

HUMAN LIVER CHIMERIC NON-HUMAN ANIMAL WITH DEFICIENT P450 OXIDOREDUCTASE AND METHODS OF USING SAME

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application claims priority to, and the benefit of, U.S. Provisional Application No. 62/355,102, filed on June 27, 2016 and U.S. Provisional Application No. 62/509,942, filed on May 23, 2017. The entire content of each of these applications is incorporated herein by reference in their entirety.

INCORPORATION BY REFERENCE OF SEQUENCE LISTING

[0002] The contents of the text file name "KARL-001-WO_ST25," which was created on June 20, 2017 and is 67 KB in size are hereby incorporated by reference in their entirety.

BACKGROUND OF THE INVENTION

[0003] Only one out of ten drugs in development gets approved for clinical use. The majority fails during clinical trials due to inefficacy or toxicity in humans. The lack of experimental animal models to accurately predict human xenobiotic metabolism is a significant limitation, which jeopardizes human lives and drives drug development costs. Hence, there is a compelling need to develop better preclinical tools. The present disclosure solves these needs in the art by providing a human liver chimeric non-human animal model and methods of using the human liver chimeric non-human animal model to predict human specific drug metabolism.

SUMMARY OF THE INVENTION

[0004] The present disclosure provides a method for preparing a chimeric non-human animal comprising human hepatocytes, the method comprising: (a) providing a non-human animal comprising a reduction or deletion of NADPH-P450 oxidoreductase (*Por*) gene resulting in reduced or absent expression of *Por* protein; and (b) transplanting human hepatocytes into the non-human animal.

[0005] The non-human animal can comprise reducing or deleting the *Por* gene resulting in reduced or absent expression of *Por* protein. The reduced or deleted *Por* gene can be a

conditional knockdown or knockout of the *Por* gene. The reduced or deleted *Por* gene can be the result of a mutation, a transgene, treatment with an exogenous substance or somatic genome engineering, including a CRISPR (Clustered regularly interspaced short palindromic repeats) system. The somatic genome engineering comprises Guide RNA (gRNA) and Caspase 9 (Cas9).

[0006] The non-human animal can comprise a floxed allele of the *Por* gene, and wherein the non-human animal is provided with a Cre recombinase sufficient to produce a conditional knockout of the *Por* gene. The non-human animal comprising the floxed allele of the *Por* gene can be provided with at least a first dose of a virus that encodes Cre recombinase. The non-human animal can be provided with at least a second dose of a virus that encodes Cre recombinase. The non-human animal can comprise the floxed allele of the *Por* gene is crossed with a transgenic non-human animal strain expressing Cre recombinase.

[0007] In a one aspect, the method of the present disclosure comprises (a) providing a non-human animal comprising a floxed allele of the *Por* gene with a first dose of a virus that encodes Cre recombinase; (b) transplanting human hepatocytes into the non-human animal; and (c) providing the non-human animal with a second dose of a virus that encodes Cre recombinase. Steps (a) and (b) can occur sequentially or simultaneously.

[0008] The non-human animal can further comprise a reduction or deletion of at least one additional gene encoding an enzyme involved in drug metabolism. The at least one additional enzyme can be a phase II drug enzyme. In one aspect, the non-human animal can further comprise a reduction or deletion of UDP-glucose 6-dehydrogenase (UGDH) gene, a reduction or deletion of Glutathione synthetase (GSS) gene, or a combination thereof.

[0009] The reduction or deletion the UGDH gene can result in reduced or absent expression of UGDH protein. The reduction or deletion the GSS gene can result in reduced or absent expression of GSS protein. The reduced or deleted UGDH gene can be a conditional knockdown or knockout of the UGDH gene. The reduced or deleted GSS gene can be a conditional knockdown or knockout of the GSS gene. The reduced or deleted UGDH or GSS gene can be the result of a mutation, a transgene, treatment with an exogenous substance or somatic genome engineering, including a CRISPR (Clustered regularly interspaced short palindromic repeats) system. The somatic genome engineering comprises Guide RNA (gRNA) and Caspase 9 (Cas9).

[0010] The non-human animal can be selected from the group consisting of primate, bird, mouse, rat, fowl, dog, cat, cow, horse, goat, camel, sheep and pig. In a preferred aspect, the non-human animal is a mouse.

[0011] The non-human animal comprising a reducing or deleting the *Por* gene can be selected from the group consisting of (i) the FRG (*Fah*^{-/-}/*Rag2*^{-/-}/*Il2rg*^{-/-}) non-human animal, (ii) a transgenic urokinase type plasminogen activator (uPA) non-human animal, which overexpress uPA under an inducible promoter, preferably a liver-restricted albumin promoter, (iii) the thymidine kinase-NOD/Shi-*scid*/IL-2R γ ^{null} (TK-NOG) non-human animal, which is a immunodeficient NOG non-human animal with transgenic expression of thymidine kinase under control of liver-restricted promoter, (iv) a non-human animal expressing an inducible Caspase 8 in the liver, and (v) a non-human animal expressing an inducible Caspase 9 in the liver.

[0012] The present disclosure also provides a chimeric non-human animal, offspring thereof, or a portion thereof, which has a chimeric liver comprising human hepatocytes, prepared by any method disclosed herein.

[0013] The chimeric non-human animal can be immunodeficient. The chimeric non-human animal substantially lacks autogenous hepatocytes. Human hepatocytes can account for any percentage of human chimerism greater than about 1%, for example at least 5%, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or at least 99% of all hepatocytes in the chimeric liver of the chimeric non-human animal. A “non-human animal” can be amphibian, reptile, avian, or a non-human mammal. The non-human animal can be *e.g.*, any non-human mammal, *e.g.*, primate, bird, mouse, rat, fowl, dog, cat, cow, horse, goat, camel, sheep or pig. In a preferred aspect, the non-human animal is a mouse.

[0014] In one aspect, the present disclosure provides a method for preparing a chimeric non-human animal comprising human hepatocytes, the method comprising steps of: (a) providing a non-human animal that allows its liver to be repopulated with human hepatocytes and comprising a non-functional NADPH-P450 oxidoreductase generated either by genome engineering or knockdown with exogenous agents such as genome engineering tools like CRISPR/Cas9 or floxed allele of the NADPH-P450 oxidoreductase (*Por*) gene with a first dose of a virus that encodes Cre recombinase or a Cre transgenic animal, thereby producing a conditional knockout

of the *Por* gene; (b) transplanting human hepatocytes into the non-human animal; and (c) providing the non-human animal with a second dose of the virus that encodes Cre recombinase. The chimeric non-human animal can substantially lack autogenous or endogenous hepatocytes and instead comprising human hepatocytes. Steps (a) and (b) can occur sequentially or simultaneously. Any non-human animal comprising mutations and/or transgenes that allow its liver to be repopulated with human hepatocytes may be used in combination with the floxed or deleted allele of the NADPH-P450 oxidoreductase (*Por*) gene or functional inactivation of the Por protein. In aspects, the non-human animal comprising mutations and/or transgenes that allow its liver to be repopulated with human hepatocytes is (i) the FRG (*Fah*^{-/-}/*Rag2*^{-/-}/*Il2rg*^{-/-}) non-human animal, (ii) a transgenic uPA non-human animal, which overexpress urokinase type plasminogen activator (uPA) in the liver under an inducible promoter and/or preferably a liver-restricted albumin promoter, (iii) the TK-NOG non-human animal, which is a immunodeficient NOG non-human animal with transgenic expression of thymidine kinase under control of liver-restricted albumin promoter, (iv) a non-human animal expressing an inducible Caspase 8 in the liver, or (v) a non-human animal expressing an inducible Caspase 9 in the liver (vi) a non-human animal expressing human heparin-binding epidermal growth factor-like receptor (HB-EGF)-like receptors under the control of a liver cell-specific albumin promoter (alb-TRECK). A “non-human animal” can be amphibian, reptile, avian, or a non-human mammal.

[0015] In one aspect, the present disclosure provides a method for preparing a chimeric mouse substantially lacking murine hepatocytes and instead comprising human hepatocytes, comprising steps of: (a) providing a mouse that allows its liver to be repopulated with human hepatocytes and comprising a non-functional NADPH-P450 oxidoreductase generated either by genome engineering by CRISPR/Cas9 mediated deletion or knockdown with exogenous agents or floxed allele of the NADPH-P450 oxidoreductase (*Por*) gene with a first dose of a virus that encodes Cre recombinase or a Cre transgenic mouse, thereby producing a conditional knockout of the *Por* gene; (b) transplanting human hepatocytes into the mouse; and (c) providing the mouse with a second dose of the virus that encodes Cre recombinase. Steps (a) and (b) can occur sequentially or simultaneously. Any mouse that allow its liver to be repopulated with human hepatocytes may be used in combination with the floxed allele of the NADPH-P450 oxidoreductase (*Por*) gene or somatic gene deletion or reduction or inactivation of the Por gene, respectively protein. In

aspects, the mouse that allow its liver to be repopulated with human hepatocytes is (i) the FRG (*Fah*^{-/-}/*Rag2*^{-/-}/*Il2rg*^{-/-}) mouse, (ii) a transgenic uPA mouse, which overexpress urokinase type plasminogen activator (uPA) under an inducible promoter, preferably a liver-restricted albumin promoter, (iii) the TK-NOG mouse, which is a super immunodeficient NOG mouse with transgenic expression of thymidine kinase under control of liver-restricted albumin promoter, (iv) a mouse expressing an inducible Caspase 8 in the liver, (v) a mouse expressing an inducible Caspase 9 in the liver or (vi) a mouse expressing human heparin-binding epidermal growth factor-like receptor (HB-EGF)-like receptors under the control of a liver cell-specific albumin promoter (alb-TRECK).

[0016] The present disclosure also provides a method for screening and identifying metabolites for any type of drugs, typically small molecule drugs, that might affect human liver functions but also any other function of the body, comprising: (a) administering a test substance to the chimeric non-human animal of the present disclosure; (b) measuring one or more values in the chimeric non-human animal to which the test substance is administered in (a); and (c) selecting a test substance that causes an increase or a decrease in one or more values measured in (b), compared with the one or more values measured in a chimeric non-human animal to which no test substance is administered or a chimeric non-human animal without deletion of the *Por* gene or a non-human animals without human chimerism. Preferably, the one or more values are selected from but not limited to the group consisting of a metabolite of the test substance, human albumin concentration, body weight curve, liver-weight-to-body-weight ratio, total albumin level, total protein level, Alanine Aminotransferase (ALT) level, Aspartate Aminotransferase (AST) level, and total bilirubin level, creatinine, Blood Urea Nitrogen (BUN), troponine, blood count, TSH and histological assessment for pathologies in the human and non-human organs. A “non-human animal” can be amphibian, reptile, avian, or a non-human mammal. The non-human animal can be *e.g.*, any non-human mammal, *e.g.*, primate, bird, mouse, rat, fowl, dog, cat, cow, horse, goat, camel, sheep or pig. Preferably, the non-human animal is a mouse.

[0017] The present disclosure further provides a method for screening for a substance that affects human liver functions, comprising: (a) administering a test substance to the chimeric mouse of the present disclosure; (b) measuring one or more values in the chimeric mouse to which the test substance is administered in (a); and (c) selecting a test substance that causes an increase or an

decrease in one or more values measured in (b), compared with the one or more values measured in a chimeric mouse to which no test substance is administered. Preferably, the one or more values is selected from the group consisting of a metabolite of the test substance, human albumin concentration, body weight curve, liver-weight-to-body-weight ratio, total albumin level, total protein level, ALT level, AST level, and total bilirubin level, histological assessment for pathologies in the human and non-human organs.

[0018] The present disclosure also provides a method for evaluating the toxicity of a test substance against human hepatocytes, comprising: (a) administering a test substance to the chimeric non-human animal of the present disclosure; (b) measuring one or more indicators in the chimeric non-human animal to which the test substance is administered in (a); and (c) evaluating the effect of the test substance on human hepatocytes using, one or more indicators measured in (b), compared with the one or more indicators measured in a chimeric non-human animal to which no test substance is administered. Preferably, the one or more indicators is selected from the group consisting of an increase or a decrease in any one or more of a metabolite of the test substance, human albumin concentration, body weight curve, liver-weight-to-body-weight ratio, total albumin level, total protein level, ALT level, AST level, and total bilirubin level, histological assessment for toxicity in the human and non-human organs. A “non-human animal” can be amphibian, reptile, avian, or a non-human mammal. The non-human animal can be *e.g.*, any non-human mammal, *e.g.*, primate, bird, mouse, rat, fowl, dog, cat, cow, horse, goat, camel, sheep or pig. Preferably, the non-human animal is a mouse.

[0019] Throughout the specification the word “comprising,” or variations such as “comprises” or “comprising,” will be understood to imply the inclusion of a stated element, integer or step, or group of elements, integers or steps, but not the exclusion of any other element, integer or step, or group of elements, integers or steps.

[0020] About can be understood as within 10%, 9%, 8%, 7%, 6%, 5%, 4%, 3%, 2%, 1%, 0.5%, 0.1%, 0.05%, or 0.01% of the stated value. Unless otherwise clear from the context, all numerical values provided herein are modified by the term “about.”

[0021] While the disclosure has been described in conjunction with the detailed description thereof, the foregoing description is intended to illustrate and not limit the scope of the disclosure, which is defined by the scope of the appended claims. Other aspects, advantages, and modifications are within the scope of the following claims.

[0022] The patent and scientific literature referred to herein establishes the knowledge that is available to those with skill in the art. All United States patents and published or unpublished United States patent applications cited herein are incorporated by reference. All published foreign patents and patent applications cited herein are hereby incorporated by reference. Genbank and NCBI submissions indicated by accession number cited herein are hereby incorporated by reference. All other published references, documents, manuscripts and scientific literature cited herein are hereby incorporated by reference.

BRIEF DESCRIPTION OF THE DRAWINGS

[0023] The patent or application file contains at least one drawing executed in color. Copies of this patent or patent application publication with color drawings will be provided by the Office upon request and payment of the necessary fee.

[0024] The above and further features will be more clearly appreciated from the following detailed description when taken in conjunction with the accompanying drawings.

[0025] Figure 1A-D shows the generation of the PIRF strain and deletion of the murine P450 (*Por*) oxidoreductase. Figure 1A is a schematic representation of deleted and transgenic loci in the PIRF strain. Figure 1B is a graph showing qPCR of *Por* mRNA upon intravenous injection of adenovirus expressing the CRE recombinase (Adeno-Cre). Figure 1C is a series of immunostaining photographs for *Por* demonstrating a gradient across the hepatic acinus with higher pericentral (cv) and lower periportal (pv) expression. Upon injection with Adeno-Cre*Por* is barely detectable. Figure 1D is a photograph of a Western blot showing the almost complete disappearance of *Por* protein.

[0026] Figure 2A-E shows the humanization of the PIRF strain and gene expression profiling upon deletion of murine P450 oxidoreductase (*Por*). Figure 2A is a series of immunostaining photographs showing humanized PIRF and FRG mice for murine *Por* (*mPor*) and human nuclei (hNuc) after injection of Adeno-Cre (2.2×10^{10} pfu/mouse) once (1x) or twice (2x). Counterstaining in merged picture using DAPI. Figure 2B is a schematic showing the experimental outline for murine and human transcriptomics from chimeric livers with or without *Por* deletion. Figure 2C is a graph showing the murine cytochrome mRNA originating from livers of the PIRF model. Figure 2D is a graph showing the human cytochrome mRNA

originating from livers of the PIRF model. Figure 2E is a graph showing the comparison gene expression of the main drug metabolizing human cytochromes in humanized, *Por*-deleted PIRF (Hu-PIRF 2x) mice with the original, isogenic human hepatocytes. Gene expression has been normalized to three murine respectively human housekeeping genes (*PSMB2*, *PSMB4* and *RAB7A* resp. *Rab7*²⁵). PIRF; *Por*^{c/c}/*Il2rg*^{-/-}/*Rag2*^{-/-}, *Fah*^{-/-}FRG; *Fah*^{-/-}/*Rag2*^{-/-}/*Il2rg*^{-/-}.

[0027] Figure 3A-E shows xenobiotic metabolism in humanized PIRF mice. Figure 3A is a graph showing the selection of abundant and decreased gefitinib metabolites upon murine P450 oxidoreductase (*Por*) deletion in non-humanized PIRF mice using mass spectrometry in the murine feces within 24 hours after intravenous injection of gefitinib. Figure 3B is a schematic showing gefitinib metabolites and know modifications. Figure 3C is a graph showing that murine *Por*-deleted, human liver chimeric PIRF (Hu-PIRF 2x) mice and control groups show the most abundant human metabolite, M4. Figure 3D is a graph showing that murine *Por*-deleted, human liver chimeric PIRF (Hu-PIRF 2x) mice and control groups show the human specific metabolite M28. PIRF; *Por*^{c/c}/*Il2rg*^{-/-}/*Rag2*^{-/-}, *Fah*^{-/-}, FRG; *Fah*^{-/-}/*Rag2*^{-/-}/*Il2rg*^{-/-}. * $p < 0.05$ using non-parametric Mann-Whitney test. Figure 3E is a graph showing a mass spectrometry analysis of PIRF liver homogenates 30 min after injection with atazanavir, a retroviral therapeutic. A major human metabolite (M15) is shown. The overall abundance of metabolites from atazanavir or gefitinib was set as 100% in each sample. The data are expressed as mean. PIRF; *Por*^{c/c}/*Il2rg*^{-/-}/*Rag2*^{-/-}/*Fah*^{-/-}, FRG; *Fah*^{-/-}/*Rag2*^{-/-}/*Il2rg*^{-/-}. * $p < 0.05$ using non-parametric Mann-Whitney test.

[0028] Figure 4A-B shows a schematic of an exemplary probe of the present disclosure. Figure 4A is a schematic showing the design of the targeting vector and modified *Por* locus. Figure 4B is a photograph of Southern blotting with three probes demonstrating proper targeting of ESC.

[0029] Figure 5 shows beta galactosidase (*lacZ*) expression from the *POR-lacZ* allele. X-gal staining of heterozygous mouse embryo and liver demonstrate expression of the galactosidase from the *Por-lacZ* allele.

[0030] Figure 6A shows a scheme of *Il2 rg*, *Rag2* and *Fah* genes showing the gRNA location (in color) and the primers used to genotype the mice. Figure 6B shows an electrophoresis gel of the PCR products obtained from genotyping. Lane 1: PCR bands using

external (Fw and Rv) primers. For *Rag2* and *Fah* heterozygotes, a wild type (arrow) and deleted (arrowhead) allele can be detected. *Il2 rg* is an X-linked gene and no founder heterozygote females were generated. Homozygotes mice for *Il2 rg*, *Rag2* and *Fah* show a single deleted allele's band of 460bp, 530bp and 200bp, respectively. Lane 2: PCR bands using one of the external (Fw or Rev) and the internal (Int) primer located between the two gRNA sites. Only heterozygotes have a clear PCR band formed from the wild type allele. *Fah* and *Rag2* homozygotes show multiple unspecific bands while *Il2 rg* homozygotes do not produce any band.

[0031] Figures 7A and 7B show the spectrum of genomic deletions in the *Il2-rg*, *Rag2* and *Fah* gene. Figure 7A is a schematic showing CRISPR/Cas9 injected zygotes of conditional *Por*^{-/-} mice sequenced to determine of deletion of DNA. Figure 7B is a schematic showing CRISPR/Cas9 injected zygotes of conditional *Por*^{-/-} mice sequenced to determine of deletion of amino acids.

[0032] Figure 8 shows lipid phenotype in the liver of PIRF mice upon deletion of the *Por* gene using an adenovirus expressing the CRE recombinase. Two weeks after injection of the adenovirus hepatocytes start to accumulated lipids. Oil-red-O stained livers have previously been validated for efficient deletion (upper panel) respectively expression (lower panel) of the *Por* gene (not shown).

[0033] Figure 9 shows clonal expansion of *Por* expressing hepatocytes in non-humanized PIRF mice. Mice were injected intravenously with adenovirus expressing CRE recombinase deleting the *Por* gene (*POR^{fl/fl}*).

[0034] Figure 10 shows the experimental setup for drug studies in humanized and non-humanized control mice. Humanized (Hu) and not-humanized PIRF and FRG mice were injected once (1x, before transplantation of human hepatocytes) or twice (2x, before and after reaching high human chimerism) before doing drug studies. Injected adenovirus expresses the CRE-recombinase, which leads to deletion of the *Por* gene in the PIRF, but not in the FRG strain (control). Orange; murine drug metabolism, white; inhibited murine drug metabolism, blue; human drug metabolism.

[0035] Figures 11A and 11B show that the conditional KO of *POR* can also be generated using a transgenic animal, which carries an expression cassette (albumin promoter) of the CRE

recombinase. Figure 11A shows that *POR* can be detected in immunofluorescence with the control group where only one allele carries a floxed *POR* sequence the other one the wild-type *POR* ($POR^{c/+}$). In Figure 11B, both *POR* alleles are floxed ($POR^{c/c}$), leading to an almost complete deletion of the *POR* gene and a barely detectable *POR* protein. *POR* (green) and nuclei (blue, DAPI).

[0036] Figure 12 depicts the expression of P450 oxidoreductase in humanized PIRF mice. Figure 12A is a bar graph showing qPCR of human and murine specific *Por* normalized to human and murine *Gapdh*, respectively. Figure 12B is a western blot image showing murine *Por* and β -actin of liver samples from the same humanized mice.

[0037] Figure 13 depicts Gefitinib metabolites upon murine P450 oxidoreductase (*Por*) deletion. Figure 13A is a bar graph showing deletion by adenoviral delivery of CRE. Figure 13B is a bar graph showing by crossing of $Por^{c/c}$ with *Alb-Cre* mice, generating an *Alb-Cre/Por^{c/c}* strain.

[0038] Figure 14 depicts the deletion of murine P450 oxidoreductase by crossing $Por^{c/c}$ with an *Alb-Cre* transgenic mouse. Figure 14A is a confocal immunostaining image showing complete *Por* deletion. Figure 14b is a western blot image showing *Por* protein liver samples from a control *Por^{c/c}* mouse and three different *Alb-Cre/Por^{c/c}* mice. Figure 14C is a bar graph showing qPCR of murine *Por* mRNA levels.

[0039] Figure 15 is a bar graph showing Gefitinib metabolite M28 in feces.

[0040] Figure 16 is a pair of bars graphs showing Gefitinib metabolite M28 in the (A) serum and (B) urine of hu-PIRF-2x mice and control groups.

[0041] Figure 17 is a Southern blot image of targeted ESCs. Figure 17A shows the use of a 5' probe, Figure 17B shows the use of a 3' probe and Figure 17C shows the use of a neomycin probe.

[0042] Figure 18 is a Western blot image showing murine *Por* and *Gadph* from PIRF mice injected with Adeno-CRE.

[0043] Figure 19A-B is a Western blot image showing murine *Por* and *Gadph* from Humanized PIRF mice (a) and $Por^{c/c}$ and (b) $Por^{c/c}/Alb-CRE$ mice.

[0044] Figure 20 is an H&E stain of liver lobe four weeks after transduction with adenovirus showing macro- and microvesicular steatosis. Left picture is a higher magnification of boxed area on right Figure 21A-B shows gene therapy vector design for liver-specific deletion by

genome engineering of drug metabolizing enzymes in humanized mice. A. *Two vector design*: *S. pyogenes* Cas9 under the control of CMV promoter in an adenoviral vector (Ad), and Adeno-Associated Virus (AAV) expressing a sgRNA targeting a drug metabolizing enzyme and co-expression of GFP on the same construct. Figure 21B. *Single vector design*: *S. aureus* Cas9 and sgRNA can be delivered on the same AAV vector (4.85 kb). HA, HA-epitope.

[0045] Figure 22 shows simultaneous deletion of murine P450 oxidoreductase (Por) and other murine enzymes involved in drug metabolism in humanized mice by somatic genome engineering. Humanized FRG mice (human albumin in murine serum >2 mg/ml) have been injected with Adeno-Associated Virus (AAV, serotype 8) expressing sgRNA targeting an early exon of murine *Por*, UDP-glucose 6-dehydrogenase (*Ugdh*) or the glutathione synthetase (*Gss*) gene (see gene therapy vector design, figure 21). AAVs have been injected (2×10^{11} GC/AAV/mouse) 1 week before injection of Adenovirus expressing Cas9 (7×10^9 pfu/Ad/mouse). Control mice have been injected with adenoviral vector only (lower row). Shown are serial sections of a representative humanized area with immunostaining for human specific Pre-ALB (transthyretin), Por, Ugdh, Gss, hALB and GFP, the latter being expressed from the AAV gene therapy vector (see gene therapy vector design, figure 21). Bar represents 50 μ m. ALB, Albumin.

[0046] Figure 23 shows genomic deletion of *por* by CRISPR/Cas9: Wild type and humanized FRG mice (human albumin in murine serum >2 mg/ml) are injected with two Adeno-Associated Virus (AAV, serotype 8) expressing sgRNA targeting consecutive early exons of murine *Por* as well as *S. aureus* Cas9 (see gene therapy vector design, figure 21). AAVs are injected (2×10^{11} GC/AAV/mouse). Control mice are injected with adenoviral vector expressing Cas9 only (7×10^9 pfu/Ad/mouse). Shown are PCR amplifications of the region spanning the two nearby targeting sites. Upon CRISPR/Cas9 mediated cutting of DNA on both target sites results in a deletion of the *por* gene leading to a smaller PCR amplicon.

[0047] Figure 24 shows humanized PIRF mouse with transgenic Alb-CRE and deletion of other murine enzymes involved in drug metabolism. *Por* was deleted by expression of CRE, but instead of adenoviral CRE, this PIRF mouse carries an Alb-CRE sequence within the murine genome. After humanization, mice were injected with Adeno-Associated Virus (AAV, serotype 8) expressing sgRNA targeting an early exon UDP-glucose 6-dehydrogenase (*Ugdh*) or the

glutathione synthetase (*Gss*) gene (see gene therapy vector design, figure 21). AAVs are injected (2×10^{11} GE/AAV/mouse) 1 week before injection of Adenovirus expressing Cas9 (7×10^9 GE/Ad/mouse). Shown are serial sections of a representative humanized area with immunostaining for human specific Pre-ALB (transthyretin), *Por*, *Ugdh* or *Gss*. Bar represents 50 μ m. ALB, Albumin.

[0048] Figure 25A-D shows troglitazone Phase II metabolites detected in liver of humanized and non-humanized FRG mice with and without *Por* and *Ugdh* deletion. Percentage of liver glucuronidated and sulfated metabolites detected in humanized livers 2 hours after i.p injection of troglitazone (600mg/kg). Figure 25A. Humanized FRG mice. Figure 25B. Humanized FRG mice after murine *Por* and *Ugdh* deletion. Figure 25C. Non-humanized FRG mice. Figure 25D. Non-humanized FRG mice with *Por* and *Ugdh* genes deleted. Sulfate metabolites of troglitazone in red and glucuronide conjugates in blue.

DETAILED DESCRIPTION OF THE INVENTION

[0049] Human liver chimeric mice have been recently introduced to predict human xenobiotic metabolism and toxicity. Despite their potential, the remaining murine liver, containing an expanded set of P450 cytochromes, makes it difficult to accurately predict human drug metabolism. Therefore, the present disclosure provides a conditional knock-out mouse of the NADPH-P450 oxidoreductase (*Por*) gene, which is the only electron donor for all murine cytochromes and if deleted, embryonically lethal¹, thereby allowing a functional inactivation of all murine cytochromes.

[0050] Any mouse comprising mutations and/or transgenes that allow its liver to be repopulated with human hepatocytes may be used in combination with the conditional knock-out allele or other genomic deletion of the NADPH-P450 oxidoreductase (*Por*) gene. In embodiments, the mouse comprising mutations and/or transgenes that allow its liver to be repopulated with human hepatocytes is (i) the FRG (*Fah*^{-/-}/*Rag2*^{-/-}/*Il2rg*^{-/-}) mouse, (ii) a transgenic uPA mouse, which overexpress urokinase type plasminogen activator (uPA) under an inducible promoter, preferably a liver- restricted albumin promoter, (iii) the TK-NOG mouse, which is a immunodeficient NOG mouse with transgenic expression of thymidine kinase under control of liver-restricted albumin promoter, (iv) a mouse expressing an inducible Caspase 8 in the liver, (v) a mouse expressing an

inducible Caspase 9 in the liver or (vi) a mouse expressing human heparin-binding epidermal growth factor-like receptor (HB-EGF)-like receptors under the control of a liver cell-specific albumin promoter (alb-TRECK). Using such mice and an adenoviral or transgenic strategy expressing CRE, an almost complete deletion of the murine *Por* gene can be generated leading to an exclusive human cytochrome metabolism.

[0051] In the uPA-SCID mouse (Rhim et al 1994; Tateno et al. 2004), the genetic cause of mouse hepatocyte ablation is uroplasminogen activator (uPA); the mouse is in the SCID immune deficient background or Rag2 (or Rag1)-/- and/or Il2rg-/- all leading to the ability to transplant and engraft human hepatocytes.

[0052] In the FRG mouse (Azuma et al 2007⁷, Bissig et al 2007⁵), the genetic cause of mouse hepatocyte ablation is fumarylacetoacetate hydrolase deficiency and mouse hepatocyte ablation is controlled by $\pm$ NTBC and/or $\pm$ low tyrosine diet; the mouse is in the Il2rg -/- and Rag2 -/- background. The FRG mouse combines immune-deficiency-mediating mutations, in the recombination activating gene 2 (Rag2) and the gamma chain of the interleukin 2 receptor (Il2rg), with a functional knockout of the fumarylacetoacetate hydrolase (Fah) gene (Azuma et al 2007⁷, Bissig et al 2007⁵). The latter gene codes for an enzyme in the tyrosine catabolic pathway and its mutation leads to an intracellular accumulation of a toxic intermediate in hepatocytes. Unlike the uPA/SCID model, the onset and severity of hepatocellular injury in FRG mice is controllable through the administration and withdrawal of the protective drug 2-(2-nitro-4-trifluoromethylbenzoyl)-1,3- cyclohexanedione (NTBC), which blocks an upstream enzyme in the tyrosine path-way and thereby prevents accumulation of the toxic intermediate.

[0053] In the TK-NOG mouse (Hasegawa et al 2011), the genetic cause of mouse hepatocyte ablation is the herpes simplex virus thymidine kinase and mouse hepatocyte ablation is controlled by $\pm$ ganciclovir; the mouse is in the Il2rg -/- and SCID background. Mouse hepatocyte ablation in this TK-NOG model was achieved through the liver-specific expression of the herpes simplex virus 1 thymidine kinase (HSVtk) in severely immunodeficient NOG mice and administration of ganciclovir (GCV), utilizing the fact that HSVtk converts the otherwise nontoxic GCV into a toxic intermediate.

[0054] In the AFC8 mouse (Washburn et al 2011), the genetic cause of mouse hepatocyte ablation is a FK508-capsae 8 fusion and mouse hepatocyte ablation is controlled by $\pm$ AP20187; the mouse is in the Il2rg -/- and Rag2 -/- background.

[0055] In the Alb-TRECK/SCID mouse (Zhang et al 2015), the genetic cause of mouse hepatocyte ablation is the human heparin-binding EGF-like receptor and mouse hepatocyte ablation is controlled by $\pm$ Diphtheria toxin; the mouse is in the SCID immune deficient background.

[0056] Sheer and Wilson, 2015 compares major features of various different liver humanized models and process of liver reconstitution in the most frequently used models to date. This reference is incorporated by reference in its entirety.

[0057] The present disclosure also provides methods of utilizing the humanized, murine *Por* deficient mice to predict human drug metabolism. In an embodiment, the FRG mouse and the conditional *Por*^{-/-} mouse was combined to generate the PIRF (*Por*^{-/-} *Il2rg*^{-/-} *Rag2*^{-/-} *Fah*^{-/-}) strain, which allows repopulation with human hepatocytes. Homozygous PIRF mice are fertile and can be repopulated with human hepatocytes generating high human chimerism (>80% human).

[0058] Human p450 cytochrome clusters contain 57 putatively functional genes and 58 pseudogenes, while the mouse cytochrome clusters are greatly expanded accounting for 102 putatively functional genes and 88 pseudogenes². This makes accurate prediction of human drug metabolism in the mouse challenging. In addition hepatotoxicity together with hypersensitivity/cutaneous reactions have the poorest correlation with animal studies yet are the most common reasons for toxicity related termination of drugs in clinical development³.

[0059] Since the liver is the main organ for drug metabolism, human liver chimeric mice are increasingly used for xenobiotic studies⁴⁻⁶. The shortcoming of humanized mice is the remaining murine liver tissue. It has been previously shown that even in mice that can achieve high human chimerism, the average humanization rate is 42%⁷. In order to functionally block the murine cytochrome metabolism, a conditional (floxed exon 3 and 4) knock-out of the NADPH-P450 oxidoreductase (*Por*) gene was generated by targeting mouse embryonic stem cells⁸ (Figure 4). Injected blastocysts with properly targeted embryonic stem cells generated mice with germline transmission of the *Por* “knock-out first” allele⁹. Expression from the targeted *Por* locus using the *lacZ* expression cassette was confirmed in the embryo and adult liver (Figure 5). The mice were then bred with a flippase expressing strain¹⁰ to generate a CRE

recombinase conditional *Por* knock-out strain. Homozygous zygotes from this strain were injected with the bacterial type II Clustered Regularly-Interspaced Short Palindromic Repeats/Cas9 (CRISPR-Cas9) system¹¹⁻¹³ targeting simultaneous deletion of critical exons of the *Il2-rg* and *Rag2* and *Fah* gene (Figure 6) to generate the PIRF strain (Figure 1A). Homozygous PIRF mice are immune deficient (T-, B- and NK-cell deficient), but healthy and fertile. Since adenoviral gene therapy vectors efficiently transduce hepatocytes *in vivo*, the *Por* gene was deleted using an adenovirus coding the CRE recombinase (Adeno-CRE). Increasing doses ($2.2 \times 10^{8-10}$ per mouse) of the virus were injected intravenously into PIRF mice. Quantitative RT-PCR of the *POR* mRNA in liver revealed efficient deletion only at high doses (Figure 1B). Immunostaining for POR (Figure 1C) confirmed these findings, while a minimal residual signal could be detected by Western blotting even at the highest dose used (Figure 1D). POR-deleted PIRF mouse livers accumulated lipids starting two weeks after adenoviral transduction (Figure 7) similar to a previously reported liver specific *Por* deletion¹⁴.

[0060] To generate human specific P450 cytochrome metabolism, human liver chimeric mice were generated by transplanting human hepatocytes^{7, 15, 16} into *Por* deleted PIRF mice. However, since a clonal expansion of residual *Por* expressing murine hepatocytes was observed in Adeno-Cre treated PIRF mice (Figure 8), some humanized PIRF (Hu-PIRF) mice were injected with an additional dose of Adeno-Cre. Immunostaining revealed that only in double injected humanized PIRF (Hu-PIRF 2x) mice an almost complete deletion of the *Por* gene could be achieved (Figure 2A).

[0061] Gene expression profiling was then performed comparing Hu-PIRF mice repopulated with the identical human hepatocytes with or without deletion of the *Por* (Figure 2B). Expression of the murine P450 cytochromes was clearly altered for half of the genes: 14 cytochromes were upregulated >1.5-fold and 18 cytochromes downregulated <0.5-fold (Figure 2c). The expression profiles of these murine cytochromes were comparable to previous work in non-humanized mice (Table 1). Table 1 shows the comparison of murine gene expression profiles of chimeric livers to previously published non-humanized mice. Gene expression of conditional (Alb-Cre) *Por* KO mice have been quantified by microarray analysis (Weng et al. 2005 J Biol Chem 280, 31686-31698 (2005)). Here, RNA-Seq was used to compare the gene expression

(Figure 2B) in humanized livers transduced with Adeno-Cre and Adeno-GFP. Table 1 lists all previously published cytochromes with values (fold changes) compared to the herein-described data set. Multiple numbers represent multiple sets of microarray probes.

[0062] Table 1: Comparison of murine gene expression profiles of chimeric livers to previously published non-humanized mice.

Murine P450 Cytochromes	Present Disclosure	Weng et al. 2005
	Change Fold (<i>Por</i> -deleted/ <i>Por</i> non-deleted mice)	
Cyp2a4	1.4	4.5
Cyp2a5	1.3	4.5
Cyp2b10	12.4	15.8/16.3/9.1
Cyp2c39	1.8	1.4
Cyp2c55	14.6	17.2
Cyp4a10	3.7	0.3/0.7
Cyp7a1	4.6	3.1/4.9
Cyp7b1	1.6	0.2/0.3
Cyp26a1	6.7	3.5
Cyp51	0.6	2.2

[0063] In the same chimeric liver, all human P450 cytochromes were down regulated upon deletion of murine *Por* with the exception of CYP2C18 (Figure 2D). Half of the human cytochromes were only slightly (<50%) reduced, and the other half including CYP3A4 and CYP2C19, were more significantly downregulated (>1.5-fold).

[0064] Not all human cytochromes take an important role in xenobiotic metabolism. From the 200 most transcribed drugs in the United States about three quarter are metabolized through P450 cytochromes, of which CYP3A4/5, 2C9, 2C19, 2D6 and 1A2 contribute to ~95% of¹⁷. These human cytochrome clusters were compared from chimeric livers (Hu-PIRF 2x) with the originating, isogenic primary hepatocytes after isolation from the donor liver. Expression levels were similar for most clusters and these important cytochromes robustly expressed in chimeric livers (Figure 3D).

[0065] To validate utility of Hu-PIRF mice for human drug metabolism, the xenobiotic metabolism of gefetinib¹⁸, an inhibitor of epidermal growth factor receptor used against lung cancer and a variety of other cancers¹⁹, was studied. Gefetinib is primarily metabolized by the P450 cytochrome system including CYP3A4 and 2D6. New gefetinib metabolites were recently

identified and demonstrated considerable differences between human and mouse liver microsomes²⁰. Gefetinib is excreted in the feces and less than 7% in the urine, irrespectively of dose, route or species^{21, 22}. Therefore, the feces of non-humanized PIRF mice was analyzed for gefetinib metabolites during the first 24-hours after intravenous injection of gefetinib. Mass spectrometry revealed a reduction of several gefetinib metabolites upon deletion of the *Por* gene, implying a *Por*-dependent P450 cytochrome deficiency for these metabolites (Figure 3A). The biggest and most relevant reduction was observed for O-desmethyl gefetinib (M4, M523595), which is by far the most abundant metabolite in human feces while rodents produce many different metabolites including M4^{21, 22} (Figure 3B). Therefore, the M4 metabolite was analyzed in murine *Por*-deleted and *Por*-expressing humanized and non-humanized control mice (Figure 9). The highest level of M4 could be detected in murine *Por*-deficient Hu-PIRF mice, where human hepatocytes preferentially metabolize gefetinib to M4 and remaining murine hepatocytes are inhibited in their drug metabolism (Figure 3C). Although murine hepatocytes preferentially produce other metabolites than M4, human specific metabolites were measured. M28 was the most abundant human metabolite, which could not be detected in the non-humanized control mice. Mass spectrometry again showed the highest level of this human specific metabolite in murine *Por*-deficient Hu-PIRF mice confirming a more human like metabolism in these mice (Figure 3D). Human xenobiotic metabolism was also determined with another drug, however, this time using liver homogenates of PIRF mice. Using human and mouse microsomes, it was previously demonstrated that atazanavir metabolite M15 is a predominately human metabolite (See, Li, F et al., "CYP3A-mediated generation of aldehyde and hydrazine in atazanavir metabolism." *Drug Metab Dispos* 39, 394-401. Mice were intravenously injected with the retroviral therapeutic and livers were harvested 30 min after injection. Results showed that M15 was 5.4-times elevated in *POR*-deleted humanized PIRF mice compared to non-deleted mice (Figure 3E) again confirming optimized human drug metabolism in this novel mouse model.

[0066] Identification of human metabolites using current experimental animal models is a major challenge. Nevertheless, identification of reactive metabolites is crucial since they drive human drug toxicity^{23, 24}. The novel humanized mouse model of the instant disclosure inhibits murine

drug metabolism without impeding on the human metabolism. Murine Por-deficient humanization can be used in combination with other repopulation models like the transgenic uPA mouse and can identify more readily human specific metabolites for a greater benefit of drug safety.

[0067] Identification of mostly human or human specific metabolites is possible with the present disclosure irrespectively of toxicity. Toxicity may be present; however this is not always the case. For instance, as shown here, gefitinib did not cause any elevation of liver enzymes, yet mainly human metabolites were identified.

[0068] The present disclosure provides a method for preparing a chimeric mouse substantially lacking murine hepatocytes and instead comprising human hepatocytes, comprising steps of: (a) providing a mouse comprising a knockout mutation in each of the *Il2-rg*, *Rag2*, and *Fah* genes and a floxed allele of the NADPH-P450 oxidoreductase (*Por*) gene with a first dose of a virus that encodes Cre recombinase, thereby producing a conditional knockout of the *Por* gene or the knockout of the *por* gene using somatic genome engineering (CRISPR/Cas9) and gene therapy vectors in *Il2-rg*, *Rag2*, and *Fah* deficient mice; (b) transplanting human hepatocytes into the mouse; and (c) providing the mouse with a second dose of the virus that encodes Cre recombinase. Steps (a) and (b) can occur sequentially or simultaneously.

[0069] The conditional knock-out POR alleles can also be generated by delivering CRE recombinase in any way known in the art. Non-limiting examples of Cre recombinase delivery include viral or non-viral gene therapy vectors. In one embodiment, the gene therapy vector is an adenovirus. Also considered are genetic delivery of Cre recombination, e.g., under a cell-, tissue-, or developmental-specific promoter or under an inducible promoter. Indeed, Cre recombinase can be activated in the murine liver in a transgenic animal with Cre expressed under the albumin or other liver specific promoter (Figure 11).

[0070] The present disclosure also provides a chimeric mouse, offspring thereof, or a portion thereof, which has a chimeric liver comprising human hepatocytes. Preferably, the chimeric mouse, offspring thereof or a portion thereof is prepared by the methods of the present disclosure. The chimeric mouse can be immunodeficient.

[0071] In the present disclosure, examples of the chimeric mouse include portions of the mouse. The term "a portion(s) of the mouse" refers to, mouse-derived tissues, body fluids, cells, and disrupted products thereof or extracts therefrom, for example (the examples thereof are not

particularly limited to them). Examples of such tissues include, but are not particularly limited to, heart, lungs, kidney, liver, gallbladder, pancreas, spleen, intestine, muscle, blood vessel, brain, testis, ovary, uterus, placenta, marrow, thyroid gland, thymus gland, and mammary gland. Examples of body fluids include, but are not particularly limited to, blood, lymph fluids, and urine. The term "cells" refers to cells contained in the above tissues or body fluids, and examples thereof include cultured cells, sperm cells, ova, and fertilized eggs obtained by isolation or culture thereof. Examples of cultured cells include both primary cultured cells and cells of an established cell line. Examples of the portions of the mouse also include tissues, body fluids, and cells at the developmental stage (embryonic stage), as well as the disrupted products or extracts thereof. In addition, an established cell line from the mouse of the present disclosure can be established using a known method (Primary Culture Methods for Embryonic Cells (Shin Seikagaku Jikken Koza (New Biochemical Experimental Lecture Series), Vol. 18, pages 125-129, TOKYO KAGAKU DOZIN CO., LTD., and Manuals for Mouse Embryo Manipulation, pages 262-264, Kindai Shuppan)).

[0072] The mouse of the present disclosure can be an immunodeficient mouse. The immunodeficient mouse of the present disclosure can be used as a host mouse for transplantation of human hepatocytes. Examples of the "immunodeficient mouse" may be any mouse that does not exhibit rejection against hepatocytes (in particular, human hepatocytes) from a different animal origin, and include, but are not limited to, SCID (severe combined immunodeficiency) mice exhibiting deficiency in T- and B-cell lines, mice (NUDE mice) that have lost T cell functions because of genetic deletion of the thymus gland, and mice (RAG2 knockout mice) produced by knocking out the RAG2 gene by a known gene targeting method (Science, 244: 1288-1292, 1989).

[0073] Moreover, the present disclosure provides a chimeric mouse having human hepatocytes. The chimeric mouse of the present disclosure can be immunologically deficient. The chimeric mouse of the present disclosure can be prepared by transplanting human hepatocytes into an immunodeficient mouse of the present disclosure.

[0074] As human hepatocytes to be used for transplantation, human hepatocytes isolated from normal human liver tissue by a conventional method such as a collagenase perfusion method can be used. The thus separated hepatocytes can also be used by thawing after cryopreservation. Alternatively, the chimeric mouse hepatocytes, which are defined as the human hepatocytes

separated by a technique such as a collagenase perfusion method from a chimeric mouse liver, in which mouse hepatocytes have been replaced by human hepatocytes, can be used in a fresh state, and the cryopreserved chimeric mouse hepatocytes are also available after thawing.

[0075] Such human hepatocytes can be transplanted into the liver via the spleen of a mouse of the present disclosure. Such human hepatocytes can also be directly transplanted via the portal vein. The number of human hepatocytes to be transplanted may range from about 1 to 2,000,000 cells and preferably range from about 200,000 to 1,000,000 cells. The gender of the mouse of the present disclosure is not particularly limited. Also, the age on days of the mouse of the present disclosure upon transplantation is not particularly limited. When human hepatocytes are transplanted into a young mouse (early weeks of age), human hepatocytes can more actively proliferate as the mouse grows. Hence, about 0- to 40-day-old mice after birth, and particularly about 8- to 40-day-old mice after birth are preferably used.

[0076] The transplanted human hepatocytes account for any percentage of human chimerism greater than about 1%, for example at least 5%, at least 10%, at least 20%, at least 30%, at least 40%, at least 50%, at least 60%, at least 70%, at least 80%, at least 90%, at least 95% or at least 99% of all hepatocytes in the chimeric liver of the chimeric non-human animal.

[0077] The present disclosure further provides a method for screening for a substance that affects human liver functions, with the use of the chimeric mouse of the present disclosure. An example of the method is an evaluation method comprising the following steps of: (a) administering a test substance to the chimeric mouse of the present disclosure; (b) measuring one or more values in the chimeric mouse to which the test substance is administered in (a); and (c) selecting a test substance that causes an increase or a decrease in one or more values measured in (b), compared with the one or more values of the chimeric mouse to which no test substance is administered.

[0078] Preferably, the one or more values are selected from the group consisting of the human albumin concentration, the body weight curve, the liver-weight-to-body-weight ratio, the total albumin level, the total protein level, the ALT level, the AST level, and the total bilirubin level, histological assessment for toxicity in the human and non-human organs.

[0079] Examples of the "test substance" in the method of the present disclosure are not particularly limited and include natural compounds, organic compounds, inorganic compounds, proteins, antibodies, peptides, and single compounds such as an amino acid, and nucleic acids, as well as compound libraries, expression products from gene libraries, cell extracts, cell culture

supernatants, products of fermenting microorganisms, extracts from marine creatures, plant extracts, extracts from prokaryotic cells, extracts from eukaryotic single cells, and extracts from animal cells. These products may be purified products or crude products such as plant, animal, or microbial extracts. Also, a method for producing a test substance is not particularly limited. A test substance to be used herein may be a substance isolated from a natural product, synthesized chemically or biochemically, or prepared by genetic engineering techniques.

[0080] The above test substance can be adequately labeled and then used as necessary. Examples of labels include radiolabels and fluorescent labels. Examples of the test substance include, in addition to the above test samples, mixtures of a plurality of types of these test samples.

[0081] Examples of test samples include and are not limited to feces, urine, blood (and any blood product, e.g., whole blood, serum, and plasma), and tissue, e.g., liver tissue. Liver tissue may be derived from a sample of a liver (e.g., a biopsy or explant) or may be derived from a whole, intact liver, e.g., that has been harvested after a mouse has been sacrificed.

[0082] Examples of a method for administering a test substance to mice are not particularly limited. Such an administration method can be adequately selected from among oral administration or parenteral administration such as subcutaneous, intravenous, local, transdermal, and enteral (intrarectal) administration, depending on the type of a test substance to be administered.

[0083] The present disclosure further provides a method for evaluating hepatotoxicity of a test substance against human hepatocytes, with the use of the chimeric mouse of the present disclosure. An example of this method is an evaluation method comprising the following steps of: (a) administering a test substance to the chimeric mouse of the present disclosure; (b) measuring one or more values in the chimeric mouse to which the test substance is administered in (a); and (c) evaluating the effect of the test substance on human hepatocytes using one or more indicators measured in (b), compared with the one or more indicators of the chimeric mouse to which no test substance is administered.

[0084] Preferably, the one or more values are selected from the group consisting of the human albumin concentration, the body weight curve, the liver-weight-to-body-weight ratio, the total albumin level, the total protein level, the ALT level, the AST level, and the total bilirubin level. Preferably, the one or more indicators are selected from the group consisting of an increase or a decrease in any one or more of the human albumin concentration, the body weight curve, the

liver-weight-to-body-weight ratio, the total albumin level, the total protein level, the ALT level, the AST level, and the total bilirubin level.

[0085] A human nucleic sequence encoding an exemplary *Por* gene of the disclosure consist or comprises, Genbank Accession number: NM_000941.2:

```

1   gaaggcgggtg gtagcgcctc agtgggtgtgg gcctgagccc tgcccagggtg cccgcagaga
61  gcagccggggc tgccagcgtt tcatgatcaa catgggagac tcccacgtgg acaccagctc
121 caccgtgtcc  gaggcgggtg ccgaagaagt atctcttttc agcatgacgg acatgattct
181 gttttcgctc  atcgtgggtc tcttaacctc ctggttcctc ttcagaaaga aaaaagaaga
241 agtccccgag  ttcacaaaaa ttcagacatt gacctcctct gtcagagaga gcagctttgt
301 ggaaaagatg  aagaaaacgg ggaggaacat catcgtgttc tacggtctcc agacggggac
361 tgcagaggag  tttgccaacc gcctgtccaa ggacgcccac cgctacggga tgcaggcat
421 gtcagcggac  cctgaggagt atgacctggc cgacctgagc agcctgccag agatcgacaa
481 cgccctgggtg gttttctgca tggccacctc cggtagaggga gacccaccg acaatgccc
541 ggacttctac  gactggctgc aggagacaga cgtggatctc tctgggtca agttcgcggt
601 gtttgggtctt gggaacaaga cctacgagca cttcaatgcc atgggcaagt acgtggacaa
661 gcggctggag  cagctcggcg cccagcgcac ctttgagctg ggggtgggag acgacgatgg
721 gaacttggag  gaggacttca tcacctggcg agagcagttc tggccggccg tgtgtgaaca
781 ctttgggggtg gaagccactg gcgaggagtc cagcattcgc cagtacgagc ttgtgggtcca
841 caccgacata  gatgcggcca aggtgtacat gggggagatg ggccggctga agagctacga
901 gaaccagaag  cccccctttg atgccaagaa tccgttcctg gctgcagtca ccaccaaccg
961 gaagctgaac  cagggaaaccg agcgccacct catgcacctg gaattggaca tctcgactc
1021 caaaatcagg  tatgaatctg gggaccacgt ggctgtgtac ccagccaacg actctgctct
1081 cgtcaaccag  ctgggcaaaa tctgggtgac cgacctggac gtctcatgt ccctgaacaa
1141 cctggatgag  gagtccaaca agaagcacc cttcccgctg cctacgtcct accgcacggc
1201 cctcacctac  tacctggaca tcaccaaccc gccgcgtacc aacgtgctgt acgagctggc
1261 gcagtacgac  tcggagccct cggagcagga gctgctgcgc aagatggcct cctcctccg
1321 cgaggccaag  gagctgtacc tgagctgggt ggtggaggcc cggaggcaca tctcggactc
1381 cctgcaggac  tgcccgtccc tgcggccccc catcgaccac ctgtgtgagc tgcctgcgag
1441 cctgcaggcc  cgctactact ccactgcctc atcctccaag gtccacccca actctgtgca
1501 catctgtgag  gtggttgtgg agtacgagac caaggctggc cgcatcaaca agggcgtggc
1561 caccaactgg  ctgcggggcca aggagcctgc cggggagaaac ggcggccgtg cgctggtgac
1621 catgttctgt  cgcaagtccc agttccgctt gcccttcaag gccaccacgc ctgtcatcat
1681 ggtgggcccc  ggcaaccggg tggcacctt cataggcttc atccaggagc gggcctggct
1741 gcgacagcag  ggcaaggagg tgggggagac gctgctgtac tacggtgcc gccgctcgga
1801 tgaggactac  ctgtaccggg aggagctggc gcagttccac agggacggtg cgctcaccga
1861 gctcaacgtg  gccttctccc gggagcagtc ccacaaggtc tacgtccagc acctgctaaa
1921 gcaagaccga  gagcacctgt ggaagtgtat cgaaggcggg gccacatct acgtctgtgg
1981 ggatgcacgg  aacatggcca gggatgtgca gaacaccttc tacgacatcg tggctgagct
2041 cggggccatg  gagcacgcgc aggcgggtga ctacatcaag aaactgatga ccaagggccg
2101 ctactccctg  gacgtgtgga gctaggggac tgctgcccc acccaccaca cagactccgg
2161 cctgtaatac  gctctcctgg ctccctcccg tagtctcctg ggtgtgtttg gcttggcctt
2221 ggcatgggag  caggcccagt gacaaaagac cctctgggac tggggtgcat cctcctcagc
2281 cccaggccca  ggtgaggtcc accggccctg ggcagcacag ccagggcct gcatgggggc
2341 accgggctcc  atgctctctg aggcctctgg ccctcggtgg ctgcacagaa gggctctttc
2401 tctctgtgta  gctgggcccc gccctccac gtgatttcca gtgagtgtaa ataattttaa
2461 ataacctctg  gcccttgtaa taaagttctg ttttctgtaa aaaaaaaaa (SEQ ID NO: 24)

```

[0086] The corresponding human amino acid sequence encoding an exemplary *Por* gene of the disclosure consist or comprises, Genbank Accession number: NP_000932.3:

```

1 minmgdshvd tsstvseava eevslfsmtd milfslivgl ltywflfrkk keevpeftki
61 qtltsstvres sfvekmkktg rniivfygsq tgtaeefanr lskdahrygm rgmsadpeey

```

```

121 dladlsslpe idnalvvfcm atygegdptd naqdfydwlg etdvdlsgvk favfglgnkt
181 yehfnamgky vdkrleqlga grifelglgd ddgnleedfi twreqfwpav cehfgveatg
241 eessirqyel vvhtdidaak vymgemgrlk syengkppfd aknpflaavt tnrklnqgte
301 rhlmhleldi sdsikiryesg dhvavypand salvnqlgki lgadldvms lnnldeesnk
361 khpfpcptsy rtalyyldi tnpprtnvly elaqyaseps eqellrkmas ssgegkelyl
421 swvvearrhi lailqdcpsl rppidhlcel lprlqaryys iassskvhpn svhicavvve
481 yetkagrink gvatnwlrak epagenggra lvpmfvrksq frlpfkattp vimvgpgtg
541 apfigfiqer awlrqqgkev getllyygc rsdedylyre elaqfhrdga ltqlnvafsr
601 eqshkvvyqh llkqdrehlw klieggahiy vcgdarnmar dvqntfydiv aelgamehaq
661 avdyikklmt kgryslvws (SEQ ID NO: 25)

```

[0087] A murine nucleic sequence encoding an exemplary Por gene of the disclosure consist or comprises, Genbank Accession number: NM_008898.2:

```

1   gggccgtggt agcgccctcag tgggtgcgggc ttgcgtccgg ccccaagtgc tcagagacct
61  acaggaccgc gcgcggtgtg tgatctggtc ggtaccgagg agcgagggtt gtgtcaccaa
121 catgggggac tctcacgaag acaccagtgc cacagtgcct gaggcagtgg ctgaagaagt
181 gtctctattc agcacaacgg acattgttct gttttctctc atcgtggggg tcctgacctc
241 ctggttcctc tttaaaaaga agaaagaaga gataccggag ttcagcaaga tccagacaac
301 ggccccacct gtcaaagaga gcagcttcgt ggaaaagatg aagaaaacgg gaaggaacat
361 tattgtattc tatggctccc agacgggaac cgcgaggagg tttgccaacc ggctgtccaa
421 ggatgcccac cgctatggga tgcggggcat gtctgcagac cctgaagagt atgacttggc
481 cgacctgagc agcctgcctg agatcgacaa gtccctggta gtctctgca tggccacata
541 cggagaaggc gacccacccg acaacgcgca ggacttctat gattggctgc aggagactga
601 cgtggacctc acgggtgtca agtttgcgtg gtttggctct gggaacaaga cctatgagca
661 cttcaacgcc atgggcaagt atgtggacca gcggtcggag cagcttggcg cccagcgaat
721 ctttgagttg gccttgggtg atgacgacgg gaacttggaa gaggatttca tcacatggag
781 ggagcagttc tggccagctg tgtgcgagtt cttcgggggt gaagccactg gggaggagtc
841 gagcatccgc cgtacgagc tcgtggtcca cgaagacatg gacacagcca aggtgtacac
901 gggtagatg ggcgtctga agagctacga gaaccagaaa ccccccttcg atgccaagaa
961 tccattcctg gctgctgtca ccacgaaccg gaagctgaac caaggcactg agaggcatct
1021 aatgcacctg gaattggaca tctcagactc caagatcagg tatgaatctg gagatcacgt
1081 ggtgtgtgac ccagccaacg actccacctt ggtcaaccag attggggaga tcctgggggc
1141 tgacctggat gtcacatgtt ctctaaacaa tctcgatgag gagtgcgaata agaagcatcc
1201 gttccctgct cccaccacct accgacggcg cctcacctac tacctggaca tcactaacc
1261 gccacgaacc aacgtgctct acgagctggc ccagtacgcc tcagagccct cggagcagga
1321 acacctgcac aagatggcgt cctcctccgg cgagggcaag gagctgtacc tgagctgggt
1381 ggtggaggcc cggaggcaca tctagccat tctccaagac taccgtccc tgcggccacc
1441 catcgaccac ctgtgcgagc tctcccggag gctgcaggcc cgctactatt ccattgcctc
1501 gtcgtcctaag gtccacccca actccgtgca catctgcgcc gtggctgtgg agtatgaagc
1561 gaagtctgga cgagtgaaca aggggtggc caccagctgg cttcggaaca aggaaccagc
1621 aggagagaat ggccgcccgg ccttggctcc catgttcgtc cgcaagtccc agttccgctt
1681 gcctttcaag cccaccacac ctgttatcat ggtgggcccc ggactgggg ttgcccttt
1741 catgggcttc atccaggagc gggcttgct tcgagagcaa ggcaaggagg tcggagagac
1801 gctgctctac tacggctgcc ggcgctcgga tgaggactat ctgtaccgcg aggagctggc
1861 gcgcttcac aaggacggcg cctcacgca gcttaatgtg gcctttccc gtgagcaggc
1921 ccacaaggct tatgttcagc aactgctcaa gagggacaaa gagcacctgt ggaagctgat
1981 ccacgaaggt ggtgccaca tctatgtctg cggggatgct cgaaatatgg ccaaagatgt
2041 gcagaacaca ttctatgaca tcgtggccga gtttggccc atggagcaca cccaggctgt
2101 ggactatggt aagaagctca tgaccaaggg ccgctactcg ctggatgtat ggagctagga
2161 gctgcgccc cccaccctc gctccctgta atcacgtcct taacttctct ctgccacct
2221 ccacctctgg tggttctctg cctgcctgga cacaggagg cccagggact gactcctggc
2281 ctgagtgatg cctcctggg ccttaggca gagcctggtc cattgtacca ggcagcctag
2341 cccagcccag ggcacatggc aagagggact ggaccacact ttgggtgatg ggtgccttag

```

2401 gtccccagca gctgtacaga aggggctctt ctctccacag agctggggtg cagccccaac
 2461 atgtgatttt gaatgagtgt aaataatttt aaataacctg gcccttgaa taaagttgtt
 2521 ttctgtta (SEQ ID NO: 26)

[0088] The corresponding murine amino acid sequence encoding an exemplary Por gene of the disclosure consist or comprises, Genbank Accession number: NP_032924.1:

1 mgdshedtsa tpeavaeev slfsttdivl fslivgvlt y wfifkkkkee ipfkskiqtt
 61 appvkessfv ekmkktgrni ivfygsgtgt aeefanrlsk dahrygmrgm sadpeeydla
 121 dlsslpeidk slvvfcmaty gegdptdnaq dfydwlqetd vdlgtgvkfav fglgnktyeh
 181 fnamgkyvdq rleqlgaqri felglgddd nleedfitwr eqfwpavcef fgveatgees
 241 sirqyelvvh edmdtakvyt gemgrlksye nqkppfdakn pflaavttmr klnqgterhl
 301 mhleldisds kiryesgdhv avypandstl vnqigeilga dldvimslnn ldeesnkhhp
 361 fpcpttyrta ltyylditnp prtnvlyela qyasepseqe hlhkmasssg egkelylswv
 421 vearrhilai lqdypslrpp idhlcellpr lqaryysias sskvhpnsvh icavaveyey
 481 ksgrvnkgva tswlrtkepa gengrralvp mfvrrksqfrl pfkpttpvim vpggtgvapf
 541 mgfiqerawl reggkevget llyygcrrsd edlylyreela rfhkdgaltq lnvafsrega
 601 hkvyvghllk rdkehlwkli heggahiyvc gdarnmakdv qntfydivae fgpmehtqav
 661 dyvkkmlmtkg rysldvws (SEQ ID NO: 27)

[0089] A human nucleic sequence encoding an exemplary II2-rg gene of the disclosure consist or comprises, Genbank Accession number: NM_000206.2:

1 agaggaaacg tgtgggtggg gaggggtagt ggggtgaggga cccaggttcc tgacacagac
 61 agactacacc caggggaatga agagcaagcg ccatgttgaa gccatcatta ccattcacat
 121 cctctctatt cctgcagctg cccctgctgg gagggtgggt gaacacgaca attctgacgc
 181 ccaatgggaa tgaagacacc acagctgatt tcttcctgac cactatgccc actgactccc
 241 tcagtgtttc cactctgccc ctcccagagg ttcagtgttt tgtgttcaat gtcgagtaca
 301 tgaattgcac ttggaacagc agctctgagc cccagcctac caacctcact ctgcattatt
 361 ggtacaagaa ctcgataat gataaagtcc agaagtgcag ccactatcta ttctctgaag
 421 aaatcacttc ttgctgtcag ttgcaaaaaa aggagatcca cctctaccaa acatttggtg
 481 ttcagctcca ggaccacagg gaaccacagg gacaggccac acagatgcta aaactgcaga
 541 atctggtgat cccctgggct ccagagaacc taacacttca caaactgagt gaatcccagc
 601 tagaactgaa ctggaacaac agattcttga accactgttt ggagcacttg gtgcagtacc
 661 ggactgactg ggaccacagc tggactgaac aatcagtga ttatagacat aagttctcct
 721 tgcctagtgt ggatgggcag aaacgctaca cgtttcgtgt tcggagccgc tttaaccac
 781 tctgtggaag tgctcagcat tggagtgaat ggagccaccc aatccactgg gggagcaata
 841 cttcaaaaga gaatcctttc ctggttgcat tgggaagccgt gggttatctct gttggctcca
 901 tgggattgat tatcagcctt ctctgtgtgt atttctggct ggaacggacg atgccccgaa
 961 ttcccaccct gaagaacctt gaggatcttg ttactgaata ccacgggaac ttttcggcct
 1021 ggagtggtgt gtctaaggga ctggctgaga gtctgcagcc agactacagt gaacgactct
 1081 gcctcgctcag tgagattccc ccaaaaggag gggcccttgg ggaggggcct ggggcctccc
 1141 catgcaacca gcatagcccc tactgggccc ccccatgtta caccctaaag cctgaaacct
 1201 gaaccccaat cctctgacag aagaacccca gggctcctgta gccctaagtgt gtactaactt
 1261 tccttcattc aaccacacct cgtctcctac tcacctcacc ccactgtggc tgatttgga
 1321 ttttgtgccc ccatgtaagc accccttcat ttggcattcc ccacttgaga attacccttt
 1381 tgccccgaac atgtttttct tctccctcag tctggccctt ccttttcgca ggattcttcc
 1441 tcctcctccc ttccctccc ttctcttctc catctaccct ccgattgttc ctgaaccgat
 1501 gagaaataaa gtttctgttg ataactcatca aaaaaaaaaa aaaaaaaaaa aaaaaaaaaa (SEQ
 ID NO: 28)

[0090] The corresponding human amino acid sequence encoding an exemplary II2-rg gene of the disclosure consist or comprises, Genbank Accession number: NP_000197.1:

```

1  mlkpslpfts llflqlpllg vglnttiltp ngnedttadf flttmptdsl svstlplpev
61  qcfvfnvey m nctwnsssep qptnlthlyw yknsdndkvq kcslylfsee itsgcqlqkk
121 eihlyqtfvv qlqdprrr qatqmlklqn lvipwapenl tlhklssesql elnwnnrfln
181 hclehlvqyr tdwdhswteq svdyrhkfs l psvdgqkryt frvrsrfnpl cgsaqhwsew
241 shpihwgsnt skenpflfal eavvisvgsm gliisllcvy fwlermpri ptlknledlv
301 teyhgnfsaw sgvskglaes lqpdysrlc lvseippkkg algepggasp cnqhspywap
361 pcytlkpet (SEQ ID NO: 29)

```

[0091] A murine nucleic sequence encoding an exemplary II2-rg gene of the disclosure consist or comprises, Genbank Accession number: NM_013563.4:

```

1  aggaaatgta tgggtgggga gggcttgttg gagagtgttg cagggttctg acacagacta
61  caccagaga aagaagagca agcaccatgt tgaaactatt attgtcacct agatccttct
121 tagtcttca gctgctcctg ctgagggcag ggtggagctc caaggtcctc atgtccagt
181 cgaatgaaga catcaaagct gatttgatcc tgacttctac agcccctgaa cacctcagt
241 ctctactct gccccttcca gaggttcagt gctttgtgtt caacatagag tacatgaatt
301 gcacttgaa tagcagttct gagcctcagg caaccaacct cagctgcac tataggtaca
361 aggtatctga taataatata ttocaggagt gcagtcacta tttgttctcc aaagagatta
421 cttctggctg tcagatacaa aaagaagata tccagctcta ccagacattt gttgtccagc
481 tccaggaccc ccagaaaccc cagagggcag ctgtacagaa gctaaaccta cagaatcttg
541 tgatccacg ggctccagaa aatctaacac tcagcaatct gagtgaatcc cagctagagc
601 tgagatggaa aagcagacat attaaagaac gctgtttaca atacttggtg cagtaccgga
661 gcaacagaga tcgaagctgg acggaactaa tagtgaatca tgaacctaga ttctccctgc
721 ctagtgtgga tgagctgaaa cggtacacat ttcgggttcg gagccgctat aaccaatct
781 gtggaagtgc tcaacagtgg agtaaatgga gccagcctgt ccactggggg agtcatactg
841 tagaggagaa tccttccttg tttgactgg aagctgtgct tatccctgtt ggcaccatgg
901 ggttgattat taccctgatc tttgtgtact gttggttgga acgaatgcct ccaattcccc
961 ccatacaaga tctagaggat ctggttactg aataccaagg gaacttttcg gcctggagt
1021 gtgtgtctaa agggctgact gagagtctgc agccagacta cagtgaacgg tctgtccacg
1081 tcagcgagat tcccccaaa ggaggggccc taggagagg gcttgagggt tctccttgca
1141 gcctgcatag cccttactgg cctcccccat gttattctct gaagccggaa gcctgaacat
1201 caatcctttg atggaacctc aaagtcctat agtcctaagt gacgctaacc tcccctactc
1261 accttgcaa tctggatcca atgctcactg ccttcccttg gggctaagtt tcgatttccct
1321 gtcccatgta actgcttttc tgttccatat gccctacttg agagtgtccc ttgccctctt
1381 tccctgcaca agccctccca tgcccagcct aacaccttcc cactttcttt gaagagagtc
1441 ttaccctgta gccagggtg gctgggagct cactatgtag gccagggttg cctccaactc
1501 acaggctatc tccccacctc tgccctcataa gagttggggg tactggcatg caccaccaca
1561 ccagcatgg tccttctctt ttataggatt ctccctccct tttctacct atgattcaac
1621 tgtttccaaa tcaacaagaa ataaagtttt taaccaatga tca (SEQ ID NO: 30)

```

[0092] The corresponding murine amino acid sequence encoding an exemplary II2-rg gene of the disclosure consist of, Genbank Accession number: NP_038591.1:

```

1  mlklslsprs flvlqllllr agwsskvlms sanedikadl iltstapehl saptlplpev
61  qcfvfnieym nctwnsssep qatnlthlyr ykvsdnttfq ecshylfske itsgcqiqke
121 diqlqytfvv qlqdpqkpqr ravqklnlqn lviprapenl tlnslssesql elrwksrhik
181 erclqylvqy rsnrdrswte livnheprfs lpsvdelkry tfrvrsrynp icgssqqwsk
241 wsqpvhwgsh tveenpslfa leavlipvgt mgliitlifv ycwlermppi ppiknledlv
301 teyqgnfsaw sgvskgltcs lqpdysrfc hvseippkkg algepgggsp cslhspywpp

```

361 pcyslkpea (SEQ ID NO: 31)

[0093] A human nucleic sequence encoding an exemplary Rag2 gene of the disclosure consist or comprises, Genbank Accession number: NM_000536.3:

```

1   attagatcag tgttcataag aacatctgta ggcacacata cacactctct ttacagtcag
61  ccttctgctt gccacagtca tagtgggcag tcagtgaatc ttccccaagt gctgacaatt
121 aatacctggt ttagcggcaa agattcagag aggcgtgagc agcccctctg gccttcagac
181 aaaaatctac gtaccatcag aaactatgtc tctgcagatg gtaacagtca gtaataacat
241 agccttaatt cagccaggct tctcactgat gaattttgat ggacaagttt tcttcttttg
301 acaaaaaggc tggcccaaaa gatcctgccc cactggagtt ttccatctgg atgtaaagca
361 taacctgtgc aaactgaagc ctacaatttt ctctaaggat tcctgctacc tccctctct
421 tcgctaccca gccacttgca cattcaaagg cagcttggag tctgaaaagc atcaatacat
481 catccatgga gggaaaacac caaacaatga ggtttcagat aagatttatg tcatgtctat
541 tgtttgcaag aacaacaaaa aggttacttt tcgctgcaca gagaaagact tggtaggaga
601 tgttcctgaa gccagatatg gtcattccat taatgtgggtg tacagccgag ggaaaagtat
661 ggggtgttctc tttggaggac gctcatacat gccttctacc cacagaacca cagaaaaatg
721 gaatagtgtg gctgactgcc tgccctgtgt tttcctgggtg gattttgaat ttgggtgtgc
781 tacatcatac attcttccag aacttcagga tgggctatct tttcatgtct ctattgccaa
841 aaatgacacc atctatatatt taggaggaca ttcacttgcc aataatatcc ggctgccaa
901 cctgtacaga ataaggggtt atcttcccct gggtagccca gctgtgaatt gcacagtcct
961 gccaggagga atctctgtct ccagtgcaat cctgactcaa actaacaatg atgaatttgt
1021 tattgttgggt ggctatcagc ttgaaaaatca aaaaagaatg atctgcaaca tcatctcttt
1081 agaggacaac aagatagaaa ttcgtgagat ggagacccca gattggaccc cagacattaa
1141 gcacagcaag atatggtttg gaagcaacat gggaaatgga actgtttttc ttggcatacc
1201 aggagacaat aaacaagttg tttcagaagg attctatttc tatatgttga aatgtgctga
1261 agatgatact aatgaagagc agacaacatt cacaacacagt caaacatcaa cagaagatcc
1321 aggggattcc actccctttg aagactctga agaattttgt ttcagtgcag aagcaaatag
1381 ttttgatggt gatgatgaat ttgacaccta taatgaagat gatgaagaag atgagtctga
1441 gacaggctac tggattacat gctgccctac ttgtgatgtg gatatcaaca cttgggtacc
1501 attctattca actgagctca acaaaccgcg catgatctac tgctctcatg gggatgggca
1561 ctgggtccat gctcagtgca tggatctggc agaacgcaca ctcatccatc tgcagcagg
1621 aagcaacaag tattactgca atgagcatgt ggagatagca agagctctac aactcccca
1681 aagagtccta cccttaaaaa agcctccaat gaaatccctc cgtaaaaaag gttctggaaa
1741 aatcttgact cctgccaaga aatcctttct tagaagggtt tttgattagt ttgcaaaag
1801 cctttcagat tcagggtgat ggaatttttg aatctatttt taaaatcata acattgattt
1861 taaaaatata tttttgttta tttaaaatgc ctatgttttc ttttagttac atgaattaag
1921 ggccagaaaa aagtgtttat aatgcaatga taaataaagt cattctagac cctatacatt
1981 ttgaaaatat tttacccaaa tactcaattt actaatttat tcttcaactga ggatttctga
2041 tctgattttt tattcaacaa accttaaaaa cccagaagca gtaataatca tgcagggtatg
2101 tttatatatta ttatataagt cttggtaaca aataacctat aaagtgttta tgacaaattt
2161 agccaataaa gaaattaaca cccaaaagaa ttaaattgat tattttgtgc aacataacaa
2221 ttcggcagtt ggccaaaact taaaagcaag atctactaca tcccacatta gtgttcttta
2281 tataccttca agcaaccctt tggattatgc ccatgaacaa gttagtttct catagcttta
2341 cagatgtaga tataaatata aatatatgta tacatataga tagataatgt tctccactga
2401 cacaaaagaa gaaataaata atctacatca aaaaaaaaaa aaaaaaaaaa aaaaaaa (SEQ ID
NO: 32)

```

[0094] The corresponding human amino acid sequence encoding an exemplary Rag2 gene of the disclosure consist or comprises, Genbank Accession number: NP_000527.2:

```

1   mslqmvtsn nialiqpqfs lmnfdgqvff fgqkgwpkrs cptgvfhldv khnhvklkpt
61  ifskdscylp plrypatctf kgslesekhq yiihggtkpn nevskdiyvm sivcknnkkv

```

```

121 tfrctekdlv gdvpearygh sinvvsyrgk smgvlfgrs ympsthrtte kwnsvadclp
181 cvflvdfefg catsyilpel qdglsfhvs akndtiyilg ghslannirp anlyrirvdl
241 plgspavnct vlpggisvss ailtqtnnde fvivggyqle nqkrmicnii slednkieir
301 emetpdwtpd ikhskiwfgs nmngntvflg ipgdnkqvvs egfyfymkac aeddtneeqt
361 tftnsqtste dpdgtstfed seefcfsaea nsfdgddefd tyneddeede setgywitcc
421 ptcddvintw vpfystelnk pamiycshgd ghvvhacqmd laertlihl agsnkyycne
481 hveiaralht pqrvlplkkp pmkslrkkg gkiltpakks flrrlfd (SEQ ID NO: 33)

```

[0095] A murine nucleic sequence encoding an exemplary Rag2 gene of the disclosure consist or comprises, Genbank Accession number: NM_009020.3:

```

1   actctaccct gcagccttca gcttggcaca aactaaacag tgactcttcc ccaagtgcgc
61  agtttaattc ctggccttgc cgaaaggatt cagagaggga taagcagccc ctctggcctt
121 cagtgccaaa ataagaaaga gtatttcaca tccacaagca ggaagtacac ttcataacctc
181 tctaagataa aagacctatt cacaatcaaa aatgtccctg cagatggtaa cagtgggtca
241 taacatagcc ttaattcaac caggcttctc acttatgaat tttgatggcc aagttttctt
301 ctttggccag aaaggctggc ctaagagatc ctgtcctact ggagtctttc attttgatat
361 aaaacaaaaa catctcaaac tgaagcctgc aatcttctct aaagattcct gctacctccc
421 acctcttctg tatccagcta cttgctcata caaaggcagc atagactctg acaagcatca
481 atatatcatt cacggaggga aaacacaaaa caatgagctt tccgataaga tttatatcat
541 gtctgtcgct tgcaagaata acaaaaaagt tactttccgt tgcacagaga aagacttagt
601 aggagatgtc cctgaaccca gatacggcca ttccattgac gtggtgtata gtcgaggga
661 aagcatgggt gttctctttg gaggacgttc atacatgcct tctaccaga gaaccacaga
721 aaaatggaat agtgtagctg actgcctacc ccatgttttc ttgatagatt ttgaatttgg
781 gtgtgctaca tcataatatt tcccagaact tcaggatggg ctgtcttttc atgtttctat
841 tgccagaaac gataccgttt atattttggg aggacactca cttgccagta atatacgccc
901 tgctaacttg tatagaataa gagtggacct tcccctgggt accccagcag tgaattgcac
961 agtcttgcca ggaggaatct ctgtctccag tgcaatcctc actcaaaca acaatgatga
1021 atttggtatt gtgggtggtt atcagctgga aaatcagaaa aggatggtct gcagccttgt
1081 ctctctaggg gacaacacga ttgaaatcag tgagatggag actcctgact ggacctcaga
1141 tattaagcat agcaaaatat gggttggaa caacatggga aacgggacta ttttcttgg
1201 cataccagga gacaataaagc aggctatgtc agaagcattc tattctata ctttgagatg
1261 ctctgaagag gatttgatgt aagatcagaa aattgtctcc aacagtcaga catcaacaga
1321 agatcctggg gactccactc cctttgaaga ctgagaggaa ttttgtttca gtgctgaagc
1381 aaccagtttt gatggtgacg atgaatttga cacctacaat gaagatgatg aagatgacga
1441 gtctgttaac ggctactgga taacatgttg ccctacttgt gatgttgaca tcaataacctg
1501 ggttccgttc tattcaacgg agctcaataa acccgccatg atctattgtt ctcatgggga
1561 tgggcactgg gtacatgccc agtgcattga tttggaagaa cgcacactca tccactgtc
1621 agaaggaagc aacaagtatt attgcaatga acatgtacag atagcaagag cattgcaaac
1681 tcccaaaaaga aacccccctt tacaaaaacc tccaatgaaa tccctccaca aaaaaggctc
1741 tgggaaagtc ttgactcctg ccaagaaatc cttccttaga agactgtttg attaatntag
1801 caaaagcccc tcagactcag gtatattgct ctctgaatct actttcaatc ataaacatta
1861 ttttgatttt tgtttactga aatctctatg ttatgtttta gttatgtgaa ttaagtgtc
1921 ttgtgattta ttgttaagta taactattct aatgtgtgtt ttttaacatc ttatccagga
1981 atgtctttaa tgagaaatgt tatacagttt tccattaagg atatcagtga taaagtatag
2041 aactcttaca ttattttgta acaatctaca tattgaatag taactaaata ccaataaata
2101 aactaatgca caaaaagtta agttcttttg tgtaataagt agcctatagt tgggttaaac
2161 agttaaaacc aacagctata tcccacacta ctgctgttta taaattttta ggtggcctct
2221 ggtttatact tatgagcaga attatatata ttggtcaata ccatgaagaa aaatttaatt
2281 ctatatcaag ccaggcatgg tgatggtgat acatgcctgt aatcctggca cttaggaagt
2341 ggaagaagga agtttgtgag tttgatgctt gttgaggtat gaccttttgc tatgtattgt
2401 agtgtatgag cccaagacc tgcttgacct agagacaaga gagtccacac atagatccaa
2461 gtaatgctat gtgaccttgc ccccggtta cttgtgatta ggtgaataaa gatgtcaaca
2521 gccaatagct gggcagaaga gccaaaagtg gggattgagg gtacctggc ttgatgtagg

```

```

2581 agggagacat gaggaagg gagaaaaag tgatggagga ggagaaagat gccatgagct
2641 agggagttaag aaagcatggc catgagtgct ggccaattgg agttaagagc agcccagatg
2701 aaacatagta agtaataact cagggttatc gatagaaaaat agatttttagt gccgtactct
2761 cccagccct agagctgact atggcttact gtaaatataa agtttgtagt tgtcttttat
2821 ccagggaacta aatggcaca ggtggagtag aaactctgga ttgggattaa atttttctac
2881 aacaaatgct ggctgggct agattttatc tcatatccga aggtgacag aacacagagc
2941 actggttaaca ttgccacctg ccatgcacaa agacctgagt ctaatactgt ggacattttc
3001 ttgaagtatc tacatgtact tctggagtga aaacatattc caacaatatg cttttgttta
3061 aatcactcac tcactttggg ccctcacatt atatcctttc aaaaatcaatg gttcaccctc
3121 ttgaaaatgc ttagccatag tccctcatct tccttaaaga cagtgtgcat ctctggaaat
3181 agtgacatgt cattcaaggt ccaatactgt gcagctctga agtatggcat taccacttta
3241 agtgaagaag gaaatatgaa catgagctca gacaaagggt tgggactatc actctcaagg
3301 aggtcttact gctaagtcct gaactgcttt cacatgaata cagaaattat aacaaaaaat
3361 atgtaatcaa taaaaagaaa actttcatat tcc (SEQ ID NO: 34)

```

[0096] The corresponding amino acid sequence encoding an exemplary Rag2 gene of the disclosure consist or comprises of gene consist of, Genbank Accession number: NP_033046.1:

```

1 mslqmvvtgh nialiqpgfs lmnfdgqvff fgqkgwpkrs cptgvfhfdi kqnhlklkpa
61 ifskdscylp plrypatcsy kgsidsdkhq yiihggtkpn nelsdkiyim svacknnkv
121 tfrctekdlv gdvepyrgh sidvvsyrgk smgvlfgrs ympstqrtte kwnsvadclp
181 hvflidfefg catsyilpel qdglsfhysi arndtvylg ghslasnirp anlyrirvdl
241 plgtpavnct vlpggisvss ailtqtnnde fvivggyqle nqkrmvcslv slgdnatieis
301 emetpdwtsd ikhskiwfgs nmngntiflg ipgdnkqams eafyfytlrc seedlsedqk
361 ivnsqstste dpgdstpfed seefcfsaea tsfdgddefd tyneddedde svtgywitcc
421 ptcddvintw vpfystelnk pamiycshgd ghwvhagcmd leertlihl egsnkyyne
481 hvqiaralqt pknpplqkp pmkslhkks gkvltpakks flrrlfd (SEQ ID NO: 35)

```

[0097] A human nucleic sequence encoding an exemplary Fah gene of the disclosure consist or comprises of gene consist of, Genbank Accession number: NM_000137.2:

```

1 gagacaaaa gtcaggtagg agcctccggg gtccctgctg tgtcaccggg acaggccgtg
61 gggggcgcca gggggcgagg gccgggctg accacagcgg ccgagttcag tctgtctctc
121 cgcacgccac cttaggcccc cagccgtgcc ggggtgctct cagcatgtcc tcatcccg
181 tggccgagga ttccgacttc cccatccaca acctgcccta cggcgtcttc tcgaccagag
241 gcgacccaag accgaggata ggtgtggcca ttggcgacca gatcctggac ctacgcatca
301 tcaagcacct ctttactggt cctgtcctct ccaaacacca ggatgtcttc aatcagccta
361 cactcaacag cttcatgggc ctgggtcagg ctgcctggaa ggaggcgaga gtgttcttgc
421 agaacttgct gtctgtgagc caagccaggc tcagagatga caccgaactt cggaagtgtg
481 cattcatctc ccaggcttct gccacgatgc acctccagc caccatagga gactacacag
541 acttctattc ctctcggcag catgctacca acgtcggaat catgttcagg gacaaggaga
601 atgcgttgat gccaaattgg ctgcacttac cagtgggcta ccatggcgt gctcctctg
661 tcgtggtgtc tggcacccca atccgaaggc ccatgggaca gatgaaacct gatgactcta
721 agcctcccgat atatggtgcc tgcaagctct tggacatgga gctggaaatg gctttttttg
781 taggcccctg aaacagattg ggagagccga tccccatttc caaggcccat gagcacattt
841 ttggaatggt ccttatgaac gactggagtg cagcagacat tcagaagtgg gagtatgtcc
901 ctctcgggcc attccttggg aagagttttg ggaccactgt ctctccgtgg gtggtgccc
961 tggatgctct catgcccttt gctgtgccc acccgaggca ggacccagg cccctgccc
1021 atctgtgcca tgacgagccc tacacatttg acatcaacct ctctgttaac ctgaaaggag
1081 aaggaatgag ccaggcggct accatagtga agtccaattt taagtacatg tactggacga
1141 tgctgcagca gctcactcac cactctgtca acggctgcaa cctgcggccg ggggacctcc
1201 tggcttctgg gaccatcagc gggccggagc cagaaaactt cggctccatg ttggaactgt
1261 cgtggaaggg aacgaagccc atagacctgg ggaatggtca gaccaggaag tttctgctgg

```

```

1321 acgggggatga agtcatcata acagggtact gccaggggga tggttaccgc atcggcctttg
1381 gccagtgtgc tggaaaagtg ctgcctgctc tcctgccatc atgagatttt ctctgctctt
1441 ctggaaacaa agggctcaag cacccttttc aacctgtgta ctggggctct ccctcgggct
1501 gtaggcctgg tccgccattc agtgacaaat aaagccattg tgctctgagg cctgcaactgc
1561 cgcagatgca gctgtgtcca cttatgatcg tgatttgatc cagtgggtca aggtgtgtaa
1621 agcctccctg ccagatatctc attaatatgt tttctcactc ttattagtga ggtcaggggt
1681 ctttgtggga ttttcttatt agacatccca ggcctcctgg tattccatgg aatttgaaaa
1741 gagactggca cctgtagtag tcagggtctc ccagagaaat agaaccaagg agaaagaaaa
1801 aaaaaaaaaa (SEQ ID NO: 36)

```

[0098] The corresponding human amino acid sequence encoding an exemplary Fah gene of the disclosure consist or comprises of gene consist of, Genbank Accession number: NP_000128.1:

```

1  msfipvaeds dfpihnlpyg vfstrgdprp rigvaigdq ldlslkhlkf tgpvlskhq
61  vfnqptlnsf mglggaawke arvflqnlls vsqarlrddt elrkcafisq asatmhlpat
121 igdytdfyss rqhatnvgim frdkenalmp nwlhlpvgyh grassvvvsg tpirrpmgqm
181 kpddskppvy gacklldmel emaffvgpgn rlgepipisk ahehifgmvl mndwsardiq
241 kweyvplgpf lgksfgttvs pwvvpmdalm pfavpnpkqd prplpylchd epytfdinls
301 vnlkgegmseq aaticksnfk ymywtmlqql thhsvngcnl rpgdllasgt isgpepenfg
361 smlelswkgt kpidlgnqgt rkflldgdev iitgycggdg yrigfggcag kvlpallps (SEQ ID
NO: 37)

```

```

1  ggggtgctaaa agaatacacta ggggtggggag gcggtcccag tggggcgggt aggggtgtgt
61  gccaggtggt accgggtatt ggctggagga agggcagccc ggggttcggg gcggctccctg
121 aatctaaagg cctcggcta gtctgatcct tgccctaagc atagtccgt tagccaacct
181 cctaccgcc gtgggctctg ctgcccgtg ctcgtcagca tgtcctttat tccagtggcc
241 gaggactccg actttcccat ccaaaacctg ccctatggtg ttttctccac tcaaagcaac
301 ccaaagccac ggattggtgt agccatcggg gaccagatct tggacctgag tgcattataa
361 cacctcttta cgggacctgc cctttccaaa catcaacatg tcttcgatga gacaactctc
421 aataacttta tgggtctggg tcaagctgca tgggaaggag caagagcatc cttacagaac
481 ttactgtctg ccagccaagc ccggctcaga gatgacaagg agcttcggca gcgtgcattc
541 acctccagg cttctgcgac aatgcacctt cctgctacca taggagacta caccgacttc
601 tactcttctc ggcagcatgc caccaatgtt ggcattatgt tcagaggcaa ggagaatgcg
661 ctgttgccaa attggctcca cttacctgtg ggataccatg gccgagcttc ctccattgtg
721 gtatctggaa ccccgattcg aagacccatg gggcagatga gacctgataa ctcaaagcct
781 cctgtgtatg gtgcctgcag actcttagac atggagtgg aaatggcttt cttcgtaggc
841 cctgggaaca gattcggaga gccaatcccc atttccaaag cccatgaaca cattttcggg
901 atggtcctca tgaacgactg gagcgcacga gacatccagc aatgggagta cgtcccaact
961 gggccattcc tggggaaaag ctttggaacc acaatctccc cgtgggtggt gcctatggat
1021 gccctcatgc ctttgtgtgt gccaaaccca aagcaggacc ccaagccctt gccatatctc
1081 tgccacagcc agccctacac atttgatatc aacctgtctg tctctttgaa aggagaagga
1141 atgagccagg cggctaccat ctgcaggtct aactttaagc acatgtactg gaccatgctg
1201 cagcaactca cacaccactc tgtaaatgga tgcaacctga gacctggga cctcttggt
1261 tctggaacca tcagtggatc agaccctgaa agctttggct ccatgctgga actgtcctgg
1321 aagggaacaa aggccatcga tgtggagcag gggcagacca ggaccttcct gctggacggc
1381 gatgaagtca tcataacagc tcaactgccag ggggacggct accgtgttg ctttgccag
1441 tgtgctggga aagtgtgcc tgccctttca ccagcctgaa gctccggaag tcacaagaca
1501 cacccttgcc ttatgaggat catgctacca ctgcatcagt caggaatgaa taaagctact
1561 ttgattgtgg gaaatgccac agaaaaaaaa aaaaaaaa (SEQ ID NO: 38)

```

[0099] The corresponding murine amino acid sequence encoding an exemplary Fah gene of the disclosure consist or comprises of gene consist of, Genbank Accession number: NP_034306.2:

```

1  msfipvaeds dfpiqnlpvg vfstqsnpkp rigvaigdqi ldlsvikhlf tgpalskhqh
61 vfdettlnnf mglggaawke araslqnlls asqarlrdk elrqraftsq asatmhlpat
121 igdytdfyss rghatnvgim frgkenallp nwlhlpvgyh grassivvsg tpirrpmgqm
181 rpdnskppvy gacrllldmel emaffvgpgn rfgepipisk ahehifgmvl mndwsardiq
241 qweyvplgpf lgksfgttis pwvvpmdalm pfvvpnpkqd pkplpylchs qpytfdinls
301 vslkgegmss aaticrsnfk hmywtmlqql thhsvngcnl rpgdilasgt isgsdpesfg
361 smlelswkgt kaidveggqt rtflldgdev iitghcggdg yrvfggqcag kvlpalspa (SEQ ID
NO: 39)

```

[00100] The following examples are provided to better illustrate the claimed disclosure and are not to be interpreted as limiting the scope of the disclosure. To the extent that specific materials are mentioned, it is merely for purposes of illustration and is not intended to limit the disclosure. One skilled in the art may develop equivalent means or reactants without the exercise of inventive capacity and without departing from the scope of the disclosure.

EXAMPLES

Example 1: Generation of the Por-floxed mouse strain

[00101] *Por* knock-out first targeting vector was purchased from the National Institutes of Health (NIH) Knock-Out Mouse Program (KOMP) (Figure 4A). The vector was linearized with the *AsiI* restriction enzyme, and DNA was electroporated into Jm8A3 mouse embryonic stem cells (ESC) (Pettitt, S.J. et al. "Agouti C57BL/6N embryonic stem cells for mouse genetic resources." *Nat Methods* 6, 493-495 (2009)) by the Mouse Embryonic Stem Cell Core at Baylor College of Medicine. Integrated clones were selected using neomycin resistance. DNA of ESC clones was digested with *NotI* restriction enzyme and screened for site specific integration by Southern blotting using DIG nonisotopic detection system (Roche Applied Biosciences) following the manufacturer's instructions (full blots in Figure 17). The 500bp-size 5' and 3' probes that bind outside the vector's homology arms were synthesized using the following set of primers.

[00102] 5' POR Fw2 GGCCTCAGAGAGGACATAGTGCCC (SEQ ID NO:1)

[00103] 5' POR Rev2 GCCCTCTGGTGTCTAGGTCCC (SEQ ID NO:2)

[00104] 3' POR Fw2 CCTCACGCAGCTTAATGTGGCC (SEQ ID NO:3)

[00105] 3'POR Rev2 GGAAGTTAAGGACGTGATTACAGGGAGC (SEQ ID NO:4)

[00106] Correctly targeted ESCs cells were injected into C57/BL blastocysts by the Genetically Engineered Mouse Core at Baylor College of Medicine. The male chimeras were bred with C57/BL albino females (Taconic) to access germline transmission of targeted ESC. To remove

the FRT-flanked LacZ and the neomycin cassette and generate a conditional POR knock-out strain, the mice were crossed with a *Rosa26-FLPe* strain (Farley, F.W., Soriano, P., Steffen, L.S. & Dymecki, S.M. “Widespread recombinase expression using FLP_{ER} (flipper) mice.” *Genesis* 28, 106-110 (2000)). Genotyping was performed by Transnetyx (Cordova, TN).

[00107] Example 2: X-Gal staining

[00108] Embryos and fresh liver sections were fixed in 4% PFA for 1 hour at 4°C and washed 2x30min in X-Gal rinse buffer (PBS 1x with 0.02% Igepal and 0.01% deoxycholate) followed by overnight incubation with X-Gal staining solution (PBS 1x with 5mM K₃Fe(CN)₆, 5mM K₄Fe(CN)₆, 0.02% Igepal, 0.01% deoxycholate, 2mM MgCl₂, 5mM EGTA and 1mg/ml of fresh X-Gal). Samples were post-fixed overnight in 4% PFA at 4°C.

[00109] Example 3: Generating of the PIRF (*Por^{c/c}-Il2rg^{-/-}Rag2^{-/-}Fah^{-/-}*) mouse strain

[00110] Six gRNA sequences targeting critical exons of the *Rag2*, *Il2-rg* or *Fah* gene were selected (Figure 1A, Figure 6 and Figure 7) using two different online tools (crispr.mit.edu and COSMID) (Cradick, T.J., Qiu, P., Lee, C.M., Fine, E.J. & Bao, G. “COSMID: A Web-based Tool for Identifying and Validating CRISPR/Cas Off-target Sites.” *Molecular therapy. Nucleic acids* 3, e214 (2014)). Complementary oligonucleotides were annealed and ligated into the DR274 vector (Addgene plasmid # 42250) (Hwang, W.Y. et al. “Efficient genome editing in zebrafish using a CRISPR-Cas system.” *Nat Biotechnol* 31, 227-229 (2013)) using standard molecular cloning techniques with the restriction enzyme BsaI (NEB) and T4 DNA Ligase (NEB). A T7 bacterial promoter sequence was inserted into the pX330-U6-Chimeric_BB-CBh-hSpCas9 vector (Addgene plasmid # 42230) (Cong, L. et al. “Multiplex genome engineering using CRISPR/Cas systems.” *Science* 339, 819-823 (2013)) upstream of the Cas9 transcription start site using standard molecular cloning techniques. DR274 vectors were cut using DraI (NEB) and gel purified using the Zymoclean Gel DNA Recovery Kit (Zymo, Cat#11-301). *In vitro* transcription of sgRNA was performed using the MEGAshortscript T7 Transcription Kit (Life Technologies, AM1354), according to manufacturer’s instructions. The resulting RNA was purified using the RNA Clean & Concentrator-5 (Zymo, R1015) and eluted in RNase-free water. Synthesis was verified by polyacrylamide gel electrophoresis. pX330 (with T7 promoter) was

digested with NcoI and NotI, and gel purified. Cas9 mRNA was synthesized from the digested pX330-T7 vector using the mMessage mMachine T7 ULTRA Kit (life tech AM1345), according to the manufacturer's protocol. Poly-adenylation was verified by denaturing agarose gel electrophoresis (1% agarose and 6.6% formaldehyde in MOPS buffer).

[00111] Zygotes from *Por c/c* mice were injected with *S. pyogenes* Cas9 mRNA (60ng/ul) and the six gRNA (15ng/uL each). All viable zygotes were implanted into 3 pseudopregnant females. To detect the deleted regions all twenty-three pups were genotyped after weaning using the following primers:

[00112] Fah Fw CTGGGTTGCATACTGGTGGG (SEQ ID NO:5)

[00113] Fah Rev AAACAGGGTCTTTGCTGCTG (SEQ ID NO:6)

[00114] Fah Int Fw ACAAAGGTGTGGCAAGGGTT (SEQ ID NO:7)

[00115] Il2 Fw CCACCGGAAGCTACGACAAA (SEQ ID NO:8)

[00116] Il2 Rev GGGGGAATTGGAGGCATTCT (SEQ ID NO:9)

[00117] Il2 Int Rev CTTCTTCCCGTGCTACCCTC (SEQ ID NO:10)

[00118] Rag2 Fw CCTCCACCTCTTCGTTATCC (SEQ ID NO:11)

[00119] Rag2 Rev AGTCTGAGGGGCTTTTGCTA (SEQ ID NO:12)

[00120] Rag2 Int Fw AGTCTGAGGGGCTTTTGCTA (SEQ ID NO:13)

[00121] Further offspring genotyping was performed by Transnetyx (Cordova, TN).

[00122] Example 4: Humanization of PIRF mice

[00123] Hepatocytes (3×10^6 /mouse) were transplanted into the murine liver of PIRF mice by splenic injections as originally described for mouse hepatocytes (Ponder, K.P. et al. "Mouse hepatocytes migrate to liver parenchyma and function indefinitely after intrasplenic transplantation." *Proc Natl Acad Sci U S A* 88, 1217-1221 (1991)). In brief, the abdominal cavity was opened by a midabdominal incision, and 3×10^6 human hepatocytes in a volume of 100 μ l PBS were injected into the spleen. Immediately after transplantation, selection pressure towards transplanted human hepatocytes was applied by withdrawing the drug nitisinone (NTBC) from the drinking water in the following steps: 2 days at 25%, then 2 days at 12% and eventually 2 days at 6% of the colony maintenance dose (100% = 7.5mg/l) prior to discontinuing the drug completely (Bissig, K.D. et al. "Human liver chimeric mice provide a model for

hepatitis B and C virus infection and treatment.” *The Journal of clinical investigation* **120**, 924-930 (2010)). Mice with clinical symptoms (hunched posture, lethargy, weight loss, etc) were put back on 100% nitisinone for a few days before once again being weaned off the drug as described above. In order to determine the extent of human chimerism, human albumin (ELISA, Bethyl laboratories) in the murine blood, having previously shown that human albumin levels correlate with the level of human chimerism assessed by immunostaining of human hepatocytes was measured (Bissig, K.D. et al. (2010)). Only mice with a human chimerism >70% were further used. Where indicated, some PIRF mice were injected intravenously with 100 μ l Adenovirus coding CRE recombinase under the CMV promoter (Ad5 CMV-Cre, 2.3×10^{11} pfu/ml, provided by the Vector Development Laboratory at Baylor College of Medicine) either 24-hours before hepatocyte transplantation and/or when reaching high human chimerism (>70%). Available hepatocyte donor information is given in Table 2. All animal experiments were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee (IACUC). All animals used for humanization (including controls) were female, due to fewer postsurgical complications.

[00124] Example 5: qPCR

[00125] Total mRNA was isolated from fresh frozen tissue samples using Purelink RNA mini kit (Invitrogen). 2 μ g of total mRNA was reverse transcribed using the qScript cDNA supermix (Quanta Biosciences) and 20ng of cDNA was used for the qPCR reactions, performed with Perfecta SYBR Green Fast Mix (Quanta Biosciences) and analyzed on ABI Prism 7900HT Sequence Detection System (Applied Biosciences). The following primers were used for *Por* mRNA amplification of PIRF mouse samples:

[00126] *mPor* Fw2 GGCCCCACCTGTCAAAGAGAGCAGC (SEQ ID NO:14)

[00127] *mPor* Rev1: CAAACTTGACACCCGTGAGGTCC (SEQ ID NO:15)

[00128] For humanized PIRF mouse liver samples, mouse *Por* and human *POR* were amplified using the following set of primers:

[00129] *mPor* Fw1: TCTATGGCTCCCAGACGGGAACC (SEQ ID NO:16)

[00130] *mPor* Rev2: CCAATCATAGAAGTCCTGCGCG (SEQ ID NO:17)

[00131] *hPOR* Fw1: CCAATCATAGAAGTCCTGCGCG (SEQ ID NO:18)

[00132] *hPDR* Rev5: ACCTTGCCCGCATCTATGTCGG (SEQ ID NO:19)

[00133] Each sample was normalized to *Gapdh/GADPH* as an internal control gene using the following primers:

[00134] *mGapdh* Fw AGAACATCATCCCTGCATCCA (SEQ ID NO:20)

[00135] *mGapdh* Rev CAGATCCACGACGGACACATT (SEQ ID NO:21)

[00136] *hGAPDH* Fw: CAGAACATCATCCCTGCCTCTAC (SEQ ID NO:22)

[00137] *hGAPDH* Rev: TTGAAGTCAGAGGAGACCACCTG (SEQ ID NO:23)

[00138] Example 6: RNA-Seq libraries

[00139] Whole-transcriptome RNA sequencing (RNA-Seq) was performed using total RNA extracted from fresh-frozen liver tissue sampled from all seven liver lobes. Total RNA was isolated using the Purelink RNA mini kit (Invitrogen). Libraries were generated from total RNA according to the manufacturer's recommendation using the TrueSeq Stranded mRNA LT kit (Illumina). The libraries were sequenced on a NextSeq 500 sequencer. The average read per sample was 17 millions. RNA-Seq TPM expression values were calculated with RSEM⁵² (version 1.2.17) using the read aligner Bowtie2⁵³ applied to the combined human and mouse NCBI Refseq (3/21/16) transcriptomes. RNA sequencing data is available from European Nucleotide Archive, ENA accession PRJEB14714. Low-abundance cytochromes (human < 20 TPM and mouse < 20 TPM) were only compared if one of the experimental groups reached >20TPM. Gene expression has been normalized to three human housekeeping genes and their murine counterparts (*PSMB2*, *PSMB4*, *RAB7A* and *VPS29*; *Psmb2*, *Psmb4*, *Rab7* and *Vps29*)⁵⁴. RNA-Seq data is available from European Nucleotide Archive, ENA accession code PRJEB14714

[00140] Example 7: Western blot

[00141] Western blotting was performed as described previously (Bissig-Choisat, B. et al. "Development and rescue of human familial hypercholesterolaemia in a xenograft mouse model." *Nature communications* **6**, 7339 (2015)). Tissue from snap frozen liver was homogenized in RIPA buffer (Sigma, cat# R0278-50ml) containing proteases inhibitors (Roche, cat# 04693159001). 30µg of total protein was electrophoresed in a NuPAGE 4-12% Bis Tris Gel

(Invitrogen, cat# NP0336BOX) and transferred to a PVDF membrane (Millipore, cat# IPVH00010). The blot was then blocked in 5% milk, followed by primary antibody incubation. Rabbit anti-Por (Abcam cat# ab13513) or mouse anti- β -actin (Sigma cat# A1978) were diluted 1:1,000 and 1:3,000, respectively (full blots in Figure 18 and Figure 19). Secondary antibodies were donkey anti-rabbit IgG/HRP and donkey anti-mouse IgG/HRP (Jackson ImmunoResearch Labs, cat# 711-035-152 and 711-035-150) used at 1:10,000 and 1:50,000, respectively. The membrane was imaged using Amersham ECL Western Blotting Detection Reagent (General Electric Healthcare Life Sciences, cat# RPN2106).

[00142] Example 8: Immunohistochemistry

[00143] 10 μ m sections from cryopreserved tissue blocks were fixed with 3% PFA for 15 minutes and incubated overnight at 4°C with the following primary antibodies: anti-Por (Abcam, cat# ab13513) diluted 1:500, anti-human Nuclei (EMD Millipore, cat# MAB1281) diluted 1:250 in PBS containing 0.2% Triton X-100 and 0.5% BSA. Secondary antibodies (1:1,000 Alexa-fluor conjugated, Molecular Probes) were incubated for 60 min at room temperature in the same buffer. Sections were mounted with Vectashield plus DAPI (Vector Labs).

[00144] Example 9: Mouse Husbandry

[00145] All mice (6-10 months old, humanized or non-humanized) were maintained under a standard 12-h dark/light cycle with water and chow provided *ad libitum*. All animal experiments were approved by the Baylor College of Medicine Institutional Animal Care and Use Committee (IACUC).

[00146] Example 10: Sample preparation for mass spectrometry

[00147] One group of mice was treated (i.v.) with gefitinib (10 mg/kg) and housed separately in metabolic cages for 16 h feces collection. Feces samples were weighted and homogenized in water (100 mg feces in 1,000 μ l of H₂O). Subsequently, 300 μ l of methanol was added to 100 μ l of the resulting mixture, followed by centrifugation at 15,000 g for 20 min. The supernatant was transferred to a new Eppendorf vial for a second centrifugation (15,000 g for 20 min). The final

concentration of agomelatine is 2 μ M. Each supernatant was transferred to an auto sampler vial for analysis (described below).

[00148] For atazanavir metabolism in liver, liver samples were harvested 30 min after the treatment of atazanavir (i.v., 30 mg/kg). Briefly, livers were weighted and homogenized in water/MeOH with the internal standard agomelatine [100 mg liver in 300 μ l of H₂O/MeOH (v/v 3:1)]. Subsequently, 300 μ l of methanol was added to 100 μ l of the resulting mixture, followed by centrifugation at 15,000 g for 20 min. The supernatant was transferred to a new Eppendorf vial for a second centrifugation (15,000 g for 20 min). The final concentration of agomelatine is 2 μ M in samples. Each supernatant was transferred to an auto sampler vial. Five μ l of each prepared sample was injected to a system combining ultra-high performance liquid chromatography (UHPLC) and quadruple time-of-flight mass spectrometry (QTOFMS) for analysis.

[00149] Example 11: Mass spectrometry (UHPLC-QTOFMS analyses)

[00150] Metabolites from gefitinib and atazanavir were separated using a 1260 Infinity Binary LC System (Agilent Technologies, Santa Clara, CA) equipped with 100 mm x 2.7 mm (Agilent XDB C18) column. The column temperature was maintained at 40 $^{\circ}$ C. The flow rate of was 0.3 mL/min with a gradient ranging from 2% to 98% aqueous acetonitrile containing 0.1% formic acid in a 15-min run. Quadrupole time of flight mass spectrometry (QTOFMS) was operated in positive mode with electrospray ionization. Ultra-highly pure nitrogen was applied as the drying gas (12 L/min) and the collision gas. The drying gas temperature was set at 325 $^{\circ}$ C and nebulizer pressure was kept at 35 psi. The capillary voltages were set at 3.5 kV. During mass spectrometry, real time mass correction and accurate mass were achieved by continuously measuring standard reference ions at m/z 121.0508, 922.0098 in the positive mode. Mass chromatograms and mass spectra were acquired by MassHunter Workstation data Acquisition software (Agilent, Santa Clara, CA) in centroid and profile formats from m/z 50 to 1000. The acquisition rate was set as 1.5 spectra per second. The method used in this study has been validated by the previous study of gefitinib metabolism in human liver microsomes³⁹. Meanwhile, the quality control samples were performed every 10 samples in the process of the sample running. Due to the authentic compounds of metabolites not available, the metabolite

identification was based on their exact mass and MS/MS fragments. The chromatograms and relative abundance of metabolite were performed on Qualitative Analysis software (Agilent, Santa Clara, CA). The relative abundance was evaluated based on integrated peak area of each metabolite.

[00151] Example 12: Statistics

[00152] Sample sizes for experiments were determined by estimated differences between groups and availability of highly humanized mice. No randomization of animals before allocation to experimental groups nor blinding of experimental groups was done. Statistical analysis was performed using PRISM version 6.0 software (Graph Pad software) using Mann-Whitney test, or ANOVA. Statistical significance was assumed with a p-value <0.05 (*). Bars in graphs represent mean $\pm$ SEM unless noted otherwise. Group size (N) represents biological sample size.

[00153] Example 13: Generation of novel mouse model for hepatocyte repopulation

[00154] In order to functionally block murine cytochrome metabolism, conditional (floxed exon 3 and 4) knock-out of the NADPH-P450 oxidoreductase (*Por*) gene by targeting mouse embryonic stem cells²⁸ was generated (Figure 4). Injected blastocysts with properly targeted embryonic stem cells produced chimeras with germline transmission of the *Por* “knock-out first” allele²⁹. Expression from the targeted *Por* locus using the *lacZ* expression cassette in the embryo and adult liver was confirmed (Figure 5). Next mice were bred with a flippase-expressing strain³⁰ to generate a CRE recombinase conditional *Por* knock-out strain (*Por*^{cre}). Homozygous zygotes from this strain were injected with the bacterial type II Clustered Regularly-Interspaced Short Palindromic Repeats/Cas9 (CRISPR-Cas9) system^{31, 32, 33} targeting simultaneous deletion of critical exons of the *Il2-rg*, *Rag2*, and *Fah* genes (Figure 6 and Figure 7) to generate the PIRF strain (Figure 1A). Homozygous PIRF mice are thus immune-deficient, lacking T, B and NK cells, but are healthy and fertile. Since adenoviral gene therapy vectors efficiently transduce hepatocytes *in vivo*, the *Por* gene was deleted using an adenovirus coding the CRE recombinase (Adeno-CRE). Increasing doses ($2.2 \times 10^{8-10}$ per mouse) of the virus were injected intravenously into PIRF mice. Quantitative RT-PCR of the *Por* mRNA in liver revealed efficient deletion only at high doses of adenovirus (Figure 1B). Immunostaining for Por (Figure 1C) confirmed these findings, although a minimal residual signal could be detected by Western blotting even at the

highest dose used (Figure 1D). *Por*-deleted PIRF mouse livers accumulated lipids starting about two weeks after adenoviral transduction (Figure 2), but in contrast to the immune competent *Alb-Cre/Por^{c/c}* strain^{25, 27}, without infiltration and lacking necrosis (Figure 20). Nevertheless, residual *Por* expressing hepatocytes had a growth advantage over the lipid-rich *Por*-deleted hepatocytes, and clonal expansion of a few *Por* expressing cells could be detected four weeks after adenoviral transduction by immunostaining (Figure 9).

[00155] Example 14: Characterization of humanized PIRF mice

[00156] Human liver chimeric mice using the PIRF strain were generated^{5, 20, 34}. To ensure cytochrome P450 metabolism would be human-specific, we injected Adeno-Cre (2.3×10^{10} pfu/mouse) before human hepatocyte transplantation and an additional dose of Adeno-Cre in some highly humanized PIRF (Hu-PIRF) mice. Immunostaining revealed that an almost complete deletion of the *Por* gene could be achieved only in double-injected humanized PIRF (Hu-PIRF 2x) mice (Figure 2A). Quantitative PCR and Western blotting corroborated the massive reduction of murine *Por* upon adenoviral delivery of CRE (Figure 12). Gene expression profiling was performed to compare PIRF mice repopulated with human hepatocytes (Hu-PIRF) that were injected with either Adeno-CRE (Hu-PIRF 2x) or Adeno-GFP (Figure 2B). Both groups were repopulated with human hepatocytes from the same hepatocyte donors (Table 2) to avoid inter-individual variations.

[00157] Table 2: Characteristics of human hepatocyte donors used in the present disclosure.

Hepatocyte	Age*	Gender	Race	BMI	Cause of death	Usage in present study
#1	24	Male	African American	20.3	Anoxia	RNAseq (chimeric mice, hepatocytes)
#2	2	Female	African American	19.6	Head trauma	RNAseq (chimeric mice, hepatocytes)
#3	45	Female	Caucasian	20.8	Anoxia	RNAseq (Chimeric mice)
#4	1.2	Female	Caucasian	20.8	Head trauma	Gefetinib metabolites
#5	18	Male	Caucasian	24.3	Cardiovascular	ATV metabolites

* in years

[00158] Expression of the murine P450 cytochromes was clearly altered for 27 out 38 genes analyzed after *Por* deletion (Figure 2C): 24 cytochromes were significantly upregulated (1.5-12.5-fold) and 3 cytochromes significantly downregulated (0.5-0.3-fold). The expression profiles of these murine cytochromes were by in large comparable to those from previous work in non-humanized, *Por*-deficient mice (Table 1)³⁵. In the human part of the same chimeric liver, human P450 cytochromes were less altered upon deletion of murine *Por* (Figure 2D). Half of the human

cytochromes were only slightly altered (0.5-1.5-fold change), while the other half were moderately upregulated (1.5-2.4-fold).

[00159] Not all human cytochromes serve an important role in xenobiotic metabolism. From the 200 most-prescribed drugs in the United States, about three-quarter are metabolized through P450 cytochromes, of which CYP3A4/5, 2C9, 2C19, 2D6 and 1A2 contribute to ~95%³⁶. Comparing these human cytochrome clusters from chimeric livers (Hu-PIRF 2x) with the originating, isogenic primary hepatocytes. For this comparison, two donor hepatocytes (Table 2) and the corresponding human (isogenic) liver chimeric mice (N=6). Expression levels were similar for most clusters, and these important cytochromes were all robustly expressed in chimeric livers (Figure 2E). Interestingly, some human clusters (*CYP1A2*, *CYP2B6*, *CYP2C19* and *CYP3A4*) were expressed at even higher levels in the chimeric liver than in primary human hepatocytes.

[00160] Example 15: Xenobiotic metabolism of humanized PIRF mice

[00161] To validate Hu-PIRF mice for human drug metabolism, xenobiotic metabolism of gefitinib³⁷, an inhibitor of epidermal growth factor receptor used against lung cancer and a variety of other neoplasia was used³⁸. Gefitinib is metabolized primarily by the P450 cytochrome system, including CYP3A4 and 2D6. Gefitinib metabolites demonstrate considerable differences between human and mouse liver microsomes³⁹, but regardless of dose, route or species, gefitinib is excreted primarily in the feces (less than 7% in the urine)^{40, 41}. The feces of non-humanized PIRF mice for gefitinib metabolites during the first 24 hours after intravenous injection of gefitinib was then analyzed.

[00162] Mass spectrometry revealed a reduction of several gefitinib metabolites upon deletion of the *Por* gene, implying a Por-dependent P450 cytochrome deficiency for these metabolites (Figure 3A and Figure 13A). Since some metabolites were not significantly altered, the possibility that residual Por activity was responsible for persistent murine P450 cytochrome metabolism system was tested. The *Por*^{c/c} strain was crossed with a transgenic mouse that expresses CRE under the Albumin promoter. Por protein in the liver of *Alb-CRE/Por*^{c/c} animals was efficiently deleted (Figure 14); nevertheless, the metabolite profile formed after gefitinib injection was comparable to that of PIRF mice with adenoviral deletion of *Por* (Figure 13). This

result similarity indicates that gefitinib has both P450-dependent and -independent drug metabolism.

[00163] The biggest and most relevant reduction was observed for O-desmethyl gefitinib (M4, M523595), which is by far the most abundant metabolite in human feces. Rodents produce many different metabolites in addition to M4^{40, 41} (Figure 3B), so the M4 metabolite in murine *Por*-deleted and *Por*-expressing humanized and non-humanized control mice was analyzed (Figure 10). The highest levels of M4 were detected in murine *Por*-deficient Hu-PIRF mice, where human hepatocytes preferentially metabolize gefitinib to M4 and the remaining murine hepatocytes are inhibited in their drug metabolism were used (Figure 3C). Next, to measure other human-specific metabolites. The most abundant human metabolite was M28, which could not be detected at all in non-humanized control mice. Mass spectrometry again showed the highest level of this human-specific metabolite in murine *Por*-deficient Hu-PIRF mice (Figure 3D and Figure 15), confirming that these mice showed liver metabolism more similar to humans.

[00164] The *Por*-deficient Hu-PIRF mouse is a novel model system for drug metabolism studies, and therefore was used to analyze different body compartments, e.g. the serum (one hour after injection) and the urine for these key gefitinib metabolites. M4 could not be detected in the urine and was massively reduced (23-fold in Hu-PIRF mice) in the serum, while M28 was detectable at lower concentrations in both the urine and the serum of Hu-PIRF mice (Figure 16A and Figure 16B). Although present at lower levels in both compartments, M28 mirrored relative abundance observed in feces (Figure 3C). These findings confirm that gefitinib metabolites are primarily excreted through the feces^{40, 41}.

[00165] To confirm human xenobiotic metabolism using liver homogenates of PIRF mice. Atazanavir, an antiretroviral drug (protease inhibitor) for treatment of human immunodeficiency virus was tested. Previous studies in human and mouse microsomes demonstrated that atazanavir metabolite M15 is a predominant human metabolite⁴². To determine levels of M15 in humanized PIRF mice, PIRF mice were intravenously injected with atazanavir and their livers harvested, 30 min after injection. M15 levels in *Por*-deleted humanized PIRF mice were 5.4 times greater than those observed in non-deleted mice (Figure 3E), again indicating that these mice metabolize drugs as humans do.

[00166] Example 16: Deletion of the UDP-glucose 6-dehydrogenase (UGDH)

[00167] Deletion of the UDP-glucose 6-dehydrogenase (UGDH) leads to depletion of UDP-glucuronate, which is the substrate of all UDP-glucuronosyl transferases (UGT). UGTs glucuronidate lipophilic drugs in the liver (phase II) and thereby contribute to biotransformation of drugs in the liver; glucuronidated drugs are more polar (hydrophilic) and more easily excreted. Deletion of UGDH is embryonically lethal and therefore needs to be deleted conditionally or by somatic genome engineering, similar to POR. Troglitazone was developed as an antidiabetic drug but withdrawn from the market due to hepatotoxicity. Interestingly, mice and humans metabolize the drug differently, meaning that humans mainly generate sulfate metabolites (main circulating metabolites) while glucuronide conjugates of troglitazone are less prevalent in humans. In contrast to mice, which generate mostly glucuronide conjugates. Hence troglitazone offers an opportunity to validate effectiveness of the approach to inhibit UDP-glucuronosyl transferases (UGT) by deletion of UDP-glucose 6-dehydrogenase (UGDH) in human liver chimeric mice in addition to the Por deletion and humanization.

[00168] Glutathione synthetase (GSS) catalyzes the second step of glutathione biosynthesis.

Glutathione is the substrate of Glutathione S-transferases (GST), which conjugates the molecule to lipophilic drugs (phase II) and thereby contribute to biotransformation of drugs in the liver.

[00169] Somatic genome engineering is used to simultaneously delete murine P450 oxidoreductase (Por) and other murine enzymes involved in drug metabolism in humanized mice. Humanized FRG mice (human albumin in murine serum >2 mg/ml) are injected with Adeno-Associated Virus (AAV, serotype 8) expressing sgRNA targeting an early exon of murine *Por*, UDP-glucose 6-dehydrogenase (*Ugdh*) or the glutathione synthetase (*Gss*) gene (see gene therapy vector design, Figure 21). AAVs are injected (2×10^{11} GC/AAV/mouse) 1 week before injection of Adenovirus expressing Cas9 (7×10^9 pfu/Ad/mouse). Control mice are injected with adenoviral vector only (Figure. 22, lower row). Results show a deletion of murine *por*, as well as *ugdh* and *gss* genes. The knockdown by CRISPR/Cas9 of the murine *por* in humanized mice is substantial but not quite as efficient as the knockdown observed with the loxP/CRE system when looking at the DNA (Figure. 23) and protein levels (Figure. 22). Also, the *por* deletion particularly in FRG mice is independent of the deletion of the other two genes (*ugdh* and *gss*), since their sgRNA (targeting molecule) are all on different AAV vectors. However,

immunostaining (Figure. 22) demonstrated that substantial amounts of cells had deletion of *por* and *gss* (Figure 22), while the deletion of *ugdh* was less efficient.

[00170] Humanized PIRF mouse with transgenic Alb-CRE and deletion of other murine enzymes involved in drug metabolism are used. *Por* is deleted by expression of CRE, but instead of adenoviral CRE, this PIRF mouse carries an Alb-CRE sequence within the murine genome. These mice efficiently repopulate with human hepatocytes as evidenced by human specific albumin > 2mg/ml in the murine blood and transthyretin (prealbumin) staining in the chimeric liver (Figure. 24). Murine *por* is efficiently deleted in the liver of these chimeric mice since the albumin promoter is expressed already in late embryonic stages in the liver. Also in these humanized PIRF mice, in addition to the *por*, *gss* and *ugdh* can also be deleted (Figure. 23).

[00171] Example 17: Analysis of troglitazone metabolites

[00172] Troglitazone metabolites (two hours after i.p. injection of 600 mg/kg troglitazone) in the livers of humanized and non-humanized FRG mice with and without *Por* and *Ugdh* deletion was analyzed. Non-humanized livers of control mice had much higher amounts of glucuronide conjugates than humans or humanized PIRF mice (Figure. 24). Furthermore, glucuronide conjugates reduced significantly upon deletion of *ugdh* and *por* in non-humanized and humanized PIRF mice. This data confirms that deletion of *ugdh* leads also to a functional impairment or abolishment of UGTs in the human liver chimeric liver.

[00173] The present disclosure provides a next generation of humanized mouse model amenable to human drug metabolism with minimal interference from the murine P450 cytochromes. The production of human metabolites for two different drugs between humanized PIRF mice and “normal” humanized FRG mice were compared. Analyses revealed higher concentrations of human metabolites in murine feces and liver homogenate in humanized PIRF mice than in FRG mice and demonstrate that these mice have humanized drug metabolism. The PIRF and FRG strains used in this study are in a mixed (C57B and 129S) genetic background. Aside from potential differences in the background to the two previously published FRG mouse strains^{5, 7}, our CRISPR/Cas9 generated knockout strains do not express any transgenes, e.g. the neomycin phosphotransferase that inactivates a wide range of aminoglycoside antibiotics. This model system is useful for early detection of reactive metabolites and is an elegant way to block a large

and confounding cluster of drug metabolizing murine enzymes. In addition to the novel mouse model provided herein, the disclosure provides (a) knocking out *Por* in a combination of multiple organs like the gut and the liver or the lung and the liver would be desirable, (b) additional deletions in other drug-metabolizing enzymes and/or achieving a *Por* deletion more efficiently. Using transgenic mice expressing Cre recombinase would require yet another crossing step into a quadruple transgenic (PIRF) mouse, however, and an early organ-specific deletion might not generate a robust strain amenable to xenotransplantation.

[00174] In summary, the present disclosure provides a novel mouse model combining human chimerism with functional deletion of all murine cytochromes by *Por* deletion. Such a murine *Por*-deficient humanization can be used in combination with other repopulation models such as the transgenic uPA mouse^{11, 21}. Studies with two different drugs in two different body compartments demonstrate that studies in humanized PIRF mice efficiently identify human metabolites.

REFERENCES

1. Olson H, *et al.* Concordance of the toxicity of pharmaceuticals in humans and in animals. *Regulatory toxicology and pharmacology : RTP* **32**, 56-67 (2000).
2. Nelson DR, Zeldin DC, Hoffman SM, Maltais LJ, Wain HM, Nebert DW. Comparison of cytochrome P450 (CYP) genes from the mouse and human genomes, including nomenclature recommendations for genes, pseudogenes and alternative-splice variants. *Pharmacogenetics* **14**, 1-18 (2004).
3. Guengerich FP, Cheng Q. Orphans in the human cytochrome P450 superfamily: approaches to discovering functions and relevance in pharmacology. *Pharmacological reviews* **63**, 684-699 (2011).
4. Mercer DF, *et al.* Hepatitis C virus replication in mice with chimeric human livers. *Nat Med* **7**, 927-933 (2001).
5. Bissig KD, Le TT, Woods NB, Verma IM. Repopulation of adult and neonatal mice with human hepatocytes: a chimeric animal model. *Proc Natl Acad Sci U S A* **104**, 20507-20511 (2007).
6. Dandri M, *et al.* Repopulation of mouse liver with human hepatocytes and in vivo infection with hepatitis B virus. *Hepatology* **33**, 981-988 (2001).
7. Azuma H, *et al.* Robust expansion of human hepatocytes in Fah^(-/-)/Rag2^(-/-)/Il2rg^(-/-) mice. *Nat Biotechnol* **25**, 903-910 (2007).
8. Hasegawa M, *et al.* The reconstituted 'humanized liver' in TK-NOG mice is mature and functional. *Biochemical and biophysical research communications* **405**, 405-410 (2011).
9. Washburn ML, *et al.* A humanized mouse model to study hepatitis C virus infection, immune response, and liver disease. *Gastroenterology* **140**, 1334-1344 (2011).
10. Suemizu H, *et al.* Establishment of a humanized model of liver using NOD/Shi-scid IL2R^{gnull} mice. *Biochem Biophys Res Commun* **377**, 248-252 (2008).
11. Heckel JL, Sandgren EP, Degen JL, Palmiter RD, Brinster RL. Neonatal bleeding in transgenic mice expressing urokinase-type plasminogen activator. *Cell* **62**, 447-456 (1990).

12. Tateno C, *et al.* Near completely humanized liver in mice shows human-type metabolic responses to drugs. *Am J Pathol* **165**, 901-912 (2004).
13. Samuelsson K, *et al.* Troglitazone metabolism and transporter effects in chimeric mice: a comparison between chimeric humanized and chimeric murinized FRG mice. *Xenobiotica; the fate of foreign compounds in biological systems* **44**, 186-195 (2014).
14. Lootens L, *et al.* Steroid metabolism in chimeric mice with humanized liver. *Drug testing and analysis* **1**, 531-537 (2009).
15. Foster JR, *et al.* Differential effect of troglitazone on the human bile acid transporters, MRP2 and BSEP, in the PXB hepatic chimeric mouse. *Toxicologic pathology* **40**, 1106-1116 (2012).
16. Xu D, *et al.* Fialuridine induces acute liver failure in chimeric TK-NOG mice: a model for detecting hepatic drug toxicity prior to human testing. *PLoS medicine* **11**, e1001628 (2014).
17. Bateman TJ, Reddy VG, Kakuni M, Morikawa Y, Kumar S. Application of chimeric mice with humanized liver for study of human-specific drug metabolism. *Drug Metab Dispos* **42**, 1055-1065 (2014).
18. Nishimura T, *et al.* Using chimeric mice with humanized livers to predict human drug metabolism and a drug-drug interaction. *J Pharmacol Exp Ther* **344**, 388-396 (2013).
19. Tanoue C, *et al.* Prediction of human metabolism of the sedative-hypnotic zaleplon using chimeric mice transplanted with human hepatocytes. *Xenobiotica; the fate of foreign compounds in biological systems* **43**, 956-962 (2013).
20. Bissig KD, *et al.* Human liver chimeric mice provide a model for hepatitis B and C virus infection and treatment. *The Journal of clinical investigation* **120**, 924-930 (2010).
21. Meuleman P, *et al.* Morphological and biochemical characterization of a human liver in a uPA-SCID mouse chimera. *Hepatology* **41**, 847-856 (2005).
22. Kato K, *et al.* Development of Murine Cyp3a Knockout Chimeric Mice with Humanized Liver. *Drug Metab Dispos* **43**, 1208-1217 (2015).
23. Nakada N, *et al.* Murine Cyp3a knockout chimeric mice with humanized liver: prediction of the metabolic profile of nefazodone in humans. *Biopharmaceutics & drug disposition*, (2015).

24. Shen AL, O'Leary KA, Kasper CB. Association of multiple developmental defects and embryonic lethality with loss of microsomal NADPH-cytochrome P450 oxidoreductase. *J Biol Chem* **277**, 6536-6541 (2002).
25. Wu L, *et al.* Conditional knockout of the mouse NADPH-cytochrome p450 reductase gene. *Genesis* **36**, 177-181 (2003).
26. Henderson CJ, *et al.* Inactivation of the hepatic cytochrome P450 system by conditional deletion of hepatic cytochrome P450 reductase. *J Biol Chem* **278**, 13480-13486 (2003).
27. Gu J, *et al.* Liver-specific deletion of the NADPH-cytochrome P450 reductase gene: impact on plasma cholesterol homeostasis and the function and regulation of microsomal cytochrome P450 and heme oxygenase. *J Biol Chem* **278**, 25895-25901 (2003).
28. Thomas KR, Capecchi MR. Site-directed mutagenesis by gene targeting in mouse embryo-derived stem cells. *Cell* **51**, 503-512 (1987).
29. Skarnes WC, *et al.* A conditional knockout resource for the genome-wide study of mouse gene function. *Nature* **474**, 337-342 (2011).
30. Farley FW, Soriano P, Steffen LS, Dymecki SM. Widespread recombinase expression using FLP_{er} (flipper) mice. *Genesis* **28**, 106-110 (2000).
31. Haft DH, Selengut J, Mongodin EF, Nelson KE. A guild of 45 CRISPR-associated (Cas) protein families and multiple CRISPR/Cas subtypes exist in prokaryotic genomes. *PLoS computational biology* **1**, e60 (2005).
32. Jinek M, Chylinski K, Fonfara I, Hauer M, Doudna JA, Charpentier E. A programmable dual-RNA-guided DNA endonuclease in adaptive bacterial immunity. *Science* **337**, 816-821 (2012).
33. Jansen R, Embden JD, Gaastra W, Schouls LM. Identification of genes that are associated with DNA repeats in prokaryotes. *Molecular microbiology* **43**, 1565-1575 (2002).
34. Bissig-Choisat B, *et al.* Development and rescue of human familial hypercholesterolaemia in a xenograft mouse model. *Nature communications* **6**, 7339 (2015).
35. Weng Y, DiRusso CC, Reilly AA, Black PN, Ding X. Hepatic gene expression changes in mouse models with liver-specific deletion or global suppression of the NADPH-cytochrome P450 reductase gene. Mechanistic implications for the regulation of

- microsomal cytochrome P450 and the fatty liver phenotype. *J Biol Chem* **280**, 31686-31698 (2005).
36. Williams JA, *et al.* Drug-drug interactions for UDP-glucuronosyltransferase substrates: a pharmacokinetic explanation for typically observed low exposure (AUC_i/AUC) ratios. *Drug Metab Dispos* **32**, 1201-1208 (2004).
37. Barker AJ, *et al.* Studies leading to the identification of ZD1839 (IRESSA): an orally active, selective epidermal growth factor receptor tyrosine kinase inhibitor targeted to the treatment of cancer. *Bioorganic & medicinal chemistry letters* **11**, 1911-1914 (2001).
38. Herbst RS, Fukuoka M, Baselga J. Gefitinib--a novel targeted approach to treating cancer. *Nat Rev Cancer* **4**, 956-965 (2004).
39. Liu X, *et al.* Metabolomics reveals the formation of aldehydes and iminium in gefitinib metabolism. *Biochem Pharmacol* **97**, 111-121 (2015).
40. McKillop D, *et al.* Metabolic disposition of gefitinib, an epidermal growth factor receptor tyrosine kinase inhibitor, in rat, dog and man. *Xenobiotica; the fate of foreign compounds in biological systems* **34**, 917-934 (2004).
41. Scheffler M, Di Gion P, Doroshenko O, Wolf J, Fuhr U. Clinical pharmacokinetics of tyrosine kinase inhibitors: focus on 4-anilinoquinazolines. *Clinical pharmacokinetics* **50**, 371-403 (2011).
42. Li F, Lu J, Wang L, Ma X. CYP3A-mediated generation of aldehyde and hydrazine in atazanavir metabolism. *Drug Metab Dispos* **39**, 394-401 (2011).
43. Baillie TA. Future of toxicology-metabolic activation and drug design: challenges and opportunities in chemical toxicology. *Chemical research in toxicology* **19**, 889-893 (2006).
44. Guengerich FP, MacDonald JS. Applying mechanisms of chemical toxicity to predict drug safety. *Chemical research in toxicology* **20**, 344-369 (2007).
45. Dalvie D, *et al.* Assessment of three human in vitro systems in the generation of major human excretory and circulating metabolites. *Chemical research in toxicology* **22**, 357-368 (2009).
46. Anderson S, Luffer-Atlas D, Knadler MP. Predicting circulating human metabolites: how good are we? *Chemical research in toxicology* **22**, 243-256 (2009).

47. Pettitt SJ, *et al.* Agouti C57BL/6N embryonic stem cells for mouse genetic resources. *Nat Methods* **6**, 493-495 (2009).
48. Cradick TJ, Qiu P, Lee CM, Fine EJ, Bao G. COSMID: A Web-based Tool for Identifying and Validating CRISPR/Cas Off-target Sites. *Molecular therapy Nucleic acids* **3**, e214 (2014).
49. Hwang WY, *et al.* Efficient genome editing in zebrafish using a CRISPR-Cas system. *Nat Biotechnol* **31**, 227-229 (2013).
50. Cong L, *et al.* Multiplex genome engineering using CRISPR/Cas systems. *Science* **339**, 819-823 (2013).
51. Ponder KP, *et al.* Mouse hepatocytes migrate to liver parenchyma and function indefinitely after intrasplenic transplantation. *Proc Natl Acad Sci U S A* **88**, 1217-1221 (1991).
52. Li B, Dewey CN. RSEM: accurate transcript quantification from RNA-Seq data with or without a reference genome. *BMC Bioinformatics* **12**, 323 (2011).
53. Langmead B, Salzberg SL. Fast gapped-read alignment with Bowtie 2. *Nat Methods* **9**, 357-359 (2012).
54. Eisenberg E, Levanon EY. Human housekeeping genes, revisited. *Trends in genetics : TIG* **29**, 569-574 (2013).

What is claimed is:

1. A method for preparing a chimeric non-human animal comprising human hepatocytes, the method comprising:
 - (a) providing a non-human animal comprising a reduction or deletion of NADPH-P450 oxidoreductase (*Por*) gene resulting in reduced or absent expression of *Por* protein; and
 - (b) transplanting human hepatocytes into the non-human animal.
2. The method of claim 1, wherein providing the non-human animal comprises reducing or deleting the *Por* gene resulting in reduced or absent expression of *Por* protein.
3. The method of claim 1, wherein the reduced or deleted *Por* gene is a conditional knockdown or knockout of the *Por* gene.
4. The method of claim 1, wherein the reduced or deleted *Por* gene is the result of a mutation, a transgene, treatment with an exogenous substance or somatic genome engineering.
5. The method of claim 4, wherein the somatic genome engineering comprises Guide RNA (gRNA) and Caspase 9.
6. The method of claim 1, wherein the non-human animal comprises a floxed allele of the *Por* gene, and wherein the non-human animal is provided with a Cre recombinase sufficient to produce a conditional knockout of the *Por* gene.
7. The method of claim 6, wherein the non-human animal comprising the floxed allele of the *Por* gene is provided with at least a first dose of a virus that encodes Cre recombinase.
8. The method of claim 7, wherein the non-human animal is provided with at least a second dose of a virus that encodes Cre recombinase.
9. The method of claim 1, comprising

(a) providing a non-human animal comprising a floxed allele of the *Por* gene with a first dose of a virus that encodes Cre recombinase;

(b) transplanting human hepatocytes into the non-human animal; and

(c) providing the non-human animal with a second dose of a virus that encodes Cre recombinase.

10. The method of claim 9, wherein steps (a) and (b) occur sequentially.

11. The method of claim 9, wherein steps (a) and (b) occur simultaneously.

12. The method of claim 7, wherein the non-human animal comprising the floxed allele of the *Por* gene is crossed with a transgenic non-human animal strain expressing Cre recombinase.

13. The method of claim 1, wherein the non-human animal further comprises a reduction or deletion of at least one additional gene encoding an enzyme involved in drug metabolism.

14. The method of claim 13, wherein the at least one additional enzyme is a phase II drug enzyme.

15. The method of claim 1, wherein the non-human animal further comprises a reduction or deletion of UDP-glucose 6-dehydrogenase (UGDH) gene.

16. The method of claim 1, wherein the non-human animal further comprises a reduction or deletion of Glutathione synthetase (GSS) gene.

17. The method of claim 1, wherein the non-human animal is selected from the group consisting of primate, bird, mouse, rat, fowl, dog, cat, cow, horse, goat, camel, sheep and pig.

18. The method of claim 1, wherein the non-human animal is a mouse.

19. The method of claim 1, wherein the non-human animal is selected from the group

consisting of (i) the FRG (*Fah*^{-/-}/*Rag2*^{-/-}/*Il2rg*^{-/-}) non-human animal, (ii) a transgenic urokinase type plasminogen activator (uPA) non-human animal, which overexpress uPA under an inducible promoter, preferably a liver-restricted albumin promoter, (iii) the thymidine kinase-NOD/Shi-*scid*/IL-2R γ ^{null} (TK-NOG) non-human animal, which is a immunodeficient NOG non-human animal with transgenic expression of thymidine kinase under control of liver-restricted promoter, (iv) a non-human animal expressing an inducible Caspase 8 in the liver, and (v) a non-human animal expressing an inducible Caspase 9 in the liver.

20. A chimeric non-human animal, offspring thereof, or a portion thereof, which has a chimeric liver comprising human hepatocytes, prepared by the method according to any one of claims 1-19.
21. The chimeric non-human animal of claim 20, wherein the non-human animal is selected from the group consisting of primate, bird, mouse, rat, fowl, dog, cat, cow, horse, goat, camel, sheep and pig.
22. The chimeric non-human animal of claim 20, wherein the non-human animal is a mouse.
23. The chimeric non-human animal of claim 20, wherein chimeric non-human animal substantially lacks autogenous hepatocytes.
24. The chimeric non-human animal of claim 20, wherein human hepatocytes account for at least 60% of all hepatocytes in the chimeric liver.
25. The chimeric non-human animal of claim 20, wherein human hepatocytes account for at least 70% of all hepatocytes in the chimeric liver.
26. The chimeric non-human animal of claim 20, wherein human hepatocytes account for at least 80% of all hepatocytes in the chimeric liver.

27. The chimeric non-human animal of claim 20, wherein human hepatocytes account for at least 90% of all hepatocytes in the chimeric liver.
28. The chimeric non-human animal of claim 20, wherein the chimeric non-human animal is immunodeficient.
29. A method for screening for a substance that affects human liver functions, comprising:
(a) administering a test substance to the chimeric non-human animal of claim 20;
(b) measuring one or more values in the chimeric non-human animal to which the test substance is administered in (a); and
(c) selecting a test substance that causes an increase or decrease in one or more values measured in (b), compared with the one or more values measured in a chimeric non-human animal to which no test substance is administered.
30. The method of claim 29, wherein the one or more values is selected from the group consisting of a metabolite of the test substance, human albumin concentration, body weight curve, liver-weight-to-body-weight ratio, total albumin level, total protein level, Alanine Aminotransferase (ALT) level, Aspartate Aminotransferase (AST) level, and total bilirubin level, creatinine, Blood Urea Nitrogen (BUN), troponine, blood count, TSH and histological assessment for pathologies in the human and non-human organs.
31. A method for evaluating the toxicity of a test substance against human hepatocytes and any other non-human organ in the non-human chimeric animal, comprising:
(a) administering a test substance to the chimeric non-human animal of claim 20;
(b) measuring one or more indicators in the chimeric non-human animal to which the test substance is administered in (a); and
(c) evaluating the effect of the test substance on human hepatocytes using, one or more indicators measured in (b), compared with the one or more indicators measured in a chimeric non-human animal to which no test substance is administered.
32. The method of claim 31, wherein the one or more indicators is selected from the group

consisting of an increase or a decrease in any one or more of a metabolite of the test substance, human albumin concentration, body weight curve, liver-weight-to-body-weight ratio, total albumin level, total protein level, ALT level, AST level, and total bilirubin level, creatinine, BUN, troponine, blood count and TSH as well as histological alterations including but not limited to necrosis and apoptosis in the human and non-human organs.