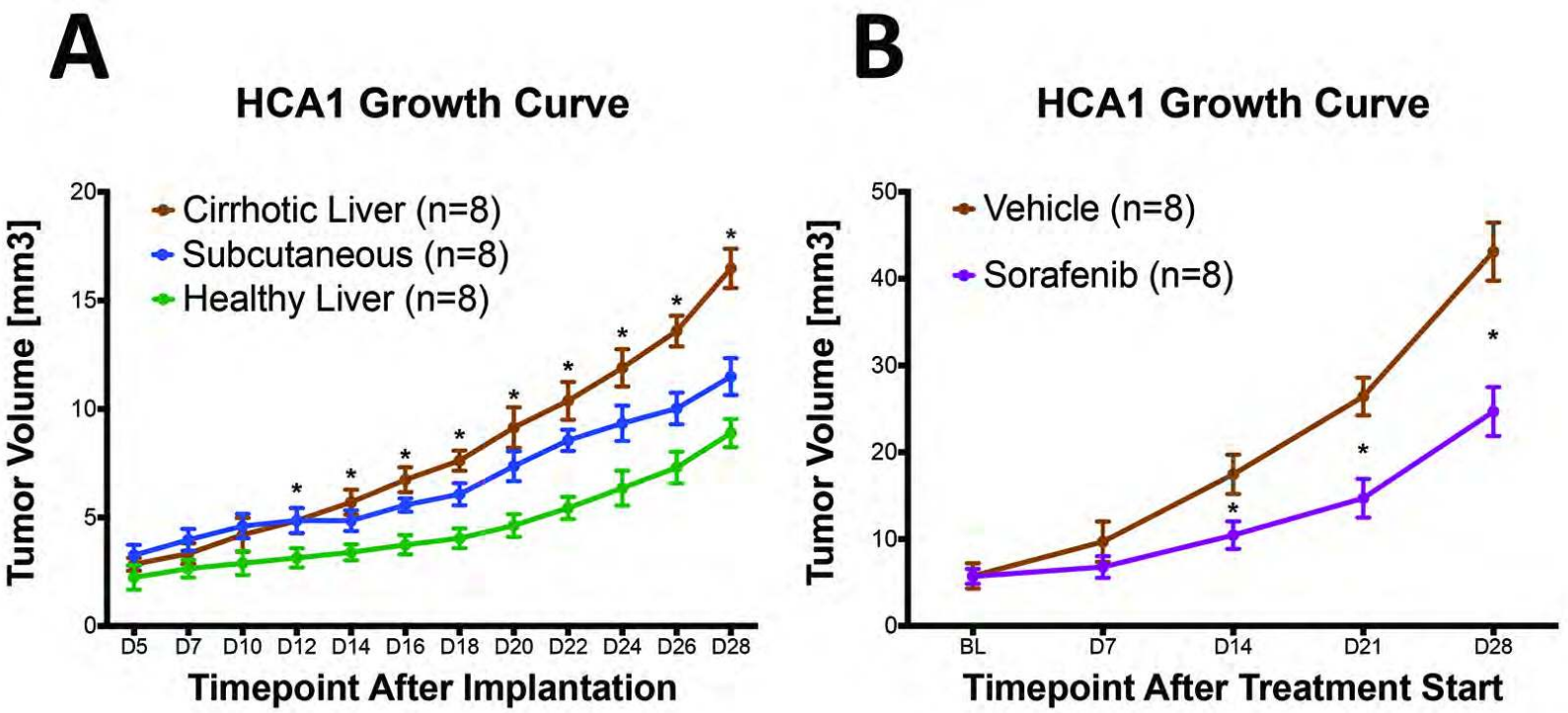
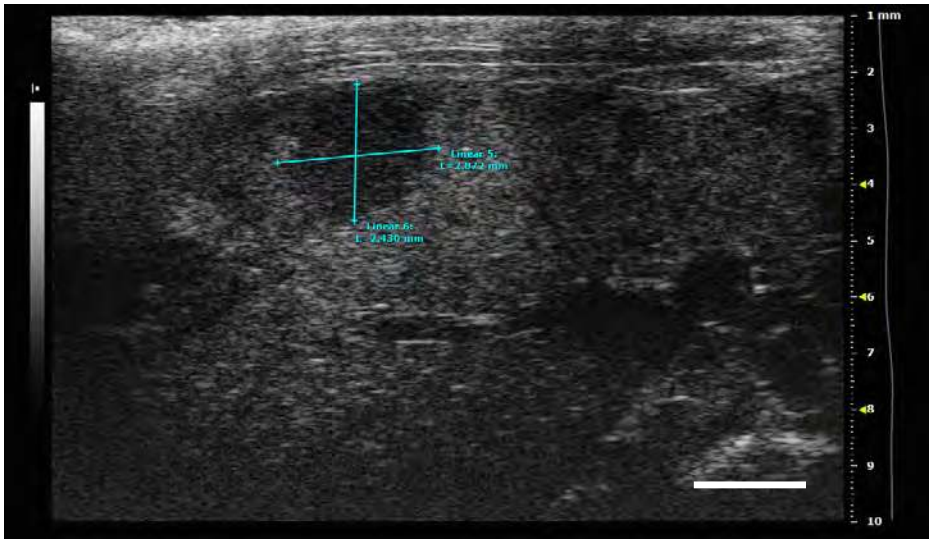

A
HCA1 Growth Curve
Cirrhotic Liver (n=8)
Subcutaneous (n=8)
Healthy Liver (n=8)
Tumor Volume [mm3]
0
5
10
15
20
D5
D7
D10
D12
D14
D16
D18
D20
D22
D24
D26
D28
Timepoint After Implantation
B
HCA1 Growth Curve
Vehicle (n=8)
Sorafenib (n=8)
Tumor Volume [mm3]
0
10
20
30
40
50
BL
D7
D14
D21
D28
Timepoint After Treatment Start

Supplementary Figure S1

A



B