HepT1-derived murine models of high-risk hepatoblastoma display vascular invasion, metastasis, and circulating tumor cells

Sarah E. Woodfield¹, Brandon J. Mistretta², Romana H. Patel¹, Aryana M. Ibarra¹, Kevin E. Fisher³, Stephen F. Sarabia³, Ilavarasi Gandhi³, Jacquelyn Reuther³, Zbigniew Starosolski⁴, Andrew Badachhape⁴, Jessica Epps⁵, Barry Zorman⁵, Aayushi P. Shah¹, Samuel R. Larson¹, Rohit K. Srivastava¹, Yan Shi¹, Andres F. Espinoza¹, Saiabhiroop R. Govindu¹, Richard S. Whitlock¹, Ketan B. Ghaghada⁴, Sanjeev A. Vasudevan¹,*

INTRODUCTION

Hepatoblastoma (HB) is the most common pediatric primary liver tumor accounting for approximately 1% of all pediatric malignancies (Czauderna et al., 2014; Darbari et al., 2003; Hiyama, 2014). Notably, the incidence of HB has significantly increased in the past decade due to its association with premature birth (Spector and Birch, 2012), and this malignancy now has the fastest growing incidence of all pediatric solid tumors (Hubbard et al., 2019). HB generally affects children under the age of 4 years and has a 5-year overall survival (OS) rate of close to 80% for all patients (Czauderna et al., 2014). Unfortunately, high-risk patients who have multifocal, treatment refractory, metastatic, or relapse disease have a 5-year OS rate of only about 40% (Meyers et al., 2009). In general, HB is a genetically simple tumor with an average of only 2.9 mutations per tumor (Eichenmüller et al., 2014). The most commonly altered genes in HB are CTNNB1 and NFE2L2 including CTNNB1 mutations or deletions in a majority of patients. Interestingly, mutations in NFE2L2 are significantly correlated with metastasis, vascular invasion, and poor outcomes (Eichenmüller et al., 2014).

A challenge in the pre-clinical laboratory environment is developing cell line and murine models that depict high-risk characteristics of disease, such as vascular invasion, multifocal tumors, circulating tumor cells (CTCs), and metastasis, for meaningful studies. Our laboratory previously published two orthotopic xenograft models using the established HepG2 and HuH-6 cell lines for intrahepatic growth of tumors, and these models recapitulate key features of disease, including generation of a robust blood supply in the tumor microenvironment, secretion of human α-fetoprotein (AFP), and immunohistochemical and transcriptomic similarity to primary tumors with expression of disease markers (Woodfield et al., 2017). At the same time, neither model showed obvious presence of high-risk features. Other standard models, including subcutaneous, splenic injection, genetically engineered (GEM), and hydrodynamic tail vein/Sleeping Beauty transposon models, also fail to consistently capture these high-risk attributes of disease (Whitlock et al., 2020). Use of patient-derived xenograft (PDX) models in the field is popular as it is thought that these models resemble primary tumors with expression of disease markers (Woodfield et al., 2017). The past decade due to its association with premature birth (Spector and Birch, 2012), and this malignancy now has the fastest growing incidence of all pediatric solid tumors (Hubbard et al., 2019). HB generally affects children under the age of 4 years and has a 5-year overall survival (OS) rate of close to 80% for all patients (Czauderna et al., 2014). Unfortunately, high-risk patients who have multifocal, treatment refractory, metastatic, or relapse disease have a 5-year OS rate of only about 40% (Meyers et al., 2009). In general, HB is a genetically simple tumor with an average of only 2.9 mutations per tumor (Eichenmüller et al., 2014). The most commonly altered genes in HB are CTNNB1 and NFE2L2 including CTNNB1 mutations or deletions in a majority of patients. Interestingly, mutations in NFE2L2 are significantly correlated with metastasis, vascular invasion, and poor outcomes (Eichenmüller et al., 2014).

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an NFE2L2 mutation. Importantly, these models show key high-risk features of disease, including multifocality, CTCs, and metastasis, and will enable meaningful studies of these phenotypes that contribute to poor outcomes for HB patients.

RESULTS

Intrahepatic injection of HepT1 cells generates invasive liver tumors

To generate HepT1-derived liver tumors, we injected two million HepT1 cells into the livers of immunodeficient NOD/Shi-scid/IL-2Rγnull (NOG) mice (see Materials and Methods). These cells were transduced with a lentiviral vector expressing the luciferase gene to allow longitudinal monitoring of tumors in living animals with bioluminescence imaging (BLI). Prior to implantation, we confirmed that the cells expressed strong luciferase activity (2-3 million relative luminescence, RLU). Intraperitoneal injection of luciferin into the tumor-bearing animals caused the cells to emit a BLI signal that could be measured overtime (Fig. 1A). Two of nine animals (22%) emitted a BLI signal at 14 days after injection of cells, and nine of nine animals (100%) emitted a signal at 30 days after injection of cells (Fig. 1A). Graph of the flux values shows their clear increase, indicating growth of tumors during the course of this experiment (Fig. 1B). We also examined tumor burden in living animals with magnetic resonance imaging (MRI, Fig. 1C) at early (4 weeks) and late (7 weeks) time points. We used contrast-enhanced MRI to examine angiogenesis of HepT1-derived tumors and found that they had grown a robust blood supply similar to tumors within humans (Fig. 1D).

After tumors were allowed to grow in animals for 7 weeks, the animals were euthanized and examined for liver tumor growth and for the presence of vascular invasion and extrahepatic disease. All animals that showed liver tumor with BL and MR imaging showed clear presence of intrahepatic tumors. Interestingly, we also found obvious presence of vascular invasion and extrahepatic disease within animals harboring HepT1-derived tumors. Shown in Fig. 2A is a gross image of a liver tumor and attached areas of vascular invasion, including intrahepatic vascular invasion (IHVI) and inferior vena cava thrombus (IVCT). To further examine the tumor and areas of vascular invasion, we performed immunohistochemical staining of formalin-fixed paraffin-embedded (FFPE) tissues with hematoxylin and eosin (H&E) (Fig. 2B). We also closely analyzed eight animals for presence of specific extrahepatic disease at time of euthanasia and found that six of the eight (75%) of animals showed clear presence of extrahepatic disease, including peritoneal metastasis, pelvic metastasis, diaphragm metastasis, and abdominal wall metastasis. To look for lung metastasis, we sectioned FFPE lung tissues from these eight animals and stained sections with H&E. We found obvious lung metastasis in one animal in the first section (Fig. 2B) and, thus, did not section the other lung tissues further. Interestingly, we were able to reimplant a whole piece of the IHVI sample into the liver of another immunocompromised animal to grow a primary liver tumor that resembled the primary tumors grown from injected cells. This was done using a previously published technique used with patient-derived xenograft (PDX) tumors in which whole pieces are implanted with an incision in the Glisson’s capsule of the mouse liver and secured with a veterinary adhesive (Bissig-Choisat et al., 2016).

Finally, with the primary tumor sample, the IHVI sample, and the IVCT sample, we grew cell lines in adherent and spheroid conditions. The primary tumor sample was grown in adherent and spheroid conditions to develop cell lines while the IHVI and IVCT samples generated two disparate adherent cell lines (Fig. 2C). Interestingly, brightfield images of early passage adherent cells in culture show that these cell lines exhibit different visual phenotypes with the primary tumor cell line resembling the parental cell line and
spreading across the plasticware while the IHVI- and IVCT-derived cells pile on top of each other as if they have lost their contact inhibition of growth (Fig. 2C).

**Mutation profiling of HepT1 parental cell line**
To verify the identity of the parental HepT1 cell line, we checked for the presence of known CTNNB1 p.Ala5_Ala80del and NFE2L2 p.Leu30Pro mutations (Eichenmüller et al., 2014). We ran a targeted next generation sequencing (NGS) custom pediatric cancer gene panel featuring 2247 coding exons of 124 genes as well as the TERT promoter. This work showed that our HepT1 cell line maintains the established CTNNB1 and NFE2L2 mutations (Fig. 3, Table S1). Interestingly, this work also revealed mutations in four other genes with established clinical significance in pediatric solid tumors, EP300, NF2, TP53, and the TERT promoter, and loss of PTEN (Fig. 3, Table S1). These mutations were confirmed with PCR and two-directional Sanger sequencing (Fig. 3) with custom-designed primer sets (Table S2) (Sumazin et al., 2017).

**Transcriptomic profiling of tissue samples and cell lines grown from tissue samples**
With this collection of eight unique HepT1-derived samples, including primary tumor (PT), IHVI, IVCT, IHVI grown as primary tumor, primary tumor adherent and spheroid cell lines, IHVI adherent cell line, and IVCT adherent cell line, we completed extensive total mRNA profiling with whole transcriptome next generation sequencing (RNA-seq). The principal component analysis (PCA) of this experiment shows three main clusters, one composed of the adherent cell lines, one containing only the IVCT sample, and one including the remaining tissue and spheroid cell line samples (Fig. 4A). While the PC2 axis shows that the samples are similar, only showing differences in 19.2% of expressed genes among the samples, PC1 is more altered with differences in 31.7% of expressed genes between the IVCT sample and the other clusters. We speculate that this is due to a small amount of murine cell contamination (<10%) in the IVCT sample, which was removed from gene expression and pathway analyses moving forward. We also compiled this data in unsupervised hierarchical clustering heat maps to compare significant gene expression among the set of tissue samples and among the set of cell line samples (Fig. 4B,C). Interestingly, the IHVI, IVCT, and primary tumor samples show clear differences in expression, and the IHVI grown as primary tumor sample more closely resembles the primary tumor sample than the IHVI sample (Fig. 4B). This work also further shows that the three adherent cell line samples are much more similar in gene expression than the spheroid sample, which more closely resembles the primary tumor sample (Fig. 4C).

In a thorough assessment of differentially expressed genes, we found that only 44 genes show a consistent >2-fold change or a <−2-fold change in each individual analysis of the IHVI, IVCT, and IHVI reimplanted samples compared to the primary tumor sample (Fig. 5A). In contrast, the IVCT and IHVI cell line samples share
249 genes changed in common when compared to the primary tumor adherent cell line (Fig. S1A). We then performed gene set enrichment analysis of the transcriptome data from the tissue and cell line samples using gene set enrichment analysis (GSEA) with the KEGG pathway enrichment database (Liao et al., 2019). GSEA on both IHVI samples compared to primary tumor and IVCT compared to primary tumor are shown in two separate bar graphs (Fig. 5B). We see many similar upregulated pathways signifying a clear transcriptomic change from the primary tumor (Fig. 5C). We also further examined the most significantly upregulated pathway among the vascular invasion tissue samples, the complement and coagulation cascades, in a hierarchical clustering heat map (Fig. 5D). Interestingly, many of the genes in this KEGG pathway belong to two gene signatures used to molecularly characterize HB to predict prognosis (Cairo et al., 2008; Sumazin Fig. 3.

CTNNB1 EP300, NF2, NFE2L2, TERT promoter, and TP53 mutations and PTEN loss detected in HepT1 cell line gDNA. (A) CTNNB1 wild-type (1187 bp) and exon 3 deletion (533 bp) alleles are detected in HepT1 cells (Lane 1, 533 bp band) while only wild-type CTNNB1 alleles are detected in A375 cells (Lane 2). An Integrative Genomics Viewer (IGV) version 2.4 representation of next-generation sequencing (NGS) pile-up up data shows the CTNNB1 deletion beginning in intron 2, spanning exon 3, and ending in intron 3, which was confirmed by Sanger sequencing. (B) IGV 2.4 views of NFE2L2, TP53, NF2, TERT promoter, and EP300 mutations detected by NGS and corresponding Sanger sequencing confirmations. (C) Copy-number changes consistent with PTEN loss were also observed, although no orthogonal confirmation was performed. Gel Lane ID: number 1=HepT1 cells, number 2=A375 cells, number 3=no template control, M=100 bp DNA ladder. Abbreviations: VAF, variant allele fraction.
et al., 2017). We performed the same comparison of the adherent primary tumor cell line to the adherent IHVI and IVCT cell lines, respectively, and to the spheroid primary tumor cell line (Fig. S1B-D). We see an overlap of 13 upregulated pathways, including the IL-17 signaling pathway that shows a >2 enrichment score. Finally, we performed this same comparison of the adherent primary tumor cell line to the primary tumor and the spheroid primary tumor cell line to the primary tumor (Fig. S2).

Detection of CTCs in the orthotopic HepT1 model
Because we saw clear presence of extrahepatic disease and lung metastasis in the original cohort of nine animals, we undertook a second experiment with HepT1 cells transduced with a lentiviral vector expressing the mCherry gene for the red fluorescent protein to look for circulating tumor cells (CTCs). We injected two animals with two million cells into the liver to generate intrahepatic HepT1-derived tumors that express mCherry. After allowing the tumors to grow for 6 weeks, we collected blood from both tumor-bearing animals and from four other unimplanted animals. We removed the plasma and red blood cells (RBCs) from the peripheral blood mononuclear cells (PBMCs) with standard density gradient centrifugation and RBC lysis and then analyzed these cells with fluorescence activated cell sorting (FACS) for mCherry to look for red fluorescent cells in the blood of the mice, which correspond to the CTC component. With both animals, we saw clear presence of mCherry-positive cells in the blood of animals (Fig. 6A,D). We also imaged the animals at this time point with MRI and found that the raw number of mCherry-positive cells found in the blood correlated with the estimated tumor volumes by MRI (Fig. 6C,D). Finally, at time of euthanasia, 10 weeks after implantation, we collected blood, separated the PBMC fraction, and plated the cells in culture to look for mCherry-positive cells. With fluorescence microscopy, we saw clear presence of mCherry positive cells from the blood of the animals (Fig. 6B), which are HepT1-derived CTCs.

Tail vein injection model of HepT1-derived liver tumors
In a final set of experiments, we generated a model of relapse HB with tail vein injection of HepT1-mCherry cells. In total, we injected six animals each with 1.5 million cells into the tail vein. Of these, four of six (66.67%) grew liver tumors, which we documented at time of euthanasia 12-13 weeks after implantation. A representative gross image of a HepT1-derived tumor grown from tail vein injection of HepT1 cells is shown in Fig. 7A, and is a representative H&E image of such a tumor is shown in Fig. 7D. These liver tumors are multifocal, as shown in the gross image of the tumor that has at least five independent tumor lesions (Fig. 7A). We also looked for lung metastasis in the animals harboring HepT1-derived liver tumors and found an obvious lung nodule in a tumor-bearing animal (Fig. 7B). We checked this nodule with fluorescence microscopy and found that it was mCherry positive (Fig. 7C), showing that it is derived from the HepT1-mCherry cells injected in the tail vein. With the animals harboring...
tumor, we collected the lungs for FFPE preservation, serial sectioning, and H&E staining. We found clear evidence of lung metastasis in all animals with a representative nodule shown in Fig. 7D. Therefore, all animals that had liver tumors generated with tail vein injection also showed clear metastasis in the lungs. We then completed transcriptomic profiling of the mCherry positive lung nodule shown in Fig. 7B and C and compared this to an mCherry-positive primary tumor. We used linear regression to fit the HepT1 primary tumor and lung metastasis transcriptomes, identifying genes with significantly divergent expression according to a z-test based on internally expressed genes. After Bonferroni multiple testing correction, no significantly differentially expressed genes were identified. Taken together, this work shows that tail vein injection of HepT1 cells leads to repeated growth of multifocal liver tumors and lung metastasis that resembles relapse and metastatic HB seen in patients.

**DISCUSSION**

Prior work with the HepT1 cell line showed that it can give rise to tumors growing in living animals. First, Pietsch and colleagues showed that subcutaneous injection gives rise to tumors (Pietsch et al., 1996). A second key study showed that injecting HepT1 cells into the spleens of animals, followed by splenectomy approximately 2 min after injection, led to multifocal intrahepatic tumors in 50% of injected animals (Ellerkamp et al., 2011). However, neither of these studies examined tumors resulting from direct intrahepatic or tail vein injection of HepT1 cells. Overall, our work led to the development of three unique models of disease, including a luciferase-expressing intrahepatic injection model, an mCherry-expressing intrahepatic injection model, and an mCherry-expressing tail vein injection model. It is notable although not surprising that injection technique led to the generation of unique models that show different attributes of disease.

The intrahepatic injection model is usable for a wide range of studies, including, in particular, traditional drug studies that examine tumor growth in living animals and tumor size and phenotype after euthanasia. Notably, this model has an implantation rate of 100% 4 weeks after injection of cells, which is particularly helpful for studies in which replicate animals are required to show significant differences. In addition, this model gives rise to vascular invasion, metastasis, and CTCs, which are key hallmarks of high-risk disease. It is possible that some of the peritoneal and diaphragmatic disease in this model is due to spillage from
injection, which is possible in any model derived from injection of cells. We did our best to minimize this (see Materials and Methods) by mixing cells with matrigel, which solidifies immediately as soon as warming to mouse body temperature, and by holding surgicel hemostatic gauze to the injection sites. It is possible that such diaphragmatic disease is seen in these animals harboring HepT1-derived tumors because of the TP53 and TERT promoter mutations present in the cell line, which are more characteristic of HCC (Cancer Genome Atlas Research Network, 2017). Primary HCC tumors are well established to give rise to abdominal metastasis in patients and mouse models (Feng et al., 2011). However, it is clear that intrahepatic injection of HepT1 cells gives rise to lung metastasis (Fig. 2B), which is the main site of extrahepatic disease observed in HB patients. A recent retrospective review of vascular invasion in 66 patients with HB showed that vascular invasion is a prognostic indicator significantly associated with a worse 3-year OS and that patients without vascular invasion showed more favorable disease characteristics (Shi et al., 2017). It is also accepted in the field of cancer biology that a major complication and cause of cancer mortality is metastatic dissemination of primary
tumor cells to distant sites by CTCs shed from the solid cancer tissue into the bloodstream of patients (Plaks et al., 2013). Thus, this model will allow particular examination of the mechanisms of these phenotypes in HB. Importantly this is the first report of HB-derived CTCs.

On the other hand, the tail vein injection model gives rise to disease that resembles relapse disease seen in patients with multifocal tumors spread throughout the liver and lung metastasis present in all animals that harbor primary liver tumors. Interestingly, in the literature, tail vein models are described as models of metastasis. When cells are injected through the tail vein, they first enter the venous circulation and confront the lung capillaries and then enter the arterial system and pass through the portal circulation. To our knowledge, most models generated with tail vein injection of cells lead to lung metastasis in the absence of primary tumors, and this modeling technique has been used with many solid tumor types including breast and lung cancers and melanoma (Gómez-Cuadrado et al., 2017). Thus, the tail vein injection model presented in this paper with HepT1 cells that leads to repeated growth of liver tumors and lung metastasis is very novel and shows a unique liver-specific tumor growth phenotype of this cell line.

Importantly, these models allow profiling of disease compartments, including primary tumor, areas of vascular invasion, and metastasis in this study but presumably also CTCs in future work. This sequencing work is particularly devoid of background noise common to such ‘screening’ experiments as all tissue and cell samples analyzed are derived from a common parental cell line. Interestingly, in the initial analysis of all eight samples, we saw clear separation of the IVCT sample. We speculate that this is due to contamination of the tumor thrombus sample with surrounding and embedded mouse blood cells. In any case, this represents less than 10% of total analyzed mRNA and was removed for the additional gene expression and pathway analyses. Notably, direct comparison of the metastasis and primary tumor samples show no significantly differentially expressed genes, and this is similar to what has been shown previously with paired primary tumor and metastasis samples from the same patients (Roessler et al., 2015). This hints at the importance of the CTCs as potentially having disparate gene expression that may drive metastasis, and our models can be used for such deep analyses of CTCs to identify genes and pathways that play a role in the metastatic cascade. Overall, deep analysis and validation of this data in future work will
likely reveal clear drivers of vascular invasion and metastasis. The transcriptome sequencing analyses presented in this work already hint at a potential role for key pathways in vascular invasion in HB, including adaptive immune regulated responses, metabolic pathways, inflammatory cytokines, and Hippo signaling, which have already been shown to be involved in this process in other cancers. For example, mounting evidence shows that alterations in metabolism allow cancer cells to survive outside their solid tumor environments in blood vessels and facilitate them colonizing distant organs to generate metastasis (Elia et al., 2018). Furthermore, the upregulated retinol metabolism, drug metabolism, PPAR signaling pathway, cysteine metabolism, cytochrome P450 metabolism, and fat digestion and absorption pathways have been implicated in high-risk, metastatic HCC tumors, supporting the validity of these HepT1-derived models (Yi et al., 2019). Interestingly, levels of all but two of the genes identified to be significantly upregulated and downregulated that are part of the complement and coagulation cascades are also previously published to be predictive of patient prognosis as part of two molecular signatures of HB (cairo et al., 2008; Sumazin et al., 2017). Finally, a specific analysis of transcriptomic data of HepT1 cells derived from the primary mouse liver tumors grown in adherent and spheroid conditions as surrogates for solid tumor primary tumor cells and floating CTCs revealed a potential role for IL-17 and TNF inflammatory cytokines in spheroid growth. Previous work has already implicated these cytokines in the support and promotion of breast cancer cell aggregate growth as spheroids (Geng et al., 2013). Overall, this work paves the way for potential future studies to search for therapeutic weaknesses of primary tumor cells, metastatic tumor cells, and floating CTCs. In studies of rare cancers, including pediatric solid tumors in general and especially HB, relevant laboratory models that accurately depict disease are even more necessary in the absence of large cohorts of patient data. These unique HepT1-derived models that exemplify high-risk features that contribute to poor survival rates will facilitate meaningful studies with the overall goal of improving patient outcomes.

**MATERIALS AND METHODS**

**Cells and culture conditions**

The HepT1 cell line used in this study was obtained from Dr Stefano Cairo (XenTech, France). It was grown at 37°C in 5% CO2 in Eagle’s Minimum Essential Medium (EMEM, Lonza, Allendale, NJ, USA) supplemented with 10% heat-inactivated fetal bovine serum (FBS, SAFC Biosciences, Lenexa, KS), 2 mM glutamine (Invitrogen, Carlsbad, CA, USA), and 100 units/ml streptomyacin/penicillin (Invitrogen). It was validated by DNA mutation analyses for known CTNNB1 and NFE2L2 mutations (see below). Cells that were harvested from tumors grown in mice or from mouse whole blood were maintained in adherent or spheroid plasticware in the same conditions as described above.

**Orthotopic mouse model with cells transduced with luciferase or mCherry**

All animal procedures used in this study were performed under an animal protocol approved by the Institutional Care and Use Committee of Baylor College of Medicine (AN-6191). In vivo studies were performed in female 8-week-old NOD/Shi-scid/IL-2Rγnull (NOG) mice (Taconic Biosciences, Hudson, NY, USA) similar to previous work (Woodfield et al., 2017). Two 10th HepT1 cells transduced with luciferase or lentiviral psi-LVRU6MP-GFP-mCherry and resuspended in 25 μl phosphate-buffered saline (PBS) mixed with 25 μl matrigel (354.230, Becton, Dickinson and Company) were injected into the right (luciferase) or left (mCherry) lobe of the liver through a right flank or midline abdominal incision, respectively. Spill of cells was minimized by holding surgical absorbable hemostat dressing (294.161, McKesson) to the site of injection as shown in Fig. 1A,B in our previous publication (Woodfield et al., 2017). For the experiment with luciferase, the mice underwent BLI beginning at 10 days after implantation and every week thereafter with the In Vivo Imaging System (IVIS, PerkinElmer, Waltham, MA, USA), and luminescence flux was recorded to assess tumor growth. After 7 weeks, necropsy was performed, intrahepatic and extrahepatic sites of tumor were noted, and samples were harvested for immunohistochemistry, RNA and DNA isolation, and to grow cell lines. For reimplantation of the intrahepatic vascular invasion area into the liver to grow a primary liver tumor, a previously published was technique used, with patient-derived xenograft (PDx) tumors in which whole pieces are implanted with an incision in the Glisson’s capsule of the mouse liver and secured with a veterinary adhesive (Bissig-Choisat et al., 2016). For the experiment with mCherry, 100 μl of blood was harvested from the facial veins of animals at 6 weeks after implantation for FACS to look for mCherry-positive CTCs. After 10 weeks, necropsy was performed, liver tumors and lung metastases were noted, and samples were harvested for immunohistochemistry. PBMCs from whole mouse blood were isolated with density gradient centrifugation and red blood cell (RBC) lysis (ACK lysing buffer, Gibco) and washed with cold 1X PBS. PBMCs were then analyzed with FACS or grown in vitro in non-adherent T-25 flasks in EMEM supplemented with 10% heat-inactivated FBS, 2 mM glutamine, and 100 units/ml streptomyacin/penicillin as described above.

**Flow cytometry**

PBMCs were analyzed with fluorescence activated cell sorting (FACS) on an Aria II (BD Biosciences) using a 355 nm UV laser to excite DAPI and a 450/50 nm bandpass filter to detect it and a 561 nm Yellow-Green laser to excite mCherry and a 610/20 bandpass filter to detect it. FSC and SSC were used to discriminate cell debris and two singlet discriminators were used to remove cells that adhered together. DAPI was used to extract dead cells. mCherry signal was measured after removal of cell clumps and dead cells. Student’s t-test (two-tailed) was used to determine statistical significance.

**Tail vein injection mouse model with cells transduced with mCherry**

All animal procedures used in this study were performed under an animal protocol approved by the Institutional Care and Use Committee of Baylor College of Medicine (AN-6191). In vivo studies were performed in male and female NOD/Shi-scid/IL-2Rγnull (NOG) mice (Taconic Biosciences, Hudson, NY, USA). 1.5×105 HepT1 cells transduced with lentiviral psi-LVRU6MP-GFP-mCherry and resuspended in 100 μl phosphate-buffered saline (PBS) were injected into the tail vein. After 12 to 13 weeks, necropsy was performed, and liver and lung samples were imaged and harvested for immunohistochemistry.

**In vivo MRI**

MRI was performed as described previously (Woodfield et al., 2017) on a 1.0 T permanent MRI scanner (M2 system, Aspect Technologies, Israel). A 35 mm volume coil was used to transmit and receive radiofrequency (RF) signal. Mice were sedated using 3% isoflurane, setup on the MRI animal setup. Body temperature was maintained by circulating hot water through the MRI animal bed, and then maintained under anesthesia at 1-1.5% isoflurane delivered using a nose cone setup. Temperature was maintained by circulating hot water through the MRI animal bed. Respiration rate was monitored using a pneumatically controlled pressure pad placed in the abdominal area underneath the animal. A long circulating liposomal-Gd blood pool contrast agent (SC-Gd liposomes) was systemically administered via the tail vein at a dose of 0.1 mmol Gd/kg and used for contrast-enhanced T1-weighted imaging (Ghaghada et al., 2017; Woodfield et al., 2017) shown in Fig. 1. High-resolution contrast-enhanced MRI (CE-MRI) was performed using a T1-weighted three-dimensional (3D) gradient echo (GRE) sequence with the following scan parameters: echo time (TE), 3.5 ms; repetition time (TR), 20 ms; flip angle, 70°; slice thickness, 0.3 mm; field of view, 54 mm; number of slices, 68; matrix, 180×180; NEX, 1; in-plane resolution, 300 μm; scan time ~5 min. Images were analyzed and processed in OsiriX (version 5.8.5, 64-bit, Pixmeo, Bernex, Switzerland). Tumor volumes were segmented and 3D volume-rendered images were generated in Slicer (version 4.4.0).
and reads were mapped to the HG38 human reference genome. Reads were mapped to the hg38 human reference genome. The Illumina sequencing adaptors were trimmed using a custom-designed SeqCap Target Enrichment probe set (Roche) targeting canonical and splicing regions (23 nt) of 2247 exons in 124 genes and the TERE promoter. NGS libraries were prepared and sequenced on an Illumina MiSeq using 600/3 chemistry with 150 bp read lengths. FASTQ files were aligned to the GRCh37 (hg19) human genome sequence using BWA v0.7.12 and NextGene v2.4.1.2 with variant calling performed by NextGene v2.4.1.2 and Platypus v0.8.1 for single nucleotide variants and small indels (<25 bp), and Pindel v0.2.5 and Delly v0.8.1 for longer indels (>25 bp). Variants were annotated with Variant Effect Predictor. Copy number variant (CNV) analysis was performed using CNVkit v.0.9.3 on BAM files with pooled normal as reference. Samples achieved on average 2.58 million unique paired-end reads with a mean coverage of 345×. Variant interpretation and classification were performed using established laboratory procedures. To confirm the mutations detected by next-generation sequencing (Table S1), PCR was performed with 125 ng of HepT1 gDNA using custom-designed primer sets (Table S2) (Sumamul et al., 2017). After 2.0% agarose gel electrophoresis to confirm the correct size of PCR products, two-directional Sanger sequencing was completed, and mutations were detected with Mutation Surveyor, version v.5.0.1 (Softgenetics, State College, PA, USA), Results of the confirmations are provided in Fig. 3.

RNA sequencing of cell lines and tumor tissues
RNA from frozen cell lines was isolated using the Direct-zol RNA MiniPrep Kit (Zymo). RNA from frozen tissue samples was isolated using the RNaseasy Plus Mini Kit (Qiagen). Samples were treated with DNase I and eluted in nuclease-free water. Extracted RNA samples underwent quality control assessment using the RNA tape on Tapestation 4200 (Agilent) and were quantified with Qubit Fluorometer (Thermo Fisher). RNA libraries were prepared and sequenced at the University of Houston Seq-N-Edit Core per standard protocols. RNA libraries were prepared with QIAseq Stranded Total RNA library kit (Qiagen) using 100 ng input RNA. Ribosomal RNA was depleted with QIAseq FastSelect HMR kit (Qiagen). RNA was fragmented, reverse transcribed into cDNA, and ligated with Illumina sequencing adapters. Size selection for libraries was performed using SPRSelect beads (Beckman Coulter), and purity of the libraries was analyzed using the DNA 1000 tape Tapestation 4200 (Agilent). The prepared libraries were pooled and sequenced using NextSeq 500 (Illumina) generating ~20 million 2×76 bp paired-end reads per sample. The primary tumor and lung metastasis samples shown in Fig. 7E were sequenced at Novogene with complementary protocols.

RNA-seq un-sequencing transcriptome analysis
The RNA-seq raw fastq data was processed with CLC Genomics Workbench 12 (Qiagen). The Illumina sequencing adaptors were trimmed and reads were mapped to the HG38 human reference genome. Read alignment was represented as integer counts by using parameters of mismatch cost 2, insertion cost 3, length fraction 0.8, similarity fraction 0.8, maximum of 10 hits for a read. Integer read counts were normalized by Trimmed Means of M-values (TMM) algorithm. After normalization, we performed differential gene expression using the EdgeR package (Robinson and Oshlack, 2010) which uses a generalized linear model linked to the negative binomial distribution to identify significance. The significance level of FDR adjusted P-value of 0.05 and a log2 fold change greater than 1.5 or less than −1.5 was used to identify differentially expressed genes. For RNA sequencing transcriptome analysis of the lung metastasis sample compared to the primary tumor sample, we used linear regression to fit the primary tumor and lung metastasis transcriptomes and approximated the distribution of residuals obtained for intermediately expressed genes as normal. We then estimated the differential expression significance of each gene by comparing its residual to this null distribution using a z-test. P-values were corrected for multiple comparisons, and P<0.05 was considered statistically significant. Fold changes were calculated relative to the primary tumor.

GSEA of cell lines and tumor tissues
GSEA was performed using WebGestalt (web-based gene set analysis toolkit) for differentially expressed genes identified by EdgeR (Liao et al., 2019). The KEGG enrichment pathway database was used to profile the genes with a minimum of five genes per category and normalized enrichment to a significance-level false discovery rate (FDR) of <0.05.

Microscopy
Brightfield images of tumor sections were taken on a DMI8 microscope (Leica, Germany) at the indicated magnifications and scales. Brightfield images of cells and fluorescent microscope images were taken on a BZ-X710 All-in-One Fluorescence Microscope (Keyence, Itasca, IL, USA) at the indicated magnifications and scales.

Acknowledgements
We would like to thank Dr Stefano Cairo (XenTech, France) for the generous gift of the HepT1 cell line. We would like to thank Dr Igor Stupin for assistance with MRI. We would like to thank Pamela Parsons in the Texas Medical Center Digestive Diseases Center for completing the immunohistochemistry experiments. This project was supported by the Cytometry and Cell Sorting Core at Baylor College of Medicine with funding from the CPRIT Core Facility Support Award (CPRIT-RP180672) and the NIH (CA17343 and 2011024574) and with the expert assistance of Joel M. Sederstrom and Amanda N. White.

Competing interests
The authors declare no competing or financial interests.

Author contributions
Conceptualization, S.E.W. and S.A.V.; methodology, S.E.W., R.H.P., and S.A.V.; validation, S.E.W., B.J.M., R.H.P., K.E.F., S.F.S., and S.A.V.; formal analysis, S.E.W., B.J.M., K.E.F., S.F.S., I.G., J.R., Z.S., A.B., J.E., B.Z., P.S., K.B.G., D.L.T., P.H.G., and S.A.V.; investigation, S.E.W., B.J.M., R.H.P., A.M.I., S.F.S., I.G., J.R., Z.S., A.B., J.E., B.Z., A.P.S., R.S.L., K.R.S., Y.S., A.F.E., S.R.G., R.S.W., P.S., and S.A.V.; resources, S.E.W., R.H.P., A.M.I., I.G., J.R., S.R.L., and K.H., and S.A.V.; data curation, B.J.M., K.E.F., I.G., J.R., J.E., B.Z., K.H., A.R., P.S., and P.H.G.; writing – original draft preparation, S.E.W., B.J.M., K.E.F., S.F.S., J.R., and A.P.S.; writing – review and editing, S.E.W., B.J.M., K.E.F., S.F.S., A.B., A.P.S., R.K.S., K.B.G., D.L.T., P.H.G., and S.A.V.; visualization, S.E.W., B.J.M., S.F.S., Z.S., A.B., and J.E.; supervision, S.E.W., A.R., P.S., K.B.G., D.L.T., P.H.G., and S.A.V.; project administration, S.E.W., R.H.P., I.G., J.R., and K.H.; funding acquisition, S.E.W. and S.A.V. All authors have read and agreed to the published version of the manuscript.

Funding
This work was supported by the Macy Easom Cancer Research Foundation Grant (S.A.V.), a Baylor College of Medicine Michael E. DeBakey Department of Surgery Faculty Research Award (S.E.W.), a U.S. Department of Defense Career Development Award [W81XWH-21-1-0396] (S.E.W.), and a Cancer Prevention and Research Institute of Texas (CPRIT) Multi-Investigator Research Award [RP180674] (S.A.V.). The Texas Medical Center Digestive Diseases Center is supported by NIH grant P30DK56338. The Cytometry and Cell Sorting Core at Baylor College of Medicine is supported with funding from the CPRIT Core Facility Support Award [CPRIT-RP180672] and the NIH [P30 CA125123 and S10 RR024574].
Data availability
The data presented in this study are available on request from the corresponding author.

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