Video Article

Generation of Subcutaneous and Intrahepatic Human Hepatocellular Carcinoma Xenografts in Immunodeficient Mice

Sharif U. Ahmed[1], Murtuza Zair[1], Kui Chen[1], Matthew Iu[1], Feng He[1], Oyedele Adeyi[2], Sean P. Cleary[3], Anand Ghanekar[1,3]

[1]Toronto General Research Institute, University Health Network

[2]Department of Pathology, University Health Network

[3]Division of General Surgery, University Health Network

Correspondence to: Anand Ghanekar at Anand.Ghanekar@uhn.ca

URL: https://www.jove.com/video/50544
DOI: doi:10.3791/50544

Keywords: Medicine, Issue 79, Liver Neoplasms, Hepatectomy, animal models, hepatocellular carcinoma, xenograft, cancer, liver, subcutaneous, intrahepatic, orthotopic, mouse, human, immunodeficient

Date Published: 9/25/2013

Citation: Ahmed, S.U., Zair, M., Chen, K., Iu, M., He, F., Adeyi, O., Cleary, S.P., Ghanekar, A. Generation of Subcutaneous and Intrahepatic Human Hepatocellular Carcinoma Xenografts in Immunodeficient Mice. *J. Vis. Exp.* (79), e50544, doi:10.3791/50544 (2013).

Abstract

In vivo experimental models of hepatocellular carcinoma (HCC) that recapitulate the human disease provide a valuable platform for research into disease pathophysiology and for the preclinical evaluation of novel therapies. We present a variety of methods to generate subcutaneous or orthotopic human HCC xenografts in immunodeficient mice that could be utilized in a variety of research applications. With a focus on the use of primary tumor tissue from patients undergoing surgical resection as a starting point, we describe the preparation of cell suspensions or tumor fragments for xenografting. We describe specific techniques to xenograft these tissues i) subcutaneously; or ii) intrahepatically, either by direct implantation of tumor cells or fragments into the liver, or indirectly by injection of cells into the mouse spleen. We also describe the use of partial resection of the native mouse liver at the time of xenografting as a strategy to induce a state of active liver regeneration in the recipient mouse that may facilitate the intrahepatic engraftment of primary human tumor cells. The expected results of these techniques are illustrated. The protocols described have been validated using primary human HCC samples and xenografts, which typically perform less robustly than the well-established human HCC cell lines that are widely used and frequently cited in the literature. In comparison with cell lines, we discuss factors which may contribute to the relatively low chance of primary HCC engraftment in xenotransplantation models and comment on technical issues that may influence the kinetics of xenograft growth. We also suggest methods that should be applied to ensure that xenografts obtained accurately resemble parent HCC tissues.

Video Link

The video component of this article can be found at https://www.jove.com/video/50544/

Introduction

Hepatocellular carcinoma (HCC) is the fifth most common cancer worldwide and the most rapidly increasing cause of cancer death in North America. The most prevalent risk factor for HCC is liver cirrhosis, most frequently occurring due to chronic viral hepatitis, alcohol misuse, autoimmune disease, or hereditary metabolic disorders [1].

Despite the heavy disease burden imposed by HCC on populations worldwide, the pathophysiology of HCC is relatively poorly understood in comparison to other common cancers such as colorectal, breast, or prostate cancer. For example, specific molecular and cellular events driving tumorigenesis remain to be clearly defined [2]. Like most other solid epithelial cancers, genomic approaches have revealed heterogeneity in the aberrations associated with HCC [3]. A number of studies have revealed disordered activity of a variety of signaling pathways involved in cell proliferation, survival, differentiation, and angiogenesis [4]. In addition, the role of cancer stem cells in HCC pathobiology remains to be clarified [5].

With a limited understanding of HCC pathophysiology, the armamentarium of effective therapies for HCC has also remained relatively limited. Early-stage patients with tumors confined to the liver are candidates for curative therapy using tumor ablation or surgical resection, though recurrence is common. For patients with more advanced disease, chemotherapy and radiation are of limited efficacy and are used primarily for disease control with palliative intent [6].

High quality *in vivo* experimental models of human HCC thus provide a valuable platform for much needed basic research into the pathophysiology of human HCC as well as for evaluation of novel therapeutic approaches. As compared with the use of cell lines or highly defined mouse models, xenografts of primary human tumors in immunodeficient mice have emerged as valuable tools for such studies since they are capable of recapitulating the human disease with high fidelity while also capturing the heterogeneity that is present within and between different patients [7,8]. To this end, we have developed a variety of methods to establish human HCC xenografts in immunodeficient mice. While the majority of published studies involving HCC xenografts describe the use of well-established human HCC cell lines for this purpose, we

have focused on optimizing our assays to generate xenografts from primary HCC specimens obtained immediately after surgical resection from patients.

Different xenografting techniques may be required for different research applications. For example, subcutaneous xenografts generated from tumor fragments are generated rapidly, are easily monitored, and may be more appropriate for local administration of novel therapeutics with convenient monitoring of tumor response. Intrahepatic xenografts may be more relevant for studies pertaining to the role of the hepatic microenvironment in HCC biology. Xenografts generated from tumor cell suspensions are necessary for the identification and characterization of tumor-initiating cell subsets or for experiments requiring *in vitro* manipulations of tumor cells prior to xenotransplantation. We have thus developed and validated the following protocols to establish subcutaneous or intrahepatic xenografts from cell suspensions or tumor fragments derived from primary human HCC specimens.

Protocol

A schematic overview of the protocol is presented in **Figure 1**.

1. Processing of Human HCC Samples

Obtain primary human HCC specimens with written patient consent and with the approval of the institutional research ethics board. These protocols have been carried out at our institution with approval from the University Health Network Research Ethics Board in compliance with all institutional, national, and international guidelines for human welfare.

Collect fresh HCC specimens as soon as possible following the surgical procedure once appropriate samples have been taken for clinical purposes. Ideally this should take place within 30 min after removal of the tissue from the patient. As illustrated in **Figure 2**, a sample of at least 1 cm^3 obtained from the periphery of the tumor is optimal, as the central portion of the tumor may be necrotic. Tumors that have not received any treatment prior to resection such as radiation, chemotherapy, or ablation are preferred in order to maximize the chance that tumor cells are viable. Handle primary human tissues in accordance with standard personal protective protocols for biohazardous material. Perform all laboratory manipulations of tumor tissues and cell preparations in a class II biosafety cabinet using aseptic techniques.

1. Place the fresh HCC sample in 10-25 ml serum-free Dulbecco's Modified Eagle Medium/Ham's F12 at 4 °C and transfer on ice to the laboratory for immediate processing and preparation of tissue fragments and/or cells for xenografting into mice.
2. Using sterile forceps, place the tumor sample in a 100 mm x 20 mm Petri dish or other suitable sterile working surface. Divide the tumor sample into fragments of approximately 2-3 mm^3 using a No.10 surgical scalpel blade. At this point, consider snap-freezing or formalin-fixing some tumor fragments for other experiments or analyses as required.
3. For xenografting of tumor fragments, place some of the HCC fragments into one or more microcentrifuge tubes containing enough Matrigel to allow the fragments to remain submerged. Keep these tubes on ice.
4. For preparation of a tumor cell suspension, use the surgical scalpel blade to mince the remaining HCC tissue as much as possible and mix with 5-10 ml of DMEM-F12 in a 50 ml conical tube depending on the volume of the minced tissue.
5. Add collagenase type IV and dispase II at final concentrations of 200 units/ml and 0.8 units/ml respectively. Pipette the mixture up and down well using a 25 ml pipette.
6. Seal the tube and incubate the mixture from step 1.6 at 37 °C in a 5% carbon dioxide incubator for 30-60 min depending on the softness of the tumor tissue. Pipette the mixture up and down a few times every 10 min to assess the progress of the enzymatic digestion.
7. After digestion is complete, pass the tumor solution through a 100 µm cell strainer. Gently mash the remaining tissue on the cell strainer using a 25 ml pipette tip to enable the maximum number of tumor cells to pass through. Collect the strained cell suspension in a 15 ml conical tube.
8. Centrifuge the tumor cell suspension at 1,200 rpm for 5 min at 4 °C.
9. Gently decant the supernatant. Depending on the size of the pellet, add 2-5 ml of ice cold 1x red blood cell (RBC) lysis buffer and gently pipette up and down to resuspend the pellet. Keep on ice for 5 min.
10. Add DMEM-F12 to a total volume of 15 ml and centrifuge at 1,000 rpm for 5 min at 4 °C to wash out the RBC lysis buffer.
11. Gently decant the supernatant and resuspend the RBC-free tumor cell pellet in DMEM-F12.
12. Count the viable cells using trypan blue exclusion either manually or with an automated cell counter.
13. Centrifuge tumor cell aliquots containing the desired number of cells for injection as described above, resuspend the resulting cell pellet in 30 µl of ice-cold Matrigel, and store on ice.

Optional: After step 1.11, we routinely deplete human CD45+ cells (leukocytes) from the bulk tumor cell suspension and/or purify subsets of tumor cells using flow cytometry or immunomagnetic beads. Detailed protocols for these techniques are well described by the manufacturers of the relevant antibodies, beads, and flow cytometers.

Note: The protocol described above can also be used to process human tumor xenografts harvested from mice in order to perform serial transplantation, substituting the xenograft tissue for the primary human HCC tissue in step 1.1. In this situation, after step 1.11, we routinely deplete infiltrating murine cells from the cell suspension using an antibody against the mouse histocompatibility antigen H2k.

2. Xenografting

Conduct all animal procedures in compliance with protocols approved by the institutional animal care committee. The procedures described herein have been completed under a specific Animal Use Protocol approved by the University Health Network Animal Care Committee in accordance and compliance with all relevant regulatory and institutional agencies, regulations, and guidelines.

Equipment for delivery of inhalational volatile anesthetic agents to small animals should be utilized according to standard operating procedures of the animal facility and research institute. Carry out all surgical procedures using aseptic technique and sterile instruments in a class II biosafety cabinet. Utilize non-obese diabetic severe combined immunodeficiency (NOD/SCID) or NOD/SCID/interleukin 2 receptor gamma chain null

(NSG) strains of mice of either sex at 6-8 weeks of age (The Jackson Laboratory, Bar Harbor, ME) [9,10]. These mice must be housed and maintained in a facility capable of providing pathogen-free conditions suitable for immunodeficient animals.

Prepare mice for surgery in a chamber supplying 5% (v/v) inhaled Isoflurane in 1 L/min of oxygen. Maintain anesthesia until there is loss of corneal and toe reflex in the animal(s). For subcutaneous xenografting, shave one or more small areas on the dorsum of the animal(s) and cleanse the skin with 70% ethanol. For intrahepatic xenografting, shave the ventral thorax and abdomen of the animal(s) from the axillae down to the inguinal region and cleanse the skin with 70% ethanol.

2.1 Subcutaneous Implantation of Tumor Fragments

1. Place the anesthetized mouse prone, maintaining inhalational Isoflurane anesthesia (2% (v/v) in 1 L/min O_2) with a mouthpiece. Apply Tear-Gel to protect the animal's eyes from traumatic injury.
2. Sterilize the dorsal shaved area(s) with Betadine surgical scrub followed by 70% ethanol and lastly with povidone-iodine solution.
3. Make a 5 mm skin incision using sterile sharp scissors.
4. Insert a closed blunt scissor gently into the subcutaneous space and spread gently to develop a pocket large enough to accommodate a tumor fragment.
5. Insert the tumor fragment prepared in step 1.3 into the subcutaneous pocket using sterile fine forceps.
6. Close the skin incision using sutures or clips.
7. Provide postoperative care to the mouse as described below.

2.2 Subcutaneous injection of tumor cells

1. Prepare the animal as described in steps 2.1.1 and 2.1.2.
2. Load the suspension of tumor cells in Matrigel prepared in step 1.13 into an insulin syringe with a 29 G 1/2 in needle.
3. Insert the needle into the subcutaneous space and discharge the contents of the syringe. Advancing the needle several millimetres along the subcutaneous plane away from the skin puncture site prevents leakage of the tumor cell suspension out of the puncture site upon withdrawal of the needle.
4. Provide postoperative care to the mouse as described below.

2.3 Intrahepatic Implantation of Tumor Fragments

1. Using a 27 G 1/2 in needle on a 1 ml syringe, administer 350 µl of sterile normal saline solution subcutaneously in the dorsum of the anesthetized animal's neck to compensate for intraoperative fluid losses. For analgesia, administer 350 µl of sterile normal saline containing buprenorphine (0.1 mg/kg) subcutaneously on the animal's flank, using a 27 G 1/2-in needle on a 1 ml syringe.
2. Place the mouse supine on a pre-heated pad with the nose and mouth positioned inside the mouthpiece to deliver maintenance inhalational Isoflurane anesthesia (2% (v/v) in 1 L/min O_2).
3. Extend the limbs and secure them with tape to the operating surface to optimize exposure of the ventral abdomen and thorax.
4. Perform the procedure under a magnifying lamp to optimize visualization.
5. Sterilize the shaved skin on the ventral abdomen and thorax with Betadine surgical scrub followed by 70% ethanol and lastly with povidone-iodine solution.
6. Using sterile sharp scissors, make a transverse bilateral subcostal skin incision and divide the muscle layers to enter the peritoneal cavity to allow adequate exposure of the entire liver.
7. Place a stitch in the skin above the xiphoid process and secure it to the mouthpiece with tape in order to allow better exposure of the liver and surrounding structures.
8. Use two cotton-tipped applicators adjacent and posterior to the liver to stabilize it.
9. Make an incision 3 mm in length and depth on the surface of the liver using a sterile No. 10 scalpel blade.
10. Immediately apply Surgicel and gentle pressure to the incision site to achieve hemostasis; remove after 60-90 sec and proceed only if complete hemostasis has been achieved.
11. Place a tumor fragment step 1.3 into the liver incision with sterile fine forceps or with an 18 G needle.
12. Apply a small piece of Surgicel over the liver incision to prevent displacement of the tumor fragment and ensure continued hemostasis.
13. Close the incision with sutures or clips.
14. Provide postoperative care as described below

2.4 Intrahepatic Implantation of Tumor Cells via Direct Injection into the Liver

1. Prepare the mouse as described in Steps 2.3.1 to 2.3.7 above.
2. Load the tumor cell suspension (prepared in step 1.13) into an insulin syringe using a 29 G 1/2 in needle.
3. With the liver stabilized using a cotton-tip applicator, insert the insulin syringe needle into the liver and advance the tip a few millimetres beyond the puncture site along a subcapsular plane.
4. Gently discharge the contents of the syringe and then remove the needle from the liver.
5. Place Surgicel over the puncture site and apply gentle pressure with a cotton-tipped applicator to prevent leakage of the tumor cell suspension and to achieve complete hemostasis.
6. Close the incision with sutures or clips and provide postoperative care as described below.

2.5 Intrahepatic Xenografting of Tumor Cells via Injection into the Spleen

1. Prepare the mouse as described in steps 2.3.1 to 2.3.5 above.
2. Using sterile sharp scissors, make a 1 cm left subcostal incision to enter the peritoneal cavity.
3. Use a cotton-tipped applicator to reflect the stomach cranially and to the animal's right side in order to expose the spleen.
4. By handling the surrounding adipose tissues with sterile fine atraumatic forceps, deliver the spleen into the incision and place a cotton-tipped applicator behind the spleen to stabilize it.

5. Using 5-0 silk suture, place a loose pre-tied knot around the spleen above the lower pole.
6. Load the tumor cell suspension (prepared in step 1.13) into an insulin syringe using a 29 G 1/2 in needle.
7. Insert the insulin syringe needle into the lower pole of the spleen and advance it past the level of the loose pre-tied knot.
8. Slowly discharge the contents of the syringe, remove the needle from the spleen, and tighten the knot to prevent any leakage of the injected tumor cell suspension.
9. Replace the spleen into the peritoneal cavity.
10. Close the incision with sutures or clips and provide postoperative care as described below.

2.6 Partial Hepatectomy to Facilitate Intrahepatic Engraftment of Human Tumor Tissue

1. Prepare the mouse as described in steps 2.3.1 to 2.3.7.
2. With sterile sharp scissors, divide the falciform ligament attached to the median lobe of the liver.
3. Using a cotton-tipped applicator, mobilize the left lobe of the liver and position a loose pre-tied knot of 5-0 silk suture around the left lobe. Advance the loose knot as close as possible towards the bilio-vascular pedicle of the left lobe and tighten the knot.
4. Excise the left lobe distal to the ligature using sterile scissors, leaving a small stump to prevent slippage of the knot and subsequent hemorrhage.
5. Using a similar technique, ligate and resect the majority of the median lobe of the liver, avoiding the gallbladder.
6. Use Surgicel and gentle pressure with a cotton-tipped applicator along the cut surfaces of the liver parenchyma to achieve complete hemostasis.
7. For intrahepatic xenografting of a tumor fragment or tumor cells, proceed with steps 2.3.8 to 2.3.11 or 2.4.2 to 2.4.5 as described above.
8. For intrahepatic xenografting of tumor cells via splenic injection, utilize cotton-tipped applicators to gently reflect the stomach cranially and to the animal's right side, exposing the spleen. Proceed with steps 2.5.4 to 2.5.9 as described above.
9. Close the incision with sutures or clips and provide postoperative care as described below.

2.7 Postoperative Care

1. Remove the mouse from the inhalational anesthesia mouthpiece.
2. Place the mouse in a cage under a heat lamp for approximately 20 min until recovered from anesthesia and mobilizing fully.
3. Repeat the buprenorphine dose every 8-12 hr during the first 2-3 postoperative days.

Representative Results

Figure 3 demonstrates the typical appearance of a subcutaneous human HCC xenograft and the corresponding histopathological appearance of the tumor. The development and growth of subcutaneous xenografts can be readily monitored by daily examination of recipient mice. The time interval between xenografting and development of a tumor may vary greatly depending on the type of tissue (tumor fragment vs. cell suspension), source of tissue (primary patient sample, passaged xenograft, or cell line), and quantity of tissue implanted (number of cells or size of tumor fragment). For example, sizable xenografts may develop from the injection of 5×10^6 cells of a well-established HCC cell line within 1-2 weeks, while 6 months may be required for the development of a similar xenograft from 1×10^5 cells obtained from a primary patient HCC sample. We have not observed subcutaneous xenograft formation later than 6 months after implantation of the tumor sample, and thus sacrifice recipient mice at this timepoint if xenografts have not developed.

Figure 4 demonstrates the typical appearances of intrahepatic human HCC xenografts achieved from direct implantation of tumor tissue into the liver as well those achieved from injection of tumor cells into the spleen. Intrahepatic xenografts cannot be readily detected by general physical examination of the recipient mouse unless the tumor is extremely large or has resulted in the development of ascites or abdominal distension. Although small animal imaging modalities such as CT scanning are available at some research institutes and could be utilized to accurately interrogate the liver of recipient mice [11], we do not consider this a widely applicable, practical, or cost-effective approach for most investigators. We have observed that the rate at which intrahepatic xenografts develop is similar to the rate at which subcutaneous xenografts develop, and is similarly affected by variables such as the type, source, and quantity of tissue implanted. Based on the performance of subcutaneous xenografts derived from the same parent tissue as that used for intrahepatic xenografts, we select a timepoint for sacrifice of the recipient mouse and examination of the liver unless clear evidence of a large intrahepatic tumor is present before this. We have observed that the addition of a partial hepatectomy to the intrahepatic xenografting procedure used improves the probability of achieving engraftment of human tumor tissue in the mouse liver, but does not seem to accelerate the rate of xenograft growth.

Table 1 summarizes the tumor engraftment rates achieved after implantation of primary human HCC tissues in immunodeficient mice using the different techniques described in this protocol. For each implantation method, the denominator of the tumor take rate reflects a unique human HCC sample. These data demonstrate that, for primary human HCC tissues, subcutaneous engraftment rates are inferior to the rates of intrahepatic engraftment achieved by intrasplenic tumor cell injection or intrahepatic tumor fragment implantation, and that partial hepatectomy appears to enhance intrahepatic engraftment. Although we have successfully utilized direct cell injection into the mouse liver to generate intrahepatic xenografts from well established human HCC cell lines (data not shown), we have not generated any intrahepatic xenografts with this method using primary human HCC samples despite the use of partial hepatectomy.

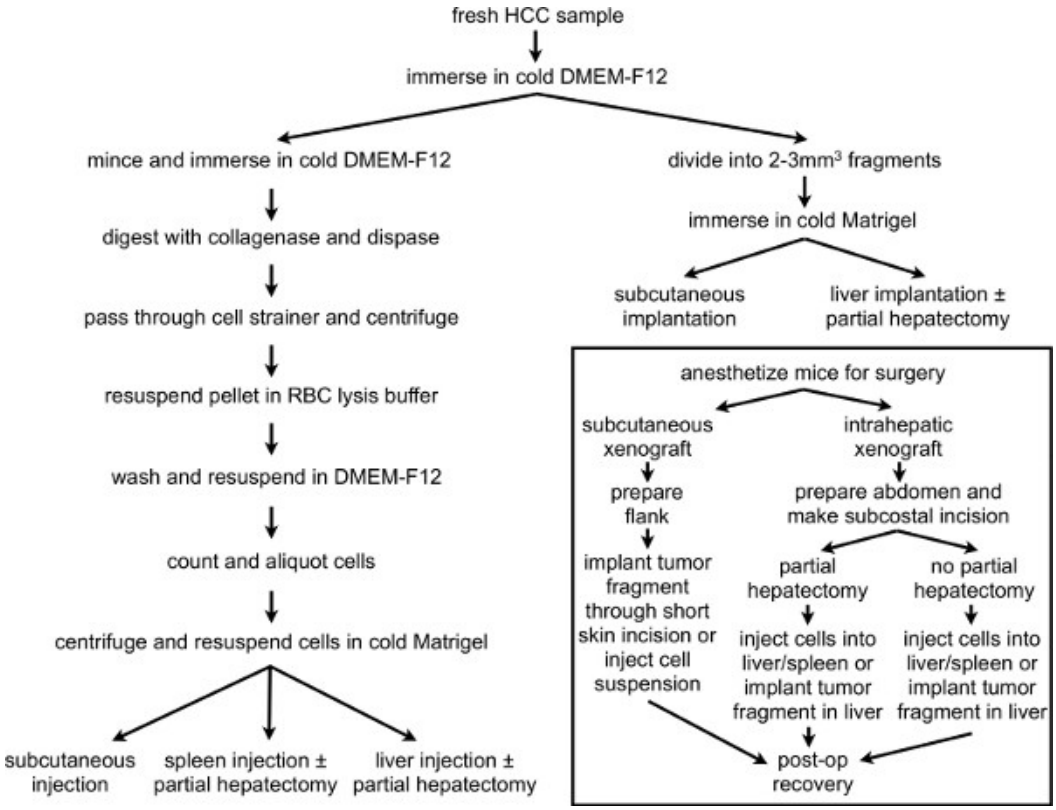

Figure 1. Schematic overview of protocol illustrating workflow for processing and preparation of HCC sample as well as subsequent xenografting options (inset).

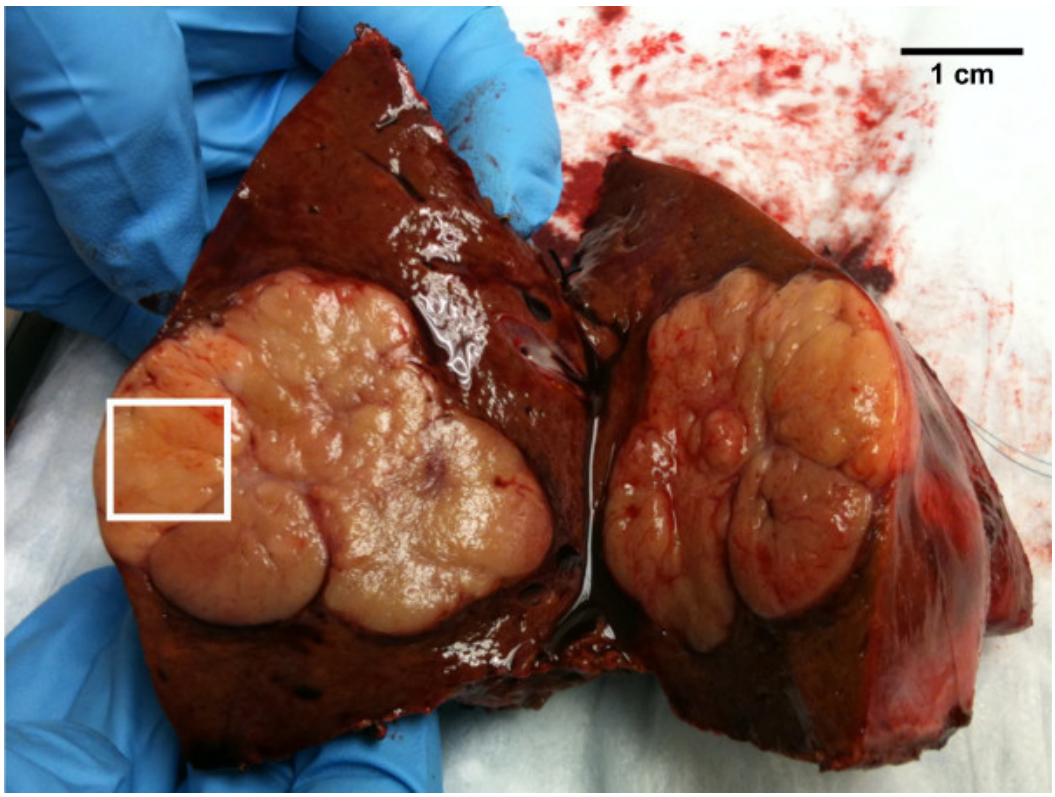

Figure 2. Liver resection specimen from a patient with hepatocellular carcinoma. This patient had not received any adjuvant therapy to the tumor. The white box indicates a viable portion of the tumor near the periphery which was obtained for xenografting. Scale bar at top right corresponds to 1 cm.

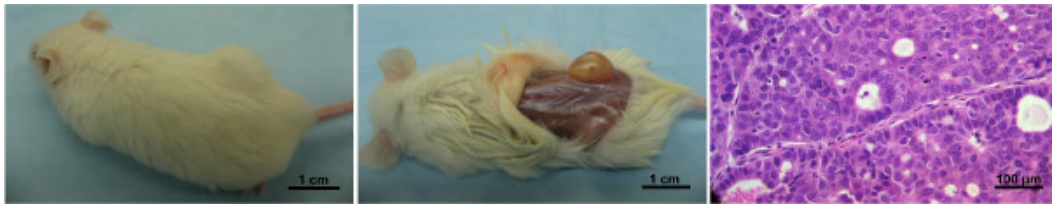

Figure 3. Human hepatocellular carcinoma xenograft generated from the subcutaneous injection of tumor cells. A) The tumor can be readily appreciated on the right flank of the animal. **B)** The tumor is seen to be distinct from the surrounding tissues after sacrificing the animal. **C)** The histology of the tumor demonstrates typical features of hepatocellular carcinoma, including hepatocyte-like cells with nuclear atypia and high nuclear-to-cytoplasmic ratio, absence of portal tracts, and distorted trabeculae with increased thickness of hepatocellular plates. Scale bar corresponds to 1 cm in panels **A** and **B**, and 100 µm in panel **C**. Click here to view larger figure.

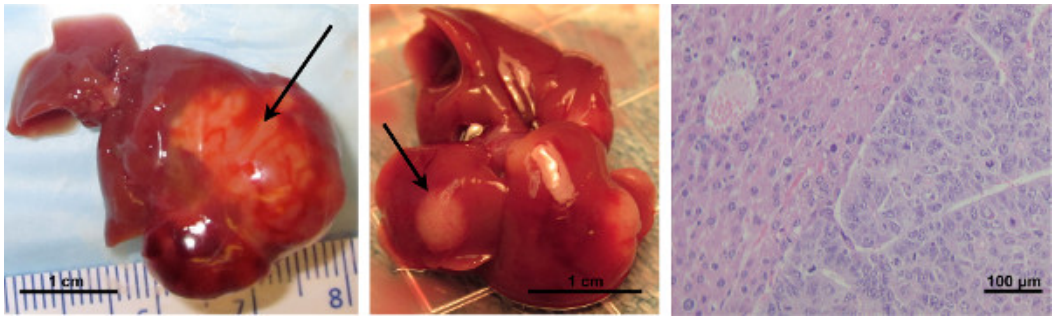

Figure 4. Intrahepatic human hepatocellular carcinoma xenografts. A) Xenograft generated from implantation of a human tumor fragment in the mouse liver. Intraparenchymal tumor indicated by black arrow. Scale of ruler at bottom of photograph in cm. **B)** Xenograft generated from injection of human tumor cells in the mouse spleen following partial hepatectomy. Intraparenchymal tumor indicated by black arrow. Scale bar corresponds to 1 cm. **C)** Histology section through margin of intrahepatic xenograft demonstrating typical features of human hepatocellular carcinoma (bottom right) and adjacent normal mouse liver (top left). Scale bar corresponds to 100 µm. Click here to view larger figure.

Implantation Site	Implantation Method	Tumor Take Rate
subcutaneous	cell suspension	6/55 (10.9%)
	tumor fragment	23/136 (16.9%)
spleen	cell suspension	3/15 (20%)*
	cell suspension + partial hepatectomy	6/14 (42.9%)*
liver	tumor fragment	6/13 (46.2%)
	tumor fragment + partial hepatectomy	2/3 (66.7%)
	cell suspension	not performed
	cell suspension + partial hepatectomy	0/8 (0%)
* proportion of samples that yielded intrahepatic tumor nodules		

Table 1. Human HCC engraftment rates in immunodeficient mice corresponding to different implantation techniques.

Discussion

We have described a variety of techniques to establish subcutaneous and intrahepatic human HCC xenografts in immunodeficient mice that can be applied to a wide variety of experimental questions and assays. While subcutaneous xenografts have been widely used to study various aspects of HCC biology, intrahepatic xenografts are rarely described in the literature. Furthermore, the majority of studies describing the use of xenografts have generated these from well established cell lines. Given the limitations of cancer cell lines in modeling human tumor biology [12], we have been motivated to validate the techniques described above using primary human HCC tissues. Although not supported by statistical analysis due to the small sample sizes of the experimental groups, our observations subjectively reveal a compelling trend towards improved engraftment rates with the addition of partial hepatectomy to the intrahepatic implantation of tumor cells or fragments.

The few studies which have rigorously attempted to utilize primary tissues have achieved successful subcutaneous engraftment with only a very small proportion of the patient samples obtained [13-15]. We have also observed that the proportion of primary human HCC samples that successfully engraft in the subcutaneous space of immunodeficient mice using the techniques described above is limited to approximately 15 %. In the human liver, HCC lesions are hypervascular, receiving their blood supply predominantly via the hepatic artery [16,17]. We speculate that the low engraftment rate of primary HCC tissues that we and others have observed may be a result of the vulnerability of fresh HCC tissues to the relative hypoxia of the microenvironment into which they are xenografted, without established arterial supply. The susceptibility of HCC tissues to rapid ischemic necrosis in the interval between surgical resection and xenografting may also account for the low engraftment rate achieved in mice.

For the generation of intrahepatic human HCC xenografts from tumor cell suspensions, we have found that injection of cells into the spleen is a technically favorable approach that results in superior rates of liver engraftment from primary human HCC samples. The spleen is a highly vascularized structure which may better support HCC cells as compared with other sites in the mouse body, and from which tumor cells appear to readily travel into the liver via the splenic and portal veins. In comparison with the liver parenchyma, into which it is technically difficult to directly inject cells without leakage or hemorrhage, the spleen has the structural capacity to accommodate the volume of the cell suspension that is injected, and can be easily ligated once the tumor cell injection is complete. Injection of cell suspensions directly into the portal vein or hepatic artery in 6-8 week old mice is not technically feasible.

We have utilized partial hepatectomy in recipient mice to facilitate intrahepatic engraftment of human HCC tissues. Although mouse models of partial hepatectomy have been widely used to study the biology of liver regeneration [18], we are not aware of any reports describing it in combination with intrahepatic tumor xenotransplantation. Since liver regeneration occurs rapidly within the first several days following partial hepatectomy, we speculated that the greatly enhanced expression of mitogenic and angiogenic stimuli within the liver parenchyma would better support the engraftment and proliferation of vulnerable human tumor tissue or cells implanted within the remaining portion of the mouse liver, as compared with a mouse liver in its "resting" state [19]. In humans, the greatest risk factor for HCC development is advanced liver fibrosis or cirrhosis, which is characterized by the development of disorganized regenerative nodules, and which has been conceptualized by some as a state of chronic liver injury and regeneration [20]. The techniques that we have demonstrated to engraft human HCC tissues into the mouse liver by activating liver regeneration may prove to be a valuable model to permit investigation into the role of the hepatic microenvironment and liver regenerative mechanisms in the pathophysiology of HCC.

While the techniques described herein are capable of generating high quality primary human HCC xenografts in immunodeficient mice, investigators contemplating the use of these protocols must consider some limitations of the model. First, our protocols incorporate minimal delay between the surgical resection of patient tissue and its subsequent processing in the laboratory, yet achieving high engraftment rates has remained a challenge; this suggests that viable implementation of these techniques requires proximity of the patient tissue collection site to the laboratory, or infrastructure that can minimize delays in transfer of fresh specimens. Second, complex intraabdominal surgical procedures on 6-8 week old immunodeficient mice must be performed by suitably trained personnel in order to achieve survival of the animals for several months before xenografts form; these procedures and subsequent animal care are both time and resource intensive. Third, the development and progression of intrahepatic xenografts cannot be readily monitored without sacrificing animals at predetermined timepoints, limiting the utility of xenografts in this location for some types of assays as outlined above. In addition, intrahepatic xenografts derived from injection of cells into the spleen may present as multiple nodules scattered throughout the liver parenchyma that are difficult to precisely quantify in terms of total tumor burden. Finally, it is important to note that intrahepatic tumor nodules resulting from the injection of cells into the spleen may selectively reflect specific subpopulations of cells that are capable of migration from the spleen to the liver and subsequent tumor initiation.

When generating xenografts from primary human HCC tissues, it is also important to confirm that the resulting xenografts resemble HCC. We have previously demonstrated that lymphomas can inadvertently develop from passenger leukocytes within xenotransplanted human HCC tissues [21]. We thus routinely deplete human CD45+ leukocytes from primary tumor cell suspensions and utilize RT-PCR, flow cytometry, histopathological assessment, and immunohistochemistry to assay xenografts for characteristics typical of HCC. When passaging established xenografts for further studies, tumor-infiltrating murine cells should also be depleted using an antibody against the mouse histocompatibility antigen H2k. If the research application involves the use of tumor fragments or the measurement of xenograft growth, analysis of the xenografts at the study endpoint should include an assessment of the degree to which the tumors are infiltrated by human leukocytes and mouse cells.

Disclosures

The authors declare that they have no competing financial interests.

Acknowledgements

This work was supported by a Canadian Institutes of Health Research Phase 1 Clinician-Scientist Award (A.G.) and an Operating Grant from the Cancer Research Society (A.G.). The authors are grateful to Dr. John Dick for his support of this project.

References

1. El-Serag, H.B. Hepatocellular carcinoma. *N. Engl. J. Med.* **365**, 1118-1127, doi:10.1056/NEJMra1001683 (2011).
2. Li, Y., Tang, Z.Y., & Hou, J.X. Hepatocellular carcinoma: insight from animal models. *Nat. Rev. Gastroenterol Hepatol.* **9**, 32-43, doi:10.1038/nrgastro.2011.196 (2012).
3. Tateishi, R. & Omata, M. Hepatocellular carcinoma in 2011: Genomics in hepatocellular carcinoma--a big step forward. *Nat. Rev. Gastroenterol Hepatol.* **9**, 69-70, doi:10.1038/nrgastro.2011.255 (2012).
4. Hoshida, Y., *et al.* Molecular classification and novel targets in hepatocellular carcinoma: recent advancements. *Semin. Liver Dis* **30**, 35-51, doi:10.1055/s-0030-1247131 (2010).
5. Ji, J. & Wang, X.W. Clinical implications of cancer stem cell biology in hepatocellular carcinoma. *Semin. Oncol.* **39**, 461-472, doi:S0093-7754(12)00114-5 [pii]10.1053/j.seminoncol.2012.05.011 (2012).
6. Villanueva, A., Hernandez-Gea, V., & Llovet, J.M. Medical therapies for hepatocellular carcinoma: a critical view of the evidence. *Nat Rev Gastroenterol Hepatol*. doi:nrgastro.2012.199 [pii]10.1038/nrgastro.2012.199 (2012).
7. Jin, K., *et al.* Patient-derived human tumour tissue xenografts in immunodeficient mice: a systematic review. *Clin. Transl. Oncol.* **12**, 473-480, doi:CLAT417 [pii] (2010).
8. Sausville, E.A. & Burger, A.M. Contributions of human tumor xenografts to anticancer drug development. *Cancer Res* **66**, 3351-3354, discussion 3354, doi:66/7/3351 [pii]10.1158/0008-5472.CAN-05-3627 (2006).
9. Shultz, L.D., *et al.* Multiple defects in innate and adaptive immunologic function in NOD/LtSz-scid mice. *J. Immunol.* **154**, 180-191 (1995).

10. Ohbo, K., *et al.* Modulation of hematopoiesis in mice with a truncated mutant of the interleukin-2 receptor gamma chain. *Blood.* **87**, 956-967 (1996).
11. Fiebig, T., *et al.* Three-dimensional *in vivo* imaging of the murine liver: a micro-computed tomography-based anatomical study. *PLoS One.* **7**, e31179, doi:10.1371/journal.pone.0031179 PONE-D-11-22768 [pii] (2012).
12. Masters, J.R. Human cancer cell lines: fact and fantasy. *Nat. Rev. Mol. Cell Biol.* **1**, 233-236, doi:10.1038/35043102 (2000).
13. Yamashita, T., *et al.* EpCAM-positive hepatocellular carcinoma cells are tumor-initiating cells with stem/progenitor cell features. *Gastroenterology.* **136**, 1012-1024, doi:S0016-5085(08)02179-3 [pii]10.1053/j.gastro.2008.12.004 (2009).
14. Ma, S., *et al.* miR-130b Promotes CD133(+) liver tumor-initiating cell growth and self-renewal via tumor protein 53-induced nuclear protein 1. *Cell Stem Cell.* **7**, 694-707, doi:S1934-5909(10)00632-6 [pii]10.1016/j.stem.2010.11.010 (2011).
15. Yang, Z.F., *et al.* Significance of CD90+ cancer stem cells in human liver cancer. *Cancer Cell.* **13**, 153-166, doi:S1535-6108(08)00009-3 [pii]10.1016/j.ccr.2008.01.013 (2008).
16. Park, Y.N., *et al.* Neoangiogenesis and sinusoidal "capillarization" in dysplastic nodules of the liver. *Am. J. Surg. Pathol.* **22**, 656-662 (1998).
17. Sigurdson, E.R., Ridge, J.A., Kemeny, N., & Daly, J.M. Tumor and liver drug uptake following hepatic artery and portal vein infusion. *J. Clin. Oncol.* **5**, 1836-1840 (1987).
18. Mitchell, C. & Willenbring, H. A reproducible and well-tolerated method for 2/3 partial hepatectomy in mice. *Nat. Protoc.* **3**, 1167-1170, doi:nprot.2008.80 [pii]10.1038/nprot.2008.80 (2008).
19. Michalopoulos, G.K. Liver regeneration after partial hepatectomy: critical analysis of mechanistic dilemmas. *Am. J. Pathol.* **176**, 2-13, doi:S0002-9440(10)60317-3 [pii]10.2353/ajpath.2010.090675 (2010).
20. Zhang, D.Y. & Friedman, S.L. Fibrosis-dependent mechanisms of hepatocarcinogenesis. *Hepatology.* **56**, 769-775, doi:10.1002/hep.25670 (2012).
21. Chen, K., Ahmed, S., Adeyi, O., Dick, J.E., & Ghanekar, A. Human solid tumor xenografts in immunodeficient mice are vulnerable to lymphomagenesis associated with Epstein-Barr virus. *PLoS One.* **7**, e39294, doi:10.1371/journal.pone.0039294 PONE-D-12-02555 [pii] (2012).

