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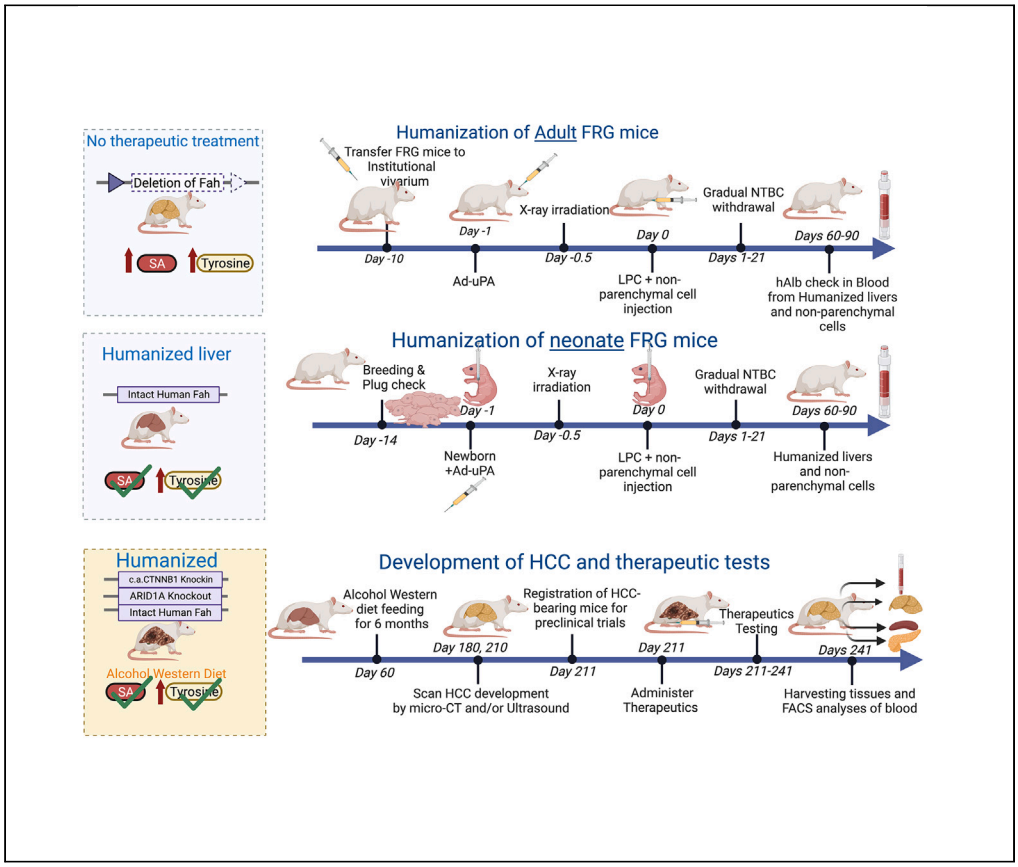
Protocol for generation of humanized HCC mouse model and cancer-driver mutations using CRISPR-Cas9



We detail procedures for generating a humanized mouse model of hepatocellular carcinoma (HCC) recapitulating genetic mutations associated with metabolic liver diseases (MLD). We humanize liver parenchymal, non-parenchymal, and hematopoietic cells. We employ CRISPR-Cas9-based ARID1A knockout and constitutively active CTNNB1 knockin combined with an alcohol Western diet to generate cancer-driver mutations commonly found in MLD-HCC patients. This HCC model facilitates the study of tumor-promoting gene-environment interactions.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Liver parenchymal, non-parenchymal, and hematopoietic cells are humanized in mice

ARID1A KO and constitutively active *CTNNB1* KI are implemented by CRISPR-Cas9 in mice

Alcohol Western diet feeding promotes HCC with ARID1A KO and/or c.a. *CTNNB1* KI

Personalized medicine trials for drugs are tested for HCC with the driver mutations

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Protocol for generation of humanized HCC mouse model and cancer-driver mutations using CRISPR-Cas9

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SUMMARY

We detail procedures for generating a humanized mouse model of hepatocellular carcinoma (HCC) recapitulating genetic mutations associated with metabolic liver diseases (MLD). We humanized liver parenchymal, non-parenchymal, and hematopoietic cells. We employed CRISPR-Cas9-based ARID1A knockout and constitutively active CTNNB1 knockin combined with an alcohol Western diet to generate cancer-driver mutations commonly found in MLD-HCC patients. This HCC model facilitates the study of tumor-promoting gene-environment interactions.

For complete details on the use and execution of this protocol, please refer to Yeh et al.[1]

BEFORE YOU BEGIN

The protocol below describes the specific steps for using humanized FRG HCC models.

Introduction

1. Development of the protocol to solve unmet needs for a preclinical HCC model.
2. Applications of the method.
3. Comparison with Other Methods with key features achieved in this model.

Development of the protocol to solve unmet needs for preclinical HCC model

The mouse HCC models frequently used are categorized into the following groups: genotoxic carcinogen models (e.g., diethylnitrosamine: DEN); oncogene overexpression models (e.g., MYC, NICD); genetic models (e.g., PTEN deficiency, MUP-uPA); and HCV/HBV transgenic mice. Various tumor-promoting diets containing alcohol, high levels of fat, cholesterol, sucrose, fructose, or in combination, are used to achieve tumor promotion by mimicking patient etiologic backgrounds. Hepatotoxins such as carbon tetrachloride are co-administered with the carcinogen to induce liver fibrosis, the common predisposing condition in HCC patients Table 1.

A major drawback of these models is mouse vs. human species differences in microanatomy, metabolism, immunity, and genetic background, which inevitably result in deviations from the HCC patient relevance. For this reason, a humanization approach of the mouse liver by human hepatocyte transplantation has been applied to the liver tumorigenesis regimen.[2,3] Further, non-parenchymal

Table 1. Comparison of humanized humanized liver models used for hematopoietic xenotransplantation

Mouse strain	Culture conditions/ matrix and/or scaffold	Source and # of transplanted	Implantation of LPC	Time of LPC transplantation	Route of LPC transplantation	Type/number of transplanted hematopoietic cells	Conditioning	Ref #
Fah−/−/Rag2−/−/ Il2rg−/− (FRG)	No culture	shipped hepatocytes	Human primary hepatocytes	1.25 × 10^9pfu of uPA adenovirus Pre-treatment	Intrahepatic	None	pretreatment with a urokinase-expressing adenovirus + X-ray	[14]
Caspase 8 under control of the albumin promoter (AFC8), which induces liver cell death, in Balb/C Rag2(−/−) γC-null mice	CD34+ Hematopoietic stem cell co-transplantation	CD34+ HSCs (0.5–1 × 10^6) and Hep progenitor cells (0.5–1 × 10^6)	hepatocyte progenitors isolate from human fetal livers	1–5 days of age	Intrahepatic	Cotransplantation of human CD34(+) human hematopoietic stem cells (HSC) and hepatocyte progenitors		[3]
Fah(−/−), Rag2(−/−), Il2r(−/−) ; FGF19 [(FRGN19(+)]	No culture	Human hepatocytes (Celsis)			intrasplenic technique	Human hepatocytes	Bile duct ligation, bile acid infusion, intestinal bile acid pool +NTBC withdrawal	[19]
uPA-SCID	No culture	5 × 10^5 human hepatocytes		8 weeks of age	Intrasplenic	None	injected intraperitoneally with 0.5 mg of TM-β1 antibody	[30]
MUP-uPA/SCID/Bg mice	No culture	Primary human hepatocytes (CellzDirect, USA) 1 × 10^6 cells or 4–6 × 10^6 hepatocytes	No LPC	8 weeks of age	Intrasplenic	None		[31]
Current studies FRG Fah-/ (Riken) crossbred with Rag2−/−;Il2rgc−/− (Jackson Lab)	Non-parenchymal cell co-transplantation		LPC isolate from human fetal livers	1–5 days of age	Intrahepatic	1 × 10^6	1.25 × 10^8 pfu/mouse Ad:uPA injection and X-ray irradiation	This article

liver cells and immune cells are humanized to reproduce a humanized tumor microenvironment (TME), by transplantation of fetal liver cells containing progenitors for these cells.[2,3]

The second weakness of the conventional models is the difficulty in examining the effects of genetic mutations of human relevance and the interactions of such mutations with a tumor-promoting environment. An ideal model should allow gain and loss of function approaches toward both genetic and environmental key variables to facilitate molecular dissections of tumor-driving gene-environment interactions.

Lastly, the existing models are not best suited for testing safety and efficacy of anti-cancer drugs due largely to the species differences alluded to above. Ideally such testing should be conducted in the model whose propensity to develop HCC is heightened by the clinically relevant gene-environment interactions.

Overview of the method

Disruption of Fah (*Fah−/−* mice) inhibits the growth of the host mouse hepatocytes due to this metabolic defect resulting in liver failure. This loss of host liver cells is permissive for engraftment and expansion by donor human hepatocytes. Two to three months after transplantation the average human hepatocyte repopulation is 70%–90% and hematopoietic chimerism is 40%–60% in bone marrow. Human non-parenchymal liver cells such as macrophages (Kupffer cells) and hepatic stellate cells are present as ~45% of this liver cell compartment.

The procedural outline and primary outcomes of humanizing HCC mice with targeted genetic mutations are summarized below. The first step in creating the model is the isolation of human liver and hematopoietic progenitors from fetal liver. These cells are transplanted via intrahepatic injection of *Fah−/−;Rag2−/−;Il2rgc-/-*: (*FRG*) immunodeficient mouse neonates at 2–3 days of age to repopulate *Fah+/+* hepatocytes, non-parenchymal liver cells and hematopoietic cells. For elimination of resident mouse liver cells to create space for human liver progenitor cell expansion, the mice are preconditioned by injection of urokinase-type plasminogen activator adenovirus (Ad-uPA) under the control of an albumin (Alb) enhancer/promoter. This allows for higher human hepatocyte repopulation in chimeric mice. To kill hematopoietic bone marrow stem cells, neonates are subjected to X-ray irradiation.

The fresh, human fetal cadaverous liver (healthy second trimester) is commercially sourced (from Advanced Bioscience Resources, Inc.). After tissue disruption, cells are recovered by low-speed centrifugation and human hepatic progenitor cells for injection are washed and collected. Adult mice (males and females) originating from a single litter are placed into an X-ray irradiation chamber in a petri dish along with a small amount of bedding material from the breeder cage. The X-ray irradiation (at 750 cGy) is applied to FRG mice. At 2 h post-irradiation of mice, primary hepatocytes and LPC + HSC are intrahepatically injected into adult mice using a syringe. If breeder FRG male mice are kept in the breeding cages, FRG males are also humanized.

The reason for humanizing breeder mice first is that after breeder females give birth, the eventual partial withdrawal and complete withdrawal of NTBC commences damage to both FRG adult and juvenile mouse livers. Survival of the mother is necessary for survival of the neonates. Otherwise, non-humanized female breeder mice will become sick, stop milk production, cease caring of neonates and eventually die after 7–10 weeks following complete withdrawal of NTBC. This paragraph summarizes breeder generation and overall general procedures.

A loss-of-function mutation of *ARID1A* and a gain-of-function mutation of *CTNNB1* commonly observed in MLD-associated HCC, are introduced in fetal hepatoblasts using a *CRISPR/Cas9*-based editing procedure prior to co-transplantation with non-parenchymal liver and hematopoietic progenitors into neonates. NTBC dosing which prevents liver damage associated with FAH deficiency,

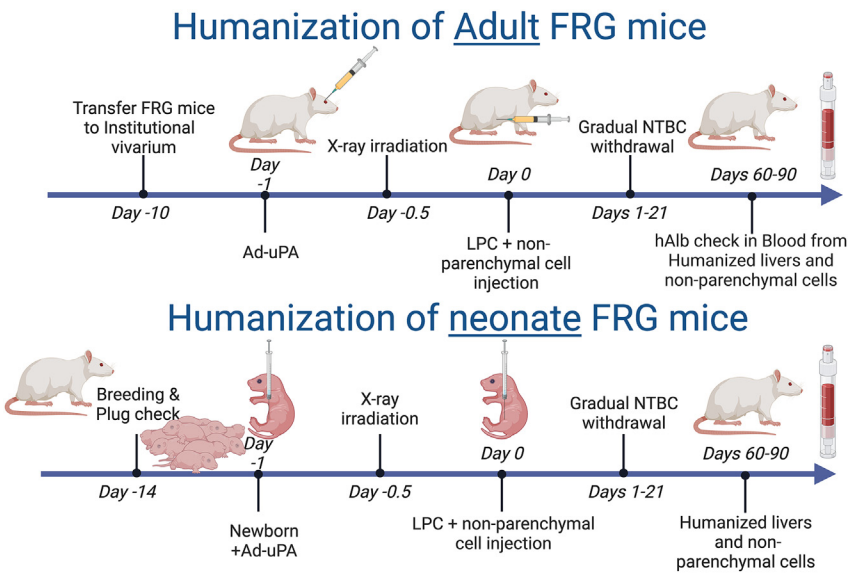

Figure 1. Time courses of humanization processes of adult and neonate FRG mice

(Top) Breeding females and male FRG mice are pre-conditioned and transplanted with human primary hepatocytes. Sixty to ninety days after transplantation, humanized livers and humanized immune systems are reconstituted. (Bottom) Breeding of humanized females and males will generate FRG neonates that are transplanted with human LPCs with non-parenchymal cells to reconstitute both humanized livers and human immune systems. FRG KO Mouse are pretreated by Ad:uPA injection and X-ray irradiation. Created by www.biorender.com (Agreement number: MZ258V2KUE).

is gradually withdrawn over three weeks to facilitate engraftment and repopulation of the transplanted liver cells. The mice are left untreated until two months of age and then placed on an alcohol Western diet (AWD) or regular chow as a control for six months. Chow-fed mice rarely develop liver tumors even with the *ARID1A* and *CTNNB1* mutations (Figure 1). By contrast, AWD feeding induces liver tumor development in 25% of mice with the *CTNNB1* mutation, 50% with the *ARID1A* mutation, and 70% with the dual mutations. Roughly half of the tumors developed in the AWD-fed mice with the dual mutations, are histologically diagnosed as HCC.

Comparison with other methods with key features achieved in the *FRG* model

Both the liver and hematopoiesis are extensively humanized after dual humanization of FRGN mice.[4] Mice transplanted with human hematopoietic stem cells can be used to study human immune responses, infections of blood cells and processes of hematopoiesis (Table 1).[4] Humanized mouse livers can model human hepatitis virus infections and drug metabolism. Dr. Grompe's and Dr. Lishan Su's groups developed a protocol for robust concurrent double-humanization of mice with human mature hepatocytes and blood (Table 1). After busulfan and Ad:uPA pre-conditioning, immune-deficient, fumarylacetoacetate hydrolase *Fah−/−;Rag2−/−; Il2rgc−/−* (*FRG*) mice on the NOD-strain background (*FRGN*) are simultaneously co-transplanted with adult human hepatocytes and hematopoietic stem cells. Other humanized models have not generated HCC development. To expedite HCC development, cancer-driver mutations were generated CRISPR/Cas9 approaches to shorten the duration of HCC development. Four months after transplantation the average human liver repopulation exceeded 80% and hematopoietic chimerism also was high (40–80% in bone marrow). High frequency of HCC development and humanization of not only hepatocytes, but also non-parenchymal cells with hematopoietic cells are innovate aspects of this humanized FRG mice models. Human macrophages (Kupffer cells) were present in the chimeric livers (Table 1).[4]

Institutional permissions

Experiments on live vertebrates or higher invertebrates must be performed in accordance with relevant IACUC and IBC protocols and national guidelines and regulations.

Description/breeding of FRG mice: Humanization of adult FRG breeder mice by surgical transplantation (steps 1–34). (Adult and neonate transplantation procedures are described separately).

Preparation of donor liver cells (steps 46-48)

Recovery of primary hepatocytes from adult liver: Adult primary hepatocyte purification.

Recovery of primary hepatocytes from fetal liver.

Humanization of liver and immune systems of FRG neonates (steps 28–33)

Mature humanized FRG mice are bred to generate pups. Although NTBC is provided in drinking water for breeding cages, published reports have shown post-natal pups in the first month of birth can survive without NTBC. For fully humanized FRG breeder mice beginning after several months, NTBC is not needed for mother mouse survival, but after four to six months, NTBC drinking water is restarted to suppress the host-graft-rejection complication effects due to a chimeric immune system, the reduction of humanized liver and/or humanized immune systems. Once breeder females are successfully humanized, NTBC treatment is no longer needed for survival.

Transplantation into adult FRG mice, breeders: Purpose: Neonate transplantation

FRG neonate mice (1–5 days of age) are placed on a warming pad or under an incandescent lamp for 1–2 min post-irradiation. The pups are restrained by grabbing mice by hand for intrahepatic injection. Please refer to SOP for intrahepatic injection. Descriptions of adult and neonate transplantation are separately described in the following sections.

Since FRG mice are deficient in FAH (fumarylacetoacetate hydrolase) they require continuous dosing with the medication 2-(2-nitro-4-trifluoro-methyl-benzoyl)-1,3 cyclohexanedione (NTBC) (pharmaceutical grade) for survival while awaiting transplantation with human hepatocytes (Figure 1). NTBC blocks the accumulation of the toxic metabolite (succinyl-acetoacetate: SA, Figure 1) arising from dietary tyrosine to prevent liver damage, so that they can be maintained in a healthy state. Since the parenteral form of NTBC for mouse use is unavailable, we rely on drug administration via drinking water at a concentration of 7.5 mg/L (Figure 1).

Human primary hepatocytes- and hepatic progenitor cells are injected into newborn pups at Day 0–2 post-partum as described above for breeder mice. After preconditioning by Ad:uPA, 24- to 48-h post-natal pups from a single litter are placed into a 100-mm2 petri dish along with a small amount of bedding material from the breeder cage and X-ray irradiated. After irradiation mice can be injected with human liver progenitors, non-parenchymal cells and hematopoietic stem cells. Alternatively, neonates can be injected with CRISPR-Cas9-mediated, genetically modified human hepatic progenitor cells (see step 54) and non-parenchymal cells (optionally with human primary hepatocytes).

During the first FRG transplantations extra care should be taken with the NTBC cycling. After the first month to prevent animal morbidity, the FRG mice are frequently weighed (3 times per week). If mouse weights drop more than 20% these mice will receive an NTBC treatment in drinking water bottles. The above-described NTBC cycling protocol can be adjusted based on your experience using this weight-based protocol, since experimental conditions may vary between labs including the proliferative capacity of hepatocytes obtained from different donor sources.

HCC development and therapeutic testing in humanized FRG mice (steps 104–107)

To mimic stepwise carcinogenesis steps through cancer initiation and promotion phases, genetic manipulations by use of CRISPR/Cas9-mediated knockout of ARID1A and/or constitutively active (c.a.) *CTNNB1* is knocked-in human LPCs and intrahepatically injected into FRG neonates with humanized livers and immune systems. After generation of the cancer-driver mutation containing humanized livers, environmental factors, such as BASH [Both ASH (Alcoholic steatohepatitis) and NASH (Non-alcoholic steatohepatitis)] are recapitulated by alcohol Western diet feeding for six months to promote the tumor development (Figure 1, Bottom row).

Important outcomes of the humanized HCC model

Therapeutic treatments for HCC can be tested with different driver mutation in humanized HCC FRG mice. This permits an examination of effects resulting from different therapeutics in response to the cancer-driver mutations:

Scanning and treatment of HCC in humanized FRG livers

Depending on the cancer driver mutation types, therapeutic efficacies may be vary. Such etiology-specific cancer driver gene-targeting drugs are used for more personalized and customized therapeutic regimens instead of conventional sorafenib, regorafenib or PD1 immune checkpoint inhibitors. Before new therapy is initiated, physicians may be able to estimate the therapeutic effects based on cancer-driver mutation types in humanized mice.

Expertise needed to implement the protocol

Virus amplification, purification and determination of titer needs prior virological expertise. Tissue digestion and cell purification techniques are used. Mouse handling and surgery needs prior experience for IACUC approval and for survival surgery procedures and instruments. CRISPR/Cas9-mediated gene manipulation of knockout and knock-in driver mutations in genome needs concurrent molecular and cellular biological expertise.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Human-specific CD45(Clone H130)+ PD7/26) (dilution rate: 1:200)	BioLegend	Cat. no.304014; Clone 2B11
Human-specific CD45 (dilution rate: 1:200)	Dako	Cat. no. IR75161
Mouse-specific CD45 (Clone 30-F11) (dilution rate: 1:200)	BioLegend	Cat. no. 103126
Human-specific CD11c (Clone Bu15) (dilution rate: 1:200)	BioLegend	Cat. no. 337208
Human-specific CD14 (Clone M5E2) (dilution rate: 1:200)	BD Bioscience	Cat. no. 558121
Human-specific CD68 (Clone KP1) (dilution rate: 1:200)	Dako	Cat. no. IR60961
Human-specific albumin (dilution rate: 1:200)	Dako	Cat. no. F011702
Human-specific hepatocyte (HepPar1, Clone OCH1E5) (dilution rate: 1:200)	Dako	Cat. no. IR62461
Human-specific α-smooth muscle actin (SMA) (Clone 1A4) (dilution rate: 1:200)	Dako	Cat. no. IR61161
Human-specific CD3 (Clone HIT3a) (dilution rate: 1:200)	BD Bioscience	Cat. no. 300306
Human-specific CD3(dilution rate: 1:200)	Dako	Cat. no. IR50361
Human-specific CD19 (Clone HIB19) (dilution rate: 1:200)	BioLegend	Cat. no. 302210
Human-specific albumin (dilution rate: 1:200)	Dako	Cat. no. F011702

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Human-specific hepatocyte (HepPar1, Clone OCH1E5) (dilution rate: 1:200)	Dako	Cat. no. IR62461
Human-specific Anti-ARID1A antibody (EPR13501] (dilution rate: 1:200)	ABCAM	Cat. No. ab182560
7-Aminoactinomycin D (7-AAD) (dilution rate: 1:200)	Invitrogen	Cat. no. A1310
LIVE/DEAD fixable yellow dead cell stain kit	Invitrogen	Cat. no. L34959
CD34MicroBead Kit, Human	Milteny	Cat. No. 130-046-702
Bacterial and virus strains		
Ad:uPA-expression under control of the *Alb* promoter)	Thomas Sisson, M.D. for current studies (Department of Internal Medicine and Department of Internal Medicine, University of Michigan)[4]	Ad:uPA
uPA adenovirus (CuRx uPA Liver Tx enhancer)	Yecuris	CuRx uPA Liver Tx enhancer
Biological samples		
Human fetal liver, 15- to 18-week gestation period	Advanced Bioscience Resources or local obstetricians with IRB approval	15- to 18-week gestation period
Newborn, 1–5 days old FRG mice (ideally 1–3 days of age)	*C57Bl/6*-background Fah−/−;*Rag2*$^{-/-}$; Il2γc$^{-/-}$ (FRG) mice, infected with Ad:uPA-expression under control of the *Alb* promoter)	
Alcohol Western Diet containing 3.5% v/v ethanol: Modified High Fat AIN-93G Purified Rodent Ethanol Liquid Diet with Anhydrous Milkfat, Lard, Corn Oil and Cholesterol Added, for Mice (1 kcal/mL) Casein (46.4 g/kg, Anhydrous Milkfat (13.9 g/kg), Lard (23.2 g/kg), Corn Oil (4.64 g/kg), Cholesterol (2.32 g/kg)	DYET	#710362
Control diet	DYET	#180724
Human frozen Bone marrow CD34+ cells: (2.5 million cells): one vial of 2.5 million BM-CD34+	AllCell	cat. no. ABM017F
Human hepatocytes: (total two vials: 5 million cells/tube × 2 tubes = at least 10 million cells): BioreclamationIVT	BioreclamationIVT	can. no. F00995
Fetal liver cells	Advanced Bioscience Resources or local obstetricians with IRB approval	1 million/mouse
0.5 million/mouse human hepatocytes	BioreclamationIVT	cat. no. F00995)
0.8 million/mouse Bone marrow CD34+ cells	AllCell	cat. no. ABM017F
2 million human hepatocytes:	BioreclamationIVT	Cat. #F00995
Chemicals, peptides, and recombinant proteins		
1.4 g/mL CsCl solution (53 g + 87 mL of 10 mM Tris-HCl pH 7.9)	Sigma-Aldrich	cat. No. 289329
1.2 g/mL CsCl solution (26.8 g + 92 mL of 10 mM Tris-HCl pH 7.9)	Sigma-Aldrich	cat. No. 289329
NTBC/SMX/TMP Sulfamethoxazole-trimethoprim (SMX/TMP; Hi-Tech Pharmacal, cat. no. 0823-16) NTBC: CuRx™ Nitisinone (Yecuris) or Nitisinone (Swedish Orphan)	Hi-Tech Pharmacal	cat. no. 0823-16
Red blood cell lysis buffer (1× ACK lysis buffer)	Invitrogen	cat. no. A10492-01
Human albumin ELISA kit	Bethyl	
RNAlater	Qiagen	
Ficoll	GE Healthcare	cat. no. 17-1440-03
Running buffer (autoMACS running buffer; alternatively, use a 2 mM EDTA, 0.5% (wt/vol) BSA/PBS solution)	Miltenyi Biotec	cat. no. 130-091-221
Rinsing solution (autoMACS rinsing solution; alternatively, use 2 mM EDTA/PBS solution)	Miltenyi Biotec	cat. no. 130-091-222
Fetal bovine serum (FBS)	Gibco	cat. no. 16000044
Penicillin-streptomycin (pen-strep)	Gibco	cat. no. 1541

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
L-Glutamine	Gibco	cat. no. 25030
Avertin (2,2,2-Tribromoethanol)	Sigma-Aldrich	cat. no. T48402
Trypan blue	Invitrogen	cat. no. 15250-061
Liquid nitrogen	Gilmore Liquid Air Company, INC	UN1977
Trizol RNA isolation kit	Thermo Fisher Scientific	cat. no. 12183555
Sirius Red/Fast Green or Masson's trichrome stain	Sigma-Aldrich	cat. no. 365548
Isoflurane	Sigma-Aldrich	cat. no. 792632
29 Ga 1 cc syringe	Thermo Fisher Scientific	
70 % EtOH	Thermo Fisher Scientific	cat. no. T08204K7CS
Rinsing solution (autoMACS rinsing solution; alternatively, use 2 mM EDTA/PBS solution)	Miltenyi Biotec	cat. no. 130-091-222
Fetal bovine serum (FBS)	Gibco	cat. no. 16000044
Penicillin-streptomycin (pen-strep)	Gibco	cat. no. 1541
BD Ultra fine Insulin syringe with BD Ultra-Fine Capacity: 3/10 mL; Length: 8 mm (5/16″); Gauge: 31G; BD Ultra-Fine II Short Needle Insulin Syringe - 31G 3/10cc 5/16″	BD Medical Systems	cat. no. 328438
Target-specific chemically modified sgRNA: Synthetic sgRNA Kit	Synthego	cat. no. A29377
Cas9 2NLS nuclease (S. pyogenes)	Synthego	cat. no. A29377
Positive control sgRNA (optional): Controls Kit	Synthego	cat. no. A29377
Neon® Transfection System	Thermo Fisher Scientific	cat. no. MPK5000
Neon Buffer T: Supplied with Neon® Transfection System	Thermo Fisher Scientific	cat. no. MPK5000
Neon™ Transfection System 10 µL Kit	Thermo Fisher Scientific	cat. no. MPK1025
Alternative: Neon Transfection System Starter Pack	Thermo Fisher Scientific	cat. no. MPK5000S
T-25 flask: Multiple vendors	Thermo Fisher Scientific	cat. no. 169900
PBS buffer: Multiple vendors (e.g., Thermo Fisher Scientific)	Thermo Fisher Scientific	cat. no. 28372
Hemocytometer or automated cell counter: Multiple vendors (e.g., Thermo Fisher Scientific)	Thermo Fisher Scientific	cat. no. AMQAF1000
TE buffer: Included in Synthego kits	Synthego	cat. No. A29377
Nuclease-free water: Included in Synthego kits	Synthego	cat. no. A29377
Human albumin ELISA with no cross-reactivity to mouse albumin	Bethyl Laboratories Inc., USA	cat. no. A80-229A
Nitisinone (NTBC,Yecuris, USA; Orfadin®, Swedish Orphan Biovitrum AB, Sweden).	Yecuris, USA	Nitisinone
Reagents for CRISPR/Cas9 gene editing		
CRISPR gRNA	Synthego	
29 Ga 1cc syringe	Thermo Fisher Scientific	
Compressed medical oxygen tank		
Isoflurane chamber/setup		
Sterile gauze squares	Thermo Fisher Scientific	
Operating scissors (sterilize by dry autoclave)	Braintree Scientific, INC	Cat. no. ST5-304
Scalpels (sterilize by dry autoclave)		
Suture 6–0, 0.7 Metric	Ethicon	13 mm with taper needle
Needle driver	Braintree	
SURGICAL® Nu-KNIT™ hemostat	Ethicon	
Human hepatocytes (fresh or cryopreserved, sold by different vendors)		
Human fetal liver, 15- to 18-week gestation period (Advanced Bioscience Resources or local obstetricians with IRB approval)	Advanced Bioresorce	1 million/mouse fetal liver cells
Human frozen Bone marrow CD34+ cells	AllCell	cat. no. ABM017F (2.5 million cells): one vial of 2.5 million BM-CD34

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REAGENT or RESOURCE	SOURCE	IDENTIFIER
Human hepatocytes: (total two vials: 5 million cells/tube × 2 tubes = at least 10 million cells)	BioreclamationIVT	cat. no. F00995
Cell and Tissue processing reagents and solutions		
NTBC/SMX/TMP ● NTBC: CuRx™ Nitisinone (Yecuris) or Nitisinone (Swedish Orphan) ● Sulfamethoxazole-trimethoprim (SMX/TMP)	Hi-Tech Pharmacal And Yecuris	cat. no. 0823-16 and CuRx™ Nitisinone
Red blood cell lysis buffer (1× ACK lysis buffer)	Invitrogen	Cat. no. A10492-01
Human albumin ELISA kit	Bethyl	
RNAlater	Qiagen	
DMEM/F-12 hepatocyte culture medium	Thermo Fisher Scientific	
uPA adenovirus) or Ad:uPA was provided by Thomas Sisson, M.D. for current studies (Department of Internal Medicine and Department of Internal Medicine, University of Michigan)[4]:	Yecuris	CuRx uPA Liver Tx enhancer
Ficoll	GE Healthcare	cat. no. 17-1440-03
Running buffer (autoMACS running buffer; alternatively, use a 2 mM EDTA, 0.5% (wt/vol) BSA/PBS solution)	Miltenyi Biotec	cat. no. 130-091-221
Avertin (2,2,2-Tribromoethanol)	Sigma-Aldrich	cat. no. T48402
Trypan blue	Invitrogen	cat. no. 15250-061
Human albumin ELISA kit (Bethyl)		
Liquid nitrogen	Gilmore Liquid Air Company	
Trizol RNA isolation kit	Thermo Fisher Scientific	
Sirius Red/Fast Green or Masson's trichrome stain	Sigma-Aldrich	
Isoflurane		
Weigh station	Thermher Scientific	
29 Ga 1 cc syringe	Thermo Fisher Scientific	
Compressed medical oxygen tank		
Gene knock-in DNA manipulation kit	Thermo Fisher Scientific	
70 % EtOH	Thermo Fisher Scientific	
Materials Required Target-specific chemically modified sgRNA: Synthetic sgRNA Kit	Synthego	
Cas9 2NLS nuclease (S. pyogenes): Synthego	Synthego	
Positive control sgRNA (optional): Controls Kit (Synthego)	Synthego	
Transfection control (optional): Recommended: pMAXGFPTM (Lonza) or pEGFP-N1 (Clonetech)	Clonetech	
Neon® Transfection System	Thermo Fisher Scientific	cat. no. MPK5000
Neon Buffer T: Supplied with Neon® Transfection System	Thermo Fisher Scientific	cat. no. MPK5000
Neon™ Transfection System 10 µL KitAlternative: Neon Transfection System Starter Pack: Thermo Fisher Scientific, Catalog #MPK5000S	Thermo Fisher Scientific	cat. no. MPK5000
T-25 flask: Multiple vendors (e.g., Thermo Fisher Scientific)	e.g., Thermo Fisher Scientific	
PBS buffer: Multiple vendors (e.g., Thermo Fisher Scientific)	e.g., Thermo Fisher Scientific	
Hemocytometer or automated cell counter: Multiple vendors (e.g., Thermo Fisher Scientific)	e.g., Thermo Fisher Scientific	
TE buffer	Included in Synthego kits	cat. no. MPK5000
Nuclease-free water	Included in Synthego kit	cat. no. MPK5000

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Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Critical commercial assays		
Gene knock-in DNA manipulation kit	Thermo Fisher Scientific	
Deposited data		
None		
Experimental models: Cell lines		
HEK293T cells	ATCC	cat. no. CRL3216
Experimental models: Organisms/strains		
FRG mice: *C57Bl/6*-background Fah−/−; *Rag2*$^{-/-}$;Il2γc$^{-/-}$ mice	This paper	Newborn, 1–5 days old (ideally 1–3 days of age)
Oligonucleotides		
Rag-2 Tm1Fwa genotyping primers: RAG2IL2Rg-Neo-3′	IDT	CCAACGCTATGTCCTGATAGCGGT (24 bp)
Rag-2 Tm1Fwa genotyping primers: RAG2IL2Rg-RG2-1	IDT	TTAATTCAACCAGGCTTCTCTCACTT (24 bp)
Common Gamma Chain Wild Type gene: Gc Sense (intron 4)	IDT	CTT TAT TGA TAA CGA TCT ATC CCT CAC CC (29 bp)
Common Gamma Chain Wild Type gene: Gc Sense Gc Antisense (intron 5)	IDT	CTC CAC TCT GCA GAG TCT ATG GAA TCC (27 bp)
Recombinant DNA		
Human c.a.CTNNB1 Donor DNA sense sequence CTNNB1 exon 3 mRNA nucleotide position1-200 (Figure 4):	IDT	CTGATTTGATGGAGTTGGACATGGCCA TGGAACCAGACAGAAAAGCGGCTGTTA GTCACTGGCAGCAACAGTCTTACCTGG ACGCTGGAATCCATGCTGGTGCCACTG CCACAGCTCCTGCTCTGAGTGGTAAAG GCAATCCTGAGGAAGAGGATGTGGATA CCTCCCAAGTCCTGTATGAGTGGGAAC AGGGATTTTCT
Transfection control (optional): Recommended: pMAXGFPTM (Lonza) or pEGFP-N1	Promega	
Software and algorithms		
ImageJ	Schneider et al.[5]	https://imagej.nih.gov/ij/
Other		
Compressed medical oxygen tank Oxygen Medical Oxygen 24CF	MEDOXE	Oxygen Compressed UN1072 690 litters
Isoflurane chamber/setup		
Scale		
Weigh station		
Engraftment needle [705SN30/51MM/12" (degree), S/O#W119704 measurement]	Hamilton	cat. no. 80508
27-gauge needles (−0.5 mL BD Micro-Fine™ IV Insulin Syringes)	Thermo Fisher Scientific	cat. no. 14-829-1D
50 mL Falcon conical tubes	Thermo Fisher Scientific	cat. no. 14-432-22
15 mL Falcon conical tubes	Thermo Fisher Scientific	cat. no. 12-565-268
5 mL tube with cell strainer snap cap (FACS-tubes)	Thermo Fisher Scientific	cat. no. 08-771-23
225 cm^2 canted neck flask, tissue-culture treated	Thermo Fisher Scientific	cat. no. 07-200-62
75 cm^2 flask, tissue-culture treated	Thermo Fisher Scientific	cat. no. 10-126-11
500 mL filter bottle (Stericup Express Plus, 0.22 μm)	Millipore	cat. no. SCGPU05RE
Iscove's modified Dulbecco's medium (IMDM)	Invitrogen	cat. no. 12440-046
Lieber-DeCarli liquid diet glass feeding tubes (50 mL)	Bio-Serv	cat. no. 9019
Tube holders, Short, Adjustable)	Bio-Serv	cat. no. 9015
Replacement Caps, Autoclavable Screw Cap, Black	Bio-Serv	cat. no. 9501

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Newborn, 1–5 days old FRG mice (ideally 1–3 days of age); *C57Bl/6*-background Fah−/−;*Rag2*$^{-/-}$;Il2γc$^{-/-}$ mice, infected with Ad:uPA-expression under control of the *Alb* promoter)		
BD Ultra fine Insulin syringe with BD Ultra-Fine Needle for Animal Use: Capacity: 3/10 mL, Length: 8 mm (5/16″), Gauge: 31G, BD Ultra-Fine II Short Needle Insulin Syringe - 31G 3/10cc 5/16″	BD Medical Systems	cat. no. 328438
Isoflurane chamber/setup		
AutoMACS cell separator (Miltenyi Biotec) or other manual/automated cell separators	Miltenyi Biotec	
Hemocytometer (or automatic cell counter)		
SW 28 Swing rotor with buckets	Beckman	
Beckman Ultracentrifuge machineGuava easyCyte mini flow cytometer (Millipore) and/or multicolor flow cytometer	Beckman	
XRAD 320 biological irradiator (precision X-ray, CT) or other X-ray irradiators		
Eppendorf Centrifuge machine #5804	Eppendorf	Rotor Cat#A4-44

MATERIALS AND EQUIPMENT

Antibody mix for human immune repopulation

Antibody mix for human immune repopulation: Combine 2 μL of human-specific CD45 FITC-labeled, 2 μL of mouse-specific CD45 PE and 0.5 μL 7-AAD in 50 μL of 2% (vol/vol) FBS/PBS solution per sample before staining. These antibodies are used for FACS staining of cells to assess human/mouse blood cell ration (chimerism).

Add 2 μL of human specific CD45 FITC.

Add 2 μL of mouse specific CD45 PE\.

Storage conditions (at 4°C and maximum time for storage for 2–3 months).

Liver digestion medium

Reagent	Final concentration	Amount
Liver digestion medium (Gibco, cat. no. 17703)		50 mL
DNase (Roche, cat. no. 10104159001)	5 mg mL^{-1}	2.5 μL
7-AAD		0.5 μL /50 μL of 2% (vol/vol) FBS/PBS solution per sample
Total	**N/A**	**50 mL**

Note on storage conditions: Freshly prepare using complete IMDM with 2.5 μL of DNase added per 50 mL.
Store at 4°C (maximum time for storage for 2–3 months).

Adult human hepatocytes with non-parenchymal cells

Reagent	Final concentration	Amount
Bone marrow CD34+ cells: AllCell Catalogue #ABM017F		0.2 million/4 mouse
human hepatocytes F00995P BioreclamationIVT (catalogue #F00995)		2 million/4 mouse
Bone marrow CD34+ cells: AllCell Catalogue #ABM017F		0.8 million/4 mouse
Total	**N/A**	**50 μL**

Note on storage conditions: Freshly prepare using complete IMDM.
Storage conditions (keep at 4°C and maximum time for storage is 2–3 months).

Cell wash buffer		
Reagent	Final concentration	Amount
Rinsing solution (autoMACS rinsing solution, Miltenyi Biotec, cat. no. 130-091-222; alternatively, use 2 mM EDTA/PBS solution)	2 mM EDTA	45 mL
Fetal bovine serum (FBS; Gibco, cat. no. 16000044)		5 mL
Penicillin-streptomycin (pen-strep; Gibco, cat. no. 1541)		0.5 mL
L-Glutamine (Gibco, cat. no. 25030)		0.5 mL
Total	**N/A**	**50 mL**
Note on storage conditions: Freshly prepare using complete IMDM with 2.5 μL of DNase added per 50 mL. Storage conditions (keep at 4°C and maximum time for storage is 2–3 months).		

Cell wash buffer		
Reagent	Final concentration	Amount
IMDM		50 mL
DNase	0.05 μL/mL	2.5 μL
7-AAD		0.5 μL /50 μL of 2% (vol/vol) FBS/PBS solution per sample
Total	**N/A**	**50 mL**
Note on storage conditions: Freshly prepare using complete IMDM with 2.5 μL of DNase added per 50 mL. Storage conditions (keep at 4°C and maximum time for storage is 2–3 months).		

Medium		
Reagent	Final concentration	Amount
IMDM (Alternatively, DMEM/F-12 can be substituted for IMDM)	1×	500 mL
Penicillin	100 U /mL	5 mL
Streptomycin	100 μg/mL	5 mL
L-glutamine	2 mM	5 mL
FBS	10% (vol/vol)	50 mL
Total	**N/A**	**500 mL**
Note on storage conditions: Freshly prepare using IMDM supplemented with 10% (vol/vol) FBS. Storage conditions (at 4°C and maximum time for storage for 2–3 months).		

⚠ CRITICAL: Working with material collected from human subjects requires Institutional Review Board (IRB) approval. Human specimens are collected after obtaining informed consent from donors.

Humanization of adult FRG breeders by surgical transplantation (steps 1–34)

At Day 2 (after birth), human hematopoietic stem cells are intrahepatically injected into adult FRG mice. The fresh human fetal cadaverous liver is commercially obtained sourced from healthy second trimester (Advanced Bioscience Resources, Inc.). After low-speed centrifugation, at 2 h post-irradiation of mice, primary hepatocytes and LPC + HSC is intrahepatically injected directly into the liver of adult mouse using a syringe (Figure 2, Top).

Expertise needed to implement the protocol

Virus amplification, purification and determination of titer need virological expertise. Tissue digestion and cell purification techniques are used. Mouse handling and surgery need expertise with IACUC approval and survival surgery tools and trainings for surgical procedures. CRISPR/Cas9-mediated gene manipulation of knockout and knock-in driver mutations in genome needs molecular and cellular biological expertise.

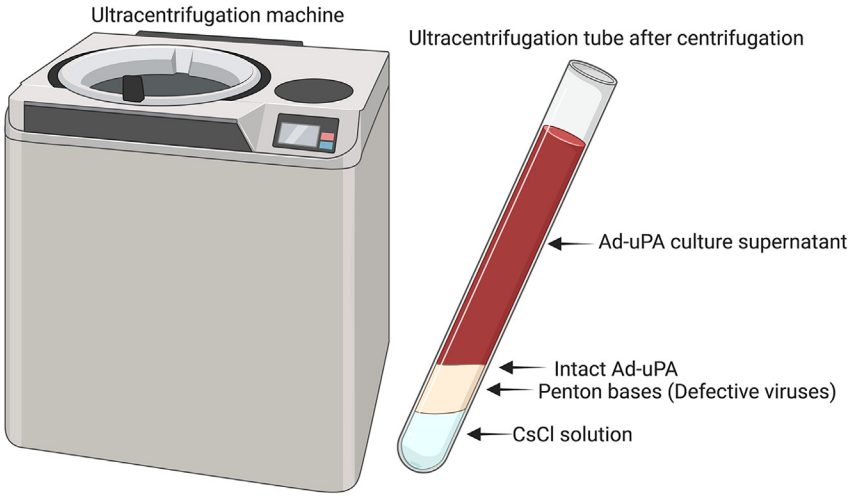


Figure 2. Ultracentrifugation separates intact Ad-uPA and penton-bases (defective viruses) by discontinuous CsCl ultracentrifugation methods

Created by www.biorender.com (Agreement number: NI258V0GH7).

Reagent setup

Antibody mix for human immune repopulation

Antibody mix for human immune repopulation: Add 2 μL of human-specific CD45 FITC, 2 μL of mouse-specific CD45 PE and 0.5 μL 7-AAD in 50 μL of 2% (vol/vol) FBS/PBS solution per sample before staining. Antibody mixture staining solution for FACS staining of blood cells collected from blood of humanized FRG mice is made as following.

STEP-BY-STEP METHOD DETAILS

Adenovirus (Ad:uPA) amplification, purification and titering (needed for in-house-preparation of Ad:uPA) [steps 1–16]:

Timing: Hands-on time: 3–9 h over 3–6 weeks, depends on the initial Ad:uPA stock virus concentration

This section describes how to amplify (from low-titer-stock to middle-titer-stock and to high-titer-stock), purify and titrate the Ad:uPA.

1. Infect HEK293A cells with seed stock Ad:uPA virus for amplification of a working stock with higher titer.

   Note: HEK293T cells do not support amplification of Ad:uPA since HEK293T cells do not have E1A and E1B (which support replication of replication defective Adenovirus that lacking E1A and E1B transgenes). HEK293A cells will complement this gene requirement.

   a. Seed a T75 flask with 1.7 × 10^6 HEK293A cells (stable transformant of Adenovirus E1A and E1B) in 4 mL of DMEM 5% FBS and incubate at 37°C overnight. Infect these HEK293A cells with 2 mL of seed Ad:uPA (low titer stock).
   b. Remove 2 mL of medium leaving only 2 mL of remaining culture medium (T25 flask).

   Note: Adsorption time is 1 h with tilting every 5 min.

   c. Add an additional 2 mL of complete medium and incubate for 5–10 days.

d. (Alternatives) Remove the medium from a 60 mm cell culture plate and add 1 mL of viral dilution per 60 mm plate instead of a T25 flask, adsorb virus by gently tilting the dish 3 times in a cross shape, add 1 mL of DMEM 5% FBS and incubate as above. For virus seed stock of 10^3–10^5 pfu/mL, use an M.O.I. of 0.01–1.1.

2. Expand serial virus for high titer working stocks.
   a. Observe if infected 293A cells are rounded and detached from culture flasks 4–14 days post-infection.
   b. Collected/pool both cells and supernatants.
   c. Freeze and thaw pellets with supernatants inside of flasks three times,
   d. Collect all cell materials.
   e. Centrifuge as above.

Note: This preparation is considered middle-titer stock.

   f. Seed approximately five million 293A cells into a fresh T75 flask and culture overnight to reach 70–80% confluency, assuming 7–9 million cells for infection.
   g. Infect 5 million cells with middle-titer stock (10 mL volume) in a new 5 × 10^6 cells/T75 flask to amplify middle-titer stock to increase Ad-uPA titer prior to creating a working stock for hepatocyte infection.

Note: Infection of 2–3 T75s at an MOI of 1 has yielded 2–3 mL of 10^9 PFU/mL. Aim for 50% confluency in flasks to give the cells room to grow. Plating of 5 × 10^6 cells/T75 yielded <50% confluency the next day. After infection, cells detach.

   h. Calculate amount of virus to add. If planing to infect with an MOI of 1, plate 6 × 10^6 cells, add 6 × 10^6 PFU Ad:uPA seed stock (no need to be exact for this).

3. Inoculate a new T150 flask with 10 million 293A cells for further expansion of virus stocks. Infect sufficient middle-titer stock preparation for an expected M.O.I of 0.3–0.7 Titer that does not need to be known at this point, but high-titer stock should have sufficient virus that is sufficient to infect almost all cells in flasks at the time of infection) The high titer stock is isolated from this flask.
   a. Use this high-titer virus stock for large scale-infection. Inoculate 3 mL of high-titer stock of Ad-uPA per T150 flask, containing 10^7 HEK293A cells. Incubate overnight. We typically use 15–20 × T150 culture flasks (initially seeded at 40 million cells per T150 flask).

Note: After 4–6 days post infection, high-titer Ad:uPA virus infection will kill 80–90% of 293A cells and cause cells to detach from the T150 flasks.

      i. If almost all cells are detached after 4–6 days post infection without reaching 100% confluency, the virus titer was sufficient to infect most of the 293A cells.
      ii. If cells do not detach from flasks during Ad:uPA infection after 6–7 days post-infection, this indicates empirically the titer is not high enough. If this is the case, return to the prior step to re-amplify for high-titer virus stock.

Pause point: At this point flasks with cell suspensions can be conveniently frozen and stored at 80°C for later virus isolation by ultracentrifugation.

4. Isolate virus by collecting infected cells by centrifugation at 1,600 *g* (3,000 rpm) for 20 min in rotor (Cat#A4-44) of #5804 Eppendorf Centrifuge.
   a. Use three freeze-and-thaw-cycles of the pelleted cells to release the intracellular Ad:uPA (freeze with liquid nitrogen or dry ice/ethanol bath).
   b. Repeat freeze-and-thaw steps.
   c. Resuspend and sonicate the cell pellets in 20 mL of non-supplemented culture medium.

d. Use 10 cycles of 30 s sonicator bursts followed by cooling 30 s on ice; avoid heating. Clarify the sonicated virus culture suspension by centrifugation at 22,000 *g* (15,000 rpm) for 15 min in Rotor Cat#A4-44 of Eppendorf Centrifuge #5804.

5. Purify Ad:uPA by discontinuous CsCl gradient centrifugation using an SW28 swinging bucket rotor.
   a. Prepare a discontinuous gradient containing 5 mL of 1.4 g/mL CsCl (53 g + 87 mL of 10 mM Tris-HCl pH 7.9) overlayed with 6 mL of CsCl 1.2 g/mL (26.8 g + 92 mL of 10 mM Tris-HCl pH 7.9).
   b. Use a 10 mL pipette to gently overlay the CsCl solutions. The final purity of the virus will depend on the quality of the gradient.
   c. Gently apply 5–8 mL of sonicated virus solution to each centrifuge tube while in a biological safety cabinet. Purify a total of up to 20 mL of viral suspension in a single centrifuge run.
   d. If the virus stock is less than 20 mL, use additional 10 mM Tris-HCl pH 7.9 to fully load the centrifuge tubes. This viral suspension should come from less than 10^9 infected cells, otherwise the gradients will be overloaded.

Note: Disruption of the CsCl layers by pipetting too quickly will negatively affect purification. The intention is to avoid making a hard pellet at the centrifuge tube bottom. A poorly formed discontinuous CsCl gradient will result in contamination of the intact virus band with penton fragments and defective virions (Figure 2). The presence of the defective particles is undesirable since these can cause hepatotoxicity and inflammatory responses after mouse injection.

6. Ensure that the tubes are well balanced before centrifugation. Centrifuge at 100,000 × *g* (23,000 rpm in SW 28) for 90 min at 4°C; allow to decelerate without braking.
7. Carefully remove the tubes from the rotor in a laminar flow hood, then secure one tube with a three-pronged clamp attached to a ring stand.
8. Aspirate most of the upper solution with impurities from the tube by use of a 10 mL pipette.
   a. Apply a piece of adhesive tape on the side of the tube where it is to be punctured. This is to prevent leakage in case the tube cracks upon puncturing.
   b. Remove the lowest, virus containing band (appears bluish-white with slight opacity) using a 5 mL syringe with an 18G needle by puncturing the tube from the side.

Note: The density of the viral band solution is about 1.345 g/mL. The area between the defective and infectious viral particle bands may appear turbid, avoid removing this area of turbidity (Figure 2).

9. Disconnect the needle from the syringe and transfer the viral suspension to a sterile 15 mL polypropylene tube. Add at least one volume of 1× TE before proceeding to the next step. This is essential to reduce the CsCl density of the solution below 1.2 g/mL.
10. Slowly load 8–10 mL of the diluted viral suspension, to the top of the gradient. Centrifuge at 100,000 × *g* (23,000 rpm with SW 28) at 4°C for 16–20 h (deceleration rate = 0) (using the same gradient conditions as earlier). After ultracentrifugation, the continuous gradient appears like the step gradient, except that there is no pellet.
11. Harvest the infectious viral band from a bottom tube puncture using a 5 mL syringe + 20 Ga needle. The high buoyancy area contains cell debris and is usually clearer (see Figure 2), although most of this debris is eliminated during the first gradient step.
12. Virus Desalting and Concentration:
    a. Choose a dialysis buffer based on the end-use of the virus and avoid buffers with glycerol if the virus is to be used in animals since these virus suspensions become difficult to inject.
    b. Avoid the use of PBS-5% sucrose buffer as that does not stabilize virus and interferes if the virus needs to be further concentrated to more than 5 × 10^{11} VP/mL.

Note: Use 10 mM Tris pH 8.0, 2 mM $MgCl_2$, 4% sucrose buffer to avoid virus precipitation and maintain virion stability for concentration to approximately 1 × 10^{13} VP/mL.[6]

13. Dialyze the viral band at 4°C with a cellulose ester membrane (Spectra/Por, Spectrum Lab, 25,000 MWCO) to remove buffer salts, mainly cesium chloride.
    a. Dialyze the virus suspension against 200 volumes of the appropriate buffer for 1 h, with three changes.

    Note: There should be no traces of CsCl remaining after three changes of dialysis buffer.

    b. Aliquot dialyzed virus suspension into Eppendorf tubes (100, 250 and 500 μL) and keep at -80°C for long-term storage; however, storage at -20°C for short periods of time is acceptable.

    Note: Repeated freeze/thaw cycles will reduce virus infectivity and should be avoided.

    Note: The purity of Ad:uPA is critical since penton base contamination in the final Ad:uPA solution is hepatotoxic and greatly increases the potential for liver damage andinflammation.

14. Plaque Plaque Assay Method: 293A cells are seeded into 96 well plates and infected with serially diluted virus stock to measure virus titers (it will take at least 10–14 days to determine the virus titer).
    a. Dilute the virus stock to be assayed in a 12-well plate. Store the plate at −20°C or −80°C for further use. Initially dilute the low-titer viral stock in 1 mL to minimize the amount of virus needed.
    b. Perform additional dilutions in a volume of 3 mL; the use of a larger volume increases reproducibility. The number of serial dilutions is a function of the type of virus stock (purified vs. non-purified).
    c. Design the dilutions to allow a count of 10–100 particles per test well. Typically, we end at a final dilution of 10^{-12}.
    d. Plate with dilutions of 10^{-7} to 10^{-12} for plaque testing.
    e. Begin dilutions by adding 100 μL of virus stock to 900 μL of DMEM + 5% FBS. Pipet up and down 5 times, discard the tip; this is dilution 10^{-1}.
    f. Pick up 300 μL of the 10^{-1} viral dilution using a new tip and add it to 2.7 mL of DMEM 5%, pipet up and down 5 times and discard the tip; this is dilution 10^{-2}.
    g. Perform serial dilutions using 300 μL of the previous dilution and 2.7 mL of DMEM + 5% FBS.
    h. Use the last 4 dilutions to infect QBI-293A (or any 293A) cells, always use a fresh tip for each virus addition.
15. Incubate for 90 min at 37°C in a cell culture incubator with 5% CO_2.
    a. Aspirate the medium and cover the cell monolayer with prewarmed 1.25% agarose mix for titer determination.
    b. Return plate to 37°C incubator.
    c. Observe the dishes regularly for plaque formation and for the need to add fresh medium.
    d. After 21 days, all plaques should be visible to the naked eye as small white spots.
16. Count the number of plaques in the dilution that gives well-isolated plaques. To calculate the plaque forming units per mL (PFU/mL), multiply the count by the dilution factor.

    Note: The anticipated virus yield ranges from 30–40 mL of 2 × 10^{11} pfu/mL to 2 × 10^{12} pfu/mL isolated from 20 × 150 cm^3 T150 flasks.

Primary adult hepatocyte recovery

Timing: 3–4 h (for steps 17–26)

This section describes how to recover primary hepatocytes from frozen samples. These are adult hepatocytes, so that this section may be referred to from a relevant following section.

17. Place the cryogenic vial into a water bath to thaw, but do not completely submerge the vial to avoid bacterial contamination of the sealing area (Thawing of cells should be rapid (<2 min) to minimize viability loss).
    a. Gently shake the vial back and forth to achieve even thawing while continuously monitoring the contents.
    b. Remove the vial from the water-bath when only a few small ice crystals remain in the vial; spray the vial with 70% alcohol, wipe dry, and proceed to the cell culture hood.
    c. Transfer the vial contents to the Recovery Media tube [ISOM's medium with 10% FBS (BD Biosciences; Corning Gentest CryoHepatocyte One-Step Purification kit)], rinse the vial with Recovery Media and combine the wash.
    d. Wash cryopreserved hepatocytes (1.5 mL) in 34.7 mL ISOM's medium (BD Biosciences; Corning Gentest CryoHepatocyte One-Step Purification kit) containing 11.8 mL Percoll.
    e. Only use low endotoxin Percoll (e.g., Percoll PLUS) and before use centrifuge at 1,300 × *g* for 1 h at 4°C to remove potential small crystals.
18. Recover cells by centrifugation at 100 *g* for 10 min (medium acceleration/low brake).
19. Carefully aspirate and discard the entire supernatant containing dead cells and cell debris without disturbing the pellet (care should be taken when aspirating dead cells after centrifugation to avoid contaminating the live cells).
    a. Move pipette tip in a circular motion, just touching the surface of the supernatant; this will assist the purification by avoiding aspiration of live cells while aspirating.
20. Add 2 mL/vial of pre-warmed Plating Media with 10% FBS by pouring along the side of the tube, avoid adding the media directly onto the cell pellet.
21. Resuspend the pellet using a gentle rocking motion. Resuspend the cells carefully (do not pipet up and down) in 10 mL ISOM's medium and centrifuge at 50 ×*g* for 5 min.
22. Add 5 mL of FBS to the Plating Media tube, prewarm the Recovery Media (DMEM/F-12 hepatocyte culture medium) to 37°C.
23. Resuspend cells carefully in 1 mL of Recovery Media (DMEM/F-12 hepatocyte culture medium) and count the cells with trypan blue for viability.

    Note: Viability should be better than 90%.

24. Use a 2 mL pipette to triturate two to three times gently to obtain a homogeneous cell suspension (do not pipet up and down) in 10 mL ISOM's medium.
25. Centrifuge at 50 × *g* for 5 min when necessary.
    a. Record cell volume, viability, and recovery.
26. Dilute resuspended hepatocytes to 10^6 cells/mL with 5–10 mL prewarmed PBS medium.
    a. (In case other experiments will be performed using plated hepatocytes, proceed with the following steps) Use a repetitive pipettor or multichannel pipet to dispense cells into BD BioCoatTM Collagen I coated plate (e.g., 400 µL/well of 24-well plate).
    b. Move the plate gently in a star pattern on a level surface to distribute the cells evenly over the bottom of the plate after cell plating is completed.
    c. Place plates in a 37°C, 5% CO_2 incubator.
    d. Remove plates from the incubator and gently rock the plates to redistribute the cells evenly in the wells every 20–30 min during the first 2 h of plating.
    e. Gently tap the edge of the plate to redistribute the cells since excessive accumulation of cells in the center of the wells can cause cell death in central regions of wells.
    f. Gently aspirate the Plating Media after 2–4 h and gently refeed cells with complete Hepatocyte Culture Media (or customized-preferred hepatocyte culture media).
    g. Keep plates in the incubator overnight for further experiments as required.

Dual humanization protocol of breeder *FRG* mice

Timing: two months (for steps 27–33)

This following section describes the dual humanization protocol of breeder *FRG* mice.

27. Co-transplant LPC and human hematopoietic stem cells into female and male *FRG* or *FRGN* (FRG mice in NOD/Scid background) breeders.[7]
    a. Transplant all breeder animals as weanlings (~ 4–7 weeks of age) by intrahepatic or intrasplenic injection of one million cells from strain-matched immune proficient adult donor animals (*C57/BL6* or *NOD/ShiLtJ*, Jackson Laboratories) or nucleated human liver cells of human adult primary hepatocytes to avoid the complications of immune deficiency during breeding.

    Note: All fetal and adult animal procedures should be approved by the local IACUC.

    b. The reconstituted immune systems have 35–60% chimerism and are not stably maintained in some mice depending on the quality of adult human primary hepatocytes[7] or mouse primary hepatocytes.
    c. Place the transplanted animals into breeding cages at six weeks of age.[7]

Pre-conditioning

Timing: 2 days (for steps 28–33)

This section describes how to pre-condition the FRG mice prior to stem cell injections to improve stem cell engraftments in these recipient animals. This is done for both breeders and experimental neonates.

28. Day -1: Dilute Ad:uPA[7] to 1.25 × 10^9 pfu/100 μL in sterile 0.9% saline or PBS (-) and filter using an Acrodisc Syringe filter (0.45 μm HT Tuffryn membrane).

    Note: For example, to make a suspension of 5 × 10^9 pfu/mL from 1.5 × 10^{11} pfu/mL virus stock, we add 50 μL stock to 15 of mL sterile 0.9% saline).

    a. Precondition neonates with SPX/TMP antibiotics and plasminogen activator urokinase-expressing adenovirus (Ad:uPA).
    b. Inject Ad:uPA (5 × 10^9 pfu/mouse) via the retro-orbital vein under isoflurane anesthesia.
    c. Inject 200 μL (1 × 10^8 pfu/20 g mouse) of diluted Ad:uPA with a BD Ultra-Fine II Short Needle Insulin Syringe - 31G 3/10cc 5/16" (BD Medical Systems: Cat #328438).
       Examples of virus dosages used for injection.
       i. 19-g mouse: 190 μL (0.95 × 10^8 pfu).
       ii. 20-g mouse: 200 μL (1 × 10^8 pfu).
       iii. 21-g mouse: 210 μL (1.05 × 10^8 pfu).
       iv. 24-g mouse: 240 μL (1.2 × 10^8 pfu).
    d. X-ra irradiate mice with 150 cGy X-ray.
29. At Day 0, intrahepatically inject human hematopoietic stem cells into adult FRG mice (See Humanization of FRG breeders). See step 33 for preparation of human fetal liver tissue prior to transplantation.
30. Maintain mice with NTBC in drinking water for adult FRG breeder female mice surgically transplanted with adult human primary hepatocytes by splenic or intrahepatic injection. House mice in a pathogen-free facility in microisolator cages.
31. Isolate human adult hepatocytes on day of liver cell transplantation.

a. Isolate human adult hepatocytes from an excess donor organ that was used for liver transplantation. The details of the isolation method are described here.[5,8,9]
b. Transfer the pelleted hepatocytes into cold University of Wisconsin solution (Viaspan).
c. Co-transplant human hepatocytes purchased from BioreclamationIVT (catalog #F00995) with isolated bone-marrow-derived cells.[7]
d. Use fresh human fetal liver of healthy full-term newborns obtained commercially. After density gradient centrifugation, E-cadherin+ LPCs and non-parenchymal cells, including CD34+ cells are injected.
e. Use optimized media and procedures to fully recover cell viability and maximize and metabolic profiles.
f. Thaw Cryopreserved human hepatocytes and remove the cryopreservation solution by centrifugation at 100×*g* for 5 min at 4°C.
g. Resuspend cell pellets in HCM media.
h. Re-centrifuge at 100 × *g* for 5 min at 4°C and resuspended in HCM at 10^7 cells/mL.
i. Resuspend hepatocytes and dilute at 1:1 ratio in 0.4% trypan blue and the cell number and determine viability using a hemocytometer.
j. Thaw cryopreserved human bone marrow CD34+ cells.
k. Remove cryopreservation solution by centrifugation at 500 × *g* for 10 min at 4°C followed by resuspension in HCM.
l. Dilute the resuspended CD34+ HSC at 1:1 ratio in 0.4% trypan blue and the viable cell number determined.
m. Centrifuge by density gradient at 2 h post irradiation
n. Resuspend CD34+ cells in sterile 0.9% saline (use 5 × 10^5 in 25–50 μL for transplantation.
o. Determine cell numbers and viability by Trypan blue exclusion in a hemocytometer.

Note: Immediately transplant immediately the received hepatocytes upon receipt, ideally within 24–48 h after isolation.

32. Inject intrahepatically the adenoviral vector expressing the secreted form of human urokinase (uPA)[10] in adult 8–12-week-old FRG mice.
33. Anesthetize the mice for cell injections using isoflurane anethesia. Mix the hepatocytes and CD34+ cell suspensions at a 1:1 volume ratio and inject 500,000 hepatocytes and 200,000 bone marrow CD34+ cells into each mouse.
    a. Draw 20 μL containing 500,000 human hepatic progenitor cells and 20,000 bone marrow cells into a 1 cc syringe fitted with a 27 G needle.

    Note: The needle should be inserted a few millimeters inside the liver taking care not back the needle out of the organ. As the liver is a

    b. Inject slowly the hepatocytes into the liver to prevent it from bursting. The organ will swell, leave the needle in for a few seconds to equalize the pressure and then slowly remove it. Apply the hemostat absorbable knit to stop the bleeding.
        i. Insert the needle a few millimeters inside the liver taking care not back the needle out of the organ.
        ii. Inject the cells with great care to prevent rupture of the organ as the liver is a delicate organ.

    Note: To achieve this, the syringe should be of high quality with a smoothly moving plunger.

    c. Gently remove the needle. Apply hemostat absorbale cotton applicater to stop any breeding.

    Note: A small amount of bleeding can occur after the needle is removed and can be blotted with sterile gauze.

d. Place the mouse back in its cage.
e. Check each mouse after 30 min to see if recovery is proceeding after transplantation.
f. Administer NTBC through drinking water for breeding cages, although published accounts report that within 1 month of birth, pups will survive without NTBC.

Note: For maintenance of humanized mice after one month, NTBC is required for viability.

g. Provide NTBC in drinking water to maintain adult FRG mice receiving human hematopoietic xenografts.

Note: These mice grow well and are fully fertile if they are continuously maintained on NTBC.

h. House these mice in a specific-pathogen-free facility using microisolator cages.
i. Do not co-house FRG neonates with the father to prevent cannibalism of the neonates after transplantation.
j. House the mother with the neonates after the transplantation procedure.

Note: If the mother senses that something is wrong with her neonates, the mother may cannibalize her neonates to provide more time for the neonates to recover.

i. Remove any residual blood droplets in the injection area by careful swabbing with water and dry with sterile gauze to avoid cannibalism.
k. Day 7: Feed mice with irradiated food and maintained on acidified, autoclaved water with or without sulfamethoxazole-trimethoprim (SMX/TMP) (7.8 mL of SMX/TMP per 250 mL of drinking water) on alternate weeks after weaning (typically 3 weeks post-partum) for the duration of the animals' lifetimes and housed $\leq$5 humanized animals per cage.
l. Decrease NTBC cyclically for the pups after transplantation and completely withdraw NTBC to allow engraftment and growth of human hepatocytes (the schedule of withdrawal of NTBC is outlined in Table 2).

Note: FRG mice grow well and are fully fertile if they are continuously provided NTBC in drinking water. Maintain breeding pairs on NTBC drinking water their entire lives. Note that after the first month NTBC dosing is required for newborn survival (See Table 2).[4]

⚠ CRITICAL: Failure to maintain mice under aseptic conditions at all times will result in lower organ reconstitution and/or will increase opportunistic infections.

Note: As the *in utero* dose is lower than the actual serum concentration, a higher concentration of NTBC is required for neonate liver reconstitution for both males and females. To avoid nursing failure, humanization of parental *FRG* mice is critical to avoid liver failure of mothers during the nursing period of neonates between day 0 to three-four weeks of age or before weaning.

Note: Diet hydrogel is an option for hydration, but without NTBC, dietary tyrosine will result in liver toxicity and damage. Neonatal pups are monitored for vitality, consequently pups that are not thriving may not survive the transplantation procedure and should be euthanized.

Breeding humanized FRG mice for neonate production and pre-conditioning

Timing: 21–40 days (for steps 34–35)

Table 2. NTBC cycling protocol

Week	M	T	W	Th	F	S	Sn
1 (with NTBC/SMX/TMP)	0	0	0	0	0	0	0
2	8	8	8	8	8	0	0
3 (with NTBC/SMX/TMP)	0	0	0	0	0	8	8
4	8	8	8	0	0	0	0
5 (with NTBC/SMX/TMP)	0	0	0	8	8	8	8
6	0	0	0	0	0	0	0
7 (with NTBC/SMX/TMP)	8	8	8	8	0	0	0
8	0	0	0	0	8	8	8
9 (with NTBC/SMX/TMP)	0	0	0	0	0	0	0
10	8	8	8	0	0	0	0
11 (with NTBC/SMX/TMP)	0	0	0	0	0	0	0
12	0	0	0	0	0	0	0
13 (with NTBC/SMX/TMP)	0	0	0	8	8	8	8
14	8	0	0	0	0	0	0
15 (with NTBC/SMX/TMP)	0	0	0	0	0	0	0
16	0	0	0	0	0	0	0
17 (with NTBC/SMX/TMP)	0	8	8	8	8	8	0
18	0	0	0	0	0	0	0
19 (with NTBC/SMX/TMP)	0	0	0	0	0	0	0
20	0	0	0	0	0	0	8
21 (with NTBC/SMX/TMP)	8	8	8	8	0	0	0
22	0	0	0	0	0	0	0
23 (with NTBC/SMX/TMP)	0	0	0	0	0	0	0
24	0	0	0	0	8	8	8
25 (with NTBC/SMX/TMP)	8	8	0	0	0	0	0
26	0	0	0	0	0	0	0
27 (with NTBC/SMX/TMP)	0	0	0	0	0	0	0
28	0	0	8	8	8	8	8

This following section describes breeding humanized FRG mice for neonate production and pre-conditioning.

34. Crossbreed nursing female *FRG* mice with humanized *FRG* males to generate *FRG* neonates 8–12 weeks post liver cell transplantation.
35. Examine females daily in the early morning after breeding for the presence of a vaginal plug (Plug-check) (preferably before 8:00 am or at the latest 9:00 am accounting for nocturnal rodent behavior).

Note: Failure to examine early in the day may result in an absent plug due to it falling out. Plug checks are needed to estimate day of conception and to predict the FRG neonate birthdate (normally twenty days after plug formation). The expected birth date is used for experimental planning to prepare reagents and pre-order human fetal livers or the isolated cells.

CRISPR/Cas9-medated gene knockout and /or knock-in of human fetal LPCs transplanted into neonate livers

Timing: 3–5 h (for steps 36–41)

36. Isolate fetal liver-derived hepatocytes (Pre-Electroporation).

Note: Transfecting unstimulated cells will generally result in lower transfection and editing efficiencies.

a. Remove red blood cells by use of red blood cell lysis buffer to negatively select cells.
b. Prepare 5 × 10^7 fetal liver-derived hepatocytes (low-speed precipitated cells or E-Cadherin+ cells by use of magnetic beads for anti-E-Cadherin Magnetic MicroBeads: Milteny) and wash in sterile 0.9% saline (alternatively 1× PBS buffer).
c. Use cell Isolation Kit as per manufacturer's protocol.

37. Setup equipment for electroporation

Note: Wear gloves and use nuclease-free tubes and reagents to avoid RNase contamination.

a. Always maintain aseptic technique and use sterile, filtered pipette tips.
b. Store all Synthego reagents according to the manufacturer's recommendations.
c. Dissolve synthetic sgRNA in TE buffer and dilute to a working concentration using nuclease-free water. Refer to Synthego.com/resources
d. RNP complexes are stable at 25°C for up to 1 h (may be stored at 4°C for up to one week, or at −20°C for up to 1 month). Note that RNPs stored at 4°C may become susceptible to contamination from microbial growth after long periods of time.

Note: Suggested Controls

i. Mock: No Cas9 or sgRNA: Wild type sequence for comparison with experimental and other negative controls. Controls for toxicity from RNP, cell death from electroporation, or possible viability issues associated with editing the specific gene of interest.
ii. Negative control: Cas9 complexed with a non-targeting sgRNA (this is the same as scrambled sgRNA) or no sgRNA: This ensures that there are no false positives due to contamination (no effect expected = wild type).
iii. Positive control: Use sgRNA that has validated high editing efficiency. This ensures that all reagents, protocol, and equipment are functioning properly.

38. Assemble RNP Complexes (2.5:1 sgRNA to Cas9 ratio).
a. Prepare 50 μM sgRNA in 1× Tris-EDTA and store at −80°C until use. Prepare 20 μM Cas9.
b. Mix RNPs as per the volumes given below for a single reaction (scale up appropriately as needed). Note: a ratio of 1:1 sgRNA: Cas9 may also be used.
RNP Components, concentration and volumes.
i. sgRNA: 50 μM (pmol/μL): 1 μL (50 pmol).
ii. Cas9: 20 μM (pmol/μL): 1 μlL (20 pmol).
iii. Total volume: 2 μL.
c. Incubate at 25°C for 10 min.

39. Count cells.
a. Gently resuspend the cells.
b. Count suspended cells using a hemocytometer or automated cell counter.
c. Alternatively cells can be incubated for 2–24 h after bead removal by use of Milteny Magnetic MicroBead kit protocol, before transfection.
d. Wash in 1× Neon buffer T (electroporation buffer).
e. Resuspend cells at a concentration of at least 3 × 10^7 cells/mL in Neon Buffer T.

40. Transfect cells.
a. Add 10 μL of cells (3 × 10^7 cells/mL) in Buffer T to each RNP sample and mix thoroughly.
b. Electroporate the cell-RNP mixture using Neon® Transfection System at the following settings: 1400 V, 10 ms, 3 pulses. Go to step 63 for intrahepatic injection of cells into FRG neonates.

Note: Work quickly and carefully to avoid leaving cells in Neon Buffer T for longer than 15 min. Avoid bubble formation.

41. Determine editing efficiency from a portion of the electroporated cells for confirmation purposes.
    a. Analyze by PCR, Sanger sequencing, and/or inference of CRISPR Edits (ICE) analysis.
    b. Visit synthego.com/resources for a Genotyping protocol (primer design, DNA extraction, and PCR) to prepare DNA for Sanger sequencing, and a protocol on how to run and interpret an ICE analysis.
    c. Conduct next-Generation Sequencing, FACS, Western blot, or functional assays.
    d. Extract DNA from the electroporated cells that have not been used for transplantation (Following protocol step 42 is for validation purposes only.

Note: These cells should not be used for transplantation into FRG mice because long-term-culturing of fetal liver cells greatly decreases engraftment efficiency).

Mouse humanization with liver and non-parenchymal cells

[Timing: 2–3 days; Hands-on Time: 3–4 h; steps: 42–45] depending on numbers of animals to transplant, 5–10 min per mouse (4 injections), 10–20 min for LPC+ non-parenchymal cell injections, 8–12 weeks for *in vivo* humanized liver and immune system reconstitution.

This section describes humanization of liver and immune systems of FRG neonates.

(Steps 45–42).

42. Day -1: Inject Adenoviral vector expressing uPA (Ad:uPA) (i.p.: 100µL in PBS) between days 0–5; 24 h before irradiation for FRG *mice*.
    a. Place one pup on the Petri dish with cotton or on the foil in the ice bucket and let the animal cool for about 1 min.
    b. Place newborn FRG pups on a gauze pad in a petri dish for irradiation and subsequent injection.

⚠ CRITICAL: This is for light anesthesia.

    c. Place the pups on a 100-mm^2 petri dish along with a small amount of bedding material from the breeder cage on a warming pad or under an incandescent lamp for 1–2 min to tranquilize neonates.
    d. Prepare an ice bucket wrapped with autoclaved aluminum foil in the cell culture hood.

⚠ CRITICAL: Be careful when handling the foil so that the side the pups are in contact with is not contaminated by unsterilized surfaces.

    e. Set up the warming light in the hood and put a cage lid covered with sterile paper towels under a warming pad (Figure 3, Left).

⚠ CRITICAL: This is for mouse recovery after cold anesthesia and injection.

        i. Rub the bedding in gloved fingers with gloves to erase rubber smell of rubber gloves.
        ii. Put a clean cage, as well as the cage the pups are in, in the hood; separate the mother from the pups, move the pups to the clean cage and move the mother back to the housing rack.
        iii. Place one pup on the foil in the ice bucket and let the animal cool for about 1 min.

⚠ CRITICAL: This is for light anesthesia.

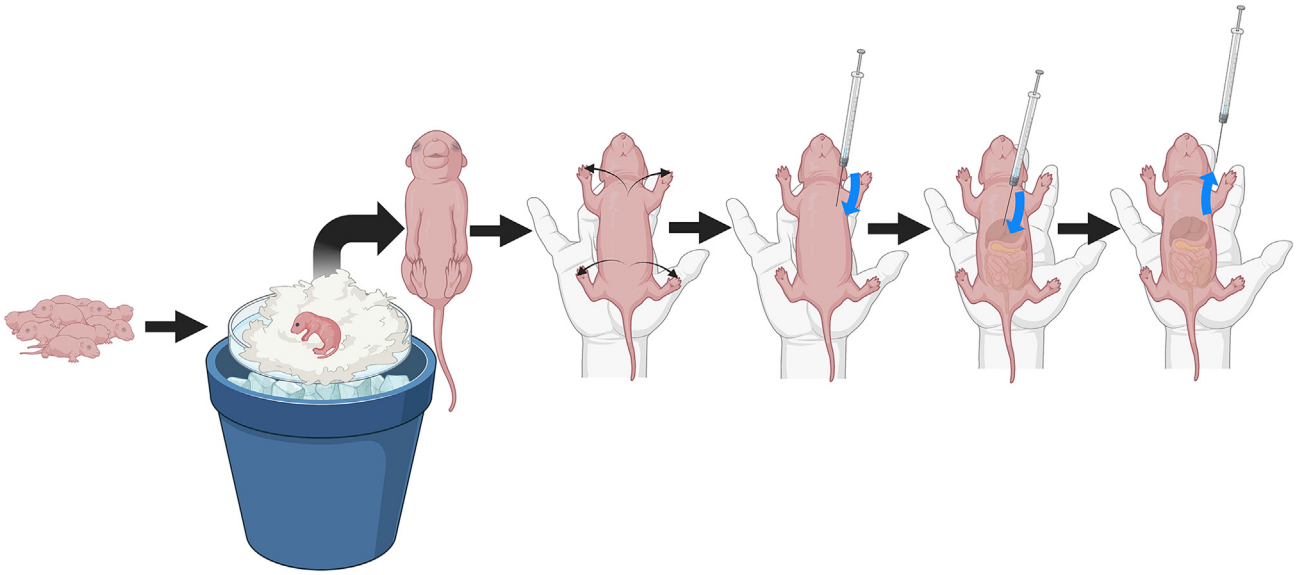


Figure 3. Neonates are put on ice to reduce mobility and grabbed by hand to inject fetal liver-derived cells with CRISPR/Cas9-based gene manipulation by use of Hamilton needles

Hamilton needle is stubbed from left shoulder area into livers of FRG neonates (1–5 days of age). Gloves are worn. Created with www.biorender.com (Agreement number: AB258UZ6K5).

f. Restrain the pup by placing your index finger between its front legs (slightly cranially to where you would expect the collarbone to be) and your thumb between its hind legs and applying gentle pressure down and outward (just enough to pull the skin a little tight without injuring the animal) (Figure 3, see the second from the far left).
   i. Begin by vertically positioning the needle to the left of the right lobe and angled toward the right lobe.
   ii. Push the needle in halfway and deposit the cells into the right lobe at the rate of 2 s per injection volume.

Note: The liver should be visible as a dark area located cranially to the stomach; it should be the largest organ in young pups.

   iii. Identify the stomach by a white color on the animal's heart side.\
   iv. Release the pressure immediately with your fingers.
   v. Remove the needle, being careful to avoid contact with your finger (Figure 3).

g. Keep neonates warm and quickly finish procedures in order to return neonates to the mother to avoid possible rejections since different temperature and smells of the neonates may cause rejection by their mother.

h. Clean the Hamilton (705SN30/51MM/12″ 25 μL) Syringe with Ethanol and PBS 3 times each before reuse (Figure 3, Right).
   i. First flush engraftment syringe with 70% ethanol three times.
   ii. Flush PBS three times (Note: Avoid use of bleach solution)

43. Day 0: Place the newborn FRG pups on a gauze pad in a petri dish for irradiation 24 h after injection of adenoviral vector. Tranquilize neonates by laying neonates in Petri dish with gauze placed on ice for 5–10 min.
44. Irradiate newborn mice over a 3–4 h-interval with a total dose 150 cGy using a 2 mm filter (field size 10 cm × 10 cm) from an X-ray source at a dose that is titrated to be sub-lethal.

Note: A total of 150 cGy is used. If FRG neonates in your vivarium facility cannot tolerate a single 150 cGy dose, two separate 75 cGy irradiation fractions can be given (75 cGy in the morning followed by the second 75 cGy dose in the afternoon).

45. Keep the field size at 10 cm × 10 cm for the irradiation.

Liver digestion and intrahepatic injection of human LPC (CRISPR/Cas9-medated gene manipulated) and non-parenchymal cells

Timing: 4–5 h; Duration: 6–7 h (for steps: 46–48; Hands-on)

This section describes liver digestion and CRISP/Cas9-mediated genetic manipulation of hepatocytes.

46. Fresh human fetal cadaverous liver can be sourced commercially from healthy second trimester fetuses (Advanced Bioscience Resources, Inc.) or obtained from your institution.
47. Obtain IRB approval for human fetal liver transplantation if your institution is providing human fetal liver tissues.
48. Collect human fetal livers into 50 mL tubes with 10 mL of Hypothermosol (Stem Cell Technology, INC) to preserve the viability and make better recovery.

⚠ CRITICAL: To mimic stepwise carcinogenesis steps through cancer initiation and promotion phases, genetic manipulations by use of CRISPR/Cas9-mediated knockout of ARID1A and/or constitutively active (c.a.) CTNNB1 are knocked-in LPCs and intrahepatically injected into FRG neonates and humanized livers and immune systems.

a. Place 24- to 48-h post-neonatal pups from a single litter into a 100-mm2 petri dish along with a small amount of bedding material from the breeder cage.
b. Place the next neonate on ice during the first pup injection is taken place (the actual injection step takes only several seconds, and the other pup can be cooled down by the time you are finished with the first one).
c. Digest to isolate liver progenitor cells (LPCs) and non-parenchymal cells.
d. Centrifuge the digested cells at 50 × *g* during 5 min.

⚠ CRITICAL: Don't keep pups away too long from the mother.

e. Pick up the bedding often.
f. Locte at the milk spot of neonates.
g. Changes gloves before you touch different parent-derived neonates to avoid cannibalism due to smells from different litters or smell of nylon gloves and to avoid attaching different smells from other group of mother and neonates. Watch if mother accept neonates for breast feeding first few days.
h. Restrain the pup by placing your index finger between its front legs (slightly cranially to where you would expect the collarbone to be) and your thumb between its hind legs and applying gentle pressure down and outward (just enough to pull the skin a little tight without injuring the neonates).
i. Inject non-parenchymal cells, including CD34+ cells (3 × 104 CD34+ cells) and CRISPR-Cas9-transfected hepatic progenitor cells (3 × 104 E-cadherin+ cells).
j. Return the neonates to respective mothers and observe if mother accept neonates for breast feeding first few days.

Digestion of human fetal livers for intrahepatic injection of human LPC, non-parenchymal cells with human hematopoietic stem cells

Timing: 12 h (for steps 49–61; Hands-on Time: 3–4 h) (Figure 2)

This section describes liver digestion of human fetal livers and intrahepatic injection of human LPC, non-parenchymal cells with human hematopoietic stem cells.

49. Warm up the wash buffer in a 37°C water bath.
50. Add 2.5 μL of DNase at 5 mg mL^{-1} (Roche, cat. no. 10104159001) for every 50 mL of liver digestion medium (Gibco, cat. no. 17703). Prepare 100 mL per liver to be digested (Catalog number: 17703034, Thermofisher Scientific).
51. Freshly prepare the Wash Buffer using complete IMDM with 2.5 μL of DNase (Catalog number: 17703034, Thermo-Fisher Scientific) added per 50 mL.
52. Insert cannula and inject digestion solution into fetal liver tissues. Pour a single 15- to 18-week gestation period human fetal liver into a 10-cm dish; use scalpels to separate the connective tissue from the parenchyma.

⚠ CRITICAL: Human tissues and isolated cells are aseptically handled in a biological safety cabinet (class II). Special precautions are required for HBV-associated HCC due to high transmission ability by needle puncture incidences.

53. Discard the connective tissue and mince the parenchymal tissue into small pieces (1–2 mm^3) with scalpels or sterile razor blades.
54. Transfer the minced tissue to a 50-mL tube with a 25-mL pipette; wash the dish with 10 mL of liver digestion solution.
55. Fill the tube with liver digestion solution to a 40 mL total volume and wrap the tube cap with Parafilm to avoid leakages and contamination. If available, use sealed buckets to prevent contamination during centrifugation.
56. Refill tube with liver digestion solution, decant the tubes, tap tubes for 6–8 times, incubate the tube in the 37°C water bath for 30 min and decant the tube several times every 5 min.
57. Allow the larger pieces to settle to the bottom; remove the supernatant portion and filter it through a 70μm filter (Corning, Inc.: 352350: 08-771-2) collect filtrate into a new 50-mL tube; place the new tube on ice.
58. Add 35 mL of liver digestion medium (Gibco, cat. no. 17703) to the tube (from step 76) containing the larger pieces of liver tissue and repeat steps 8 and 9.
59. Centrifuge the two tubes with liver cells at 252*g* for 10 min at 4°C.
60. Decant the supernatant, resuspend the cells with 40 mL of wash buffer and combine the tubes into one 50-mL tube.
61. If viability is less than 60%, delay transplantation. Go to step 62 or 66 for constitutely active CTNNB1 CRISPR/Cas9 knockin procedures (Step 62) and/or ARID1A gRNA (Steps 66-68) electroporation into cells.

Constitutively active (ca) CTNNB1 knock-in procedures

Timing: 7-12 days (for step 62)

This section describes how to introduce c.a.*CTNNB1* in the human stem cell genome by use of CRISP/Cas9-mediated donor DNA insertion techniques.

This section describes procedures for constitutively active (ca) *CTNNB1* knock-in.

62. Synthesize donor DNA and left gRNA and right gRNA.
    a. β-Catenin ssDNA
    b. The reference human CTNNB1 exon 3 amino acid sequence that is frequently mutated to create a stabilized β-CATENIN:
    >EMBOSS_001_1: DLMELDMAMEPDRKAAVSHWQQQSYLDSGIHSGATTTAPSLSGKGNPEEE DVDTSQVLYEWEQGFS.

c. Human c.a.CTNNB1 Donor DNA sense sequence CTNNB1 exon 3 mRNA nucleotide positions 1–200:
CTGATTTGATGGAGTTGGACATGGCCATGGAACCAGACAGAAAAGCGGCTGTTAGTCAC
TGGCAGCAACAGTCTTACCTGGACGCTGGAATCCATGCTGGTGCCACTGCCACAGCTCCT
GCTCTGAGTGGTAAAGGCAATCCTGAGGAAGAGGATGTGGATACCTCCCAAGTCCTGTAT
GAGTGGGAACAGGGATTTTCT (Note: The underlined Gs are base substitutions to convert artificial mutation sequences that substitute Serine or Threonine residues to Alanine to make the non-phosphorylatable, stable mutant of β-Catenin to stabilize β-CATENIN, which results in constitutively-active β-CATENIN).
d. β-CATENIN AA: 1–90: 1 matqadlmel dmamepdrka avshwqqqsy ldSgihSgat tTapSlsgkg npeeedvdts qvlyeweqgf sqsftqeqva gknidgqyamc.a.CTNNB1 AA: 1–90: 1 matqadlmel dmamepdrka avshwqqqsy ldAgihAgat tAapAlsgkg npeeedvdts qvlyeweqgf sqsftqeqva gknidgqyam
e. Left sg-CTNNB1: AATACTGTTTCGTATTTATA
f. Right sg-CTNNB1: AGTCCTTCACTCAAGAACAA

CRISPR Editing Human fetal LPCs with RNPs using Neon Electroporation

Timing: 3 h (for steps 63–66)

This protocol describes how to deliver by electroporation of stimulated human primary LPCs with ribonucleoprotein (RNP) complexes that consist of purified Cas9 nuclease duplexed with chemically modified synthetic single guide RNA (sgRNA).

63. Mix gRNA and the annealed donor DNA (Human c.a.*CTNNB1* Donor DNA sense sequence CTNNB1 exon 3 mRNA nucleotide positions 1-200) with recombinant Cas9 proteins.
64. Incubate solution at 25°C for 15 min to make RNA-Cas9 protein complexes (RNP).
65. Electroplate RNA complexes into target LPCs. Accomplish RNP delivery using the Thermo Fisher Neon™ Transfection System.
66. Incubate the electroplated cells in a 37°C incubator with 20% CO_2 concentration.
    a. Electroporate RNP complexes into these LPCs (Refer to steps 81-step 85),
    b. Intrahepatically inject these electroporated cells into *FRG* neonates (1–5 days of age) (Refer to steps 75-83).
    c. Keep neonates with nursing mother *FRG* mice in the same cages.

    Note: In case of extensive cell death after electroporation, Ficoll-pack-based live cell isolation will be performed. To avoid extensive cell deaths, the optimal cell concentration (1 million cells per 200 μL) is required to avoid the extensive cell death.

    Note: If the RNP complex formation is not properly formed, the knock-in or knockout efficiency will be reduced. To avoid a false positive or unsuccessful knock-in or knockout, after electroporation, a small fraction of cells should be cultured to test the knock-in and/or knockout efficiency. These control follow-up experiments will exclude false-positive electroporation and serve as a good quality control step.

    d. Place NTBC-containing water bottles continuously until completely removed after seven days eventual decrease of NTBC concentration.
    e. Install NTBC-containing water bottles in mouse cages and use the schedule in Table 2 for water and NTBC changes.
    f. Add antibiotics to NTBC-containing water bottles for avoidance of bacterial infection in immunocompromised FRG mice.

⚠ CRITICAL: Once human immune systems are reconstituted after 8–12 weeks post electroporation of hematopoietic cell-containing non-parenchymal cells, these FRG mice acquire relatively normal immune systems. Feeding antibiotics to completely humanized FRG mice at this point is optional.

g. Eight weeks after stem cell intrahepatic injection, begin feeding FRG mice with alcohol Western diet for six months to accelerate HCC development by synergistic interactions between cancer driver mutations and environmental factors i.e., alcohol Western diet feeding.

ARID1A knockout CRISP/Cas9 by use of sg-RNA electroporation and cell injection into humanized FRG mice

Timing: 2–4 h (for steps: 67–71)

This section describes how to disrupt ARID1A in human liver progenitor cells by use of two gRNAs, donor DNA and recombinant Cas9 proteins via gRNA electroporation and cell injection into humanized FRG mice.

67. Rehydrate the Multi-guide ARID1A sgRNA.

⚠ CRITICAL: Synthego's multi-guide ARID1A sgRNA ships dry at ambient temperature and remains stable for several weeks at 25°C. Dried ARID1A sgRNA may be stored at -20°C for long-term storage (up to 3 years).

⚠ CRITICAL: Be sure to work in an RNase-free environment by use of RNAse-free water (DEPC-treated DW: treat water with 0.1% of diethyl pyrocarbonate at 37°C overnight in cell culture incubator): DEPC-treated DW is further autodclaved) and frequent use of 70% ethanol to wipe out the surfaces of gloved hands, pipetters and filter tips.

Note: The quantity of material present is measured by UV absorbance spectroscopy at a wavelength of 260 nm prior to dehydration.

a. Briefly centrifuge the tube containing dried multi-guide sgRNA to ensure the pellet is collected at the bottom.
b. Reconstitute gRNA in diluent. Rehydrate 1.5 nmol multi-guide ARID1A sgRNA (1–3 sgRNAs/tube) in 15 μL nuclease-free buffer (1× TE buffer: 10 mM Tris-HCl, 1 mM EDTA, pH 8.0) and pulse vortex for 30 s to ensure complete mixing.
   i. The final concentration of the sgRNA is 100 μM (100 pmol/μL). The sgRNA combines crRNA+TracRNA.

⚠ CRITICAL: For microinjection: It is critical to only hydrate and dilute sgRNA in a nuclease-free 1× microinjection buffer (e.g., 10 mM Tris-HCl, 0.1 mM EDTA, pH 8.0).

c. Store rehydrated ARID1A sgRNA at -20°C. Under these conditions, the ARID1A sgRNA is stable for up to 3 years, if not repeatedly thawed.

68. Dilute the Multi-guide ARID1A sgRNA.
   a. Depending on the application, use 1 × TE buffer or nuclease-free water to dilute the multi-guide ARID1A sgRNA to a working stock in a sterile microcentrifuge tube.
      i. Synthego's nucleofection protocol requires a concentration of 30 μM multi-guide sgRNA (see Example Dilution below).
      ii. For Synthego's lipofection protocol, a working concentration of 3 μM is needed.
      iii. The term "sgRNA" is used for crRNA+TracRNA. Conditions to produce sgRNA after annealing crRNA and tracRNA are included by the vendor.

b. Example mixture: Add 6 μL of 100 μM multi-guide ARID1A sgRNA to 14 μL of nuclease-free water to make a total volume of 20 μL of 30 μM multi-guide sgRNA (30 pmol/μL). The term "sgRNA" is used for crRNA+TracRNA. Conditions to get sgRNA after annealing crRNA and tracRNA is included.
c. Use the diluted multi-guide ARID1A sgRNA or store at -20°C for up to 3 months (or 6 months if not repeatedly thawed).

69. Transfect cells by use of either Synthego's nucleofection or lipofection reagent with this kit and the Gene Knockout Kit v2. All protocols are available at Synthego.com/resources.

Note: The positive control multi-guide ARID1A sgRNA provided in this kit is sufficient for about eight transfections when using Synthego's nucleofection protocol and about 384 transfections when using Synthego's lipofection protocol.

70. Analyze knockout efficiency to quantitatively assess Synthego's Inference of CRISPR Edits (ICE) using Sanger sequencing data. The software compares the sequence traces of amplicons generated from genomic DNA isolated from both the edited and unedited pools of cells.
71. Transfect CRISPR/gRNA complexes into cells.

Note: The efficiency with which mammalian cells are transfected with gRNA varies according to cell viability and human subjects and the transfection reagent used.

a. Use up to 2 μg TrueCut Cas9 Protein v2 and 400 ng gRNA per well in 24-well format.
b. Expect the highest editing efficiency with a 1:1 M ratio of gRNA to TrueCut Cas9 Protein v2.
c. Add recombinant Cas9 proteins and mix with gRNA solutions. Incubate at 25°C to make RNA-Cas9 protein complex (RNP).
d. Use 30–70% confluent cell cultures on the day of transfection when using lipid-mediated delivery or 70–90% confluence for electroporation using the Neon Transfection System.

Note: The highest editing efficiency is expected at a 1:1 molar ratio of gRNA to TrueCut Cas9 Protein v2. We use up to 2 μg TrueCut Cas9 Protein v2 and 400 ng gRNA per well in a 24-well format. Add recombinant Cas9 proteins and mix with gRNA solutions. Incubate at 25°C to make RNA-Cas9 protein complex (RNP).

Note: Cells recover two days after electroporation. If electroporation is performed in low cells concentrations, the potential for cell death becomes more obvious. To avoid excessive cell deaths of electroporated human fetal liver-derived cells, use optimal concentrations of RNA-Cas9 protein with high of cell concentration in the electroporation cuvettes. These precautions will ensure good cell viability with optimum cell metabolic activities.

Note: For an overview of the factors that influence transfection efficiency, refer to the "Transfection Basics" chapter of the Gibco Cell Culture Basic Handbook, available at thermofisher.com/cell culture basics.

e. Use the TrueGuide Positive Controls (for example, human AVVS1, CDK4, HPRT1, or mouse Rosa 26, that are commercially available from Synthego, INC.) and negative control gRNA (non-coding) to determine gRNA amount and transfection conditions that give the optimal gene editing efficiency with highest cell viability.
f. Include the TrueGuide Positive and Negative sgRNA and crRNA Controls that are available separately from Thermo Fisher Scientific. For more information, refer to thermofisher.com/trueguide.

g. The cell number and other recommendations provided in the following procedures are starting point guidelines based on the cell types we have tested.
h. Prepare a master mix of components to minimize pipetting error for multiple wells, then dispense the appropriate volumes into each reaction well.
i. Prepare extra volume to account for any pipetting variations when making a master mix for replicate wells.
   i. TrueCut Cas9 Protein v2/gRNA (ng/pmoles): 80 ng/pmoles.
   ii. Number of cells/10-μL reaction ($\times 10^3$): 1500×10^3/10-μL reaction.
   iii. Neon electroporation conditions: 1200 V/30 ms/1 pulse (#7).
j. Collect up to half of the electroporated/transfected cells to isolate genomic DNA for only confirmation purpose. The remainder of the electroporated cells will be intrahepatically transplanted after 4 days post electroporation.

Note: Cells need at least two days recovery after electroporation. If electroporation is performed at low cell concentration, the potential for cell death increases. To avoid excessive cell deaths of human fetal liver-derived cells, use the proper concentration of electroporated sgRNA-Cas9 protein, and a higher cell concentration in the electroporation cuvettes.

Characterization of edited clones for confirmation purposes *in vitro*

Timing: 5–6 h (for steps: 72–74)

The transduced cells used for characterization are not used for neonate injection since extended culturing decreases engraftment efficiency due to the possibility of differentiation effects

This section describes characterization of edited clones for confirmation purposes in an *in vitro* system.

72. Analyze single cell clones for purity and for desired genotypes (homozygous or heterozygous alleles). Methods that can be used include genotyping PCR, qPCR, next-generation sequencing, or western blotting. Grow single clones in complete media and incubate for 6–10 days before splitting into two plates (one for DNA isolation, followed by exome-Sequencing to determine the knockout efficiency of ARID1A targeted loci).
73. Verify the gene editing efficiency of the control target and select the condition that shows the highest level of editing efficiency for future screening experiments before proceeding with downstream applications.
   a. Use the GeneArt Genomic Cleavage Detection Kit or perform Ion Torrent next generation sequencing or a Sanger sequencing–based analysis.
   b. Electroporate RNP complexes into cells by Neon electroporation systems and incubate in cell culture incubator at 37°C.
   c. Design and order target-specific primer sets for the GCD assay through SYNTHEGO's GeneArt CRISPR Search and Design tool, available at thermofisher.com/crisprdesign.
   d. To perform the GCD assay for the positive control, design the primers for target specific primer sets for the GCD assay by use of Invitrogen Custom DNA Value or Standard Oligos, available from Thermofisher.com/oligos.
   e. Use primers listed in key resources table (KRT).
   f. Set up the GCD assay in a 96-well plate format and analyze multiple gRNA-treated samples in parallel on a 2% E-Gel 48 agarose gel (48-well).
   g. Pick the clones that show the highest cleavage efficiency to use in your experiments. Note that the clone that shows the highest cleavage efficiency may not always be the clone with the highest expression.

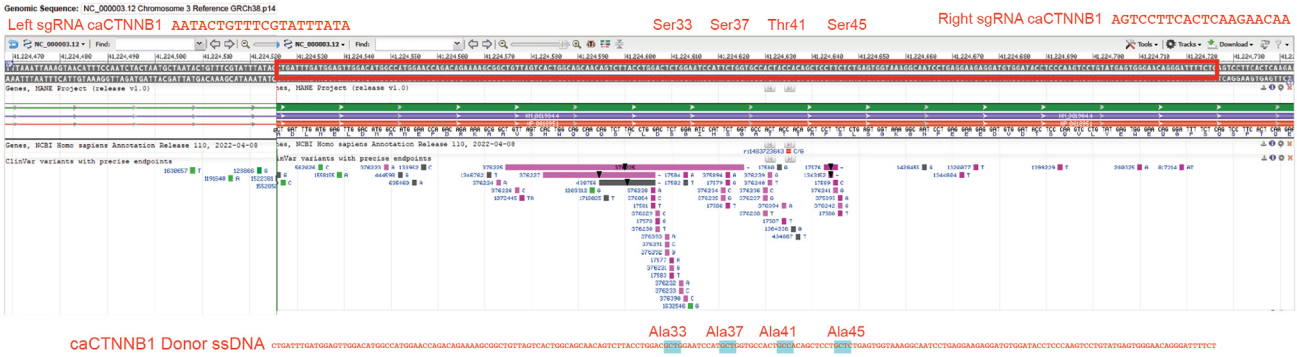


Figure 4. Constitutively active (ca) *CTNNB1* knock-in strategy

CTNNB1 genomic coding loci and donor DNA, Left and Right sg-*CTNNB1* binding sites are shown in the designated genomic loci. Human fetal LPCs are co-injected with two modified pX330 plasmids carrying sgRNAs targeting *CTNNB1* (termed sg-*CTNNB1)* and a 200-nucleotide ssDNA oligonucleotide containing four hot-spot-non-synonymous-mutations (Ser33, Ser37, Thr41, Ser45) in HCC patients that harbors serine/threonine substitution to alanine (Bottom) that abolish phosphorylation and to stabilize β-CATENIN.

h. See the GeneArt Genomic Cleavage Detection Kit User Guide for more information and detailed protocols.
i. Estimate the CRISPR-Cas9-mediated editing efficiency in a pooled cell population.

Note: While the genomic cleavage detection (GCD) assay provides a rapid method for evaluating the efficiency of indel formation following an editing experiment, next-generation sequencing (NGS) of the amplicons from the edited population or Sanger sequencing of amplicons cloned into plasmids give a more accurate estimate of the percent editing efficiency and indel types.

74. Analyze sequence by use of one of three options (a) Sanger sequencing, (b) bar-coded next-generation sequencing (NGS), or (c) deep sequencing of CRISPR modified ARID1A loci.
    a. For Sanger sequencing-based editing efficiency analysis, refer to Thermofisher's application note referenced at thermofisher.com/sangercrispr.
        i. Perform PCR amplification of targeted *CTNNB1* genetic loci to amplify exon 3 region of *CTNNB1* to examine whether c.a.*CTNNB1* exon 3 sequences are knocked in by plasmid-based sequencing (Figure 4).
        ii. Perform limited dilution to obtain single cell clones in 96 well plates to isolate single cell clones.
        iii. Incubate cells at 37°C for 6–10 days to allow single cell growth before splitting cells into duplicate plates (one is for DNA isolation and DNA sequencing and the other is for expansion and storage or used for experiments. Flanking regions of CRISPR/Cas9 targeted loci are designed for sequencing primer designs.
        iv. Amplify these flanking region-bound primers for *ARID1A* CRISPR/Cas9 targeted genetic loci to validate the gene mutagenesis. In alternative methods, the isolated genomic DNA is subjected to exome-sequencing to determine the proporion of CRISPR/Cas9-targeted ARID1A loci.
        v. Compare gene sequence between wild type mock-transfected cells vs. gene knock-in clones after sequence results are determined.
        vi. Expand the positive colonies for *in vitro* experimental purpose into three to four T75 culture flasks for cryogenic storage in cell storage media (final concentration of 10% DMSO, 40% of FBS mixed with 50% of cell culture suspension). These can be used once knock-in sequences are confirmed
    b. Barcoded next-generation sequencing (NGS): Use barcoded target-specific amplicon primers and perform multiplex analysis using several gRNA-treated samples in parallel.

Note: If you are experienced in next-generation sequencing (NGS) and analysis. Multiplex analysis using NGS is especially useful when using the custom arrayed plate format for TrueGuide Synthetic gRNA transfections. For more information on *NGS* analysis, refer to Ion Torrent targeted sequencing solutions at thermofisher.com/ionapliseqsolutions.

c. Deep sequencing of CRISPR modified ARID1A loci.
   i. PCR amplify the genomic region of ARID1A using Herculase II high-fidelity polymerase and gel purify. Libraries are made from 50 ng of the PCR products using the Nextera protocol and sequenced on the Illumina MiSeq (150 bp, paired-end reads) or the HiSeq2500 (100 bp, paired-end reads) instruments.
   ii. Process data according to standard Illumina sequencing analysis procedures. Briefly, reads are mapped to the PCR amplicons as reference sequences using Burrows–Wheeler Aligner with custom scripts.
   iii. Map reads to the PCR amplicons as reference sequences using Burrows-Wheeler Aligner with custom scripts.

Note: Insertions and deletions are crosschecked against reference using VarScan2. Indel phase is calculated as the length of insertions or deletions modulus.

   iv. Calculate the rate of β-Catenin donor integration as a donor allele frequency.
   v. Insertions and deletions are crosschecked against reference genome using VarScan2. Indel phase is calculated as the length of insertions or deletions modulus.
   Sequence two to five biological replicates for *in vivo* liver samples. One DNA sample is sequenced for *ARID1A* in human hepatic progenitor cells and for ARID1A off-target sites in sg-*ARID1A*-treated liver.
   vi. Calculate the indel frequency within 10-nucleotide regions (20 nucleotides total) flanking *ARID1A* target site to compare the editing efficiency at ARID1A off-target sites.
d. Perform plasmid-based sequencing or next-generation sequencing to examine the knockout efficiency for ARID1A knockout.
e. Perform limited dilution of electroporated cells to obtain single cell isolates in 96 well plates for expansion of knockout cell clones.
f. Once several knockout clones are identified, freeze a portion ofof ongoing cultured clones in cryostat tubes for future recovery. The remaining half of cells are used for experiments.
g. Prepare cryogenic storage by 1:1 dilution of cell suspension in complete media: 20% DMSO in 80% of FBS solution (Final concentration of DMSO 10% and 40% of FBS and 50% of cell suspension).
h. Freeze cryogenic tubes at -80°C for 2 h before transfer to and liquidfreezer.
i. Sequence the genomic DNA for ARID1A orβ-catenin mutagenesis to validate CRISPR/Cas9 gene editing.
j. Once several clones have been identified with the expected mutations (e.g., ARID1A), test if ARID1A protein levels were changed as per Western blot analyses of expanded single clones using anti-human ARID1A antibody.

Note: As human and mouse ARID1A sequences are very similar, extra care is needed to obtain the appropriate antibodies for human ARID1A protein detection. Several anti-ARID1A protein antibodies Human-specific Anti-ARID1A antibody [(EPR13501), ABCAM, cat. No. ab182560] are only human specific, but do not detect mouse ARID1A thus are ideal to distinguish between humanized ARID1A protein levels vs. endogenous mouse ARID1A protein levels.

Transdermal human fetal liver cell intrahepatic transplantation

Timing: 2 h; steps: 75–83

This section describes transdermal human fetal liver cell intrahepatic transplantation.

75. Irradiate 1- to 5-d-old newborn *FRG* mice in Petri dishes on gauze with a single dose of 150 cGy using an X-ray irradiator. Please refer tp omstructions for intrajepatic injection as described above.
    a. Place FRG neonates from a single litter into an X-ray irradiation chamber along with a small amount of bedding material from the breeder cage. Please refer to SOP for intrahepatic injection as above.
    b. Place FRG neonate mice on a warming pad or under an incandescent lamp for 1–2 min post-irradiation.
    c. Restrain the pups by grabbing mice with hands for intrahepatic injection into a FRG neonates.
    d. Place FRG neonates in the X-ray irradiation chamber in a petri dish for irradiation and subsequent injection.
    e. Please refer to SOP for intrahepatic injection.

⚠ CRITICAL: Mice less than 1–2 day of age have lower survivability and mice older than 5 days of age are difficult to transplant because it is difficult to see the liver through the skin.

76. Electroporate LPCs with CRISPR/Cas9 RNP complexes and left gRNA, right gRNA with donor DNA for c.a.CTNNB1.
77. Day 1: At 2 h post irradiation, place the pups on a warming pad or under an incandescent lamp for 1–2 min. Anesthetize mice with isoflurane before receiving cell injections.
78. Take the digested cells to the vivarium.
79. After irradiation, place post-natal pups from a single litter onto a 100-mm^2 petri dish along with a small amount of bedding material from the breeder cage (field size 10 cm × 10 cm).
    a. Place the neonates on ice to cool them to induce anesthesia prior to intrahepatic injection.

    Note: Since the actual injection step takes only several seconds, stage neonates sequentially for transplantation to minimize the overall time needed for the procedure.

    b. For injection, restrain the pup by placing the index finger between the front legs (slightly cranially to where you would expect the collarbone to be) with the thumb between its hind legs with application of gentle downward pressure in a posterior direction to pull the abdomen skintight for injection. Take care to avoid injury to the neonates. Inject intrahepatically with a 25–50-µL volume of LPCs (3 × 104 E-cadherin+ cells) and non-parenchymal cells (supernatant from low-speed centrifugation).
    c. Allow neonates to recover after injections by putting on heat pad and put them back to mother's cages.
    d. Take extra care with the NTBC cycling.
    e. Weigh frequently the FRG mice (3 times per week) during the first FRG transplantations to prevent animal morbidity.

    Note: If mouse weights drop more than 20% these mice will receive an NTBC treatment in drinking water bottles. The above-described NTBC cycling protocol can be adjusted based on your experience using this weight-based protocol, since experimental conditions may vary between labs including the proliferative capacity of hepatocytes obtained from different donor sources.

80. Remove the first pup and place the next on ice (the actual injection step takes only a few seconds, and the other pup can be cooled down by the time you are finished with the first one). Two to three days of age pups are safe for injection.

a. Clean the Hamilton (705SN30/51MM/12″ 25 μL) Syringe with Ethanol and PBS 3 times each (Figure 3, Right).
   i. First flush engraftment syringe with 70% ethanol three times.
   ii. Flush PBS three times (Note: Avoid use of bleach solution)
b. Take 25–50 μL of cells (500,000 cells: 0.5 million cells) (better for injection than 50 μL). Make sure no bubble in syringes. *Draw 50 μL containing 500,000 human hepatocytes into a* Hamilton (705SN30/51MM/12″ 25 μL) Syringe.
c. Spray the hands with 70% ethanol.
d. Grab neonates to open up abdomen area nicely and tightly.
e. Rub the abdominal skin surface of the mouse with 70% ethanol-containing cotton.
f. Pull back arms.
g. Remove bubbles inside syringe. (Needle should be ready before grabbing neonates).
h. Identify two milk spots. The liver is above the milk spots on the left side.
i. Stretch the mouse's skin and slowly insert the needle through the skin into the liver.
j. Inject very slowly.

Note: If the liver is perforated, the food coloring is visible in the peritoneal cavity.

k. Insert the needle with bevel up between ribs and skin (Inject needle from above the rim bone: collarbone, the clavicle) in right hand side of neonates).
   i. Insert subcutaneously needle for 10 mm.
   ii. If you pass rib cage, bend the needle to inject the edge of needle into liver (to stab the liver) under the lib cage.
   iii. Insert needle.
   iv. Target the edge of needle just right-hand side of belly button. If you insert needle in belly button area, you will damage intestine.
   v. If you insert too deep, you will damage genital tract. (If you inject subcutaneous area, skins will bubble up) (not too fast, not too slow). *Slowly inject the complete volume into the liver, wait a couple of seconds to allow the pressure to equalize and then slowly remove the needle.*
l. Begin by vertically positioning the needle slightly to the left of the right lobe and angled slightly toward the right lobe; push the needle in about halfway, and then deposit the cells into the right lobe at the rate of 2 s per injection volume.
m. Identify the stomach by a white color on the animal's heart side.

Note: The liver should be visible as a dark area located cranially to the stomach; it should be the largest organ in young pups.

n. Release the pressure immediately with your fingers. Thereafter, remove the needle, being careful to avoid contact with your finger.
o. Repeat Steps until the last mouse has been injected; place the pups back in their nest and return them to the mother.
p. Perform similar procedures as stated above on control mice using vehicle (wash buffer without cells; nontransplanted *FRG* controls).
q. Use commercially available human primary hepatocytes and human HSCs as controls.
r. Rub the hands again and again with bedding from the original cage.
s. Hold the pups on backside smoothly with two fingers so that an animal cannot move.
t. Curve the needle 30° up and inject the solution.
u. Rub the mouse abdomen areas with 70% ethanol-containing cotton swabs and place it back into its housing.
v. Repeat Steps a.-u. until the last neonates have been injected; place the pups back in their nest and return them to the mother.
w. Perform similar procedures as stated above on control mice using vehicle (wash buffer without cells; non-transplanted *FRG* controls).

81. Gradually decrease the concentration of NTBC (1.6 mg/L, day 0–2; 0.8 mg/L, day 3–4; 0.4 mg/L, day 5–6) and completely withdraw one week after transplantation.

Note: After transplantation, the cyclic decrease of NTBC and complete withdrawal regimen allows engraftment and growth of human hepatocytes. *FRG* mice grow well and are fully fertile if they are continuously provided NTBC in drinking water. Breeding pairs are maintained on NTBC drinking water their entire lives. Note that after the first month NTBC dosing is required for newborn survival (See Table 2).

82. Euthanize these 12 week of age *FRG* adult mice after 8–12 weeks post-electroporation, followed by intrahepatic injection into *FRG* neonates.
83. Test for human Albumin immunostaining and test for circulating human Albumin in serum to demonstrate whether CRISPR/Cas9-genetically manipulated LPCs and non-parenchymal cells are successfully reconstituted and humanized.

Follow-up and analysis of humanization

Timing: 3–4 h: steps: 84–87

This section describes humanization with KO liver and non-parenchymal cells. (See previous sections for detailed instructions).

84. Draw blood for humanization chimerism analyses by use of FACS: At 8 weeks after transplantation, bleed to test human albumin concentration in serum and presence of human CD45 by FACS. Repeat this for the second and third generations of pups from at least two-pairs of breeders.
    a. Draw 20 μL blood from a mouse using a 1 cc syringe sporting and a 29G needle.
    b. Return the mouse back to its cage.
    c. Check each mouse after half an hour to see if they recovered well from the transplantation.
    d. Resuspend the pellet using a gentle rocking motion. When necessary, use a 2 mL pipette to titrate two to three times very gently to obtain a homogeneous cell suspension.
    e. Measure cell volume, viability, and recovery.
    f. Dilute resuspended hepatocytes to 10^6 cells/mL.
    g. Use repeat pipet or multichannel pipet to dispense cells into BD BioCoatTM Collagen I coated plate (e.g., 400 μL/well of 24-well plate).
    h. After cell plating is completed, gently move the plate in a star pattern on a level surface to distribute the cells evenly over the bottom of the plate. See illustration below.
    i. Place plates in a 37°C, 5% CO_2 incubator.
    j. Every 20–30 min during the first 2 h plating, remove plates from incubator and gently rock the plates to redistribute the cells evenly in the wells.

    Note: Gently tapping the edge of the plate may also be helpful to redistribute the cells. Excessive accumulation of cells in the center of the wells can cause cell death.

    k. After 2–4 h, gently aspirate the Plating Media and gently refeed cells with complete Hepatocyte Culture Media (or customer preferred hepatocyte culture media).
    l. Keep plates in the incubator overnight for further experiments as required.
85. Withdraw NTBC for the *FRG* mice for 7 days after transplantation. This is followed by 5 days of NTBC treatment (8 mg/L).
    a. Start a long NTBC cycle.
    b. Withdraw period of 21 days followed by 5 days of NTBC treatment.

c. Apply periodical NTBC cycling protocols to rescue the liver failures if any of injected neonates become sick.
d. Maintain the long NTBC cycle until the mice have high Albumin levels and are ready to be used in experiments as illustrated in Table 1A.[4]
e. Repeat this regimen once and is followed by two cycles of another withdrawal of 7 days and a shorter NTBC treatment of 4 days.
f. Withdrawal NTBC by 3 days of treatment the next two rounds of 7 days.
g. Decrease the concentration of NTBC gradually (1.6 mg/L, day 0–2; 0.8 mg/L, day 3–4; 0.4 mg/L, day 5–6: Details is described in Table 1A) and completely withdraw one week after transplantation.[4]

⚠ CRITICAL: As NTBC withdrawal in FRG mice results in gradual hepatocellular injury and eventual death after 4–8 weeks, all animals are maintained with 2-(2-nitro-4-trifluoromethyl-benzoyl)-1,3 cyclohexanedione (NTBC)-containing drinking water at a concentration of 7.5 mg/L. NTBC is administered through drinking water for breeding cages since any literatures demonstrate that within 1 month after birth, pups survive without NTBC. But for more than 1-month-maintenance, NTBC is required. Mice given NTBC laced drinking water will require separate water bottles for their cages.

86. Day 7: Feed animals autoclaved or irradiated food and maintain them on acidified autoclaved water with or without sulfamethoxazole-trimethoprim (SMX/TMP) (7.8 mL of SMX/TMP per 250 mL of drinking water) in water bottles on alternate weeks for the duration of the animals' lives.

⚠ CRITICAL: The purpose of SMX/TMP special water is for prophylaxis so that *FRG* mice do not have B, T and NK cells and have defective dendritic cells although human hematopoietic lineage cells are injected as part of the non-parenchymal cell population from human fetal livers (it will take time to fully reconstitute functional human immune systems). These SMX/TMP water must be continued throughout their life spans. The purpose of feeding alcohol western diets is to generate steatosis and liver cancer development in the humanized mouse liver.

87. Initially feed Nitisinone (NTBC) drinking water (16 mg/mL) for the first week and the water is changed bi-weekly.

Note: Afterwards, the concentration of NTBC is gradually decreased (1.6 mg/L, day 0–2; 0.8 mg/L, day 3–4; 0.4 mg/L, day 5–6) and completely withdrawn one week after transplantation. These NTBC drinking water is fed for 0–6 days for humanized pups.

a. After transplantation, withdraw NTBC after three days of treatment the next two rounds of 7 days. Withdraw NTBC from the *FRG* mice for seven days.
b. This is followed by five days of NTBC treatment (8 mg/L).
c. Repeat this regimen once and is followed by two cycles of another withdrawal of 7 days and a shorter NTBC treatment of four days.
d. Start a long NTBC cycle. This consists of a withdrawal period of 21 days followed by five days of NTBC treatment.
e. Maintain the long NTBC cycle until the mice have high albumin levels and are ready to be used in experiments as illustrated in Table 1.[4]

Note: During the first FRG transplantations extra care is taken with the NTBC cycling. To prevent animal morbidity, the FRG mice are weighed frequently (up to three times per week). If mouse weight drops more than 20% these mice will receive an NTBC treatment. The above-described NTBC cycling protocol is adjusted based on your experiences using a

weight-based protocol since experimental conditions between labs and the proliferative capacity of hepatocytes from different donors may vary.

Humanization analysis and follow-up

Timing: 4–8 weeks (for steps 88–97)

Timing: 2 days: (for step 98 for hitology section preparation)

This section describes the validation of liver humanization and follow-up.

88. After 8–12 weeks post-intrahepatic injection, feed Alcohol Western diet to humanized mice for six months to generate HCC.
89. Monitor feeding groups of humanized FRG mice for behavior and palpation of abdomen areas to detect any harder objects in liver areas for human HCC development.
90. Four months after alcohol Western diet feeding, scan palpation-based hard object-positive *FRG* mice exhibiting liver hardening by micro-CT and ultrasound live animal imaging to identify and quantify the volumes of liver tumors and hyperplasias.
91. Once tumors are detected, register these *FRG* mice for drug treatment to test how these therapeutic drug candidates effectively reduce liver tumor sizes.
92. Use both females and males of humanized *FRG* mice for comparison of HCC incidence in females and males to avoid biological variables.

Note: After successful electroporation and gene knockout, approximately 20–40% of cell clones are mutated in targeted ARID1A genetic loci.

93. Isolate genomic DNA from parts of liver tumor, followed by PCR amplification of targeted loci to examine ether liver tumor genomic DNA contain *ARID1A* mutations or c.a.*CTNNB1* mutagenesis to validate whether HCC are developed from *ARID1A* knockout clones and/or c.a.*CTNNB1*-knockin hepatic progenitor cells.

Note: Once gene sequencing is validated, these cancer-driver mutagenesis with alcohol Western diet feeding are ideal tools to accurately access the drug efficacy in each driver mutations groups.

94. Starting at six-to-eight weeks post transplantation, collect two microliters of whole blood via the saphenous vein and immediately diluted into ELISA diluent buffer.
95. Human albumin ELISA: Analyze plasmas of the transplanted mice for the presence of human albumin following the protocol of the human albumin ELISA quantitation kit, starting six weeks after transplantation.
    a. Monitor human albumin levels using the Bethyl Laboratory Quantitative Human Albumin ELISA Kit.
    b. To measure human liver reconstitution, perform human albumin ELISA using plasma and the human albumin ELISA kit (Bethyl) according to the manufacturer's procedures.
96. Determine the kinetics of the appearance of human albumin in the mouse plasma from the quality of the human hepatocytes at the time of transplantation and the engrafted hepatocyte efficiency to integrate into the liver parenchyma.
    a. Albumin > *2 mg/mL: indication of successful engraftment of human hepatocytes.* If levels of human albumin are above 2 mg/mL 14 weeks after transplantation, those mice with human albumin levels are successfully engrafted. Whole blood albumin levels of > 4 mg/mL correlate with repopulation indices > 90%.[4]

b. 0.5 mg/mL: The detection limit of human albumin ELISA. If human albumin levels are below the detection limit of 0.5 mg/mL at the first analyses, it does not immediately indicate a failed transplantation. Continue to monitor albumin levels.
c. *0 mg/mL: Failed transplantation procedures:* Low to no human albumin: The transplantation has failed. If low to no human albumin is detected or still *below the detection limit* by 12–*14* weeks after transplantation, the transplantation *procedure* has failed.

Note: If human albumin levels are below the detection limit of 0.5 mg/mL at the first analyses, it does not immediately indicate a failed transplantation. The kinetics of the appearance of human albumin in the mouse plasma is influenced by the quality of the human hepatocytes at the time of transplantation and their efficiency to integrate into the liver parenchyma. If human albumin levels are still below the detection limit after 14 weeks, then the procedure has likely failed.

97. Bleed to test human albumin concentration and human CD45 positive cells by FACS at eight to 12 weeks after transplantation. Repeat this for the second and third generations of pups from the two-pairs of breeders.
98. Histology and immunocytochemistry to confirm humanized parenchymal and non-parenchymal cells.
    This section describes determining the ratio of humanized hepatocytes and non-parenchymal cells by use of immunohistochemistry.
    a. Perform FAH immunohistochemistry.[11]
    b. Stain sections of humanized mouse liver with Hematoxylin & Eosin (H&E) and examine histology by assistance of board-certified pathologists to distinguish between hyperplasia, low/middle/high grade of HCC with quantification of the degrees of differentiations (i.e., poorly-differentiated vs. well-differentiated HCCs).
    c. Use the HepPar antibody (DAKO) according to the manufacturer's specifications.
    d. Plate liver cells on collagen type1-coated 6-well plates for immunocytochemistry of hepatocytes from humanized mouse. Fix the attached cells with 4% paraformaldehyde for 15 min and block with 5% skim milk. Use rabbit anti-FAH (Grompe lab), goat anti-human albumin (Bethyl), goat anti-mouse albumin (Bethyl) as primary antibodies at dilution of 1/200.
    e. Use Alexa Fluoro 488 anti-goat IgG (Invitrogen) or Alexa Fluoro 555 anti-rabbit IgG (Invitrogen) as secondary antibodies.
    f. Capture the images with an Axiovert 200 microscope and a Nikon digital camera.

Confirmation analyses of human liver and immune system reconstitution

Timing: 2–3 months: steps 99–103

This section describes methods to determine chimerism between human and mouse of immune systems and hepatocytes.

99. Wean animals at 3–4 weeks of age and house at $\leq$4–5 humanized animals per cage.
100. Feed animals with autoclaved/irradiated food and maintain mice on acidified autoclaved water with or without SMX/TMP (7.8 mL of SMX/TMP per 250 mL of drinking water). If SMX/TMP is needed include in drinking water on alternate weeks for the duration of their lives.
101. At 16 weeks after transplantation, warm FRG-hu HSC/Hep and control mice with a heat lamp and bleed them by making a single 5-mm cut (nick) on the tail vein with a sterile scalpel. Collect 100 μL of blood per mouse in 1.5-mL sterile microcentrifuge tubes containing 100 μL of 20 mM PBS-EDTA and place it on ice; samples can be kept on ice for up to 12 h.
102. Spin collected blood–PBS/EDTA solution at 469*g* for 10 min at 4°C using a centrifuge. The top portion (plasma) can be used to examine human liver reconstitution by ELISA or FACS as described earlier. The pellet [peripheral blood mononuclear cells (PBMCs)] can be used to measure human immune reconstitution.

103. Resuspend the pellet (PBMCs) in red blood cell lysis buffer (1× ACK lysis buffer), incubate for 5 min at 25°C, spin cells at 469*g*, repeat this procedure and resuspend in 2% (vol/vol) FBS/PBS containing human CD45, mouse CD45 and 7-AAD antibody mixture. Examine the human immune reconstitution (percentage of human $CD45^+$cells/total $CD45^+$ cells) using flow cytometry analysis.

Note: If the pellet is red, repeat steps 138 and 139 until the pellet is yellow or white to ensure removal of red blood cells.

Alcohol western diet (AWD) feeding to promote HCC development and therapeutic testing in humanized FRG mice

Timing: six months (for steps 104–107) (Figure 5)

After generation of the cancer-driver mutation containing humanized livers, environmental factors, such as BASH (both ASH and NASH) can be recapitulated by alcohol Western diet feeding for six months to promote the tumor developments.

104. Feed alcohol WD for six months to promote liver cancer development through gene-environmental interactions after confirmation of humanization of livers.
    a. Collect blood from facial vein (once) BEFORE alcohol WD feeding (facial vein route is only used for uninfected mice): Required: 4–5 mm lancet or 28–30-gauge needle, small blood collecting tube, clean work surface, and mouse.
    b. Achieve hemostasis by using a gauze and applying into facial vein to stop the bleeding for 30–60 s.
    c. Collect blood from the orbital sinus (One time), one week AFTER alcohol WD feeding:
    d. Anesthetize mice by us of isoflurane vaporizer machine (1–4%). Use Isoflurane to anesthetize mice at four months of age (one week after alcohol WD feeding). For the RO blood collection, apply proparacaine to the eye before blood collection for local anesthesia.
    e. Use orbital sinus route for blood collection from mice instead of facial vein.
    f. Gently insert tip of the pipette below the eye at approximately a 45° angle into the space between the globe and the lower-eyelid. Take care not to occlude the trachea with your thumb. Insert the tip of a soft plastic pipette (o.d. of 1.2 mm) into the corner of the eye socket underneath the eyeball, directing the tip at a 45° angle toward the middle of the eye socket.

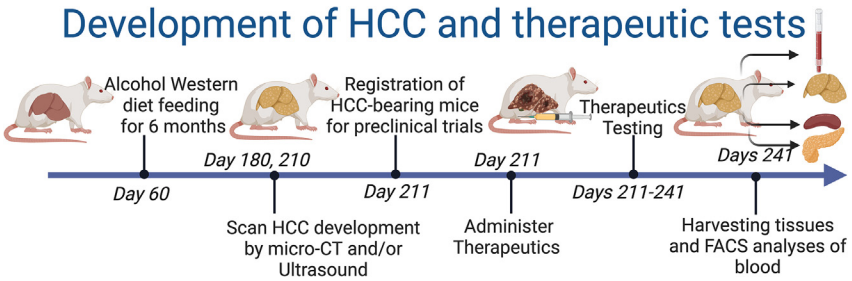


Figure 5. Development of HCC and therapeutic tests
After humanized livers are confirmed, humanized *FRG* mice are fed alcohol Western diet for a maximum six months to generate HCCs. After four and five months following alcohol WD feeding (Days 180 and 210), micro-CT and/or ultrasound is performed to monitor HCC development. Once HCC is detected, HCC-harboring FRG mice are registered for therapeutic testing and after a 4-week treatment, all mice are euthanized. Created by biorender.com (Agreement Number:).

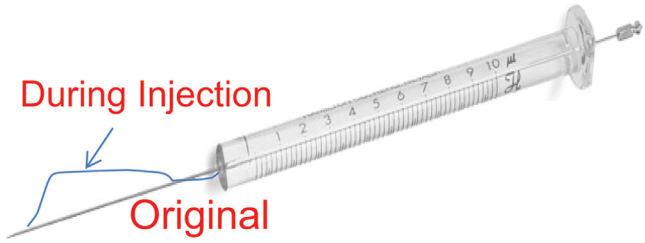


Figure 6. Hamilton needle is stubbed from left shoulder area into livers of FRG neonates (1–5 days of age)

g. Feel the pipette rest on the orbit, at this point gently twist the pipette between our thumb and forefinger. Rotate the pipette between your fingers during forward passage; do not move it from side to side or front to back. Apply gentle downward pressure and then release until the vein is broken and blood is visualized entering the pipette.

Note: Blood flows into the pipette once the sinus/plexus has been ruptured.

h. Withdraw slightly and allow the pipette to fill when a small amount of blood begins filling the pipette.

Note: Do not let the pipette come out of the eye socket. If the pipette is not withdrawn slightly, it may occlude the vein and blood will not flow freely.

i. Use multiple pipettes as needed to collect the total volume. Cover the open end of the pipette with the tip of finger before removing it from the orbital sinus to prevent blood from spilling out of the tube.

Note: Bleeding usually stops immediately and completely when the pipette is removed. It may be necessary to apply gentle pressure on the eyeball for a moment by closing the skin above and below the eye using first finger and thumb. It is recommended that sample collection not be repeated on the same eye for at least two weeks. We will place each pipette into an opened collection tube as they fill.

j. Release tension on the animal and gently hold a gauze pad over the eye until the bleeding has stopped.
k. Either tap the pipette on the edge of the tube to empty or place a small pipette tip on the end of a syringe to carefully blow the sample out, it will clot very quickly.
l. Watch the animal carefully until it begins to recover from anesthesia and move about, then you can return it to its cage and move on to the next animal.
m. Apply triple antibiotic ointment post-retroorbital bleed to prevent infection.

105. Feed mice either control liquid diet or alcohol Western diet and acidified sulfamethoxazole-trimethoprim (SMX/TMP) water for 6 months.
    a. Feed Alcohol Western diet (DYET#710362) containing 3.5% (v/v) ethanol or control diet (DYET#180724) by use of Lieber-DeCarli liquid diet glass feeding tubes (Bio-Serv: Catalogue #9019, 50 mL) in tube holders (Bio-Serv Cat #9015, Short, Adjustable) in the FRG model (Figure 6).
    b. Replace caps (Bio-Serv Cat #9501, Autoclavable Screw Cap, Black) to seal feeding tubes if it is loose to avoid the leakage of liquid diet.

Note: Alcohol Western diet feeding will increase numbers of human leukocytes ($CD45^+$, $CD3^+$ and $CD4^+$, plasmacytoid dendritic cells, macrophages and T regulatory cells) in the liver T cell immune response after mice responded to a challenge infection as previously reported.[12]

106. Collect retro-orbital blood one week after alcohol WD feeding.
107. Feed either normal or Alcohol fed diet for four weeks, afterwards initiation of drug treatment.

Therapeutic efficacy assessment by use of different cancer-driver mutant HCC FRG mice

Timing 4–5 weeks (for steps 108–110) (Figures 5 and 6)

This section describes the therapeutic treatments for HCC-bearing humanized FRG mice.

108. Follow up for one month after four weeks of drug treatment.
109. Scan by Micro-CT and take ultrasound images.
110. Collect blood by terminal cardiiac puncture (Endpoint approximately Day 196): Anesthetized mice (by Ketamine/Xylazine) prior to blood collection. Blood Collection Technique from Facial Vein BEFORE (once) humanization/repopulation. 4–5 mm lancet or 28–30-gauge needle, small blood collecting tube, clean work surface, and mouse. Anesthetics. Hemostasis is achieved by using a gauze and applying it to facial vein to stop the bleeding for 30–60 s.

Annealing temperature 60°C is better for the specificity (Figure 7).

PCR reaction master mix

Reagent	Amount
DNA template	5 μL (30 ng/μL × 5 μL: 150 ng
Advantage DNA Polymerase	1 μL
10 mM FAH WT Fw1	1 μL
10 mM FAH WT/MT Rev	1 μL
10 mM FAH MT Fw2	1 μL
10× Buffer	5 μL
ddH2O	36 μL
Total	50 μL

Note: Present PCR reactions with the following conditions:

PCR cycling conditions

Steps	Temperature	Time	Cycles
Initial Denaturation	94°C	2 min	1
Denaturation	94°C	30 s	35 cycles
Annealing	60°C	30 s	
Extension	72°C	1 min	
Final extension	72°C	10 min	1
Hold	4°C	Forever	

Genotyping of homemade-*Fah−/−; Rag2−/−; IL2Rgc−/−* (FRG) mice

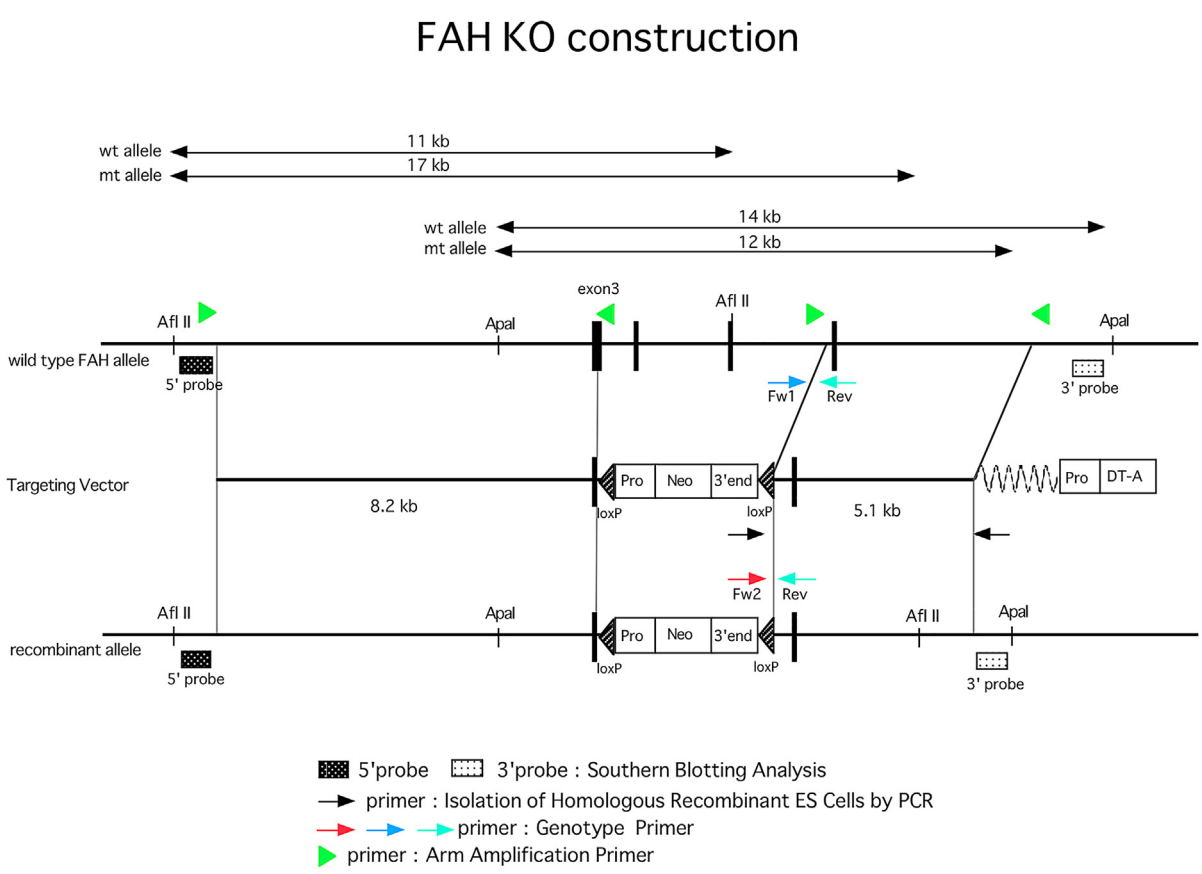
111. *Rag2−/− IL2Rgamma−/−* mice (Taconic: cat#4111, purchased on Oct. 1, 2010)

For Rag-2 genotyping.

Rag-2 Tm1Fwa genotyping primers.

112. RAG2IL2Rg-Neo-3′: CCAACGCTATGTCCTGATAGCGGT (24 bp)
113. RAG2IL2Rg-RG2-1: TTAATTCAACCAGGCTTCTCTCACTT (24 bp)
114. RAG2IL2Rg-RG2-3: GCCTGCTTATTGTCTCCTCCTGGTATG (27 bp)

Rag2 WT: 973 bp.



Genotyping

FAH WT Fw1 : AGGCCTAACCTCTTGCTTCATTCA

FAH WT/MT Rev: ATCGGGGTTCCAGATACCAC

FAH MT Fw2 : CCAGCTCATTCCTCCCACTC

wild type allele: 809bp

mutant type allele : 433bp

94 ℃ 2 min
94 ℃ 30 sec
58 ℃ 30 sec
72 ℃ 1 min
30 cycle
72 ℃ 15 min

Figure 7. Targeting of mouse Fah loci and their genotyping using PCR primer binding sites

Annealing temperature 60°C is better for primer specificity.

Rag2 KO: 1,107 bp.

For Rag-2 genotyping	
Reagent	Amount
DNA template	5 μL (30 ng/μLX 5 μL: 150 ng)
Advantage DNA Polymerase	1 μL
RAG2IL2Rg-Neo-3′	1 μL
RAG2IL2Rg-RG2-1	1 μL
RAG2IL2Rg-RG2-3	1 μL
10× Buffer	5 μL
ddH_2O	37 μL
Total	50 μL
Fah Wild Type gene PCR reaction master mix	

Note: Present PCR reactions by use of Advantage DNA polymerase with the following conditions:

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	5 min	1
Denaturation	94°C	30 s	35 cycles
Annealing	60°C	30 s	
Extension	72°C	1 min	
Final extension	72°C	10 min	1
Hold	4°C	Forever	
Amplicon is 500bp.			

Common gamma chain wild type gene

Timing: 3–4 h (for step 115c)

Gc Sense (intron 4) CTT TAT TGA TAA CGA TCT ATC CCT CAC CC (29 bp)

Gc Antisense (intron 5) CTC CAC TCT GCA GAG TCT ATG GAA TCC (27 bp)

PCR reaction master mix	
Reagent	Amount
DNA template	5 µL (30 ng/µL × 5 µL: 150 ng
Advantage DNA Polymerase	1 µL
Gc Sense (intron 4)	1 µL
Gc Antisense (intron 5)	1 µL
10× Buffer	5 µL
ddH_2O	37 µL
Total	50 µL

Note: Present PCR reactions with the following conditions:

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	94°C	4 min	1
Denaturation	94°C	60 s	40 cycles
Annealing	67°C	60 s	
Extension	72°C	2 min	
Final extension	72°C	10 min	1
Hold	4°C	Forever	
Amplicon is 500bp.			

Overall **Timing:** Total time is 8-9 months

Humanization of adult FRG breeder mice by surgical transplantation (steps 1–41).

a. Adenovirus (Ad:uPA) amplification, purification and titer determination (needed for in-house-preparation of Ad:uPA) [steps 1–26; 3–7 weeks] (Figure 2)

i. Human adult hepatocyte purification
ii. Preparing parental *FRG* breeder mice and breeding

b. Precondition *FRG* neonates by Ad:uPA injection and X-ray irradiation [Steps 28-33]
i. Digestion and preparation of human fetal liver cells

c. Primary hepatocyte recovery [steps 31]
i. Transplantation and eventual withdrawal of NTBC in drinking water

d. Confirmation ***analyses*** of human liver and immune system reconstitution

Dual humanization protocol of breeder *FRG* mice [steps 28-33]

Timing: 2 months

Timing: 21–40 days (for step 116)

Timing: 3–5 h (for step 117)

Timing: 2–3 days (for step 118)

Timing: 3 h (for step 118ei)

Timing: 12 h (for step 118eii)

Timing: 2–4 h (for step 119)

Timing: 2–3 months (for step 120)

Timing: 6 months (for step 121)

Timing: 4–5 weeks (for step 122)

Breeding humanized FRG mice for neonate production and Pre-conditioning [steps 34, 35] (Figure 2)

Days post-transplantation	Procedure
0	Mice in Breeding Cage
1	Ad-uP injection
2	X-ray irradiation
2.5	Intra-hepatic injection HSCs and LPCs (LPC)
10	Withdrawal of NTBC
18–21	Weaning of mice

Note: NTBC + water feeding throughout (day 0–10).

CRISPR/Cas9-medated gene knockout and /or knock-in of human fetal LPCs transplanted into neonate livers [step 36–58]

Humanization of liver and non-parenchymal cells [steps: 42–58; Hands-on Time: 3–4 h] depending on numbers of animals to transplant, 5–10 min per mouse (4 injections), 10–20 min for LPC+ Non-parenchymal cell injections, 8–12 weeks for *in vivo* humanized liver and immune system reconstitution

Typical Time schedules of the day before transplantation and transplantation date.

a. One day before transplantation (Late afternoon around 7:00 pm on the day before transplantation: Retro-orbital injection of Ad:uPA to pre-condition recipient FRG neonates.
b. 7:00 am Start works and wait for tissue delivery and reagent preparation
c. 8:00 am Start to receive and digest tissues
d. 8:50 am Leave to X-ray irradiator (150 cGy for FRG neonates): We may adjust a radiation dose for pups due to a size difference.
e. 9:30 am Take them back to vivarium
    i. CRISPR Editing Human fetal LPCs with RNPs using Neon Electroporation [steps 50–51;]
    ii. Liver digestion of human fetal livers and intrahepatic injection of human LPC, non-parenchymal cells with human hematopoietic stem cells. [steps 52–85; Hands-on Time: 3–4 h]

ARID1A knockout CRISP/Cas9 by use of sg-RNA electroporation and cell injection into humanized FRG mice. [steps: 63-66] (Figure 6)

Confirmation analyses of human liver and immune system reconstitution: step 72-74 and 84-103:

Alcohol Western Diet (AWD) feeding to promote HCC development [step 104–107]

Therapeutic efficacy assessment by use of different cancer-driver mutant HCC FRG mice [step 108–110]

EXPECTED OUTCOMES

Humanized AWD-promoted HCC mouse model provides the translational relevance to humans because of its similar pathology, genetic background, tumor microenvironment (TME) and immunity.[13] Human fetal liver cells were transplanted into *FRG* mice that were previously established, but with a different genetic locus knock out locus.[14] Human fetal livers were used as the source of parenchymal and non-parenchymal liver cells and hematopoietic progenitors for transplantation.[14] *FRG* pups (2–3 days old) were transplanted with fetal liver cells after X-ray irradiation for liver reconstitution beginning after withdrawal of NTBC at day 10 (Figure 6B). The mutations in *CTNNB1* and *ARID1A* frequently observed in alcohol-associated HCC,[15] were introduced into hepatoblasts by CRISPR/Cas9 technology. *ARID1A* was efficiently knocked out by electroporation with directed sgRNA-recombinant Cas9 protein complexes (Synthego) or knock-in of *CTNNB1* activation mutations in exon 3 by using of donor DNA with right/left sgRNAs (Figures 8A and 6B). When humanized *FRG* mice were subjected to alcohol Western diet feeding they developed HCCs, especially in the *CTNNB1* or *ARID1A* mutant hepatoblast groups, six months post-HCV infection with or without alcohol Western diet feeding (Figure 8B). Examination of representative histology showed a significant resemblance to tissues of patient HCC (Figure 8B, Bottom). ARID1A protein levels were quantified by Western blot analyses in livers derived from humanized mice fed alcohol WD (Figure 8C). The tumor histology of these mice was similar to human HCCs, indicating the validity of this model for studying HCC. These results indicated that mutations in *ARID1A* promoted alcohol-associated HCCs while activating mutations in *CTNNB1* promoted alcohol WD-mediated HCCs these mice as is also seen in different human cancers. Our results indicated that alcohol Western diet enhanced the onset of hepatocarcinogenesis (Figure 8D). Without genetic manipulation of LPCs, tumor incidence with Alcohol WD (AWD) feeding was less than 8% (Figure 8D). To promote tumor incidence of humanized FRG mouse models and to mimic stepwise-genetic mutagenesis as initiation process, clinically relevant alcohol-associated cancer driver mutagenesis of c.a.*CTNNB1* and/or ARID1A functional knockout were introduced in human LPCs and transplanted into FRG mouse livers. With these mutations, liver tumor incidence in chow-fed humanized FRG mice was 10% by 6 months, which increased to 70% resulting from AWD feeding (Figure 8D). Nearly 80% of the liver is humanized in the humanized FRG mouse livers.

Tumor incidence of ARID1A mutant liver tumors is 30–40%. Tumor incidence with c.a.CTNNB1 mutagenesis are 20–30%. Tumor incidence of humanized FRRG mice with both ARID1A

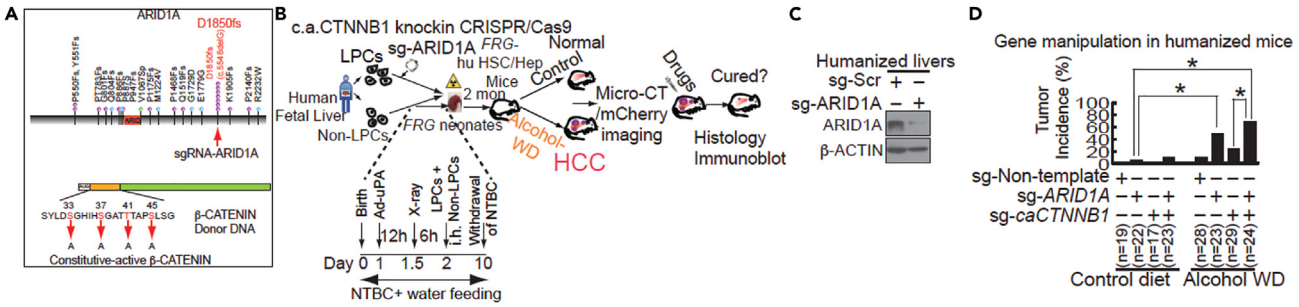


Figure 8. Tumor progression and poor survival rate in HCC patients with high expression of NANOG and EZH2

(A) CRISPR-Cas9-based gene editing generated β-Catenin constitutively-active alanine substitutions as indicated (Ser33, Ser37, Thr41, Ser45). *ARID1A* and *CTNNB1* (β-catenin) mutations by sgRNA and donor DNA. Distribution of the mutations detected by Exome-seq in the coding region of *ARID1A* gene is shown in cancer from a literature.[16]

(B) Humanized HCC Mouse Model. The diagram depicts the humanized HCC model production procedure (1) transplantation of human fetal liver cells into FRG neonates; (2) NTBC withdrawal; (3) Ad:uPA injection; (4) X-ray irradiation; (5) hepatoblast gene editing; (6) Intrahepatic injection of both LPCs with non-parenchymal cells; (7) Withdrawal metabolic rescue drug NTBC from drinking water. Humanized *FRG*-hu-Hep/HSC were fed alcohol Western diet (WD) for tumor development. Days refer to the procedure timeline.

(C) Immunoblot of *ARID1A* of livers collected from mice shown in (D).

(D) Alcohol Western diet (WD) feeding promoted HCC in humanized *FRG*-hu-Hep/HSC mice. Tumor incidence in mice, effects of alcohol and western diet (WD) as indicated. Liver tumor incidence rate markedly increases by *ARID1A/CTNNB1* mutations and alcohol Western alcohol diet feeding. Part of Figure is being published after acceptance in Cell Death Discovery article (1) and another pending manuscript.

KO + c.a.CTNNB1 is 60–70% incidence range. Therefore, these double mutagenesis groups can be used for alcohol-associated HCC preclinical mouse HCC models effectively evaluate drug efficacy in mice with combined humanized HCC and humanized tumor microenvironment with humanized immune system. These triple humanized components will accurately access the drug efficacy that can be often underestimated or overestimated in regular mouse HCC mouse models because of differences between human HCC-human TME vs. mouse HCC-mouse TME including significant differences in may signaling pathways.

Important outcomes of the humanized HCC model

Therapeutic application of HCC can be tested in different driver mutation backgrounds in humanized HCC *FRG* mice to examine differential therapeutic effects based on these cancer-driver mutations. Depending on the cancer-driver mutation types, therapeutic efficacies can be epected to vary. Thus, etiology-specific cancer-driver gene-targeting drugs can be used formore personalized and customized therapeutic regimens rather these conventional sorafenib, regorafenib or PD1 immune checkpoint inhibitors. Before new therapy is initiated, physicians may be able to estimate the therapeutic effects based on identification of cancer-driver mutation types.

For example, a β-CATENIN inhibitor is far more effective in c.a.*CTNNB1* HCC FRG mice while the same treatment displayed less effective efficacy in *ARID1A* knockout HCC FRG mouse groups. PRC complex component inhibitor, such as EZH2 inhibitor effectively reduced HCC incidence and tumor sizes while ARID1A mutant HCC FRG mice are more registrant resistant to PRC2 complex inhibitors. These driver-mutation HCC specific therapeutic stratification will eventually establish made-to-order therapeutic agents. Therefore, tumor DNA sequencing can be used as a companion diagnostic tools to stratify the HCC patient groups for the purpose of employing drugs expected to be effective against a specific driver mutation background. These cancer-driver mutation specific human HCC preclinical trials will shorten the clinical trial duration and use of biomarker-guided therapeutic stratification will lower clinical trial costs and shorten trial duration. As we observed, both *ARID1A* KO FRG mice and c.a.*CTNNB1* knock-in *FRG* mice are more susceptible for liver tumor development after alcohol Western diet feeding for six months.

QUANTIFICATION AND STATISTICAL ANALYSIS

Statistical considerations

For *in vitro* culture studies, Student's t-test was used to analyze data using Statistical software.

Statistical analysis

Experimental data are presented as the mean ± standard deviation (SD). All statistical analysis was performed using a two-tailed Student's t test and Chi squared test. Differences were considered statistically significant when P values were less than 0.05. Error bars reflect standard errors.

LIMITATIONS

Intestinal farnesoid × receptor (*Fxr*) gene expressions are induced in murine intestine of hFRGN mice: *Fgf15* and small heterodimer partner (*Shp*) were observed.[17] Proteomics revealed persistence of remnant murine proteins (cytochrome P450 7α-hydroxylase (*Cyp7a1*) and other enzymes and transporters) in h*FRGN* livers and suggest the likelihood of mouse liver enzyme activity.[17] When compared to normal human liver tissue, h*FRGN* livers showed lower SHP mRNA and higher CYP7A1 (3-fold increase) protein expression. Consequently occurence of tβ- and tα-muricholic acid–mediated inhibition of the FXR-SHP cascade and miscommunication between intestinal Fgf15 and human liver FGFR4 is observed.[17] Dysregulation of hepatocyte proliferation and bile acid homeostasis in h*FRGN* livers led to hepatotoxicity, gallbladder distension, liver deformity, and other extrahepatic changes have occured, making questionable the use of the preparation for drug metabolism studies.[17]

Other limitations of *FRG* mice is overestimatation of the tumorigenesis effects and underestimation of the therapeutic ability due to overwhelming liver growth signaling pathways.[18] Our described protocol in this article has repopulated the human liver in the mouse intestinal system that secretes mouse FGF15 (human orthologue is FGF19) that is not recognized by human FGF19 receptor on humanized hepatocytes that cannot induce CYP7A1 of *FRG* mice,[18] leading to significantly larger liver/body weight ratios (hepatomegaly).

Another pitfall is that detection of tumors and nodules may sometimes lack not accuracy because of non-detection by use of micro-CT or ultrasound since mouse livers are smaller and less than 3–5 mm tumor nodules may challenge accurate estimation of tumor sizes and locations. The concern may lead to false tumor negativity. Tumor detection may be enhanced by use of fluorescence labeling via infection with dsRed-lentivirus and use of live animal imaging. These methods, however, can detect tumor locations, but accurate tumor volume estimation needs to be improved for future analyses.

TROUBLESHOOTING

Problem 1

If more than tolerated Ad:uPA are injected into FRG neonates, these mice will die within several days with liver failure (steps 1–26).

Potential solution

Ad:uPA doses must be properly tittered and injected properly. Insufficient Ad:uPA virus injection will lead to insufficient humanization processes. Therefore, proper Adenovirus tittering and dilution are critical to avoid the lethal effects of Ad:uPA.

Problem 2

Infection of animals with various pathogens, including bacteria, will result in very low human reconstitution and illness.

Potential solution

Use drinking water containing sulfamethoxazole-trimethoprim (SMX/TMP) (7.8 mL of SMX/TMP per 250 mL of drinking water). Maintain a clean facility and a germ-free colony through steptic procedures, including use of autoclaved bedding and water .

Problem 3

If only hepatic progenitor cells are implanted wihtou other non-parenchymal cells, these mismatched FGF19 issues may arise although our described *FRG* mouse protocol in this article have repopulated the human liver in mouse intestinal system that secretes mouse FGF15 (human orthologue is FGF19) that is not recognized by human FGF19 receptor on humanized hepatocytes that cannot induce CYP7A1 of *FRG* mice,[18] leading to significantly larger liver/body weight ratios (hepatomegaly) (step 27).

Potential solution

To solve these disadvantages, a novel host strain possessing *FRG* has been established by several groups. We have also produced *FRG* mice and successfully transplanted human hepatocytes to create stable chimeric livers. The latter animals were efficiently generated in large numbers and with human liver cell replacement exceeding 70% efficiency.[18] The replacement index (RI) of human hepatocytes in the mouse liver is calculated from chimeric mouse livers immunostained with human specific cytokeratin 8/18 (hCK8/18) antibodies. The RI is observed to correlate with the concentration of human albumin in mouse blood.[18] Thus, the RI can also be estimated from human albumin levels in mouse plasma. Approximately 70% efficiency of the transplant take efficiency in mice transplanted with human hepatocytes exhibited >70 % RI[22]. Instead of FRG mice, use of FRGN19 mice may resolve this confounding factor based on FGF15 and FGF19 cytokine-receptor interaction difference, leading to defective intestinal FGF19-medated CYP7A1 expression and stimulation of FGF19 receptor on hepatocytes which transactivates CYC7A1 to normalize the liver size and liver/body weight ratios.

As Fibroblast Growth Factor (FGF) promotes and maintains liver size in humanized livers of FRGN mice, FRGN mouse livers are significantly larger than normal livers of C57Bl/6 mice and those of FGF19 transgenic (Tg)+ FRGN mice (FRGN19).[19] An increase in the circulating bile acid pool elevates portal venous bile acid concentration in non-Tg humanized livers.[19] FGF19 production in FRGN19 mice reduces (cytochrome P450 7α-hydroxylase (Cyp7a) in transplanted human hepatocytes, correcting a bile acid signaling short circuit in mice expressing Fgf15 only, which is not recognized by human hepatocytes. Correction of aberrant bile acid signaling was confirmed by measuring the bile acid pool, markedly enlarged in FRGN mice (a phenomenon not seen when livers are repopulated with mouse donor hepatocytes) and restored near-normal levels in FRGN19þ mice.[19] To resolve this issue, the gene for human FGF19 (ortholog to mouse Fgf15), including regulatory sequences can be expressed is transgenic mice that can be crossbred with the FRGN mice to create FRGN19þ mice.[19] Livers from FRGN19b normalized to 7.8% of body weight, and their bile acid pool and signaling more closely resembled that of control FRGN19þ mice.[19] Healthy human liver does not produce FGF19, however nonparenchymal cells from cholestatic livers produce FGF19.[19] In mice with humanized livers, expression of an FGF19 transgene corrects bile acid signaling defects, resulting in normalization of bile acid synthesis, the bile acid pool, and liver size.[19] Liver size is regulated by the size of the bile acid pool that the liver must circulate.[19]

Problem 4

It may cost too much money to synthesize template DNAs, left gRNA, and right gRNA.

Potential solutions

Addgene plasmids pX330 Ctnnb1.1 and pX330 Ctnnb1.2 can be used for template plasmids as template plasmids for *in vitro* mutagenesis reactions to change from mouse c.a.*CTNNB1* sequences into

human c.a.*CTNNB1* sequences since almost all sequences are identical between human and mouse in this exon 3 of CTNNB1 (pX330 backbone expressing sgRNA targeting Ctnnb1 to edit mouse β-Catenin by *in vitro* mutagenesis.[20] Expresses Cas9 from CBh promoter).

Problem 5

Storing CRISPR-KO/Knock-in-cells for future use is desired (steps 62-66 for c.a.CTNNB1 and ARID1A KO for steps 67-71).

Potential solution

Split cells into two groups (one for analysis and one for cell culture). If you choose to determine the efficiency of gene knockout and/or knock-in efficiency, hepatic progenitor cells derived from mouse fetal livers can be expanded without feeder cells. In contrast, hepatic progenitor cells derived from early-fetal livers (second trimester human livers) require interaction with feeder cells for *in vitro* expansion. Purified hepatic progenitor cells from early-fetal livers have properties distinct from those from mid-fetal development livers.[21]

Problem 6

As CRISPR KO/Knock-in cells are directly injected, knockout or knock-in efficiency is not to be determined, KO or KI is not measured in transduced cells (steps 62-66 for c.a.CTNNB1 and ARID1A KO for steps 67-71).

Potential solution

The transduction efficiency must be determined. If *in vitro* knockout or knock-in efficiency is to be determined, the following protocols for fetal hepatic progenitor cell culture are published.[22] Refer to Steps 72–74.

Problem 7

FAH humanization was not confirmed.

Potential solution: FAH enzyme assay

- Use human FAH (fumarylacetoacetate hydrolase: fumarylacetoacetase) ELISA Kit (MBS9332637: MyBioResource, INC.) to quantify the FAH activities in humanized FRG liver vs.
- Use Fah−/− (non-humanized control liver) for comparison.
- Prepare fumarylacetoacetate enzymatically from homogentisic acid[23] since Fumarylacetoacetate is not commercially available. Incubate Fumarylacetoacetate with cytosolic liver fractions from recipient liver, and disappearance speed was measured spectroscopically at 330 nm.
- Use wild-type and $Fah^{-/-}$ livers as positive and negative control, respectively.

Problem 8

Human fetal livers are not available, human primary hepatocytes only are injected, but very poor engraftment efficiency is observed (step 31).

Potential solution

Adult primary hepatocytes can be alternatively transplanted, but engraftment efficiency is limited. Human non-parenchymal cells are required for efficient human hepatocyte engraftment since interactions between hepatic progenitor cells and mesenchymal cells or mesenchymal stem-like cells support liver development. The fetal liver has several mesenchymal cell types, which are the origin of stellate cells and portal myofibroblasts.[24,25] Mesenchymal stem/progenitor cells reside in the adult bone marrow, adipose tissue, skeletal muscle, skin, umbilical cord, dental pulp, and amniotic fluid.[26] These mesenchymal stem/progenitor cells are also found in fetal liver tissues. In the bone

marrow, mesenchymal stem cells serve as the hematopoietic stem cell niche.[27,28] Thus, mesenchymal cells can be purified from fetal livers.[29]

Mesenchymal cell surface proteins *Dlk1midPDGFRα+* (platelet-derived growth factor receptor (PDGFR)α-positive) cells have a mesenchymal morphology and mesenchymal stem/progenitor cell-like properties in the parenchymal region of mouse E13.5–14.5 fetal livers. Dlk1midPDGFRα+ cells mesenchymal cells support the proliferation of hepatic progenitor cells through production of soluble paracrine factors.

E13 mouse livers are minced and digested with collagenase buffer. CD45 +Ter119+ cells are eliminated using MACS negative selection. Next Dlk1 + hepatic progenitor cells are purified using MACS flow cytometry beads. Purified Dlk1+ hepatic progenitor cells are cultured for the proliferation and differentiation analyses. Progenitor cells are cultured on mitomycin C-treated MEF feeder cells at a low density (since mitomycin C arrests MEF growth and avoids contamination of MEFs in the final transplanted LPCs). For hepatic maturation culture in the differentiation assay, progenitor cells are cultured on gelatin-coated dishes with hepatic maturation factors (OSM and EHS matrices).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Keigo Machida (keigo.machida@med.usc.edu).

Materials availability

This study did not generate/analyze datasets/code.

Data and code availability

Not applicable.

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AUTHOR CONTRIBUTIONS

Conceptualization, methodology, K.M., H.T.; validation, formal analysis, investigation, data curation, Y.Z., K.M.; writing – original draft, S.M.T., H.T., Y.Z., K.M.; supervision, funding acquisition, K.M., H.T.; Y.Z., S.M.T., H.T., K.M., data curation, writing – review and editing.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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