Concerning the localization of Cygb, we demonstrated that Cygb was expressed on fibroblasts and myofibroblasts. Cygb was not expressed in the epithelial cells. These results were commonly observed in both an acute gastric mucosal injury model and chronic gastric ulcer model. Moreover, double positive spindle shaped cells for Cygb and HIF-1α were abundantly present at the marginal zone of gastric ulcer, whereas Cygb-immunoreactive cells were not observed in the vicinity of vWF-positive cells. Cygb expression was faint in the regenerative tissues with angiogenesis. From day 11 to 18, Cygb and HIF-1α protein levels were gradually decreased from the peak level observed at day 11. However, VEGF mRNA level was increased in a time-dependent manner from day 11 to 18. VEGF expression peaked at day 18. These characteristic changes of HIF-1α and VEGF expressions during gastric ulcer healing are consistent with the previously reported data [15]. These results suggest that the area with angiogenesis which is mediated by VEGF has not already been under hypoxic condition. In contrast, a hypoxic condition in the ulcerated area triggers HIF-1α induction followed by Cygb induction in the mesenchymal cells like fibroblasts. Accordingly, Cygb is likely to function as a transit oxygen supplier, oxygen sensor, and scavenger of reactive oxygen species in the relatively hypoxic lesion in which angiogenesis has not been completed. Cygb functions as an intracellular oxygen transporter because this protein has 40 % amino acid sequence homology with myoglobin [7]. Fago et al. [6] demonstrated that O_2 binding to Cygb was pH-independent and exothermic throughout the temperature range investigated. These results showed that Cygb might be involved in O_2-requiring metabolic processes. Previous reports showed Cygb might function as a scavenger of reactive oxygen species [4, 5]. Xu et al. [27] demonstrated that an increased expression of Cygb in hepatic stellate cells protected the cells from oxidative stress when exposed to nitrolotriacetate and arachidonic acid, two compounds which induced lipid peroxidation. Besides the heme, other components may also contribute to scavenging for reactive oxygen species (ROS), like reactive thiols, which are readily accessible to oxidizing agents. The exact mechanism by which Cygb protects the cell against oxidative stress-induced cell death remains to be elucidated. In addition, Cygb triggers the transformation of fibroblasts to myofibroblasts that express well-developed actin stress fibers and stimulates collagen production at the inflammatory sites [3]. The extracellular matrix (ECM), including collagen, fibronectin, and laminin, plays an important role in cell adhesion, migration, and proliferation during wound healing [28, 29]. Cygb is associated with ECM synthesis in the healing process and the present time course of Cygb expression was consistent with those of ECM expression needed for ulcer healing in our previous report [24]. Cygb may also play a role as an introducer of tissue fibrosis during the late phase of gastric ulcer healing. Unfortunately we could not investigate the precise role of Cygb in this study. Though Cygb and HIF-1α were colocalized in the cytoplasm of the spindle-shaped cells, there is no direct evidence to indicate that HIF-1α binds to HRF motifs of the *CYGB* gene. Further studies are needed to clarify these problems.

In conclusion, Cygb was mainly expressed on myofibroblasts and fibroblasts and it may be involved in the healing process of gastric mucosal injuries in the late phase when angiogenesis has not been developed. This finding may be helpful for elucidating the mechanism(s) underlying the healing process. Cygb targeting therapy may provide a new strategy for the treatment of various ulcerative diseases.

Conflict of interest None.

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EXPERT OPINION

1. Introduction
2. Requirements for the host to be hepatocyte-humanized
3. Hepatocyte-humanized mouse and its utilization
4. Improved hepatocyte-humanized mouse
5. Hepatocyte and immune cell dually humanized mouse
6. New technologies to increase the usability of the humanized murine model
7. Expert opinion

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A mouse with humanized liver as an animal model for predicting drug effects and for studying hepatic viral infection: where to next?

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Introduction: The mouse is a common model used in evaluating drug metabolism and hepatitis infectivity. However, these models have limited value due to species difference in hepatic functions, leading to the creation of the chimeric mouse 12 years ago. These models were unique in that their hepatocytes had been replaced with human (hu) hepatocytes (dubbed the 'first-generation chimeric mouse'). Since then, the chimeric mouse has become a practical tool for this area of studies. However, some shortcomings have also been recognized. One major shortcoming is that the mouse cannot mimic hu-liver diseases due to immunodeficiency and also it is unable to provide sufficient amounts of blood for analysis compared to the rat. There are also issues around donor-to-donor variability of hu-hepatocytes such as variable engraftment efficiency.

Areas covered: This review provides the current status of the first-generation chimeric mouse. Furthermore, the authors review studies intended to create a 'second-generation of the chimeric mouse' in which inflammation/immune-response cells as well as hepatocytes are humanized. A brief comment is also made on studies aiming at producing chimeric rats. Finally, the authors consider induced pluripotent stem cells (iPS cells) as new sources of hu-hepatocytes.

Expert opinion: The authors believe that the current rapid progress in the field of biotechnology should enable us to create a mouse model with a humanized liver that is made by iPS-derived hu-hepatocytes and hu-immune cells. This development will provide researchers with a model that will be able to effectively mimic human liver disease under experimental conditions.

Keywords: chimeric mouse, drug testing, hepatitis infection model, mouse with humanized liver

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1. Introduction

Ideal drugs and medicines are defined as natural or artificial substances that are effective at improving disease conditions in humans without inducing any harmful outcomes. Tremendous efforts and costs are required for pharmaceutical companies to successfully produce such drugs for public use. Conventionally, rodents, usually rats and mice, have been utilized for research and development (R&D) of new drugs and medicines. However, there are often cases in which such candidates fail to exhibit the effects and safety predicted from the animal experiments during

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Article highlights.

- Humanization of the mouse liver has greatly benefited drug metabolism prediction in humans.
- Mice with humanized liver have also proven to be a precious tool to study infection and proliferation of hepatitis viruses.
- Human hepatocytes propagated in humanized mice could prove to be a useful source of human hepatocytes for *in vitro* drug-testing.
- Mice with humanized liver and immune/inflammation systems will be available and could capture the effects of hepatitis and hepatic fibrogenesis when their livers are exposed to stress.
- Technologies for creating humanized mice using human iPS cells will be available, in the future, and these will increase the utility of humanized mice for drug testing.

This box summarizes key points contained in the article.

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preclinical tests. This phenomenon occurs because rodents are not necessarily identical to humans regarding the metabolic and toxicological profiles of drugs (species differences).

Generally, R&D consists of two types of studies: preclinical and clinical trials of candidate drugs, which represent testing in animals or *in vitro* (with cultured cells) and in humans, respectively [1,2]. Data concerning the drug's feasibility and safety pharmacodynamics are collected during preclinical development. It is said that the success rate of tested compounds that gain approval is ~ 10% [3]. However, the number of drugs that can survive for a sufficient period of time as drugs for public use is quite few; the success rate in this sense is estimated to be as low as < 0.01% [4]. As a whole, one of the major causes of these failures is the unpredictability of efficacy in animal models due to rodent–human species differences. Therefore, the development of accurately predictable animal models is greatly required in pharmaceutical areas. The liver is the prime site of drug metabolism of orally administered drugs, which predominantly express genes of the three cytochrome P450 (CYP450) families (CYP1, CYP2 and CYP3) [5], the prime contributors for eliminating most clinical drugs [6].

There have been two straightforward approaches to decrease the mouse (m)–human (hu) species differences in drug metabolism and to increase the predictability of metabolic profiles of a given test drug based on data from mouse studies. One is to create mice in which major hu-genes involved in drug metabolism such as those of CYPs are incorporated (gene-humanized [*h*] mice). The other is to create a mouse (the so-called a chimeric mouse) with the liver in which hepatic parenchymal cells (hepatocytes) are replaced with hu-counterparts. The present review focuses on the hepatocyte-*h*-mouse and describes its present status with regard to the methodology to generate them, the advantages and limitations in their usage and the necessity of methodological developments to generate a new type of *h*-mice that could solve the problems associated with the currently available chimeric mice. Reviews are available to readers who are interested in the gene-*h*-mouse [7,8]. A review is also available that comparatively summarizes the present status of studies of both gene- and hepatocyte-*h*-mice [9].

The first chimeric mouse was created in 2001 as a m-model for studying the mechanisms of infection and the propagation of hu-hepatitis viruses [10,11]. Methodological improvements enabled us to reproducibly and stably generate chimeric mice at high levels of chimerism and in sufficient numbers for preclinical tests [12]. As a result, these mice have often been utilized in medical and pharmaceutical studies, and much knowledge has been accumulated regarding their usefulness and limitations. Several attempts have been made to improve the methods for producing chimeric mice, and new technology to manipulate hu-cells has been developed since the advent of the chimeric mouse. These situations have enabled researchers to create a new type of liver-*h*-mouse, in which not only hepatocytes but also blood cells responsible for inflammation and immune reactions are humanized (liver with dual chimerism). The original and new types of *h*-mouse are dubbed 'first-generation' and 'second-generation' chimeric mice, respectively, in this review. Previously, we reviewed the characteristics of the first-generation chimeric mice mainly focusing on the growth of hu-hepatocytes, the interactions between hu-hepatocytes and host non-parenchymal cells, drug metabolism and the propagation of hepatitis viruses [13-16]. In the present article, the current status of the first-generation chimeric mouse is reviewed with special emphasis on studies to overcome its limitations. We also comment on hu-induced pluripotent stem cells (iPS cells) as a cell type of great promise for a new source of hu-hepatocytes.

2. Requirements for the host to be hepatocyte-humanized

Hepatocyte-humanization requires two features of a mouse as host. First, the host must be immunodeficient, which allows it to accept xenogeneic cells when properly transplanted. Second, its hepatocytes must be damaged, which allows xenogeneic hepatocytes to engraft and colonize the host liver by proliferating at the expense of the host's damaged counterpart cells [17]. Thus far, four types of hepatocyte-*h*-mice have been developed as the major first-generation models, which differ from each other in the ways of conferring a combination of immunodeficiency and hepatocytic injury on the host mouse. Two research groups independently generated the first workable hepatocyte-*h*-mice in 2001 [10,11]. Regarding the immunodeficiency requirement, one group [10] used recombination-activating gene-2 (Rag-2)-knockout (KO) mice, and the other [11] adopted severe-combined immunodeficient (SCID) mice as reported by Bosma *et al.* [18]. The mature B and T lymphocytes play key roles in the adaptive immune system. Developing lymphocytes restrictedly express two RAG product enzymes, known as RAG1 and RAG2, which play an

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important role(s) in the rearrangement and recombination of the genes of immunoglobulin and T-cell receptor molecules during the process known as VDJ recombination [19]. Thus, RAG1 and RAG2 are crucial to the generation of mature B and T cells. The former group utilized this mechanism of differentiation of lymphocytes and chose mice with a $Rag2^{-/-}$ background but not those with a $Rag1^{-/-}$ background [10]: it was reported that hu-hepatocytes did not successfully repopulate the damaged liver of $Rag1^{-/-}$ mice in which fumarylacetoacetate hydrolase (FAH) gene had been deleted [20]. As described below, the absence of the Fah gene leads to an accumulation of toxic tyrosine catabolites within the hepatocytes. $Rag2^{-/-}$ mice (Balb/C-line) were also excellent recipients of hu-hematopoietic xenografts when combined with Il2rg-deficiency [21,22]. The $Rag2^{-/-}$ mice lack mature lymphocytes due to their inability to initiate VDJ rearrangement [23]. SCID mice also lack mature B and T cells due to an inactivating mutation in the catalytic subunit of a DNA-dependent protein kinase ($Prkdc^{scid}$) [24], but other hematopoietic cell types develop and function normally, including monocytes/macrophages, neutrophils, natural killer (NK) cells and dendritic cells (DCs) [25].

Later, an additional immunodeficient m-strain, NOG, was utilized as a host. These mice lack not only mature T and B lymphocytes but also NK cells [26]. Non-obese diabetic (NOD) mice are known to exhibit the spontaneous development of autoimmune insulin-dependent diabetes mellitus [27], and NOD-related strains were developed at the Shionogi Research Laboratories as NOD/Shi mice. NOD/Shi mice were crossed with the SCID mice, the offspring (NOD/Shi-*scid*) of which were mated with interleukin-2 receptor g-chain gene-KO (C57BL/6J-IL-$2Rg^{null}$) mice. The resulting m-strain is highly immunodeficient and is called NOG (NOD/Shi-*scid* IL-$2Rg^{null}$) [28].

Regarding the requirement of hepatocyte damage, genetically liver-injured m-line, albumin (Alb) promoter/enhancer-driven urokinase-type plasminogen activator (Alb-uPA) gene-transgenic ($Tg_{Alb\text{-}uPA}$) mice were originally developed to generate mice with damaged livers. This line carries a tandem array of four murine urokinase genes [29] and has long been utilized to generate hepatocyte-*h*-chimeric mice. The mice of this line are mated with one of the above three types of immunodeficient mice strains (Rag-2-KO, SCID and NOG). The liver of the $Tg_{Alb\text{-}uPA}$ mouse becomes severely hypofibrinogenemic after birth because the hepatocytes of $Tg_{Alb\text{-}uPA}$ mice overproduce urokinase under the control of the Alb promoter/enhancer, which accelerates death of hu-hepatocytes through multiple undefined mechanisms involving extracellular matrix decomposition [30]. Fah has also been utilized as a hepatocyte-damaging agent. In previous studies, mice were triple-mutated for Fah, Rag2, and the common g-chain of the interleukin receptor ($Fah^{-/-}/Rag^{2-/-}/Il2rg^{-/-}$ mice) [20,31]. These mice lack B-, T- and NK-cells, and, thus, their immunodeficiency is more complete compared to $Rag^{2-/-}$ or SCID mice. The hepatocytes of these mice are damaged due to the absence of the Fah gene, which leads to an accumulation of toxic tyrosine catabolites within the hepatocytes [32]. This genetically determined toxicity is preventable by oral administration of 2-(2-nitro-4-trifluoro-methylbenzoyl)-1,3-cyclohexane-dione (NTBC), which blocks hydroxyphenylpyruvate dioxygenase activity upstream of Fah and therefore prevents the accumulation of hepatotoxic metabolites. As noted above, when hu-hepatocytes are introduced to damaged livers in uPA-Tg- or $Fah^{-/-}$-mice, the donor cell chimerism takes place exclusively in the liver, because first hu-hepatocytes exhibit high affinity to their original (liver) tissues, and second damages are induced exclusively in the liver in these gene-manipulated model animals.

To date, the immunodeficient uPA mouse has been best characterized and most frequently utilized as the host for xenogenic hepatocyte transplantation. A high level of hu-chimerism can be obtained when homozygous uPA mice are used as hosts, though these mice suffer from infertility. There is a method to overcome this disadvantage. A research group transplanted healthy non-Tg-m-hepatocytes into uPA homo-mice [33]. As a result, the group found that their livers were totally repopulated with the normal hepatocytes, and importantly, the mice themselves became normal in body weight, life span and fertility. Fah-deficient mice are said to have some advantages over uPA-overexpressing mice [20]. In the former, the time and extent of liver disease are controllable by administering and withdrawing NTBC12 depending on an investigator's study design. The former m-line contains a Fah gene deletion, and thus it does not revert back to wild-type by transgene inactivation, which is often problematic in the latter m-line [34]. Originally, we administered a complement inhibitor during the establishment processes of the hepatocyte-*h*-mice with the uPA/SCID background [12], which could be a potential source of pharmacological interference and limits their usage in virological applications. However, currently established lines of uPA-Tg/SCID mice are able to accept hu-hepatocytes at high levels of replacement (replacement index [RI] > 80% in average, the ratio of the repopulated hu-hepatocytes to the total number of host (m)- and hu-hepatocytes in the host liver) without treatment of the complement inhibitor. The possibility of cell fusion in the hu-hepatocyte repopulation process could not be ruled out in uPA-overexpressing mice [20,] because liver repopulation by embryoid body-derived monkey hepatocytes in uPA-Tg-mice was due almost entirely to cell fusion between the hu- and m-hepatocytes [35]. However, in our accumulated experiences, such cell fusion has never been observed in hematoxylin and eosin-stained histological examinations in which m-hepatocytes were clearly distinguishable by their intense eosinophilicity in the hepatocyte-*h*-mouse. These host hepatocytes were all single cells, degenerative, and did not locate in the regions where hu-hepatocytes were repopulated in the form of nodules [12]. These repeated and reproducible histological observations accumulated to date enable us to rule out the possibility of cell fusion or to conclude that if fused cells exist, they are rare.

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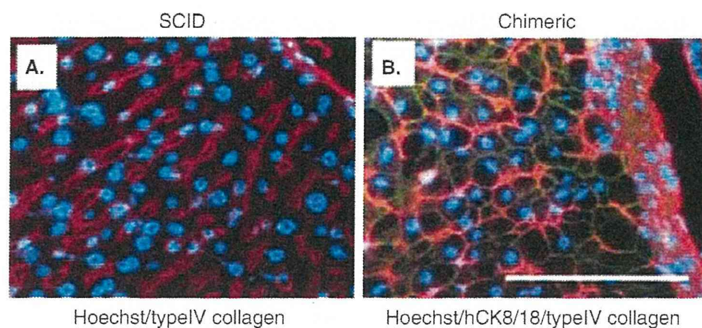

Figure 1. Illustration of assembly of hu-hepatocytes in a twin-cell plate fashion in the chimeric m-liver. Liver tissues of SCID mice were subjected to Hoechst staining (blue) and immunostaining for type IV collagen (red) (**A**). Similarly, liver tissues isolated at 13 - 15 weeks post-transplant from chimeric mice with 5-year-old male hepatocytes were subjected to Hoechst staining (blue), immunostaining for type IV collagen (red) and hu-specific CK8/18 (yellow) (**B**). The m-hepatocytes in SCID m-liver are organized in a single-cell plate fashion, but hu-hepatocytes in chimeric m-liver are organized in a twin-cell plate fashion, reflecting their highly proliferative potential as reported previously [75]. Bar represents 100 μm.
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3. Hepatocyte-humanized mouse and its utilization

When transplanted, hu-hepatocytes engraft and actively colonize the livers of immunodeficient and liver-injured mice [12]. In the 8 years of experience, since the original report in 2004 [12], many improvements have been made in our hepatocyte-*h*-mouse-producing system, and, as a result, the chimeric mice in our facility usually attain RI > 90%, ~ 2 months after transplantation [13]. Recently, *h*-livers in this model that had been produced by transplanting hu-hepatocytes from 2- to 10-year-old males or females to 2- to 4-week-old homozygotic uPA/SCID mice were characterized in details with respect to morphology and gene expression profiles [36], which is summarized as follows. The hu-Alb monitoring in host blood was performed in 54 mice with 5-year-old male hepatocytes for 8 - 16 weeks following transplant. Of these animals, 50 remained alive beyond 10 weeks of age (> ~ 7 weeks post-transplantation). Most of the surviving hosts (~ 90%) secreted > 7 mg/ml hu-Alb into the blood at 8 - 16 weeks after the transplant, indicating RIs > ~ 75%. The hepatocytes and their nuclei were smaller in the chimeric mice than in the SCID mice, as are those in a hu-body. Type IV immunostaining showed that hu-hepatocytes in the *h*-mice are arranged in a manner of 'twin-cell' plates and not in single-cell plates as in normal livers (**Figure 1**).

The occupancy of parenchymal cells (mostly hu-hepatocytes) in the chimeric m-liver was higher (~ 2-fold) compared to that of m-hepatocytes in the SCID m-liver. A fluorescent probe (indocyanine green) was injected into the mice to determine the blood flow in the liver. Blood was collected at appropriate time points after injection to determine the concentrations of the probe. The data obtained showed that there was no difference in the overall blood flow within the liver between chimeric and SCID mice, but the blood cell flow rate in the sinusoids was slower (~ 50%) in the chimeric m-liver than in SCID m-liver, which might be explained by the enlargement (> ~ 2-fold) of the former liver compared to the latter liver. No disturbance of the microcirculation was observed in the chimeric m-liver despite the slower sinusoidal blood cell flow. The hu-hepatocytes in the chimeric m-liver were not found in conditions of hypoxia, but were found in conditions of normoxia in spite of the lower blood cell flow. This result might be related to the fact that the oxygen consumption rate of hu-hepatocytes in the chimeric m-liver was slower (~ 20%) than that of m-hepatocytes in SCID m-liver, although the rate was still higher (~ 2-fold) than that of the donor hepatocytes. Microarray profiles showed that ~ 80% of the ~ 16,600 tested probes were within a twofold range difference between hu-hepatocytes in the chimeric m-livers and those in hu-livers. Immunohistochemical and electron microscopic examinations of the chimeric m-liver showed normal construction of the sinusoids by hu-hepatocytes and m-hepatic sinusoid cells (Kupffer cells, stellate cells and sinusoidal endothelial cells), as detailed in **Figure 2**.

Hepatocyte-*h*-mice exhibiting such characteristics described above are becoming a general tool in laboratories to understand and characterize hu-livers. We first demonstrated that the *h*-m-liver mimics hu-phenotypes at a level appropriate for pharmacological studies, and thus, these hepatocyte-*h*-mice can be used not only for developing new medicines but also for examining biological and pathological mechanisms in the hu-liver, including hepatitis infection and propagation.

3.1 Drug testing and screening

We demonstrated that the expression profiles of major CYPs involved in Phase I drug metabolism by hu-hepatocytes of the *h*-mice are similar to those of donors and are inducible

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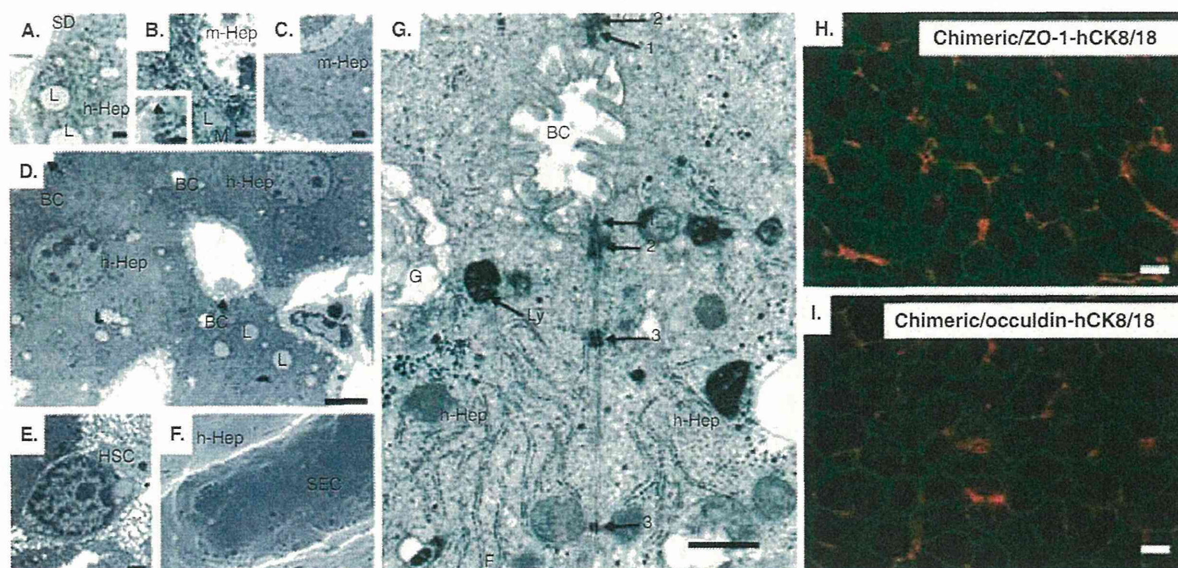

Figure 2. Illustration of structural features of chimeric m-liver. The *h*-regions of a chimeric m-liver were characterized with electron microscopy compared to non-*h*-regions in the same individual. Liver tissues were examined by transmission electron microscopy (**A - E, G**), scanning electron microscopy (**F**) and immunohistochemistry (**H** and **I**). Histochemical sections from chimeric liver tissues were stained for ZO-1 and hCK8/18 (**H**) and for occuldin-1 and hCK8/18 (**I**). The hu-hepatocytes in the *h*-regions are featured by the presence of abundant glycogen and large lipid droplets in the cytoplasm (**A**). The m-hepatocytes that retain the transgene (uPA gene) are diseased and contain abundant small granules (**B**). The m-hepatocytes that lose the uPA gene become normal (**C**). Normal sinusoidal structures are elaborated by hu-hepatocytes and m-non-parenchymal cells as seen in (**A**), in which the space of Disse is clearly discernible. The hu-hepatocytes adhere to each other and form bile canaliculi on the apical surface (**D**). The basal surfaces of hu-hepatocytes develop abundant microvilli, facing the thin m-sinusoidal endothelial cells (**D**), which are fenestrated (**F**). A hepatic stellate cell with lipid droplets is shown in the space of Disse (**E**). Bile canaliculi are organized by junctional complexes, consisting of tight junctions, adherence junctions and desmosomes (**G**). Tight junction proteins such as ZO-1 (**H**) and occludin-1 (**I**) are involved in these adhesions. Occasionally, bile canaliculi are also formed between hu- and m-hepatocytes (**G**). Bar represents 1 μm in (**A - C, E, F**); 5 μm in (**D**); 10 μm in (**H**) and (**I**); 0.5 μm in (**G**).

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BC: Bile canaliculi; BL: Blood cell; HSC: Hepatic stellate cell; L: Lipid droplets; M: Mitochondria; SD: Sinusoid; SEC: Sinusoidal endothelial cell; G: Golgi complex; Ly: Lysosome.

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by known CYP species-specific drugs [13,15,16]. Similarly, major enzymes involved in Phase II drug metabolism, such as uridine 5′-diphospho-glucuronosyltransferase (UGT), sulfotransferase (SULT), *N*-acetyltransferase (NAT), and glutathione *S*-transferase, are appreciably expressed in hu-hepatocytes [13]. These Phase II enzymes are known to participate in the metabolism of a drug group that bind to PXRs or constitutive androstane receptors, allowing these receptors to regulate some Phase II enzyme genes on ligation with drugs. Human-type Phase II metabolism is thought to function in these mice because a study showed that a conjugated form of a metabolite of an anabolic steroid, metandienone, which is known to be processed through Phase II (conjugation), was detected in chimeric m-urine [37]. The capacity of drugs to be transported through the hepatocyte cell membrane is an essential feature of effective drugs, and this consists of recognition and intake of drugs as well as secretion of the drugs. The extrahepatic-to-hepatic transportation that is responsible for drug recognition and intake is performed by transporters, such as organic cation transporter 1, organic anion transporting polypeptide (OATP) 1B1 and OATP1B3. Adenosine 5′-triphosphate-binding cassette (ABC) proteins are responsible for hepatic-to-bile-duct transportation, which occurs in drug excretion. Currently available data indicate that these drug transporter systems are also humanized in the chimeric mouse, although the data related to humanization of hepatic drug transportation are quite limited [13].

To determine whether the *h*-chimeric mouse may be utilized as a m-model to examine the excretory pathway of a drug, a study was conducted using cefmetazole as a probe drug, which in humans is mainly excreted in the urine [38]. Non-*h*-mice (uPA$^{wild/wild}$/SCID mice) excreted 23.7 and 59.4% of the drug in urine and feces, respectively. In contrast,

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the *h*-mice excreted it mostly in urine (81 and 5.9% in urine and feces, respectively). Thus, the chimeric mouse could be a useful small animal model in drug excretory studies. A drug excretion test on urine is commonly used among athletes to check their possible utilization of prohibited substances [39]. The above-cited research group used *h*-mouse to examine the metabolism of anabolic androgenic steroids that could be utilized by athletes hoping not only to enhance their performance but also to decrease the probability of a positive result during anti-doping testing [37]. Some of the steroids in use among athletes are 'designer steroids' – clandestine drugs synthesized outside the official channels, which indicates that the ability of a method to discover the right markers among their metabolites is more critical to drug testing in sports than to its overall detection limit [39]. Anti-doping investigators experience difficulties in obtaining reference substances to identify steroids and in having appropriate experimental systems to study the metabolic pathways of steroids due to their clandestine nature.

Methandione, a 17α-methylated anabolic steroid, was appropriate for validating the use of the chimeric m-model for identifying steroid metabolites in urine, because its metabolism had been thoroughly investigated in hu-liver both *in vivo* and *in vitro* [37]. This anabolic steroid was orally administered to chimeric mice and urine samples were collected 24 h after administration for analysis with liquid chromatography-tandem mass spectrometry. Seven metabolites among nine reported metabolites were identified and quantified in this study. Non-chimeric mice were found to metabolize this compound quite differently from humans; only two metabolites were detected. In contrast, six of the seven metabolites were detected in *h*-mice in a similar relative abundance as in humans, which enabled the authors to conclude that hepatocyte-*h*-mouse appear to be a suitable small animal model for the investigation of human-type metabolism of anabolic steroids. A similar urinary excretion study with the *h*-uPA/SCID m-model was conducted using 4-androstene-3,17-dione as a model steroid [40]. The obtained profiles of its metabolites were significantly different from those of non-chimeric mice and resembled known hu-profiles, which supports the usefulness of this m-model to predict the metabolites in urine tests for anabolic androgen [39].

Since our advocacy work is to recommend hepatocyte-*h*-mice as a useful animal model for predicting hu-drug metabolism, several studies have been published in which the chimeric mice were treated with various candidate drugs, and their metabolism was compared to the metabolism in non-*h*-mice and in hu-livers. Systematic and thorough review of these studies should be valuable to understand not only the advantages but also the limitations of the chimeric mice; however, such a review is beyond the scope of the present paper. Therefore, only some of the recent studies on this line are introduced herein as representatives. Troglitazone, a ligand (agonist) of PPAR-γ, has been reported to exhibit anti-fibrotic and anti-tumorigenic actions [41]. Hepatocyte-*h*-mice (Alb-uPA/SCID chimeric mice) were utilized to examine metabolic profiles of troglitazone, such as its pharmacokinetics (PK) and biotransformation, and it was found that its metabolic profiles in the chimeric mice are similar to the reported hu-data [42]. Ibuprofen and (*S*)-naproxen are nonsteroidal anti-inflammatory drugs that are metabolized through passages in the liver, including not only CYP450 but also non-CYP450 enzymes, such as UGT, SULT and amino acid NAT for taurine conjugation [43]. These drugs were administered to *h*-mice (uPA/SCID type), and their metabolites in the urine were determined and compared to hu-data. The obtained results indicate that hepatocyte-*h*-mice should be helpful in predicting the quantitative metabolic profiles of drugs mediated by CYP450 and non-CYP450 in the liver [43]. Another study was conducted in which the usability of a *h*-mouse for predicting PK parameters of drugs in humans was examined globally [44]. For this purpose, the chimeric mice with ~ 80% RI were given 13 selected model compounds that are metabolized by CYP450 and/or non-CYP450 enzymes. These compounds included as follows: mirtazapine and warfarin, which are mainly metabolized by CYP450; diclofenac, ibuprofen and naproxen, which are metabolized by both UGT and CYP450 and 6-deoxypenciclovir, fasudil, sulindac and zaleplon, which are metabolized by aldehyde oxidase (AO).

The expression levels and metabolic activities of CYP450 and non-CYP450 enzymes in the livers of the utilized *h*-mice were similar to those in humans. Comparison between humans and the *h*-mice of the clearance of these compounds together with their elimination half-life showed good correlations. From these and other results, the cited authors strongly suggested the usefulness of the chimeric mice for semi-quantitative prediction of the PK characteristics of candidate drugs in humans. AO is a molybdo flavoprotein [45] that catalyzes the metabolism of not only aldehydes but also nitrogen heterocycles [46]. AO-dependent drug metabolism has been receiving increasing attention because new chemicals metabolized by AO seem to be increasing in number [47]. AO is one of the enzymes whose expressions and activities differ among species, and they are generally high in primates and low in rodents [48]. The 6-(2-amino-4-phenylpyrimidine-5-yl)-2-isopropylpyridazin-3(2H)-one (FK3453), an AO-dependently metabolized drug, has been developed as a novel adenosine A1/2 dual inhibitor for the treatment of Parkinson's disease [49], but its development was suspended due to extremely low systemic exposure in a clinical study, despite encouraging results in animal experiments.

During R&D phases of this drug, species differences in oxidative metabolism of the aminopyrimidine moiety of FK3453 were examined using 'human-chimeric mice with humanized liver' (i.e., hepatocyte-*h*-mice) and 'rat-chimeric mice with rat hepatocytes' [46]. The obtained results are consistent with the expectations from the previous studies with humans and rats. AO activity of the human-chimeric m-hepatocytes was higher than that of the rat-chimeric m-hepatocytes; higher

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concentrations of hu-specific AO-generated FK3453 metabolites were detected in urine and feces after administration of FK3453 to the human-chimeric mice compared to the rat-chimeric mice. Additionally, the total clearance of the human-chimeric mice was twofold higher than that of the rat-chimeric mice. Thus, it seems that hepatocyte-*h*-mice may be utilized for predicting the metabolic profile of AO-dependent drugs in humans.

However, there have also been some reports that showed that the hepatocyte-*h*-m-model does *not* reproduce the hu-data on drug clearance. Bruton's tyrosine kinase (BTK), a member of the Tec family of nonreceptor tyrosine kinases, is expressed in all types of hematopoietic lineage cells, except plasma cells, NK cells and T lymphocytes [50]. BTK has been known to play critical roles in the development, differentiation and proliferation of B-lineage cells [51]. Studies using selective inhibitors for BTK suggested BTK as an attractive target for the treatment of rheumatoid arthritis (RA) and B-cell-related diseases. (*R*)-*N*-(3-(6-(4-(1,4-dimethyl-3-oxopiperazin-2-yl)phenylamino)-4-methyl-5-oxo-4,5-dihydropyrazin-2-yl)-2-methylphenyl)-4,5,6,7-tetrahydrobenzo[b]thiophene-2-carboxamide (GDC-0834) is one such BTK inhibitor [52]. Considering its potential usefulness as a therapeutic drug for RA and the suggested presence of species differences in the major route of its metabolism between humans and other preclinical animal species, a research group investigated the metabolic profiles of GDC-0834 in mouse, rat, dog, monkey, human and a hepatocyte-*h*-m-model [53]. This drug appeared to be predominantly metabolized to an inactive metabolite (M1) via amide hydrolysis in a NADPH-independent manner in humans. GDC-0834 exhibited low clearance in the chimeric mice compared to humans. Although it seems that the metabolic pathways of GDC-0834 have not yet been characterized in detailed, a major pathway is considered to be CYP-independent, which might explain the failure of the *h*-m-model in reproducing the hu-metabolism of this compound. In any case, the mechanistic elucidation of this unpredictability would greatly contribute in improving the hepatocyte-*h*-m-model as a more reliable tool for the preclinical testing of medicines.

3.2 Research for hepatic viral infection

One of the major obstacles for studies in deciphering the mechanisms of pathogenesis induced by hepatitis viruses (hepatitis C virus [HCV] and hepatitis B virus [HBV]) is the lack of a suitable small animal model to replicate the infection in humans and the lack of an *in vitro* experimental system of cultured hu-liver cells in which these viruses can infect hepatocytes, propagate, egress and induce diseased states in the liver. The HCV life cycle consists of the following five major sequential processes: i) entry into hepatocytes by receptor-mediated endocytosis; ii) initiation of viral genome (RNA) translation to generate a polyprotein that is processed into 10 individual gene products by host-encoded and virally encoded proteases; iii) viral genome replication; iv) production of infectious virions utilizing the gene products, the core protein (C), the envelope glycoproteins (E1 and E2) and non-structural proteins, such as p7, NS2, NS3 and NS5A; and v) escape from the host [54]. The requirement of many host (hu-hepatocyte) factors in these processes is considered to be the cause of species-specific viral and host interactions and, thus, makes it difficult to replicate the viral-to-host interactions in m-model. Some of these host factors are permissive both in m- and hu-hepatocytes, but some are restricted in the latter [54,55]. Before the advent of the chimeric mouse, there had been no animal model that faithfully recapitulates this life cycle except chimpanzees, which could not be a practical experimental model for general use because of ethical and cost issues. There have been studies on *in vitro* model of hepatitis viral infection, such as the HCV/Huh-7.5 cell system [56], but no practically usable *in vitro* models are found by which researchers can investigate viral infection and pathogenesis of wide ranges of genotypes and subtypes of hepatitis viruses.

The chimeric mouse is a practical experimental model that meets this need and is susceptible to hu-hepatotropic pathogens, including different genotypes and subtypes of HBV and HCV. Studies on infection by HCV/HBV and its inhibition are vividly progressing, utilizing the several types of first-generation hepatocyte-*h*-mouse. Infection with HBV and HCV was comparatively studied using $Fah^{-/-}Rag2^{-/-}Il2rg^{-/-}$ mouse as hosts of hu-hepatocytes [57]. A higher rate of liver chimerism was attained by a high transplantation dose (3×10^6 to 5×10^6 hu-hepatocytes/mouse); the average RI was 42% with a highest RI = 95%. It was shown that HBV was able to infect not only the hosts with high RIs but also those with a very low RI (< 2%), reflecting the highly infectious nature of HBV and contrasting to how HCV did not infect mice with low hu-chimerism (< 10%). It was also shown that HCV is capable of propagating in these chimeric animals and is sustainable for more than half a year. These HCV-bearing mice were seemingly without any symptoms of disease. It is of practical importance that HCV can be passaged from one chimeric mouse to another with viral titers and dynamics similar to those achieved with inoculation with patient serum.

We utilized the *h*-mice as an animal model of long-term infection of cloned HBV to comparatively investigate the infectiousness of different sources of HBV [58]. A high-level viremia (~ 10^{10} copies/ml) was observed in mice inoculated with HBV-positive hu-serum samples. The level of viremia increased with the increasing RI of hu-hepatocytes. The virus produced in HepG2 cell lines that transiently or stably transfected with 1.4 genome length HBV DNA also showed high levels and long-lasting viremia. Passage experiments showed that the serum of these mice contained infectious HBV. Lamivudine, a known anti-HBV drug, effectively reduced the level of viremia in these infected mice. These results and those of other research groups show that the *h*-m-model is a useful tool for the study of HBV virology and the evaluation of anti-HBV drugs. Similarly, the chimeric

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mice inoculated with full-length genotype 1b HCV clone from serum samples of a patient with severe acute hepatitis developed viremia at 2 weeks post-infection, which persisted for > 6 weeks [59]. The m-sera were found to contain infectious HCV. A mathematical model analysis of viral kinetics was presented based on the measurements of the HCV clearance rate in the chimeric m-model and the virion production rate in the engrafted cells [60]. This model predicted that 1 g of the *h*-liver could produce at least 10^8 virions/day, a comparable value to the hu-liver, supporting an assertion that the chimeric mouse is able to reproduce viral kinetics at an appreciably faithful level.

Furthermore, this model appears to contribute to the evaluation of a clinical treatment regimen for HCV infection. Peg-interferon (IFN) plus ribavirin combination therapy is a currently used effective treatment regimen for patients with chronic HCV. Although IFN has been the most reliably effective anti-HCV medicine, it is expensive and intolerable for some patients because of severe side effects, which has stimulated R&D to develop a new therapeutic strategy without IFN. Hepatocyte-*h*-mice were utilized to examine whether a short-term combination therapy with the HCV NS3-4A protease inhibitor telaprevir and the RNA polymerase inhibitor MK-0608 with or without IFN eradicates HCV from infected mice [61]. The results showed that mice treated with a triple combination therapy of telaprevir, MK-0608 and IFN became negative for HCV RNA soon after commencement of the therapy, and HCV RNA was not detected in serum of these mice 12 weeks after cessation of the therapy. Mice became negative for HCV RNA 1 week after the beginning of the therapy without administration of IFN when treated with a high dose telaprevir and MK-0608 combination therapy for only 4 weeks. Effectiveness of ME3738 (22β-methoxyolean-12-ene-3β , 24-diol), a derivative of soyasapogenol B, which had been thought to inhibit replication of HCV by enhancing IFN-β production, was tested using chimeric mice [62]. HCV-infected *h*-mice were treated with ME3738 and/or IFN-α for 4 weeks. The measurements of m-serum HCV RNA titer, HCV core antigen and IFN-stimulated gene (ISG) expression in the liver showed that ME3738 inhibited HCV replication, enhancing the effect of IFN-α to increase ISG expression. Thus, it was suggested that the combination of ME3738 and IFN is therapeutically useful for patients with chronic HCV.

Telaprevir is a potent inhibitor of HCV NS3-4A protease [63] and, therefore, has been clinically used as an anti-HCV drug. However, this drug has a therapeutically serious problem, that is, drug-resistant strains emerge during therapy. It is thought that this problematic phenomenon could be used as a measure of whether a hepatocyte-*h*-m-model faithfully mimics hu-liver regarding host-to-virus interactions. Chimeric mice were injected with serum samples obtained from a patient who had developed viral breakthrough during telaprevir monotherapy with strong selection for resistant mutations [64]. Under IFN therapy, mice infected with the resistant strain developed only low-level viremia, and the virus was successfully eliminated. In contrast, telaprevir monotherapy in viremic mice resulted in breakthrough with selection for mutations that confer resistance to telaprevir, as is observed in patients. This study showed that the chimeric m-model is able to reproduce a rapid emergence of telaprevir-resistant HCV by mutation from the wild-type strain of HCV, which strengthens the notion that the chimeric mouse is valuable and useful in predicting therapeutic aspects of HCV–hepatocyte interactions in the hu-liver.

4. Improved hepatocyte-humanized mouse

The uPA has been regarded as a powerful and effective agent to induce liver injury in host mice for hu-hepatocyte chimerism. However, uPA-Tg-mice exhibit problems regarding low breeding efficiency, a high risk of neonatal lethality and a narrow time window for donor hepatocyte transplantation [20], as mentioned above. To solve these problems, a new m-model was proposed in which uPA is not constitutively overexpressed but inducible only during donor hepatocyte repopulation [65]. Tg-mice were generated in which the reverse tetracycline transactivator (rtTA) was driven by the m-Alb promoter (Alb-rtTA mice). Alb-rtTA mice were back-crossed with SCID mice and used as host for hepatocyte transplantation. These mice were confirmed to express rtTA exclusively in the livers. Parallel to generating the Tg-mice, recombinant adenoviruses (Ad.TRE-uPA) were prepared, in which the uPA gene was located downstream of the tetracycline response element (TRE).

The authors generated a Tet-on-regulated Alb-uPA/SCID m-model by injecting recombinant Ad.TRE-uPA. The advantage of this model over the Alb-uPA/SCID m-model is that liver damage is not constitutive, but it is inducible when necessary by administering Dox to the mice. Immunofluorescent staining for uPA was performed against Dox-treated 6- to 8-week-old Alb-rtTA mice that had been transfected with Ad.TRE-uPA for 2 days. More than 90% of the hepatocytes were positive for uPA. Histological examinations of the DOX-treated Tg-mice 4 days after the adenovirus administration showed that hepatocytes were severely damaged. Changes of uPA and ALT (alanine transaminase, a marker of the extent of liver damage) in blood were determined at different time periods for up to 13 days following one single injection of appropriate amounts of Ad.TRE-uPA. The uPA began to increase at day 2, reached a peak at day 3 and decreased to basal levels at day 9. ALT levels increased in a similar fashion, but the days until peak levels and the days until returning to basal levels were delayed ~ 1 day compared to those of uPA. These results indicate that researchers can utilize Alb-rtTA mice as a model in which liver injury is arbitrarily inducible according to their experimental schedules by Tet-on-regulated uPA. These model mice were transplanted with enhanced green fluorescent protein (EGFP)-labeled m-hepatocytes and were thereafter given Ad.TRE-uPA weekly. The donor hepatocytes

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