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

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

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 backcrossed 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

gradually and steadily replaced the uPA-damaged host hepatocytes and occupied ~ 90% of the whole hepatocytes ~ 6 weeks post-transplantation. Although the repopulated liver has not been characterized in detail, this m-model could be utilized to generate hepatocyte-*h*-mice without the problems related to constitutive overexpression of uPA.

Major urinary protein (MUP) genes are expressed at high levels in hepatocytes in mice and are considered to be involved in binding pheromones or other small molecules, thereby facilitating their secretion and excretion, and their expression does not begin until mice are 2 to 4 weeks old [66]. This suggests the usefulness of their promoter sequences for driving the expression of uPA gene specifically in hepatocytes of mice that are older and more mature compared to the expression driven by the Alb promoter/enhancer. Thus, it is expected that uPA-mediated neonatal lethality could be eliminated in this model [67] and that the time window for hu-hepatocytes is more flexible, which permits more efficient repopulation by donor cells. Considering these advantages, MUP-uPA-Tg mice were generated and characterized [67]. As predicted, the expression of uPA was significantly delayed in some lines of the Tg-mice and was used for transplantation experiments of normal healthy m-hepatocytes. The donor hepatocytes were transplanted between 2 and 7 weeks of recipient age, and recipient mice were sacrificed 8 weeks later. The repopulation ranged up to 80% in young recipients. Some recipient mice homozygous for the MUP-uPA transgene displayed 93% repopulation by donor cells. It remains to be studied whether these mice could be utilized as hosts to generate hepatocyte-*h*-mice.

There are practical disadvantages in using triply mutated $Fah^{-/-}/Rag^{2-/-}/Il2rg^{-/-}$ mice as immunodeficient hosts, including the high mortality during colony breeding and cell transplantation surgery. These disadvantages motivated a research group to utilize doubly mutated $Fah^{-/-}/Rag^{2-/-}$ as hosts, which had lower mortality rates (~ 60%) during colony breeding than $Fah^{-/-}/Rag^{2-/-}/Il2rg^{-/-}$ mice [68]. The research group fortified their low xeno-repopulation capacity due to the intactness of NK cells with two known pharmacological immunosuppressors, anti-asialo GM1 and FK506, the latter of which is also known to induce hepatocyte proliferation [69]. The $Fah^{-/-}/Rag^{2-/-}$ mice were given anti-asialo GM1 and FK506 by intraperitoneal injection and through drinking water, respectively. The single treatment with anti-asialo GM1 was quite effective in decreasing NK cells (~ 6% of the original level). The chimerism of anti-asialo-GM1-treated $Fah^{-/-}/Rag^{2-/-}$ mice ranged from ~ 3 to 32% at 12 weeks post-hu-hepatocyte-transplantation, which was still lower than those of $Fah^{-/-}/Rag^{2-/-}/Il2rg^{-/-}$ recipients. The combined treatment of both increased the repopulation to as high as ~ 67% (ranging from ~ 10 to 67%) at 12 weeks post-transplantation, which was comparable to that of $Fah^{-/-}/Rag^{2-/-}/Il2rg^{-/-}$ mice. The repopulated hu-hepatocytes recovered the metabolic functionality and showed infectivity against HBV. However, this model has still not obtained high levels of repopulation, and phenotypes of its hu-hepatocytes are required to be sufficiently characterized in relation to those in the hu-liver.

5. Hepatocyte and immune cell dually humanized mouse

The hepatocyte-*h*-mouse has been proven to be useful for predicting metabolic patterns of drugs in hu-liver and for investigating infection and propagation of HCV/HBV. However, the currently available chimeric mouse cannot be a liver disease model for drug/hepatitis-induced inflammation, fibrogenesis and hepatocytic carcinogenesis due to the lack of hu-cells responsible for inflammatory and immune reactions. Therefore, it is an important challenge to create the hepatocyte-*h*-mouse, which can be used as a convenient tool for developing drugs/medicines that suppress the initiation of liver diseases or that eliminate the diseased liver, which are the *bona fide* achievements of medical and pharmaceutical treatments in hospitals. Generally, the immune system functions as a major network for protecting the body from pathogens and deleterious drugs by detecting exogenous entities and inducing inflammatory reactions. Thus, it is logical that researchers endeavor to solve the limitations of the first-generation hepatocyte-*h*-m-model by reconstructing the hu-immune system in the hepatocyte-*h*-mouse [70].

Although it is known that T cells, in particular $CD4^+$ (helper) and $CD8^+$ (cytotoxic) T cells (CTL), play prime roles in HCV control and clearance [71], very little is understood about the immune response to the viral infection, which led a research group to undertake a study to generate a mouse engrafted with hu-liver cells and a functional hu-immune system [72]. A suicide gene and hu-embryonic liver cells were utilized to delete the host (m-) hepatocytes and to humanize both hepatocytes and immune cells, respectively. Caspase 8 is a member of the cystein-aspartic acid protease (caspase) family, which is involved in apoptotic alterations of cells induced by Fas and various apoptotic stimuli. Caspases exist as inactive proenzymes composed of large and small protease subunits. The activation of the proenzyme requires its cleavage to each of the subunits and their dimerization. FKBPv [Phe36Val mutant FKBP (FK506 binding protein)/AP20187 (a FK1012 analog)] has been utilized as a 'dimerization agent' [73]. AP20187, a prodrug, binds to FKBPv with ~ 1000-fold greater affinity than to endogenous FKBP and dimerizes it. The cited research group made a gene construct (AFC8 gene) by fusing the catalytic domains of hu-caspase 8 to FKBPv, which induces conditional apoptosis of a targeted cell type as originally reported by Pajvani *et al.* [74]. In this case, the construct was designed such that it is driven by the Alb promoter/enhancer for targeted expression in hepatocytes. The hu-hepatocyte progenitor cells and $CD34^+$ cells as hematopoietic stem cells were each isolated from 15 – 18-week-old fetal liver tissues, the purity being > 95%, and both were co-injected into the liver of < 5-day-old newborn immunodeficient Tg-Rag2-γC-null mice with the AFC8 transgene that

had been preconditioned by irradiation with X-ray. Then, the mice were treated with AP20187 to induce apoptosis in the host hepatocytes. The hu-Alb$^+$-hepatocytes were significantly increased in the liver as expected, and they expressed CYP2E1, CYP2C9 and UGT2B7, although the average RI was lower (~ 15% between 5 and 16 weeks after transplant) than those of the conventional hu-hepatocyte chimeric mice. As predicted, these mice included hu-immune cells in all lymphoid organs, including the liver, indicating that they were doubly *h*-mice in terms of their liver and immune system. However, functional hu-B cells were reported not to be present, indicating that this model is on the way of technical completion, because antibodies have been shown to play important roles in the pathogenesis of HCV infection and viral persistence [75].

To examine the infectivity toward HCV, AFC8 mice were inoculated with patient HCV isolates (genotype 1a), and their livers were examined for the presence of HCV genomic RNA 1 – 4 months post-infection. Approximately 50% showed its presence with variable levels, although not in the blood, which is most likely due to a low repopulation rate of hu-hepatocytes. In addition to the viral infection, hu-immune cells infiltrated into the liver of HCV-infected AFC8 mice, including CD45$^+$ leucocytes, CD68$^+$ macrophages, CD3$^+$ T cells and other multiple hu-leukocyte subsets, although functional B cells were not present as described above. ALT levels were elevated in HCV-infected mice, but not in HCV-non-infected control mice. These facts together strongly suggest that hu-immune cells are actively involved in the process of liver injury, a state of HCV-induced hepatitis that has not been reproduced in the first-generation chimeric mouse, the immunodeficient mouse singly humanized for hepatocytes. In this process, hu-T cells were thought to play primary roles as predicted from clinical data [71], because CD4$^+$ T cells or CD8$^+$ T cells isolated from HCV-infected mice express higher levels of effector cytokines, such as IL2, IFNγ and TNFα, when stimulated *in vitro* with phytohemagglutinin (PHA) than those from non-infected mice. Importantly, HCV-infected AFC8 mice with hu-hepatocytes and hu-immune cells developed severe fibrosis throughout the liver parenchyma as indicated by the appearance of αSMA$^+$-cells (activated stellate cells), but this event did not occur in those with hu-immune cells or hu-hepatocytes alone. Interestingly, the livers of these mice showed elevated mRNA expressions of hu-COL1A1 and TIMP1, but not their m-counterparts. This observation suggests that the hu-hepatic stellate cell progenitors present in the hu-hepatocyte progenitor fraction from 15- to 18-week-old fetal liver tissues developed into mature hu-stellate cells in the host liver and were activated by HCV proteins released from HCV-infected hu-hepatocytes, which supports the notion that HCV infection causes the activation of the hepatic stellate cells, leading to hepatic fibrosis [76]. It is also considered that host (m-) stellate cells are not involved in the fibrogenesis induced by HCV infection of hu-hepatocytes due to the lack of pathophysiological interactions between hu-hepatocytes and m-stellate cells. Similar species-specific interactions between hepatocytes and stellate cells were reported in liver growth of hepatocyte *h*-uPA/SCID mice [14,77]. This pioneering work is undoubtedly a promising first step for further development of a m-model permissive for not only HV infection but also for pathogenesis triggered by interactions between HV and hu-immune cells. Several improvements remain to be made on this dually chimeric m-model. HCVs were variably detected at low levels in liver tissues but not in the blood, which might be due to insufficient RIs (~ 10 – 30%) of the donor cells, as prior studies have reported a high level of HCV infection among chimeric mice with > 50% RI [64,78]. The livers of AFC8 mice are considered to have acute injury, whereas those of the first-generation chimeric mice with high RIs have chronic injury induced by uPA [17] or Fah [32]. It remains to be clarified whether acute or chronic types of liver injury affect the extent of repopulation of xenogeneic hepatocytes [75].

Generally, xenobiotics induce immuno-inflammatory reactions in host tissues. The first-generation hepatocyte-chimeric mice are immunosuppressed, which hampers researchers from studying the hepatic responses induced by xenobiotics under the immuno-ethio-pathogenic conditions, such as viral hepatitis. This limitation is a critical issue for the first-generation hepatocyte-chimeric mice because, for example, hepatitis viruses act as antigens and induce chronic inflammatory-immune reactions in the hu-body and result in severe diseases unless appropriate treatments are given. The necroinflammatory reaction plays a central role in hepatitis virus elimination. We previously demonstrated that the chimeric mouse with high RIs is an ideal model for investigating the mechanisms of HBV viral replication under conditions close to the human liver *in vivo* and for testing a candidate drug effective in inhibiting the viral infection and replication [58-62]. However, it is apparently not an ideal model for investigating the virus-induced inflammatory reactions due to the lack of hu-blood cells.

Apart from hepatitis viruses, xenobiotics generally induce inflammatory-immune reactions in host tissues when administered to humans. The conventional (first-generation) hepatocyte-chimeric mouse is not an animal model for studying hu-hepatic responses induced by xenobiotics, which proceed under appropriate immuno-ethio-pathogenic conditions. A direct and simple approach to overcome this fault is to introduce hu-inflammatory-immune cells into the SCID mouse [79]. CD8$^+$ T cells and NK cells are thought as major players in the elimination of infected cells. We first transplanted hu-hepatocytes into uPA/SCID mice, obtained mice whose livers were highly repopulated with the donor cells and then infected these mice with HBV. After establishing HBV infection, we additionally injected hu-peripheral blood mononuclear cells (hu-PBMCs) into the mice and analyzed liver pathology and infiltrating hu-immune cells with flow cytometry. As expected, hu-hepatocytes showed severe degeneration only in HBV-infected mice transplanted with

hu-PBMCs, and these HBV-infected hu-hepatocytes were eliminated by NK cells and DCs. This was completely prevented in mice treated with anti-Fas antibodies or by depletion of DCs, but not by depletion of $CD8^+$ CTLs. This study clearly demonstrated that DC-activated NK cells induce massive HBV-infected hepatocyte degeneration through the Fas/FasL system and may indicate new therapeutic implications for acute severe/fulminant hepatitis B. Based on this study, it can be said that the introduction of the inflammation/immune-responsible hu-cells into the first-generation chimeric mouse is a promising approach to overcome limitations that are inherent in the conventional hepatocyte-*h*-mouse.

There was another trial to generate hepatocyte-*h*-mice bearing hu-blood cells, in which immunotolerant hosts were utilized instead of immunosuppressive hosts [80]. This trial was based on the well-established mechanism of development of the immune system, by which auto-reactive T-cell clones are completely deleted from the immune repertoire during fetal development [81]. Chimeric m-embryos were generated using normal (immunotolerant) mouse fetuses or blastocysts as hosts and human cord blood $CD34^+$ cells as donors. To make chimeric fetuses, the uterus was exposed by a single abdominal incision, and the donor cells were intraperitoneally inoculated. The chimeric blastocysts made by microinjecting the donor cells were transferred into foster mothers. The livers were immunohistochemically examined to detect hu-hepatocytes using hu-specific antibodies against hu-hepatocyte-specific antigen (HepPar1), hu-Alb and hu-α-1-antitrypsin, or for determining the expression levels of the hu-Alb gene. These examinations showed that hu-cord blood $CD34^+$ cells were able to integrate into preimmune m-liver and give rise to liver cells expressing hepatocyte markers for at least 4 weeks after birth. Although this study provides a possible approach for generating immunocompetent mice harboring hu-hepatocytes, several important issues remain unanswered. These include the identification of these hu-hepatocyte-like cells as functional true hepatocytes, the extent of repopulation of hu-hepatocytes and the extent of immune competency of the mouse.

6. New technologies to increase the usability of the humanized murine model

In the above sections, we have reviewed the hepatocyte-*h*-m-model as a tool for preclinical testing of medicines and for investigating the mechanisms of hepatic virus infections. Although its usefulness has been increasingly recognized among researchers in the related areas, its shortages limit its robust and general use in pharmaceutical and medical arenas. One such shortage is related to its size. Availability of sufficient amounts of blood samples is preferred for the animal model for drug testing. Currently, researchers are able to utilize hu-hepatocytes provided from commercial sources as donor cells. However, these hu-hepatocytes have problems around donor-to-donor variability such as variable engraftment efficiency. Recent progressions in biotechnology are expected to provide us with new sources of host animals and hu-hepatocytes for drug testing, which could alleviate these issues.

6.1 Rats as a host for a humanized animal model

The mouse has been a frequently utilized animal model to study the biology and pathology of the vertebrate liver because of its appropriate size for experimental handlings, its well-characterized genetic background, the availability of a variety of lines and the applicability of commonly utilized genetic and genomic manipulations. However, the rat has traditionally been a major animal model in the area of pharmacological and pharmaceutical studies for the development of new medicine because the rat has an advantage over the mouse in the availability of sufficient blood samples for investigating the PK of drugs. Abundant data regarding the effectiveness, toxicity and the metabolic profiles of drugs and medicines have accumulated from studies with this murine species. Therefore, generation of a rat model with a *h*-liver has been a goal after the establishment of the basic technology to produce hepatocyte-*h*-m-model, but it has been hampered due to the lack of an appropriate method to create a rat with dysfunctional immune systems. A recently developed novel gene-targeting technology based on utilization of zinc-finger nucleases (ZFNs) solved this difficulty, and it is a useful tool to arbitrarily insert or delete base pairs in a targeted gene [82]. ZFNs are made by fusing a specific DNA binding domain containing the tandem zinc finger motifs with a non-specific cleavage domain of the restriction endonuclease *FokI*. When a ZFN construct is introduced into cells, the ZFNs target genes in a site-specific manner and create double-stranded breaks, which are then repaired via non-homologous end joining – a mutagenic process that results in the insertion/deletion of base pairs. Thus, ZFN technology enables researchers to inactivate a targeted gene in living cells, including rat embryonic cells [83]. A research group led by Serikawa prepared ZFN plasmids to generate rats that lack both $Prkdc^{SCID}$ gene and interleukin-2 receptor γ (*Il2rγ*) gene which were referred to as F344-scid γ rats [84]. The obtained rats showed severe immunocompromised phenotypes including the abolishment of NK cells. The double-KO rats aged 5 years were pretreated with retrosine, a pyrrolizidine alkaloid that is toxic to hepatocytes [85], to damage the host liver and were transplanted with hu-hepatocytes. Successful engraftment and repopulation of hu-hepatocytes were indicated by increase of hu-Alb levels in the blood and appearance of small clusters of hu-CK8/18-positive cells in the liver. Currently, these hepatocyte-*h*-rats have not been sufficiently characterized biologically and pathologically and RIs of hu-hepatocytes are not high enough for practical uses, but these rats could be an promising *in vivo* model for the preclinical testing of new drugs, taking advantage of their easier handling and the

availability of a larger volume of blood samples due to larger body size compared to the m-counterpart.

6.2 iPS cells as a source of human cells for liver humanization

When necessary, hu-hepatocytes are generally obtained in hospitals under ethical and clinical regulations in quite limited amounts for specific clinical and therapeutic purposes, but not for studies and experiments such as drug testing. There are hu-hepatocytes that are available from commercial sources that are authorized to collect specified hepatic biopsies from hospitals under the regulations and the requirements set by authorities. Even in this case, the quantity of available hu-hepatocytes is not abundant, and they are usually expensive. In addition, they display lot-to-lot individual variability such as viability and variable engraftment efficiency. These hu-hepatocytes are mostly refractory to genetic manipulations due to their poor proliferative potential. Rapid developments of technologies to differentiate stem cells or progenitor cells into hepatocyte-like cells are changing these unavoidable and unfavorable situations. Several types of stem cells/progenitor cells were the targets of novel sources of hu-hepatocytes, such as embryonic stem cells (ES cells), bone marrow mesenchymal stem cells, peripheral blood stem cells and iPS cells, among which iPS cells are especially worthy of being commented on herein because they are the most promising and versatile potential sources of hu-hepatocytes for drug testing.

A protocol for generating hu-hepatocytes from hu-iPS cells generally consists of two consecutive procedures: the first is for induction of hu-fibroblasts to hu-iPS cells, and the second is for differentiation of hu-iPS cells to hepatocyte-like cells. Takahashi and Yamanaka first demonstrated that only a few defined factors, that is, Oct3/4, Sox2, c-Myc and Klf4, are able to reprogram the differentiated state of m-embryonic or adult fibroblasts to a undifferentiated state, in which these cells exhibit the morphology and growth properties of ES cells and express ES cell-marker genes. These ES-like cells were dubbed iPS cells [86]. It was also shown that primary, genetically unmodified, diploid hu-fibroblasts (IMR90 fetal fibroblasts) were reprogrammed to ES-like cells by a combination of OCT4, SOX2, NANOG and LIN28 [87]. The second protocol was a procedure in which hu-iPS cells obtained from, for example, foreskin fibroblasts, were induced to differentiate into hepatocyte-like cells with a combination of activin A, BMP4/FGF2, HGF and OSM [88]. The hu-iPS cells were induced into definitive endoderm – the endoderm that forms organs in the embryo itself – by activin A treatment. These cells then acquired hepatic specification under the influence of BMP4/FGF2. HGF treatment caused the cells with hepatic specification to become hepatoblasts, which then differentiate to hepatocyte-like cells when treated with OSM.

The hepatocyte-like cells generated were thought to be 'hu-hepatocyte-like' in that they shared many of the morphological characteristics associated with 'genuine' hepatocytes and expressed the representative hu-hepatocyte biomarkers such as hu-Alb and, importantly, a series of genes encoding Phase I and Phase II enzymes, whose expression is characteristic of a fully differentiated hepatocyte. The hepatocyte-like cells secreted hu-Alb in the medium and displayed several hepatic functions, including accumulation of glycogen, metabolism of indocyanine green, accumulation of lipid, active uptake of low density lipoprotein and synthesis of urea. When injected into liver lobes of newborn mice, these hepatocyte-like cells formed hu-Alb-positive foci throughout the injected lobe, indicating that they exhibit the inherent capacity to integrate into the hepatic parenchyma *in vivo*. However, they do not appear to entirely replicate mature liver functions, because the expression levels of hepatocyte-specific enzymes were lower in most cases compared with adult liver samples. Utilizing hu-iPS cell lines generated from the hu-embryonic lung fibroblast cell line MCR5, Takayama *et al.* showed that FoxA2 and HNF1a are useful in generating metabolically functional hepatocytes [89].

Hepatic viral permissiveness will currently be one of the most important issues for the iPS cell-derived hu-hepatocytes, as it was for the hepatocyte *h*-chimeric mouse. Several attempts have been reported to optimize requirements for differentiating hu-iPS cells into functional mature hepatocytes *in vitro* in relation to viral permissiveness. There have been a few studies that demonstrated that iPS cell-derived hu-hepatocytes are permissive to the viral infection *in vitro*. The hu-iPS cells were produced from foreskin fibroblasts, differentiated to hepatocyte-like cells as described above [88] and challenged with genotype 2a HCV with Gaussia luciferase reporter gene [90]. The results were remarkable, showing that the hepatocyte-like cells not only allowed HCV to enter and replicate but also allowed HCV to escape to the culture medium as infectious virions, indicating that iPS cell-derived hu-hepatocyte-like cells are a promising experimental tool to replicate the entire viral life cycle *in vitro* of at least genotype 2a. Furthermore, these cells exhibited antiviral inflammatory responses on HCV-infection by upregulating the expression of genes such as IFN γ-inducible protein 10 (CXCL-10), IFN-inducible T-cell, a chemoattractant (CXCL-10), TNF-α and IL-28B (a member of type III IFN [IFN-l]). These HCV-dependent expressions of innate antiviral cytokine genes could provide iPS cell-derived hu-hepatocyte-like cells with an advantage as an *in vitro* experimental tool for HCV infection and replication over the hu-hepatoma cell line Huh-7 described above. There was also a study that demonstrated the permissiveness of hu-iPS cell-derived hepatocyte-like cells to HCV [91]. Therefore, iPS cell-derived hu-hepatocyte-like cells could be a novel cell culture model for investigating the entire life cycle of HCV, including inflammatory responses to the viral infection, and could contribute to studies on the relationships between host genetics and viral pathogenesis.

Primary hu-fetal liver cell cultures seem to exhibit a similar permissiveness to HCV as iPS cell-derived hepatocytes [92]. A hepatocyte-enriched fraction was prepared from hu-fetal

livers at 16 – 24 week of gestation by collagenase digestion and centrifugation, cultured and used as primary liver cultures for HCV-infectivity tests. HCV replication in these cells was less robust than that in the Huh-7 line. Analyses of gene expression that are associated with IFN-induction and signaling showed that cell culture-produced HCV robustly activated expression of CXCL10 and CXCL11, variably activated IFN-λ (IL-29 and IL-28B) and ISGs such as viperin, IFITM1 and IFIT2, but did not activate IFN-β and TNF-α. These results suggest that innate antiviral mechanisms (especially INF-λ-associated pathways) in primary cells limit HCV replication or spread, which could explain a less robust viral replication in these cells than in Huh-7 lines. However, inductivity of IFN-λ and ISGs by HCV implies that HCV infection of fetal hu-liver cell cultures may provide a useful model for the study of gene induction by HCV *in vivo*, as does the infection of iPS cell-derived hepatocyte-like cells. In addition, the apparent commonness in the induction of antiviral IFN-related cytokines on HCV infection between fetal hu-hepatocytes and hu-iPS cell-derived hepatocyte-like cells suggests an association between their plausible immaturity and permissibility of HCV. It is well known that, paradoxically, sufficiently differentiated primary hepatocytes are generally reluctant to allow HCV to replicate *in vitro*. The hu-iPS cells are thought to be useful as a source of hu-hepatocytes for both *in vitro* and *in vivo* studies. Currently available hepatocyte-*h*-chimeric mice are those with genuine hu-hepatocytes in the liver. It is quite plausible that *h*-chimeric mice bearing hu-iPS cell-derived hepatocyte-like cells in the liver will be generated in the near future and these mice can be utilized as preclinical test animal model.

Regenerative capabilities of m-hepatocyte-like cells that had been differentiated *in vivo* from m-iPS cells were evaluated utilizing genetically FAH (a tyrosine-degrading enzyme)-deficient mice as an experimental model for liver regeneration, which causes the liver diseases, such as tyrosinemia type I [93]. FAH-deficient mice in a pure C57BL/6 background were bred with C57/6 R26R to label the host blastocyst-derived cells in the FAH-deficient neonates. These mice suffer from liver failure in the neonatal period unless treated with NTBC [32,94] as described above. The iPS cells were generated by repeated transfection of m-embryonic fibroblasts with Oct4, Sox2, Kif4 and c-Myc, and they were injected into blastocysts obtained from FAH-deficient mice. The foster mothers bearing these blastocysts were treated with NTBC during pregnancy. When the neonates were 6 days old, the foster mothers were deprived of NTBC. The contribution of iPS cell-derived cells (iPS-hepatocytes) was determined using PCR amplification of the Fah allele in genomic DNA in the digits and livers. Pups derived from FAH-deficient blastocysts that had not been injected with iPS cells rapidly deteriorated. However, those injected with iPS cells and with high levels of digital chimerism continued to thrive. They showed various levels of liver repopulation with FAH^+-hepatocytes, reaching ~ 100% repopulation at 70 days after birth. These FAH^+-hepatocytes were negative to Rosa26 reporter staining, indicating that they resulted from direct iPS cell differentiation, but not from fusion of the iPS cell progeny with blastocyst-derived host hepatocytes.

These mice were challenged with two-third partial hepatectomy at 28 days and 70 days after birth to determine the regenerative capacity of their livers. As a result, it was found that iPS-hepatocytes responded to the hepatectomy with characteristics of normal liver regeneration. The results reported from this study clearly indicate that iPS cells are able to differentiate into mature and functional hepatocytes *in vivo* following the embryonic hepatocyte-differentiation program, and thus provide a practical and experimental basis and rationale to generate hu-iPS-hepatocyte-*h*-chimeric mice for drug testing and HCV/HBV-related studies. To this end, it is necessary to create an alternative method in which hu-iPS cells can be incorporated into the m-liver as normally differentiated hepatocytes, which does not include a procedure of hu-iPS cell-injection into the host blastocysts. It is also possible to create a hepatocyte-*h*-m-model by transplanting hu-iPS cell-derived hepatocytes into the livers of young immunodeficient and liver-injured mice, such as the uPA/SCID mouse. To the authors' knowledge, studies are actively being undertaken to achieve this goal, although no trial has been reported in which sufficiently high levels of RI of the donor cells were attained.

7. Expert opinion

The time 'distance' from the discovery of a candidate chemical to the creation of a clinical drug is becoming shorter, and the risk of failing to produce a reliable and effective medicine is decreasing as knowledge of science and technology increases and deepens. The hepatocyte-*h*-m-model is one example of such relations between principles and applications in the world of science and technology. Before the generation of the hepatocyte-*h*-m-model, there was a complete separation between R&D (preclinical) and clinical tests, which were mostly conducted with animal-derived or human-derived systems in laboratories and with completely hu-systems in hospitals, respectively. Since the generation of the hepatocyte-*h*-m-model, a living experimental model of hu-liver functions, this clear separation has become diffuse and obscure due to the involvement of hu-liver-like functions in the m-liver. In this overview, the current status of the hepatocyte-*h*-mouse is described, including its usability for drug testing, such as predicting metabolic profiles and PK in humans and studies of the mechanisms of hepatitis viruses. Accumulating data from these studies indicate that the chimeric mouse is largely faithful in reproducing metabolic profiles of drugs and their kinetics in humans, especially those that are processed through CYP 450 enzymes if their RIs are high (> ~ 70%).

Although there is still much work to do for improving the currently available hepatocyte-*h*-mice to increase their

usability, it is thought that the basic technologies have been established and as described in this review, the first-generation hepatocyte-*h*-mice practically meets the requirements from laboratories for drug development and for researches of hepatitis viruses. Therefore, the researchers' interests are now moving from the first-generation chimeric mouse model to the second-generation chimeric model. Challenges have been robustly undertaken for improving hepatocyte-*h*-mice (first-generation chimeric mice) and overcoming their shortcomings, such as the absence of hu-immune cells required for inducing inflammations and immunological reactions. These innovative research trials have been creating second-generation chimeric mice with dual chimerism (hepatocytes and inflammation/immune-response cells), with which researchers could examine drug- or hepatitis virus-induced inflammation and hepatic fibrogenesis. Hopefully, in the near future, researchers could generate the second-generation *h*-mice that develop hepatocytic carcinogenesis under appropriate experimental conditions. Modern progressions in biotechnology are also making large contributions to the usability and availability of animal models to understand drug metabolism, toxicity and effectiveness, which includes the creation of liver-*h* rats and generation of chimeric mice with 'artificial hu-hepatocytes' using the patients' fibroblasts and iPS cell technology.

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Declaration of interest

K Yoshizato and C Tateno are employees of PhoenixBio Co., Ltd.

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