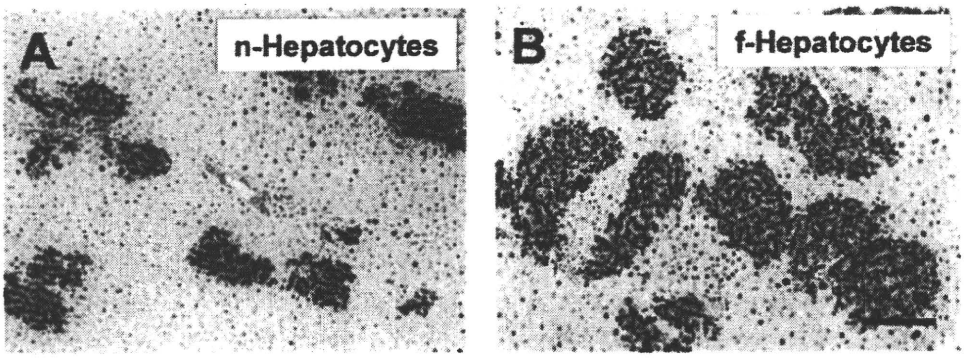

Figure 2. Colonies formed by transplanted hepatocytes. (A) DPPIV$^+$ n-hepatocytes and (B) DPPIV$^+$ f-hepatocytes were transplanted into the livers of retrorsine/PH DPPIV$^-$ rats. Liver sections were prepared from the rats 21 days after transplantation. Cryosections of the livers were subjected to DPPIV histochemical staining. The transplanted hepatocytes were stained black. Scale bar: 200 μm.

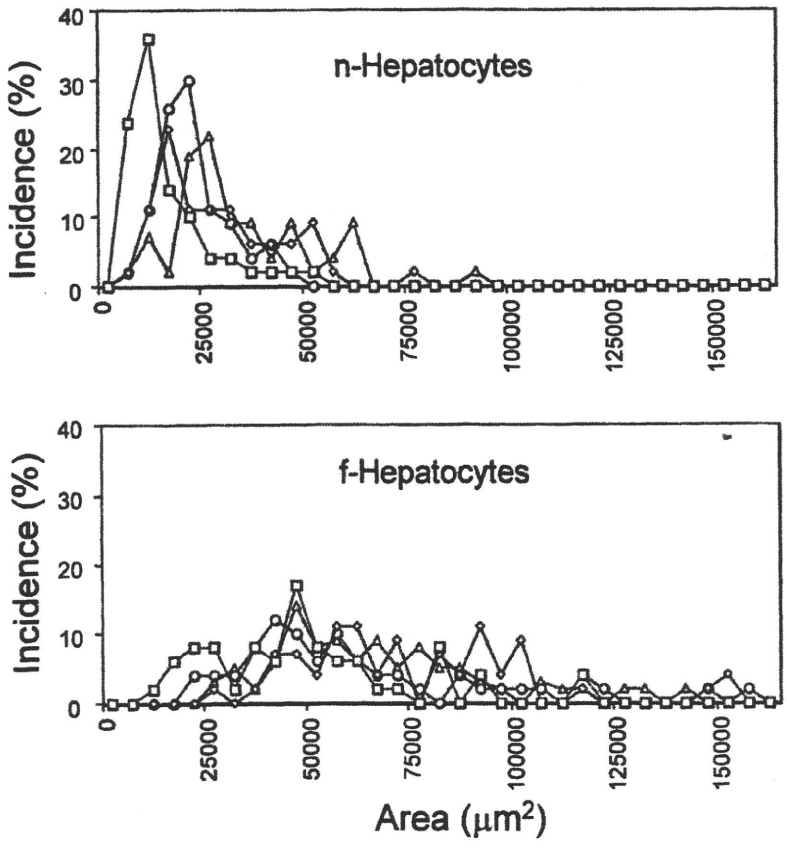

Figure 3. Size distribution of the hepatocyte colonies. Forty regions were selected randomly from DPPIV-stained sections for four individuals as shown in Figure 2 and were photographed. The colony area was measured on the photographs using NIH ver. 1.62. Each line represents the proportion (%) of the area measured for each of the four animals. f-Hepatocytes occupied more area than n-hepatocytes.

Table 1. The Mean Area and Volume of Colonies Formed by Transplanted Hepatocytes

Transplanted Cells	Area (μm^2)	Volume (μm^3)	Number
n-Hepatocytes	26,387 ± 1,015 *	3,580,888 ± 212,285 *	211
f-Hepatocytes	64,450 ± 2,016	13,207,708 ± 628,909	206

Hepatocytes were transplanted into the livers of rats, which were killed 21 days after transplantation. The areas of colonies were measured for four different rats transplanted with hepatocytes under the identical experimental conditions, as shown in Figure 2. The mean ± SE of areas and volumes of colonies was calculated from these measurements.
*$p < 0.05$. The p-values were determined by Mann-Whitney rank sum test.

Cell Phenotype in Colonies Formed by Transplanted Hepatocytes

The cells in the colonies of n- and f-hepatocytes were characterized in terms of the expression of four lineage-specific markers of liver cells: albumin, α-SMA, CK19, and GST-P. The cells in colonies of both types of hepatocytes expressed albumin at a high level, comparable to that of the surrounding host hepatocytes (Fig. 4A–D). Neither CK19 nor GST-P was expressed in the cells of colonies formed by both types of hepatocytes (data not shown), supporting the notion that f-hepatocytes do not show preneoplastic or bile duct epithelial cell phenotypes. These results strongly suggest that f-hepatocytes retain the phenotype of normal hepatocytes and maintain the normal phenotype throughout replication. In addition, α-SMA$^+$ cells (activated stellate cells) were not observed in the f-hepatocyte-transplanted livers (Fig. 4G, H) as in the n-hepatocyte-transplanted livers (Fig. 4E, F).

Gene Expression in f-Hepatocytes

Previously, we had identified 10 SH-associated genes: *p55cdc, Sta, CYP17, Pge2r, Psti, Cdc2, Cx26, Mcak, rat EST 207254*, and an unknown gene (*ab088476*) (2). The mRNA expression levels of all of these genes except *rat EST 207254* were determined using real-time RT-PCR in n-, f-, and r-hepatocytes (Fig. 5B, Table 3) and were compared with our previous data, which are shown in Figure 5A. The expression levels of the SH-associated genes were much higher in SHs than in LHs (Fig. 5A). The expression levels of four genes (*ab088476, Pge2r, Cx26*, and *Psti*) were similar between n- and f-hepatocytes. By contrast, five genes (*CYP17, p55cdc, Cdc2, Mcak*, and *Sta*) were expressed at much higher levels in f-hepatocytes than in n-hepatocytes. It was noteworthy that the overall expression profile of n-hepatocytes was similar to that of r-hepatocytes (Fig. 5B).

DISCUSSION

Fibrotic livers have been characterized mainly with respect to activated stellate cells, which are known as extracellular matrix (ECM)-producing cells, and little is known about the nature of hepatocytes in the fibrotic liver. Mito et al. reported that hepatocytes from fibrotic liver exhibited greater proliferation than hepatocytes from normal liver when transplanted into the spleen, despite the injection of approximately 1/200 of the amount of normal cells (10). Histological observations revealed that transplanted hepatocytes from cirrhotic livers differentiated into plates two to several cells thick, whereas plates of one-cell thickness prevailed with the transplantation of normal hepatocytes. One interpretation of this observation is that cirrhotic hepatocytes are still in the regenerating phase. Hepatocytes from cirrhotic livers retained normal functions such as glycogenesis and albumin synthesis (10). On electron micrographs, collagen fibers in the space of Disse were observed 1 year after the transplantation of hepatocytes from cirrhotic livers, but were not present with normal hepatocyte transplantation (10). In the present study, we characterized hepatocytes isolated from the fibrotic liver for the first time in

Table 2. Mean Cell Number in a Colony

Transplanted Cells	Cell Diameter (μm)	Cell Number per Colony Area	Cell Number per Colony Volume	Cell Divisions During 21 Days
n-Hepatocytes	22.8 ± 0.5	110 ± 12	1,217 ± 206	9–11
f-Hepatocytes	20.3 ± 0.3	209 ± 21	3,131 ± 481	11–13

Data are expressed as the mean ± SE.

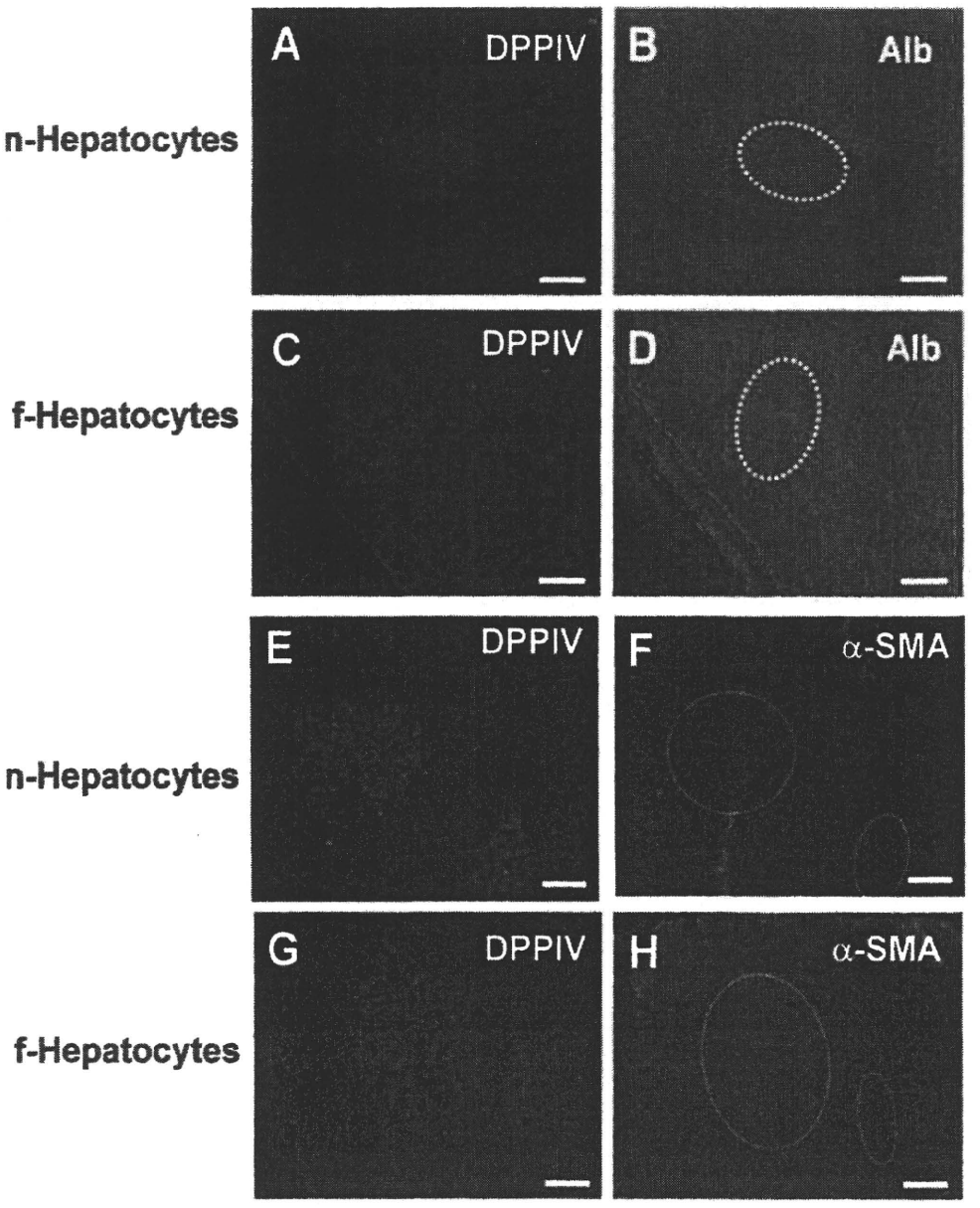

Figure 4. Phenotypes of the cells in the colonies formed by transplanted hepatocytes. DPPIV$^-$ n-hepatocytes (A, B, E, and F) and DPPIV$^+$ f-hepatocytes (C, D, G, and H) were transplanted into retrorsine/PH-treated DPPIV$^-$ rats. Serial liver cryosections were prepared 21 days after transplantation and were stained for DPPIV (A, C, E, and G), albumin (B and D), and α-SMA (F and H). The colonies formed by the transplanted hepatocytes are localized as DPPIV$^+$ regions (A, C, E, and G). These colonies are each marked by broken lines on the corresponding immunostained serial sections (B, D, F, and H). The DPPIV$^+$ cells were all albumin$^+$. There were no α-SMA$^+$ cells in the livers transplanted with both n- and f-hepatocytes. Scale bar: 100 μm.

terms of their growth potential in vitro and in vivo (in the retrorsine/PH rat liver), FACS profile, cell size, growth potential, and gene expression.

The growth potential of hepatocytes is heterogeneous: the hepatocyte with the greatest proliferative ability forms a colony consisting of more than 100 cells within 10 days, whereas the hepatocyte with the lowest ability does not divide (19). The adult rat liver contains a minor population of hepatocytes called SHs, which have a smaller size and higher replicative potential than LHs (6,17,19). Previously, we had used FACS and centrifugal elutriation to isolate highly proliferative SHs as

cells with low autofluorescence and low granularity (2). In the present study, most of the f-hepatocytes had low autofluorescence and low granularity, similar to SHs. Therefore, we investigated whether f-hepatocytes have characteristics similar to SHs in terms of size, growth potential in vitro and in vivo, and gene expression.

The average diameter of f-hepatocytes (20.1 ± 0.2 μm) was comparable to that of n-hepatocytes (22.1 ± 0.2

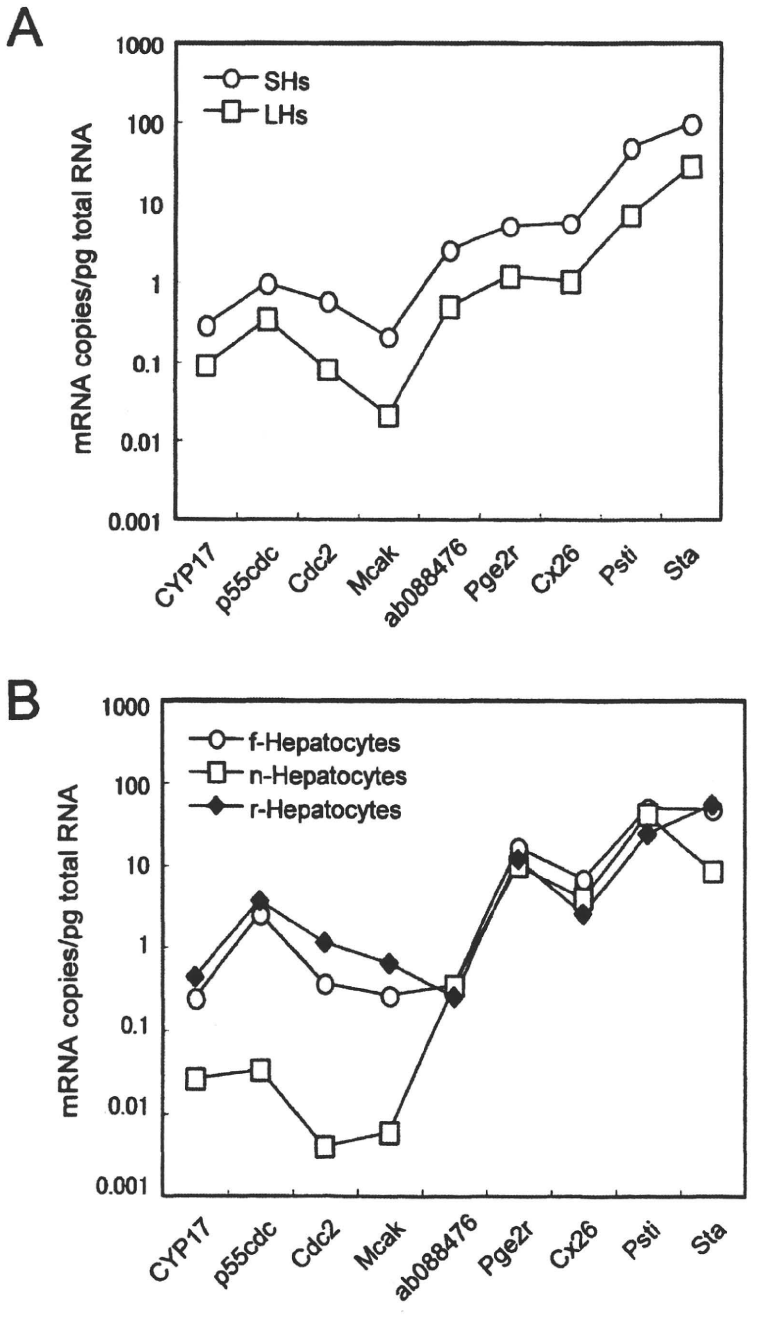

Figure 5. Expression of the SH-associated genes in hepatocytes. (A) Gene expression levels in SHs and LHs. This graph is drawn using the mRNA expression data presented in Tables 4 and 5 of Asahina et al. (2). (B) The gene expression levels in n-, f-, and r-hepatocytes were determined by measuring the mRNA copy number using real-time RT-PCR.

Table 3. Expression Levels of SH-Associated Genes in n-, r-, and f-Hepatocytes

	mRNA Copies/pg Total RNA		
Gene*	n-Hepatocytes	f-Hepatocytes	r-Hepatocytes
CYP17	0.026 ± 0.000	0.239 ± 0.147	0.430 ± 0.216
p55cdc	0.032 ± 0.022	2.443 ± 3.138	3.527 ± 1.044
Cdc2	0.004 ± 0.004	0.360 ± 0.424	1.092 ± 0.397
Mcak	0.006 ± 0.008	0.259 ± 0.308	0.627 ± 0.197
Unknown (*ab088476*)	0.343 ± 0.566	0.339 ± 0.281	0.252 ± 0.032
Pge2r	9.770 ± 8.698	16.452 ± 11.455	11.557 ± 1.699
Cx26	3.870 ± 0.967	6.690 ± 2.426	2.481 ± 0.267
Psti	40.961 ± 9.499	48.189 ± 11.954	23.374 ± 9.560
Sta	8.429 ± 7.905	46.431 ± 36.094	52.897 ± 21.741

The primers used for real-time RT-PCR have been reported elsewhere (2). Each value represents the mean ± SD of three different rats.
*Genes identified as differentially expressed using a cDNA microarray and representational difference analysis in SHs (2).

μm) but was larger than that of SHs (17.4 ± 0.0 μm). The growth of f-hepatocytes in vitro was compared with that of n-hepatocytes. Previously, we had shown that SH proliferation was about four times that of LHs in vitro (17). The f-hepatocytes showed growth ability similar to that of n-hepatocytes. SHs were also highly proliferative in vivo (about three times compared with LHs), as determined by colony-forming ability after transplantation into the livers of the retrorsine/PH rats (6). Here, we also estimated the growth potential of hepatocytes using this model. The engraftment index obtained in this study at 48 h after transplantation was 9.9% for f-hepatocytes and 9.3% for n-hepatocytes, and did not differ significantly. The colonies formed by transplanted cells at 21 days were examined in all of the residual livers of the recipients and showed that f-hepatocytes were more proliferative (about 2.5 times) than n-hepatocytes.

From the data, we concluded that f-hepatocytes have higher growth ability than n-hepatocytes in vivo but not in vitro. At present, there is no explanation for this apparent discrepancy between the in vivo and in vitro growth abilities of the two types of hepatocytes. However, it seems that the culture conditions used in the present study were not sufficient for f-hepatocytes to exhibit their full proliferation potential.

Ten SH-associated genes had been identified previously (2). We examined whether f-hepatocytes also express the SH-associated genes, and we compared the expression of SH-associated genes between f- and r-hepatocytes. The f-hepatocytes expressed five of the nine SH genes, at similar levels as SHs. Importantly, the overall expression profile of f-hepatocytes resembled that of r-hepatocytes.

The expression of marker proteins specific to hepatocytes, preneoplastic hepatocytes, and bile duct epithelial cells was characterized in f-hepatocytes. When rats were treated with CCl_4 for more than 7 weeks, morphologically recognizable GST-P$^+$ preneoplastic nodules were observed in some of the treated rats (7). In the present study, the cells in colonies formed by transplanted f-hepatocytes were albumin$^+$ but CK19 and GST-P$^-$, like n-hepatocytes, showing that the cells in the f-hepatocyte colonies expressed hepatocyte markers but not bile duct or preneoplastic markers. In addition, stellate cells did not express α-SMA in the f-hepatocyte colonies. There seems to be a regulatory mechanism in vivo under which f-hepatocytes stably express hepatocytic phenotypes but do not express biliary phenotypes.

In this study, we demonstrated that hepatocytes surrounded by ECM in the fibrotic liver retained high growth potential. f-Hepatocytes can be used instead of n-hepatocytes for hepatocyte transplantation, although the yield of hepatocytes from a fibrotic liver is fewer than that from a normal liver. However, it remains to be determined whether human f-hepatocytes have similar normal and proliferative phenotypes as rat f-hepatocytes, when isolated from the livers of fibrotic patients and transplanted into normal recipients. If they show these phenotypes, fibrotic livers could be a useful source of hepatocytes for the treatment of liver damage using hepatocyte transplantation.

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Expert Opinion

In vivo modeling of human liver for pharmacological study using humanized mouse

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The liver occupies a central place in the treatment of the substances taken into the body. If we could devise an *in vivo* or *in vitro* model that perfectly mimics the naturally-created human (h) liver, the work required for making effective and safe medicines would become easier and could be undertaken more cost effectively than it is currently. Considering the advantages of *in vivo* modeling over *in vitro* modeling under the current technological state of life sciences research, we have created an experimentally workable *in vivo* h-liver model, a liver-humanized mouse, in which host hepatocytes are largely replaced with healthy normal h-hepatocytes. Xenogenic h-hepatocytes are capable of constructing a histologically normal liver by collaborating with mouse-nonparenchymal cells in an elaborately organized manner. Considering its potential use for drug development, we have extensively characterized the mouse regarding the infectivity toward h-hepatitis viruses, activities of h-enzymes in Phase I and II of drug metabolisms, and h-hepatocyte-related drug transporters. These studies indicate that the humanized mouse liver mimics h-phenotypes at a level appropriate for pharmacological studies, and, thus, can be used not only for developing new medicines, but also for examining biological and pathological mechanisms in the h-liver.

Keywords: human hepatocytes, humanized mouse, immunodeficient mouse, *in vivo* drug metabolism

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1. Introduction

The body takes in and treats natural and artificial substances from the surrounding environment; some of the processed materials are used for life activities, while others are excreted from the body. The liver plays a central role in the processing of internalized substances (metabolism). The nominal functions of the liver are performed by a group of cells called parenchymal cells, or hepatocytes. It is well known that there are interspecies differences in the hepatic metabolic patterns of a given xenobiotic. Thus, the analytical results obtained from experiments using hepatocytes from rodents, such as rats and mice, are not always relevant in predicting the responses of human (h)-hepatocytes, indicating that h-hepatocytes are required to examine the metabolism and toxicity of a given chemical when the purpose of the study is to understand the reactions of the h-liver. However, there are also difficulties in using h-hepatocytes as an analytical tool.

In creating an *in vivo* replica of the liver, a normal, healthy human would be the ideal model; however, humans cannot serve as experimental targets. Perhaps the simple and easiest way to use h-hepatocytes for such purposes is to isolate them from an appropriate source, cultivate them for propagation and utilize them in the desired experiments. However, in doing this there are several problems,

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including the limited number of normal h-livers available, the reluctance of h-hepatocytes to replicate *in vitro* and the attenuation of a normal h-hepatocytic phenotype under *in vitro* culture conditions.

Importantly, such *in vitro* characterization of hepatocytes is insufficient to correctly understand hepatocyte function *in situ*, because their biological features such as growth, proliferation and expression of phenotypes are regulated in a complex and intricate manner by other types of liver cells (nonparenchymal cells), including resident vascular endothelial cells, stellate cells and Kupffer cells (macrophages), in addition to non-resident cells, such as immune-responsive cells. These cells are tightly associated, not only structurally, but also functionally, and constitute a well-organized biological entity, the liver. That is, hepatocytes fulfill their given tasks in the context of this specialized and unique community of different cell types. It is clear that even the most advanced modern biotechnological techniques cannot create a suitable *in vitro* environment to enable isolated populations of hepatocytes to function as they would *in vivo*; however, such cells can mimic some of their *in vivo* functions.

We accept that *in vitro* experimentation is superior to its *in vivo* counterpart in terms of understanding complex biological phenomena in that cause and effect relationships among the factors involved can be examined in a point-by-point manner. However, we are still far from the time when such complete *in vitro* experimentation will be possible. Currently, *in vivo* experimentation is considered to be better than *in vitro* experimentation for examining the normal phenotypes of hepatocytes, although the interpretation of results obtained in this way is not always simple because of the complex nature of the interactions between populations of various cell types.

Given these considerations, we sought to develop a small *in vivo* animal model for use in studying the biological and pharmaceutical phenotypes of h-hepatocytes. Additionally, for both *in vivo* and *in vitro* modeling, the development of technologies capable of producing large numbers of normal h-hepatocytes is required because normal h-hepatocytes are not readily available to researchers due to their obviously limited source. Generally, normal hepatocytes are not conducive for replicating *in vitro*, despite their high proliferative potential *in vivo*. If we could create such an animal model, h-hepatocytes could be readily propagated in a host liver.

To accomplish our goal to generate a small animal model with h-liver that can be utilized for investigation of drug metabolism, we utilized an immunodeficient and liver-injured mouse as a host. Such a mouse will be tolerant of receiving h-hepatocytes in its liver. The engrafted h-hepatocytes would be stimulated to actively proliferate in the liver because the host hepatocytes have been injured and they would repopulate the liver by expelling the not-replicable injured mouse (m)-hepatocytes. With this idea, we undertook intensive laboratory works investigating whether the xenogenic h-hepatocytes can actually collaborate with m-nonparenchymal cells in a proper fashion and reconstruct a normal liver both functionally and histologically. These works resulted in generating a mouse whose hepatocytes are mostly of human origin.

We next undertook works to test whether the chimeric liver is practically humanized and suitable for predicting h-type drug metabolism. These continuous endeavors in collaborations with researchers in pharmacological, pharmaceutical and medical areas have enabled us to launch a 'humanized mouse factory' that produces homogeneous mice in a large scale whose livers are largely repopulated with h-hepatocytes and made them available to researchers on demand for drug testing. In our experience, we are able to conclude that the factory mouse can contribute to the needs for high-throughput predictive models required for drug discovery. In this article, we overview the development in producing liver-humanized mice and their usefulness for research and development (R&D) activities in discovering new medicines that are suitable to humans.

Although this humanized mouse provides an ideal *in vivo* model of h-liver compared to other currently available ones, it has some problems to be solved in the future due to the lack of other types of h-cells and endocrinological h-factors that affect the metabolic activities of hepatocytes. Including these issues, we present our opinions regarding studies that have not been explored enough yet, but are important in making the liver humanized mouse a much better tool for pharmaceutical researches.

2. Ample regenerative capacity of hepatocytes *in vivo*

In adults, the liver is functionally very active, but largely quiescent in terms of proliferation. The turnover rate of h-hepatocytes is around 1 year in rodents [1,2]; however, the same organ is ready for rapid regeneration if the liver mass is reduced. Generally, the weight of an organ is closely related to body weight [3]. The ratio of liver:body weight ($R_{L/B}$) in humans is about 2.4 – 2.6% [4]. If the $R_{L/B}$ falls below this range, residual hepatocytes in the G0 phase of the cell cycle enter G1 and progress to S phase within 24 h. The hepatocytes will continue to replicate until the $R_{L/B}$ reaches 2.4 – 2.6%. This weight loss-induced regeneration is conserved through life, although the capacity decreases somewhat with age.

The exceptionally high regenerative capacity of the liver *in vivo* suggests a means for the abundant propagation of h-hepatocytes, starting with small numbers of h-hepatocytes in the liver of an appropriate model animal. In terms of the best species for this type of experimental design, mice or rats are preferable because they are commonly used in the laboratory. A key requirement for this type of experimental design is the availability of an immunodeficient mouse or rat whose liver is damaged and, thus, whose hepatocytes are in a proliferative phase.

If a rodent model satisfying these requirements is available, we could propagate h-hepatocytes by engrafting them in the animal model liver. The engrafted h-hepatocytes would then

proliferate and form colonies that would continue to expand, replacing damaged host hepatocytes, until the completion of the replacement. Regarding the immunodeficiency requirement, mice are superior to rats in that several mouse strains with defective immune systems have been characterized, whereas there is a dearth of immunodeficient rat strains. However, mice are less preferable from the viewpoint of generating large numbers of hepatocytes because there are far fewer cells in the small livers of mice.

3. Propagation of congenic and xenogeneic hepatocytes in a mouse model

An ideal mouse model for amplifying h-hepatocytes was discovered in a study of neonatal bleeding disorders. Albumin (Alb) promoter/enhancer-driven urokinase (Alb-uPA) gene-transgenic ($Tg_{Alb\text{-}uPA}$) mice carrying a tandem array of four murine urokinase genes controlled by the Alb promoter overproduced urokinase in their hepatocytes [5]. As a result, the livers became severely hypofibrinogenemic, which accelerated hepatocyte death through multiple undefined mechanisms involving extracellular matrix decomposition [6].

In this Tg mouse line, the functional liver deficit was thought to result in the chronic stimulation of liver growth [7]. Indeed, in hepatocytes with a stochastic deletion of the deleterious transgene, selective hepatocyte replication and expansion was observed with restoration of the liver. This event occurred most commonly in mice hemizygous for the transgene. In these animals, transgene expression in the hepatocytes was abolished because of a DNA rearrangement that affected the transgene tandem array, permitting the individuals to survive beyond birth with plasma uPA concentrations gradually returning to normal by 2 months of age. Transgene-deficient cells behaved like normal hepatocytes in transgene-active hepatocyte-induced regenerative environments, forming clonal colonies called hepatic nodules. These nodules expanded, replacing the surrounding transgene-active cells that could not replicate because of cellular damage, and eventually replaced the entire liver.

Based on this study, $Tg_{Alb\text{-}uPA}$ mice may be useful for examining the replicative capacity of hepatocytes from mice [8] and other mammals with acquired immunotolerance. Thus, when xenogeneic hepatocytes, including h-hepatocytes, were transplanted into this Tg-mouse model, the cells could be propagated at the expense of pre-existing resident hepatocytes [9].

Rhim *et al.* [10] introduced the Alb-uPA transgene into immunotolerant nu/nu mice by mating $Tg_{Alb\text{-}uPA}$ mice with Swiss athymic nude (*nu/nu*) mice, generating immunotolerant $Tg_{Alb\text{-}uPA}$ mice ($Tg_{Alb\text{-}uPA}$/NUDE mice). Rat (r) liver cells were transplanted into the livers of $Tg_{Alb\text{-}uPA}^{+/+}$/NUDE mice homozygous for the transgene. Host livers that had not been transplanted with r-liver cells were pale (white) in color. In contrast, those with r-liver cells consisted of white and red regions, with the white regions representing areas composed only of transgene-expressing host cells and the red regions representing areas composed only of transgene-deleted host m-cells, repopulated r-cells or both. Immunohistochemical analysis using antibodies against r-hepatocytes confirmed that the red regions consisted primarily of r-hepatocytes. The completely regenerated Tg m-livers resembled normal m-livers in terms of their color, shape and size. Southern blot DNA band analysis demonstrated that up to 56% of the DNA was of rat origin, in accordance with the parenchymal cell occupancy rate in the liver and supports the idea that the host liver was chimeric, with r-parenchyma and m-nonparenchyma, including vessels, bile ducts and associated connective tissues.

The weight ratio of liver:body ($R_{L/B}$) was around 7%, similar to that in the non-transgenic control mice (~ 6%), indicating that the r/m-chimeric livers were able to terminate growth normally. The successful generation of a healthy mouse with a chimeric liver indicates that r-parenchymal and m-nonparenchymal cells can communicate with each other to reconstitute a functional liver, despite the species difference. It is known that hepatocytes initiate and terminate proliferation under the influence of nonparenchymal cells [11]. Thus, the normal progress and termination of r/m-chimeric liver regeneration indicates that r-hepatocytes produce surface proteins that interact correctly with soluble m-factors, the m-extracellular matrix and m-surface proteins on m-nonparenchymal cells.

Together, these studies indicate that constitutive expression of the uPA transgene in resident m-hepatocytes generated a selective environment that favored the growth of not only endogenous m-hepatocytes with a normal (non-transgenic) phenotype [7], but also of transplanted congenic [8] and xenogeneic hepatocytes [9,10,12], raising the possibility that the liver of a uPA-Tg mouse could be reconstituted with h-hepatocytes [10]. This possibility was verified independently by two groups in 2001 [13,14] in studies of hepatitis virus infectivity.

4. Propagation of h-hepatocytes in a mouse model

Three types of immunodeficient mice have been used as hosts for h-hepatocytes. The first studies to produce a mouse with a h-hepatocyte/mouse (h/m)-chimeric liver were reported simultaneously, with one using recombinase-activating gene-2 (RAG-2)-knockout mice as the immunodeficient host [13] and the other [14] using severe combined immunodeficient (SCID) mice [15] that lacked mature B and T cells due to an inactivating mutation in the catalytic subunit of a DNA-dependent protein kinase ($Prkdc^{scid}$) [16]. Recently, an additional immunodeficient mouse strain, NOG, was used as a host; these mice lack not only mature T and B lymphocytes, but also NK cells [17]. Non-obese diabetic (NOD) mice are prone to the spontaneous development of autoimmune insulin-dependent diabetes mellitus [18]; related strains were developed at the Shionogi Research Laboratories as NOD/Shi mice. NOD/Shi mice were crossed with the SCID mice. Highly

immunodeficient mice, called NOG (NOD/Shi-*scid* IL-2Rγ^{null}) mice, were generated by backcrossing the resulting NOD/Shi-*scid* mice with IL-2 receptor γ-chain gene-knockout (C57BL/6J-IL-2Rγ^{null}) mice [19].

These three immunodeficient mice strains (RAG-2 knockout, SCID and NOG) were used as partner mouse lines for mating with the genetically liver-injured uPA-Tg mouse line. h-Hepatocytes were engrafted in the livers of these immunodeficient and liver-injured mice. h-Hepatocyte transplantation studies with uPA/RAG-2-knockout and uPA/SCID mice successfully generated mice harboring h-hepatocyte-repopulated livers. The extent of h-hepatocyte repopulation could be periodically monitored by measuring the h-Alb levels in the host blood and quantified as the replacement index (RI, the ratio of engrafted h-hepatocytes to the total number of hepatocytes (m- and h-hepatocytes) in the host liver) by calculating the ratio of the area occupied by a h-specific hepatocytic protein, such as h-cytokeratin (CK) 8/18, to the entire area in immunohistochemical sections of the host liver tissues.

In early studies, the attained RIs were between about 15 and 50% in the uPA/RAG-2-knockout and uPA/SCID mouse experiments, respectively. Three years after these two pioneering studies, we conducted additional studies using mice with h-hepatocyte-repopulated livers in an attempt to increase the RI as much as possible and to examine the possibility of using them to predict h-type metabolism and the excretion of xenobiotics [20].

As a result, we were able to generate chimeric mice having livers with RIs as high as 96%. Similarly, Meuleman *et al.* [21] made an h-liver in a uPA/SCID mouse with an RI around 90%. Suemizu *et al.* [17] transplanted h-hepatocytes into the livers of NOG mice and followed the engraftment and proliferation of the h-hepatocytes therein, generating chimeric mice with RIs up to 80%.

5. Human versus murine properties of h-hepatocyte-repopulated mouse livers

In our studies, ~ 10^6 normal viable h-hepatocytes per mouse were typically transplanted into the livers of uPA/SCID mice at 20 – 30 days after birth [20]. The h-hepatocytes engrafted the livers at rates as high as 96% and progressively repopulated them, as shown by increases in hAlb in the host blood. The actual growth of h-hepatocyte colonies was visualized and quantified in liver tissue sections from chimeric mice that had been immunohistologically stained with h-specific anti-CK8/18 antibodies. The engrafted h-hepatocytes started to proliferate around 7 days after transplantation and formed colonies, which gradually became larger, replacing the damaged host m-hepatocytes, and were nearly confluent at around day 80, when the RI reached 100% (**Figure 1A**). Histological analyses showed a well-organized hepatic architecture with normally located portal and central veins formed by m-cells, separated by intervening hepatic plates formed by h-hepatocytes (**Figure 1B**). The emergence of hepatic parenchymal cells in mice that are primarily h-hepatocytes raises an interesting biological question: do the h-hepatocytes in mouse livers remain h-hepatocytes or do they adopt the characteristics of m-hepatocytes to conform to the m-liver environment?

h-Hepatocytes are under the control of m-nonparenchymal cells in the m-liver. Thus, it is apparent that the h-hepatocytes can no longer be truly authentic h-hepatocytes, as they would be in an h-liver. Likewise, the m-liver occupied by h-hepatocytes is no longer an authentic m-liver, like the liver of a normal mouse. The h-hepatocyte host is immunotolerant and able to accept h-proteins physically, but may not accept them functionally. It is important to know how far removed the h-hepatocytes are from authentic h-hepatocytes, and how far removed the chimeric host liver is from an authentic m-liver, because the interest in creating an entity that adequately mimics an authentic h-liver for biological and pharmacological studies drove us to start this project in the first place. The last question is also important because if the degree of difference is too large, the host will not harmonize with the h-hepatocytes and will die.

6. Ability of h-hepatocytes to reconstruct an m-liver sufficiently to support mouse life without losing their h-hepatocyte phenotype

In our studies of h-hepatocyte-repopulated mice, we used actively growing young mice as hosts. These mice were able not only to survive, but also to grow, though relatively slowly, during the experimental period, reaching a body weight > 50% of the original and an RI > 90% [20]. Thus, h-hepatocytes can function like m-hepatocytes and support host growth.

It appears that most of the m-proteins responsible for host growth may be replaced with the corresponding h-proteins from h-hepatocytes, suggesting that h-hepatocytes are functionally accepted as m-hepatocytes in the m-liver community, enabling h-hepatocytes to communicate with m-nonparenchymal cells to such an extent that the liver can support the growth of a young mouse. Several lines of experimental evidence support this assertion. The host liver of the uPA/SCID mouse is congenitally damaged, and the mice showed lower levels of blood Alb and abnormally high levels of alanine aminotransferase, a biochemical indicator of liver damage, before h-hepatocyte transplantation.

The repopulation of h-hepatocytes in the liver increased the blood Alb concentration and decreased the alanine aminotransferase level, indicating that h-hepatocytes in place of m-hepatocytes improved m-liver function [20]. This is consistent with the results of histological analyses. In chimeric m-liver sections stained for type IV collagen, laminin, stabilin (a liver endothelial cell marker), BM8 (a Kupffer cell marker) and desmin (a hepatic stellate cell marker), highly organized liver structures made by xenogeneic hepatocytes and m-nonparenchymal cells were observed (data submitted). Interactions between hepatocytes and stellate cells are

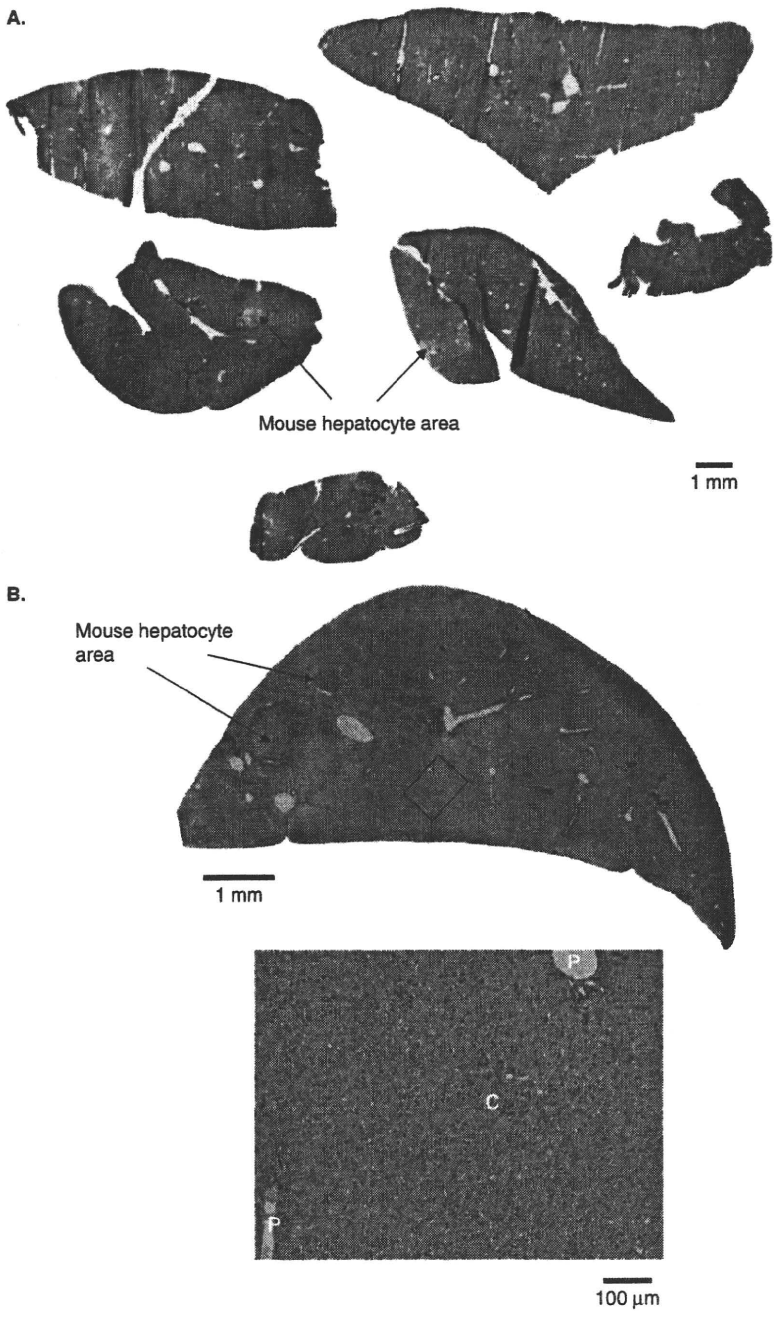

Figure 1. Appearance of m-livers constructed by xenogeneic cooperation between h-hepatocytes and m-liver nonparenchymal cells. A. h-Hepatocytes (10^5 cells) from a 6-year-old girl were transplanted into a uPA/SCID mouse. The mouse was sacrificed 77 days post-transplantation when almost all of the m-hepatocytes had been replaced with h-hepatocytes (RI = 98.8%). The six liver lobules were sectioned for h-CK8/18 staining to locate regions occupied by h-hepatocytes. Small m-hepatocytic regions remained (indicated by arrows). **B.** h-Hepatocytes (7.5×10^5 cells) from a 9-month-old boy were transplanted into a uPA/SCID mouse. The mouse was sacrificed 80 days after transplantation, when the RI reached 82%. The largest liver lobule was sectioned for h-Alb staining to locate regions occupied by h-hepatocytes. Some areas of m-hepatocytes remained (arrows, upper panel). The region enclosed by the rectangle is enlarged to show the cellular architecture (lower panel).

Bars: 1 mm for the upper panel and 100 µm for the lower panel.

C: Central vein; h: Human; m: Mouse; P: Portal vein; RI: Replacement index; SCID: Severe combined immunodeficient.

important in both physiological and pathological conditions in the liver [22]. The reconstructed sinusoidal structures between the h-hepatocytes and m-stellate cells were immunohistologically visualized by staining of these two types of cells with antibodies against h-CK8/18 and m-desmin, respectively (**Figure 2**). Our results indicate that the livers of the chimeric mice with a high RI consisted of parenchymal cells (mostly h-cells and a small percentage of m-cells), m-nonparenchymal cells and m-ECMs. Therefore, it is apparent that xenogeneic interactions between the h-parenchymal and m-nonparenchymal liver cells supported the construction of an m-liver that was seemingly normal in terms of histological structure and biochemical function. Meuleman *et al.* [21] showed the formation of functional bile canaliculi connected to mouse canaliculi by electron microscopy. There was a good correlation between the RI and mRNA expression levels of such housekeeping genes as hAlb and h-transferrin, supporting the notion that transplanted h-hepatocytes were functional [23]. In our experience, mice with > 6 mg/ml hAlb in their blood have an RI > 70%.

That h-hepatocytes are accepted by the m-nonparenchymal community and are able to construct a liver that biochemically and morphologically resembles an m-liver and is capable of supporting mouse life indicates that basic features of hepatocytes are common to humans and mice, despite the big species difference.

Many hepatocyte proteins are required to support the growth and maintenance of a mouse, and h-hepatocyte proteins appear to be functionally recognized as m-hepatocyte proteins in mice. We suggest that h-hepatocytes are able to express most h-proteins even in the quite different m-liver environment. That is, the autonomy of h-hepatocytes is maintained in a xenogeneic environment under immunotolerant conditions. It appears that h-hepatocytes can keep their autonomy as h-hepatocytes in an m-liver without disrupting the life of the mouse, at least for the time periods studied (up to 80 days after transplantation). This conclusion is our rationale for the use of h-hepatocyte-chimeric mice as an experimental tool for studying the biology and pharmacology of h-hepatocytes. However, many details of this supposition remain to be demonstrated experimentally.

7. Infection of a chimeric m-liver with human hepatitis viruses and the propagation thereof

The infectivity of human-specific viruses, such as hepatitis B virus (HBV) and HCV, in h/m-chimeric mice and the propagation of such viruses provides a criterion for determining whether the chimeric m-liver is 'humanized'. Additionally, if these mice are sensitive to such viruses, they may be useful as infectious disease animal models, because human liver diseases caused by HBV and HCV have been studied extensively worldwide in the search for effective antiviral medicines [24]. Moreover, rodents are not useful animal models, due to the strict species specificity of viruses [25]. Further, h-hepatocytes in culture are not sensitive to such viruses.

Two groups sought to inoculate chimeric mice with hepatitis virus-infected h-serum. They found that the h/m-chimeric mice were not only infected by the viruses, but could also be hosts for viral propagation; one for HBV, using h-hepatocyte-chimeric Rug-2-knockout mice [13], and the other for HCV, using h/m-chimeric uPA/SCID mice [14]. In the HCV infection study, the homozygous animals were superior to their hemizygous counterparts, due to a substantial advantage in terms of the magnitude and duration of h-hepatocyte engraftment in the former. Viral replication was confirmed by the detection of negative-strand viral RNA in the transplanted livers. HCV proteins were localized to h-hepatocyte colonies, and the infection was serially passaged through three generations of mice, confirming both the synthesis and release of infectious viral particles.

Subsequently, we also assessed the infectivity of HBV in the chimeric mice [26]. The mice were inoculated with h-serum containing HBV. High-level viremia was observed in mice inoculated with HBV-positive h-serum for up to 22 weeks. Passage experiments showed that the serum of the mice contained infectious HBV. As in the case of the HCV infection study, the degree of viremia tended to be higher in those mice with a greater RI. Further, it was demonstrated that lamivudine, an anti-HBV drug, effectively reduced the degree of viremia in the infected mice, indicating that chimeric mice are a useful model for the development and evaluation of anti-human hepatitis virus drugs.

8. Humanization of drug metabolism in the chimeric m-liver

Humans take in many natural and artificial materials from their surrounding environment, including foods, nutrients and drugs. The biochemical treatment of foreign substances, or xenobiotics, is one of the major tasks of the liver, and this is achieved somewhat differently in mice and humans. Thus, the metabolic pathway induced by a xenobiotic administered to a chimeric mouse could be an important criterion in evaluating the humanness of a chimeric m-liver. This criterion is probably also of major commercial significance with respect to the R&D of efficient and effective new drugs for humans.

Conventionally, rodents, especially rats, have been used as animal models to study the metabolism and safety of a candidate drug; if a chimeric mouse is sufficiently humanized in terms of drug metabolism, it may provide an innovative new model valuable for R&D leading to the development of new medicines. When administered, a xenobiotic is taken up largely by hepatocytes and distributed intracellularly, metabolized and secreted, via the bile or urinary duct, through the processes of absorption, distribution, metabolism, and excretion (ADME). Each step in the ADME of a drug involves multiple genes and their corresponding proteins, and is species-dependent. Further, the steps in these processes are interdependently regulated; thus, the pharmacokinetics of a drug is species-dependent, determined by parameters resulting from these interactive

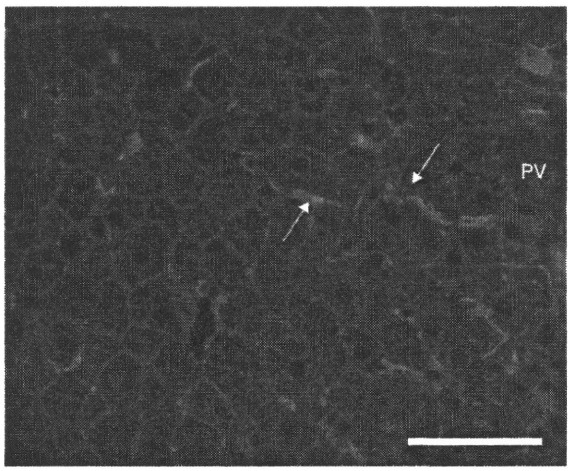

Figure 2. Chimeric sinusoids formed by the elaborate collaboration of h-hepatocytes with m-nonparenchymal cells.
A liver section prepared from a liver lobule of a chimeric mouse was double immunostained with h-CK8/18 (green) for h-hepatocytes and desmin (red) for m-stellate cells (indicated by arrows).
Bar: 10 μm.
h: Human; m: Mouse; PV: Portal vein.

processes. The potential for differences in the ADME of a drug between humans and rodents means that caution must be taken in order to correctly extrapolate the pharmacokinetics of xenobiotics in rodents to those in humans.

Xenobiotics are metabolized in hepatocytes by xenobiotic-metabolizing enzymes (XMEs) in two phases. Phase I involves oxidative enzymes while Phase II involves conjugating enzymes that create more stable, hydrophilic derivatives [27]. Drugs, toxicants and chemical carcinogens are processed in Phase I primarily by the cytochrome P450 (CYP) and flavin-containing monooxygenase superfamilies, with the former being especially important in eliminating most clinical drugs. Thus, we examined the expression profile of CYP enzymes in chimeric m-livers to assess the degree of humanization.

8.1 Phase I drug metabolism

Among the known CYP families, four families, CYP1 – 4, are known to play primary roles in xenobiotic metabolism. In particular, CYP3A4 is the most abundantly expressed CYP in human liver and metabolizes more than 60% of all therapeutic drugs [27]. CYP2D6 is also important in drug R&D; it is believed that 70% of the drugs on the market are metabolized by these two enzymes [27].

From the viewpoint of humanization, CYP isoforms CYP2C8, CYP2C9, CYP2C18 and CYP2C19 are useful targets for study because all are found in the h-liver, but are absent from m- and r-livers [28,29]. Western blot analyses using h-specific antibodies against CYP2C9 of hepatocytic microsomal fractions from h/m-chimeric mice with an RI > 34% showed positive signals, in contrast to those from chimeric mice with an RI < 28% or control mice (mice not transplanted with h-hepatocytes) [20]. CYP2C9 has diclofenac 4′-hydroxylation activity. Microsomal fractions from the chimeric mice showed diclofenac 4′-hydroxylase activity, the degree of which depended on the RI of the mouse. These positive results support our expectation that h-hepatocytes in chimeric livers retain h-type pharmacologic activity toward administered drugs.

As mentioned above, CYP2D6 metabolizes a large number of clinically used drugs [30,31], and there is a prominent difference in this CYP between mice and humans, making this enzyme a good test material for judging whether an animal model is useful and reliable for the study of h-type drug metabolism [32,33]. The CYP2D subfamily in humans has a single active member, CYP2D6. Rats and mice carry at least five genes, but none encodes a protein with the same enzymatic activity as its h-counterpart [32,34]. Debrisoquin is an h-CYP2D6 substrate that is largely metabolized to 4′-hydroxydebrisoquin in a reaction inhibited by quinidine, an h-CYP2D6 enzyme inhibitor. When debrisoquin was administered to the chimeric mice, 4′-hydroxydebrisoquin was detected in the blood of the animals, and its levels were decreased in mice pretreated with quinidine [35]. This result further suggests that the chimeric m-livers were humanized.

Additional support for the human-ness of the h-hepatocytes in the chimeric mouse livers was obtained in an experiment in which we selected six major CYP subfamilies with primary roles as XMEs, CYP1A1, 1A2, 2C9, 2C19, 2D6 and 3A4, and compared their mRNA and protein expression profiles between chimeric mouse and donor livers [20]. All of the RI-dependent mRNAs were detected. Mice with a greater RI value generally showed much stronger h-CYP expression than did mice with a lower RI value.

Our results showed that the h-hepatocytes in the chimeric mice expressed all six h-CYP genes in a semi-normal manner, as in the human body (**Figure 3**). The h-CYP1A and h-CYP3A4 subfamilies are known to specifically respond to 3-methylcholanthrene (3-MC) and rifampicin, respectively [36]. To address whether the chimeric m-livers retained the expected reactivity against these specific inducers, the mice were treated with 3-MC (**Figure 3A**) or rifampicin (**Figure 3B**). Neither 3-MC nor rifampicin induced the expression of any of the six hCYPs in uPA/SCID mice that had not been transplanted with h-hepatocytes, indicating the specificity of these inducers for h-hepatocytes [37]. 3-MC enhanced the mRNA expression of CYP1A1 and CYP1A2 10- and 6-fold, respectively, but not of the other four CYPs examined. Rifampicin enhanced the expression of h-CYP3A4 in the chimeric mice sixfold, but not of the other five hCYPs tested. Rifabutin, an analogue of rifampicin, also specifically induced h-CYP3A in the chimeric m-livers, but not murine Cyp3a [36]. The CYP3A4-induction potency in the chimeric mice is useful for drug testing because, as mentioned above, many drugs are substrates of CYP3A4 and, thus, its induction decreases the

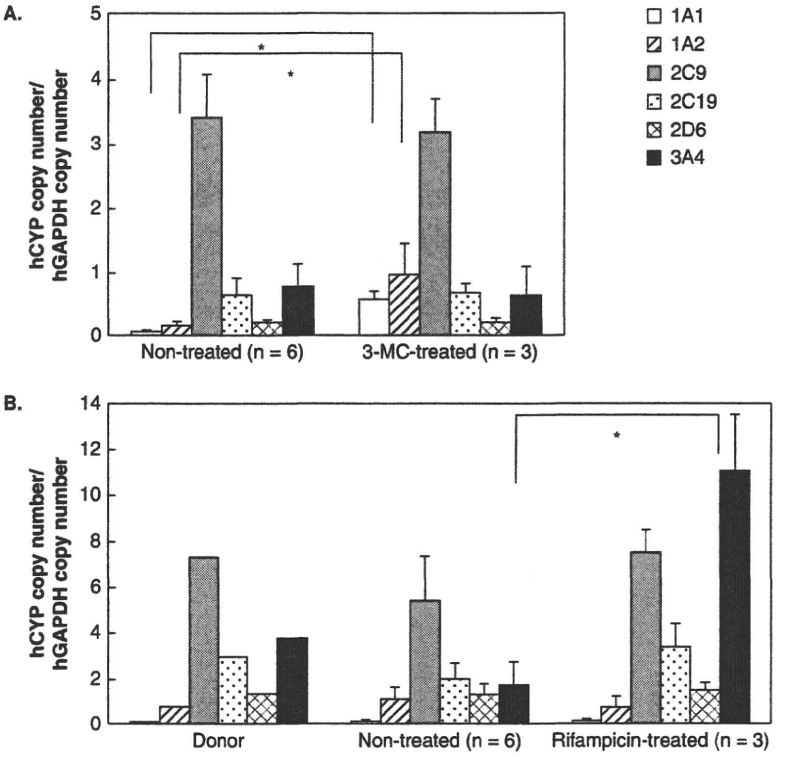

Figure 3. Expression profiles of the CYP1-3 subfamily genes in the chimeric mice. A. Chimeric mice made with h-hepatocytes from a 12-year-old boy were sacrificed – 70 days post-transplantation. Some mice were treated with 3-MC at 20 mg/kg body weight/day for the last 4 days. The mRNA expression of six CYP subfamilies, 1A1, 1A2, 2C9, 2C19, 2D6 and 3A4, was quantified by quantitative real-time reverse transcriptase-PCR using h-specific primers. **B.** Chimeric mice made with h-hepatocytes from a 9-month-old boy were sacrificed – 70 days post-transplantation. Some mice were treated with rifampicin at 50 mg/kg body weight/day for the last 4 days. Liver tissues were also obtained from the donors. The mRNA expression of six CYP subfamilies was determined as in **A.** The results shown are the average of the tested samples. Thin vertical bars indicate s.d.. The donor expression levels represent the averages of duplicate determinations for the same sample.

*Significant difference ($p < 0.01$) between the indicated measurements.

The graphs are modified from those published previously [20].

3-MC: Methylcholanthrene; h: Human.

pharmacological potency of drugs [27]. Thus, h-hepatocytes appear to be able to maintain their autonomy in an m-liver environment, at least as far as the CYPs we studied are concerned.

The specific induction of CYP by rifampicin and 3-MC in h-livers is accomplished through complex and specific cell-surface and subcellular signaling networks. Rifampicin is a ligand for pregnane X receptor (PXR), which forms a heterodimer with retinoid X receptor a (RXRa) forming a complex (rifampicin/PXR/RXRa) that upregulates CYP3A4 gene expression by binding to a xenobiotic response element composed of a direct repeat of α and β half-sites separated by four nucleotides [38]. Rifampicin is a potent activator of human and rabbit PXR, but has little activity toward rat or mouse PXR [39]. That the chimeric m-livers were responsive to rifampicin suggests that h-specific PXR/RXRa-dependent intracellular signaling is also at work in the chimeric m-livers, again supporting the notion that the liver data for the h/m-chimeric mice faithfully reflect those for humans. 3-MC is a ligand of aryl hydrocarbon receptor (AHR), and its complex with AHR (AHR/3-MC) is known to upregulate the genes CYP1A1, CYP1A2 and CYP1B1 by binding to their xenobiotic response elements together with AHR nuclear translocator [40]. Our studies suggest that these known ligand-activated receptor signaling pathways are functional in h/m-chimeric mouse livers. Although our data related to CYP expression, drug profiles and regulation by inducers and inhibitors are not comprehensive, our current opinion is that h-hepatocytes in mice do not lose their intricate intracellular signaling networks, which are specialized for h-hepatocyte drug metabolism; thus, hepatocyte-humanized mice may prove to be a useful animal model for studying the h-type signaling pathways that regulate xenobiotic-induced gene expression.

8.2 Humanization of the Phase II pathways of drug metabolism

The contribution of Phase II conjugation to the clearance of a drug is said to be ~ 30% [41]; in particular, compounds with polar groups are primarily metabolized in this way. The major hepatic Phase II enzymes in humans are UDP-glucuronosyltransferase (UGT), sulfotransferase (SULT), *N*-acetyltransferase (NAT) and glutathione *S*-transferase (GST), which are responsible for glucuronidation, sulfation, acetylation and glutathione conjugation, respectively. hUGT, hSULT, hNAT and hGST were expressed at the mRNA level while UGT2B7, SULT1E1, SULT2A1 and GSTA1 were expressed at the protein level in the chimeric m-livers, with a correlation between the level of expression and the RI of the mice [42]. The activities of related enzymes, including morphine 6-glucuronosyltransferase and estrone 3-SULT, were also detected in an RI-dependent manner. The protein contents and enzymatic activities of the Phase II-associated enzymes in chimeric m-livers with high RIs (~ 90%) were similar to those in the donor livers. We also compared the mRNA expression profiles of 26 Phase II h-enzymes, including members of the GST, SUL, NAT and UGT families, between the livers of chimeric mice with RIs between 71 and 89% and donors in a systematic and comprehensive manner [23]. All of the genes tested were detectable in the chimeric mice. Although the expression levels of the tested genes (65%) were significantly lower than in the donors (30 – 55% of the level in the donors), we suggest that the Phase II drug biotransformation is appreciably humanized in h/m-chimeric mice.

There are groups of clinically used drugs that bind to PXRs or constitutive androstane receptors. These ligand-activated PXRs and constitutive androstane receptors are involved in the regulation of some Phase II XME genes, including SULT1A and UGT1A [43] and GST and UGT1A [44,45], respectively. Considering the semi-normal expression profiles of the Phase II XME genes and proteins, it is likely that these ligand-activated transcriptional regulators are functional in h/m-chimeric m-livers; however, additional, direct analytical data are needed to confirm this.

8.3 Humanization of drug transportation

Generally, the recognition and intake of a drug and its secretion occur on and in the cell membrane and are the initial and final steps of proper drug handling by hepatocytes. Studies of humanization related to the membrane-associated aspects of drug treatment in chimeric mice are quite limited compared with humanization at the intracellular level, despite the general recognition of their physiological and pathological importance. Drug transportation in the liver is largely performed by two systems. The first, extrahepatic-to-hepatic transportation, involves transporters such as organic cation transporter 1, organic anion transporting polypeptide (OATP) 1B1 and OATP1B3. The second is hepatic-to-bile-duct transportation, and it involves, among others, adenosine 5′-triphosphate-binding cassette (ABC) proteins, including P-glycoprotein, bile salt export pump (ABCB11), breast cancer resistance protein (ABCG2) and multidrug resistance-associated protein 2 [46]. The former transporters are located on the sinusoidal membrane, and are responsible for the intake of drugs into hepatocytes, while the last are on the canalicular membrane, and are responsible for the biliary excretion of metabolites. The h-genes encoding these transport systems were preferentially expressed compared with their m-counterparts in chimeric mice with RIs > 60% [46]. Cefmetazole, a cephalosporin antibiotic, is excreted without chemical modification via urinary and biliary pathways. Humans use the former as the dominant pathway [47], whereas the latter has been demonstrated in rats [48] and mice [46]. Before receiving h-hepatocytes, the host mice excreted cefmetazole predominantly via the biliary pathway. The urinary pathway was dominant in chimeric mice with RIs > 60% [46].

We also examined the expression levels of 21 human transporter genes, including ABC and OATP, in the livers of chimeric mice and compared them with those in donor livers [23]. The chimeric mouse:human expression ratio for 62% of the 21 genes tested in the chimeric livers ranged from 0.35 to 0.75. We also found that when treated with fibrates, amphipathic carboxylic acids used to treat metabolic disorders and as hypolipidemic agents, the expression of multidrug-resistance P-glycoprotein 3 in the chimeric mice increased [49]. Although data related to the drug transporters in chimeric m-livers are limited, the available data suggest that chimeric livers are substantially humanized and could be useful for investigating h-type drug transport systems in drug R&D.

9. Expert opinion

To researchers who recognize the value of a liver-humanized mouse in predicting the metabolic pathway and safety of a drug in h-livers, information concerning the ADME profiles of various drugs is of great importance. To date, h-hepatocyte-chimeric mice have been largely evaluated in relation to the 'M' (CYP-associated metabolism) of ADME. It should also be remembered that most chimeric mouse-associated studies have considered these mice to be an innovative and useful *in vivo* animal model for studying the mechanisms of infection and propagation by human hepatitis viruses, for comparing the infectious potential of viral subtypes and for the screening of candidate antiviral drugs. Since the introduction of liver-humanized mice to the research environment, almost a decade has passed. During this time, interest in using chimeric mice as *in vivo* model for examining the ADME of drugs, in place of humans and conventional rodents, has gradually increased because researchers in the drug-hunting field increasingly are appreciating their usefulness. Based on our accumulated experience, we propose that these mice are significantly and appreciably humanized in terms of their hepatic phenotype (at least in terms of the factors we have examined thus far) and, thus, that the mice represent a reliable and promising animal model that may be more useful in predicting the

metabolism and efficacy of a drug in humans than any other currently available model. In chimeric mice, the liver is largely occupied by h-hepatocytes; however, it retains its structural and functional integrity as a liver while the h-hepatocytes retain their autonomy in the murine hepatic environment. Accordingly, researchers can investigate the response to a drug as if they are working with an h-liver, without being limited to just one or a few related genes or proteins, as in an h-gene-targeted Tg mouse model.

However, we are still in an initial stage of scientific characterization of chimeric mice from various aspects of interest. Above all, human pharmacokinetic scalability of the humanized mouse is one of major themes that has not been systematically studied yet, but should be extensively examined, because allometric scaling has been generally used in the prediction of human pharmacokinetics from animal species. Practically, information on drug–drug interactions estimated by, for example, changes in 'area under the curve', is essential for developing new medicines in laboratories, but has been still poor in public. The accumulations of more experience and experimental data will reveal the advantages and limitations of the hepatocyte-humanized mouse.

In commercial R&D activities for effective drug discovery, a large number of homogeneous small animal models with h-type metabolic activities are at once required on demand. We began the large-scale production of homogenous populations of hepatocyte-humanized mice with high RIs 5 years ago to meet the need for high-throughput models in drug discovery. The product mice will facilitate academic and industrial research activities aimed at examining the h-type metabolism of new drugs and the mechanism of h-HCV infection and propagation, with the goal of discovering new anti-HCV drugs. Currently, we are able to produce about 200 chimeric mice with an RI of 70% per month. The cost for testing a drug using a humanized mouse is now much higher than that with a conventional animal, but will considerably decrease as the need increases.

Several problems specific to h-hepatocyte-chimeric mice remain. Currently, chimeric mice carry only a hepatocyte population of human origin; all other cells are of murine origin. Parenchymal cells perform their functions with the support of nonparenchymal cells. Chimeric mice lack nonparenchymal cells of human origin. Some interactions between h-parenchymal hepatocytes and m-nonparenchymal cells might proceed as they normally would in a homogeneic situation, but others might not.

Additionally, it is known that endocrine regulation is necessary for hepatocytes to achieve normal metabolic homeostasis and to reestablish normal conditions when metabolic parameters move outside the normal range due to endogenous or exogenous causes. The chimeric livers are under the influence of the murine endocrine system. However, it is known that some m-hormones, such as growth hormone (GH), are not able to act on h-cells, due to an inability to form a hormone-receptor complex between mGH and h-hepatocytes [50]. Consistent with this, h-hepatocytes repopulated the host livers at about a sixfold higher rate than in the control mouse livers when the animals were given hGH. The expression of such liver growth-associated human genes as IGF-1, STAT-3, Cdc 25A and cyclin D1 was enhanced. This simple experiment explicitly demonstrates that h-hepatocytes are under GH-deficient conditions, which is obviously not physiological. If we are concerned only with GH deficiency, we can solve this problem simply by treating the chimeric mice so as to establish a physiological concentration of hGH. However, it is also apparent that there are other conditions and factors that distinguish the h-hepatocytes in chimeric m-livers from authentic h-hepatocytes.

We are entering a time when h-liver-chimeric mice will be much improved and their h-hepatocytes will be able to more fully express authentic h-hepatocyte phenotypes. This next generation of chimeric mice will facilitate research activities with both medical and pharmaceutical purposes, adding to our understanding of human hepatitis-induced diseases and speeding up the discovery of new drugs. They will also bolster our awareness of the uniqueness and similarities between h-hepatocytes and those of other mammals.

Declaration of interest

We have had two independent and nearly overlapping occasions to provide an overview of our studies and experiences with human liver-chimeric mice. The two articles share considerable content, although we have managed to distinguish the viewpoint of this article from the other (submitted to *PPAR Research*).

Our study of chimeric mice with livers composed of human and rat hepatocytes was supported by the joint program of the Yoshizato Project in Cooperative Link of Unique Science and Technology for Economy Revitalization (CLUSTER) of the Japanese Ministry of Education, Culture, Sports, Science and Technology (2002 – 2009); by the Hiroshima Prefecture and Hiroshima University 21st Century COE Program for Advanced Radiation Casualty Medicine (2003 – 2008); and by a Research on Advanced Medical Technology, Health and Labor Sciences research grant from the Ministry of Health, Labor and Welfare of Japan (2001 – 2004).

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CYP1A1 and CYP1A2 expression: Comparing 'humanized' mouse lines and wild-type mice; comparing human and mouse hepatoma-derived cell lines

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Benzo[*a*]pyrene hydroxylase, ethoxyresorufin *O*-deethylase, acetanilide 4-hydroxylase and methoxyresorufin *O*-demethylase as CYP1A1 and CYP1A2 substrates
2,3,7,8-Tetrachlorodibenzo-*p*-dioxin (TCDD, dioxin) as P450 inducer
Mouse Hepa-1c1c7 cell culture
Human HepG2 cell culture

ABSTRACT

Human and rodent cytochrome P450 (**CYP**) enzymes sometimes exhibit striking species-specific differences in substrate preference and rate of metabolism. Human risk assessment of CYP substrates might therefore best be evaluated in the intact mouse by replacing mouse *Cyp* genes with human *CYP* orthologs; however, how "human-like" can human gene expression be expected in mouse tissues? Previously a bacterial-artificial-chromosome-transgenic mouse, carrying the human *CYP1A1_CYP1A2* locus and lacking the mouse *Cyp1a1* and *Cyp1a2* orthologs, was shown to express robustly human dioxin-inducible CYP1A1 and basal versus inducible CYP1A2 (mRNAs, proteins, enzyme activities) in each of nine mouse tissues examined. Chimeric mice carrying humanized liver have also been generated, by transplanting human hepatocytes into a urokinase-type plasminogen activator*(+/+)*_severe-combined-immunodeficiency (*uPA/SCID*) line with most of its mouse hepatocytes ablated. Herein we compare basal and dioxin-induced CYP1A mRNA copy numbers, protein levels, and four enzymes (benzo[*a*]pyrene hydroxylase, ethoxyresorufin *O*-deethylase, acetanilide 4-hydroxylase, methoxyresorufin *O*-demethylase) in liver of these two humanized mouse lines versus wild-type mice; we also compare these same parameters in mouse Hepa-1c1c7 and human HepG2 hepatoma-derived established cell lines. Most strikingly, mouse liver CYP1A1-specific enzyme activities are between 38- and 170-fold higher than human CYP1A1-specific enzyme activities (per unit of mRNA), whereas mouse versus human CYP1A2 enzyme activities (per unit of mRNA) are within 2.5-fold of one another. Moreover, both the mouse and human hepatoma cell lines exhibit striking differences in CYP1A mRNA levels and enzyme activities. These findings are relevant to risk assessment involving human CYP1A1 and CYP1A2 substrates, when administered to mice as environmental toxicants or drugs.

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Introduction

The human and mouse genomes comprise 57 and 102 protein-coding cytochrome P450 (**CYP**) genes, respectively, each divided into 18 families (**Nelson et al., 2004; Nebert et al., 2004; Nebert and Dalton, 2006**). The mammalian *CYP1* gene family encodes three enzymes in both human and mouse—CYP1A1, CYP1A2 and CYP1B1. While the *CYP1A* and *CYP1B* subfamily ancestors diverged from one another ~480 million years ago, *CYP1A2* arose as a duplication event from the *CYP1A1* gene about 420 million years ago. Thus, land animals (including birds) carry both *CYP1A1* and *CYP1A2*; on the other hand, fish genomes do not contain the *CYP1A2* gene (**Nelson et al., 1996**).

It was originally noted that alteration of a single amino-acid in a CYP protein could change dramatically its catalytic activity from coumarin to testosterone hydroxylation (**Lindberg and Negishi, 1989**). Similarly, numerous other examples have shown that human and rodent CYP1A2 orthologs, having important amino-acid differences, can display striking species-specific variability in the rates by which certain substrates are metabolized (**Turesky, 2005**). For example, human and mouse CYP1A2 differ by 3- to 7-fold in catalyzing ethoxyresorufin *O*-deethylation (**Aoyama et al., 1989**) and uroporphyrinogen oxidation (**Nichols et al., 2003**).

It therefore can be difficult to extrapolate toxicity or cancer data from rodent studies to human risk assessment. For this reason, we and others have generated "humanized" *hCYP1A1_1A2* transgenic lines in which either mouse *Cyp1a1* or *Cyp1a2* (**Jiang et al., 2005; Cheung et al., 2005; Derkenne et al., 2005**) or both mouse *Cyp1a1* and *Cyp1a2* (**Dragin et al., 2007; Shi et al., 2008**) orthologs are

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ablated. In addition, a global approach for making a humanized mouse has been developed by transplanting human hepatocytes into the urokinase-type plasminogen activator(+/+)_severe-combined-immunodeficiency (*uPA/SCID*) mouse, which otherwise is immunodeficient and undergoes liver failure; these chimeric mice no longer develop liver failure, but rather the mouse liver comprises >70% human hepatocytes that propagate successfully and retain normal pharmacological functions such as drug metabolism (Tateno et al., 2004; Katoh et al., 2008). Eight human P450s (CYP1A2, 2A6, 2C8, 2C9, 2C19, 2D6, 3A4, 3A5), 35 other Phase I enzymes, and four classes of Phase II conjugating enzymes (UDP glucuronosyltransferases, glutathione *S*-transferases, *N*-acetyltransferases, and sulfotransferases) have been shown to be functional in chimeric mice (Katoh et al., 2008). Because each chimeric mouse will reflect the liver profile and genetic makeup of the human donor's hepatocytes, interindividual and ethnic differences in drug metabolism will undoubtedly exist. Nevertheless, variations of xenobiotic-metabolizing enzymes as well as other enzymes, receptors, transporters, transcription factors, and any other drug target located in human liver—might effectively be studied in such chimeric mouse lines.

Mammalian CYP1A1 basal mRNA is known to be negligible, resulting in no detectable CYP1A1 protein in any tissue, whereas basal levels of CYP1A2 mRNA and protein are relatively high in liver but generally low (protein undetectable on Western immunoblot) in nonhepatic tissues; induction by a CYP1 inducer such as chemicals in cigarette smoke or 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (**TCDD**; **dioxin**) increases CYP1A1 and CYP1A2 mRNA and protein levels (Eaton et al., 1995; Nebert et al., 2004). Recently, two humanized *CYP1A1_1A2* lines were compared with C57BL/6J (**B6**) inbred mice with regard to expression of CYP1A1 and CYP1A2 mRNA levels following TCDD pretreatment. Maximally-induced mRNA concentrations of mouse CYP1A1 were ~10 times higher in liver and lung and ~100-fold greater in kidney than those of human CYP1A1 (Shi et al., 2008). On the other hand, maximally-induced mRNA levels of mouse CYP1A2 in liver were <2-fold higher than those of human CYP1A2. Maximally-induced mRNA levels of human CYP1A2 in liver were ~12 times higher than those of human CYP1A1, whereas maximally-induced mRNA levels of mouse CYP1A2 in liver were ~3-fold greater than those of mouse CYP1A1 (Shi et al., 2008).

These data caused us to query how "physiologically" relevant these human mRNA levels might be, in the intact mouse. Do these humanized mouse lines actually reflect "average" *CYP1A1* and *CYP1A2* gene expression that might be expected among individuals in a human population, or is this expression abnormally low or high? One should be able to shed some light on this, by comparing precise copy numbers of basal and TCDD-induced CYP1A1 and CYP1A2 mRNA (combined with quantification of protein levels and enzyme assays) in the humanized mouse lines versus wild-type mice.

Those who oppose the use of laboratory animals, and recommend instead that everyone utilize cells in culture, often declare that studies with cultured cell lines can provide information that would accurately reflect what is found in the intact animal. We therefore have compared the above-mentioned CYP1A1 and CYP1A2 parameters in liver from the humanized mouse lines and wild-type mice with those in human versus mouse hepatoma-derived established cell culture lines. The present study addresses these questions. Answering these questions should be beneficial, before launching into human risk assessment studies using such humanized mouse lines.

Material and methods

Mice. C57BL/6J (B6) mice were purchased from The Jackson Laboratory (Bar Harbor, ME). Development of the humanized *hCYP1A1_1A2_Cyp1a1/1a2(−/−)-Ahr^{b1}* transgenic line has been detailed (Dragin et al., 2007). Chimeric mice bearing human hepatocytes were generated using *uPA(+/+)/SCID* mice as the host (Giannini et al., 2003) and characterized (Tateno et al., 2004; Katoh et al., 2008); their human hepatocyte-replacement rates were between 73% and 83%. All experiments involving mice adhered to the Guidelines for Animal Experiments and Use Committee of the Nihon University School of Medicine.

Treatment of mice. Mice were treated with intraperitoneal TCDD (25 μg/kg for 24 h), versus corn oil vehicle alone for untreated. At least three groups ($N = 3$ each time) were studied to ensure reproducibility.

Cell cultures and treatment. The human HepG2 established cell line was derived from a hepatoblastoma (Dearfield et al., 1983). The mouse Hepa-1c1c7 line was derived from a C57L/J hepatoma (Bernhard et al., 1973). Cultured cells were treated with 10 nM TCDD for 24 h before total RNA isolation.

Reverse transcription. Total RNAs from samples were prepared by the acid guanidine thiocyanate-phenol/chloroform method (Tavangar et al., 1990). The cDNAs were synthesized using the ImProm-II Reverse Transcription system (Promega, Madison, WI) (Inaba et al., 2007).

Quantitative real-time PCR (qRT-PCR). We used the primers listed in Table 1. The qRT-PCR was performed in an ABI PRISM 7000 Sequence Detection System™ (Applied Biosystems), using Power SYBR Green PCR Master Mix (Applied Biosystems). Individual CYP1 mRNA abundance was determined, using the standard-curve method (from 10^1 to 10^8 copies/μL), as previously described by K. Livak (PE-ABI; Sequence Detector User; Bulletin #2) (Winer et al., 1999). Each sample was normalized to mouse glyceraldehyde-3-phosphate dehydrogenase (**GAPDH**) mRNA.

CYP1A mRNA copy numbers. Transcripts from the human *CYP1A1* and *CYP1A2* and the mouse *Cyp1a1* and *Cyp1a2* genes were quantified by fitting qRT-PCR data to a curve generated from cloned RNAs (cRNAs) for each CYP1. Briefly described, templates for cRNA synthesis were produced by PCR on cDNA constructs from each CYP1A cDNA that had been cloned into pcDNA3.1(+) (Invitrogen), using T7 RiboMAX™ Express Large-Scale RNA Production System (Promega) (Uno et al., 2006). The cRNAs were used to generate a standard curve in the PCR reactions from which mRNA copy numbers from qRT-RNA measurements could be extrapolated.

Western immunoblot analysis. Mice were euthanized by carbon dioxide asphyxiation followed by cervical dislocation. The liver was excised, and microsomes were prepared as previously described (Dalton et al., 2000). Protein concentrations were determined by the bicinchoninic acid method (Pierce Chemical Co.; Rockford, IL), according to details provided by the manufacturer. Microsomal proteins were separated on sodium dodecylsulfate (0.1%)–polyacrylamide (12%) minigels. Separated proteins were transferred to nitrocellulose membranes. Western immunoblot analysis was performed using goat polyclonal anti-rat CYP1A1/1A2 antibody; this antibody (Daiichi Pure Chemicals, Tokyo, Japan) recognizes both the human and mouse CYP1A1 and CYP1A2 proteins. We used alkaline phosphatase-conjugated secondary antibodies (Kirkegaard Perry Lab., Gaithersburg, MD) and the Alkaline

Table 1
Primer pairs used in qRT-PCR.

Gene	Forward primer	Reverse primer
hCYP1A1	5′-AAGGGGCGTTGTGTCTTTGT-3′	5′-ATACACTTCCGCTTGCCCAT-3′
hCYP1A2	5′-ACAAGGGACACAACGCTGAA-3′	5′-AGGGCTTGTTAATGGCAGTG-3′
mCyp1a1	5′-CCTCATGTACCTGGTAACCA-3′	5′-AAGGATGAATGCCGGAAGGT-3′
mCyp1a2	5′-AAGACAATGGCGGTCTCATC-3′	5′-GACGGTCAGAAAGCCGTGGT-3′
mGapdh	5′-TGCACCACCAACTGCTTAG-3′	5′-GATGCAGGGATGATGTTC-3′

h, human; m, mouse.