

Acknowledgements

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References

- Cheng TKH, Waxman DJ. 2006. Synthetic drugs and natural products as modulators of constitutive androgen receptor (CAR) and pregnane X receptor (PXR). *Drug Metabolism Reviews* 38:51–73.
- Dresser GK, Spence JD, Bailey DG. 2000. Pharmacokinetic–pharmacodynamic consequences and clinical relevance of cytochrome P450 3A4 inhibition. *Clinical Pharmacokinetics* 38:41–57.
- Gentile DM, Tomlinson ES, Maggs JL, Park K, Back DJ. 1996. Dexamethasone metabolism by human liver in vitro. Metabolite identification and inhibition of 6-hydroxylation. *Journal of Pharmacology and Experimental Therapeutics* 277:105–112.
- Katoh M, Matsui T, Nakajima M, Tateno C, Soeno Y, Horie T, Iwasaki K, Yoshizato K, Yokoi T. 2005a. In vivo induction of human cytochrome P450 enzymes expressed in chimeric mice with a humanized liver. *Drug Metabolism and Disposition* 33:754–763.
- Katoh M, Watanabe M, Tabata T, Sato Y, Nakajima M, Nishimura M, Naito S, Tateno C, Iwasaki K, Yoshizato K, Yokoi T. 2005b. In vivo induction of human cytochrome 3A4 by refabutin in chimeric mice with a humanized liver. *Xenobiotica* 35:863–875.
- Katoh M, Yokoi T. 2007. Application of chimeric mice with a humanized liver for predictive ADME. *Drug Metabolism Reviews* 39:145–157.
- Lin J. 2006. CYP induction-mediated drug interactions: in vitro assessment and clinical implications. *Pharmaceutical Research* 23:1089–1116.
- McGinnity DF, Parker AJ, Soars M, Riley RJ. 2000. Automated definition of the enzymology of drug oxidation by the major drug metabolizing cytochrome P450s. *Drug Metabolism and Disposition* 28:1327–1334.
- Minagawa K, Kasuya Y, Baba S, Knapp G, Skelly JP. 1986. Identification and quantification of 6 β -hydroxydexamethasone as a major urinary metabolite of dexamethasone in man. *Steroids* 47:175–188.
- Nelson DR, Koymans L, Kamataki T, Stegeman JJ, Feyereisen R, Waxman DJ, Waterman MR, Gotoh O, Coon MJ, Estabrook RW, Gunsalus IC, Nebert DW. 1996. P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics* 6:1–42.
- Rendic S. 2002. Summary of information on human CYP enzymes: Human P450 metabolism data. *Drug Metabolism Reviews* 34:83–448.
- Tateno C, Yoshizane Y, Saito N, Kataoka M, Utoh R, Yamasaki C, Tachibana A, Soeno Y, Asahina K, Hino H, Asahara T, Yokoi T, Furukawa T, Yoshizato K. 2004. Near completely humanized liver in mice shows human-type metabolic responses to drugs. *American Journal of Pathology* 165:901–912.
- Tomlinson ES, Maggs JL, Park BK, Back DJ. 1997. Dexamethasone metabolism in vitro: species difference. *Journal of Steroid Biochemistry and Molecular Biology* 62:345–352.
- Zurbonsen K, Bressolle F, Solassol I, Aragon PJ, Culine S, Pinguet F. 2004. Simultaneous determination of dexamethasone and 6 β -hydroxydexamethasone in urine using solid-phase extraction and liquid chromatography: application to in vivo measurement of cytochrome P450 3A4 activity. *Journal of Chromatography B* 804:421–429.



Review

Chimeric mice with humanized liver

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Abstract

Recently, chimeric mice with humanized liver were established by transplanting human hepatocytes into an urokinase-type plasminogen activator^{+/+}/severe combined immunodeficient transgenic mouse line. The replacement with human hepatocytes is more than 80–90% and is higher than any other chimeric mouse reported previously. In drug development, the liver is one of the most important organs because it is mainly involved in the pharmacokinetics of drugs and is frequently damaged by many drugs due to the accumulation of drugs and/or metabolites. The pharmacokinetics could affect the efficacy and toxicity of a drug, and thus prediction of the human pharmacokinetics is important for developing new drugs without adverse reactions and toxicity. Extrapolation from experimental animals or *in vitro* studies to the human *in vivo* pharmacokinetics is still difficult. To date, human hepatocytes and liver microsomes are recognized as better tools and are frequently used to estimate the human pharmacokinetics. We thought that chimeric mice with humanized liver could become a new tool for estimating the human toxicity and pharmacokinetics. At first, metabolism, which plays an essential role in pharmacokinetics, was investigated in the chimeric mice. In the liver of the chimeric mice, human drug metabolizing enzymes were found to be expressed and to reflect the capacities and genetic polymorphism of the donor. In an *in vivo* study on metabolism, human specific metabolites could be detected in the serum of the chimeric mice indicating that the chimeric mice could be used as an *in vivo* model to address human metabolism. These results suggested that the chimeric mice could overcome the species differences in drug metabolism and be used to evaluate drug toxicity due to genetic polymorphism. The reasons for drug interaction are often enzyme induction and inhibition. By the treatment with a typical inducer of cytochrome P450 (P450), which is the central drug-metabolizing enzyme, P450s expressed in the liver of the chimeric mice were found to possess induction potencies. After the treatment with a specific inhibitor of human P450, the area under the curve of the P450 metabolite was significantly decreased in the chimeric mice but not in the control mice. Therefore, it was indicated that the chimeric mice could be useful for assessing drug interactions *in vivo*. Moreover, drug excretion was determined to be humanized because cefmetazole was mainly excreted in urine both in the chimeric mice and humans but in the feces in control *uPA^{-/-}/SCID* mice. Drug transporters expressed in the liver of the chimeric mice were also humanized.

In this review, studies of the chimeric mice with humanized liver, particularly on metabolism and excretion, are summarized and the possibility of using the chimeric mice is proposed for the advanced prediction of human pharmacokinetics and toxicity.

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Keywords: Humanized model; Drug-metabolizing enzyme; Transporter; Drug interactions; Genetic polymorphism

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1. Introduction

There is large interindividual variability in the efficacy and toxicity of drugs, which may be mainly caused by differences in the drug pharmacokinetics. The pharmacokinetics can be determined by ADME (absorption, distribution, metabolism, and excretion), especially drug metabolism. Drug metabolism consists of the phase I reactions (oxidation, reduction, and hydrolysis) and phase II reaction (conjugation), which occur predominantly in the liver. Therefore, the liver is the essential organ to determine the drug pharmacokinetics. One of the phase I enzymes, cytochrome P450 (P450, CYP), plays a central role in drug metabolism. P450 can metabolize various compounds including xenobiotic and endogenous compounds (Nelson et al., 1996). In addition, bioactivation leading to toxicity can sometimes be initiated by some P450s. Recently, the phase II reaction has been well studied since a parent drug and/or phase I metabolites are frequently excreted after conjugation. Furthermore, several drug-metabolizing enzymes have been shown to be polymorphic (Ingelman-Sundberg, 2002; Miners et al., 2002) indicating that genetic polymorphism can affect differences in the drug pharmacokinetics.

The mechanism of drug interactions can be often explained by the induction and inhibition of drug metabolizing enzymes. Serious drug interactions involving P450 have been reported (Dresser et al., 2000; Niemi et al., 2003). The QT prolongation caused by the inhibition of CYP3A4 by a coadministered drug resulted in the withdrawal of terfenadine and cisapride from the market. The prediction of adverse drug reactions is essential in drug development.

There are many reports regarding drug interactions and toxicity of various drugs, although the drugs were approved only after their safety was thought to have been confirmed in preclinical and clinical studies. In the preclinical stage, the pharmacokinetics of a drug candidate are investigated using human-derived sources or experimental animals. The results from experimental animals and in vitro studies sometimes wrongly predict the human pharmacokinetics and toxicity. Many researchers have made much effort to overcome such difficulties including those caused by species differences. Nowadays, human liver microsomes and human hepatocytes in primary culture are recognized as better tools and are frequently used during drug development. Human liver microsomes can be stored for a few years without the loss of enzyme activities but cannot be used to evaluate the induction potencies. Human hepatocytes express all the drug metabolizing enzymes, but a novel technique for culture is needed to avoid decreas-

ing the enzyme activities. Such in vitro models have various advantages and limitations, as have been described previously (Gomez-Lechon et al., 2003; Rodrigues and Rushmore, 2002).

To develop an artificial human liver is one of the best approaches for predicting human pharmacokinetics and safety. An urokinase-type plasminogen activator (uPA)^{+/+}/severe combined immunodeficient transgenic mouse line, in which the liver could be replaced by 80–90% with human hepatocytes, was established in Japan (Tateno et al., 2004). In this review, basic researches concerning drug metabolism and drug interactions are summarized for the application of chimeric mice in drug development and toxicology.

2. Generation of chimeric mice with humanized liver

In 2001, chimeric mice with partially humanized liver were described by Dandri et al. (2001) and Mercer et al. (2001). In the former report, the livers in uPA/recombinant activation gene-2 mice could be repopulated with approximately 15% human hepatocytes (Dandri et al., 2001). In the latter, a portion of the liver in uPA^{+/+}/SCID mice was replaced with human hepatocytes (Mercer et al., 2001). Since they were investigating on hepatitis virus, their chimeric mice with low replacement might be sufficient to achieve their purposes. However, the livers of such chimeric mice are not suitable to investigate human ADME in mice in vivo. In 2004, Tateno et al. (2004) succeeded in generating chimeric uPA^{+/+}/SCID mice, in which the livers could be replaced by more than 90% with human hepatocytes. The uPA^{+/+}/SCID mice at 20–30 days after birth were injected with human hepatocytes through a small left-flank incision into the inferior splenic pole (Tateno et al., 2004). We transplanted 7.5×10^5 hepatocytes into the livers of the uPA^{+/+}/SCID mice, and monitored their growth by determining the concentration of hAlb in the host serum. After transplantation using hepatocytes from 9-month-old Caucasian male into 19 uPA^{+/+}/SCID mice, serum human albumin (hAlb) concentrations in 14 mice reached 5 mg/ml around 60 days post-transplantation (Fig. 1). All mice survived for up to 60 days post-transplantation. To identify human hepatocytes in the chimeric livers, liver sections of mice were subjected to human specific cytokeratin 8/18 (hCK8/18) immunostaining (Fig. 2). The anti-hCK8/18 antibodies reacted specifically with hepatocytes and bile duct cells in the human livers, but did not react with the livers of the host mice. The replacement index (RI) of the mouse liver that received the human hepa-

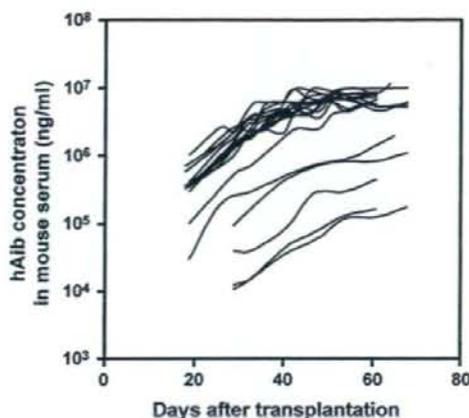


Fig. 1. Changes in the hAlb concentration in mouse serum. Nineteen uPA^{+/+}SCID mice were transplanted with 7.5×10^5 hepatocytes from 9-month-old Caucasian male. The hAlb concentrations increased in all the transplanted mice, and were >5 mg/ml in 14 mice among them.

cytes was determined as the ratio of the area occupied by hCK8/18-positive hepatocytes to the entire area examined in immunostained sections using all lobules. The RI values correlated well with the serum hAlb concentrations of chimeric mice ($y = 0.0413e^{0.0676x}$, where $y =$ hAlb concentration (mg/ml) and $x =$ RI (%), $r = 0.92$). The correlation formula suggests that mice with >5 mg/ml hAlb concentration should have RI > 70%. Chimeric mice more than 11 weeks old would be used for experiments because the hAlb concentration will have reached a plateau. This chimeric mouse line has many advantages. By measurement of the hAlb concentration, it is easy to estimate the degree of replacement by human hepatocytes. Hepatocytes from various donors and cryopreserved hepatocytes could be used to generate such chimeric mice. Therefore, interindividual variabilities of pharmacokinetics and toxicity can be evaluated by the chimeric mice with hepatocytes from different donors.

Table 1
Correlation between enzyme activities and hAlb concentrations in chimeric mice with humanized liver

Isoform	Enzyme activity	Correlation (<i>r</i>)	
		Donor A	Donor B
CYP2C8	PTXOH	0.93*	0.69
CYP2C9	DICOH	0.98**	0.93**
CYP2D6	DBOH	0.97**	0.72
CYP3A4	DEXOH	0.97**	0.87*
UGT2B7	Morphine 6-glucuronosyltransferase activity	0.93*	0.93***
SULT1A1	Troglitazone sulfotransferase activity	0.82	0.71*
SULT1E1	Estrone 3-sulfotransferase activity	0.90*	0.89**

Donor A chimeric mice ($n = 5$); donor B chimeric mice ($n = 7$). Donor A, 12-year-old Japanese male; donor B, 9-month-old Caucasian male. * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. PTXOH: paclitaxel 6 α -hydroxylase activity; DICOH: diclofenac 4'-hydroxylase activity; DBOH: debrisoquine 4'-hydroxylase activity; DEXOH: dexamethasone 6-hydroxylase activity.

3. Drug metabolism in chimeric mice with humanized liver

3.1. Cytochrome P450

The most important drug metabolizing enzyme involving phase I reactions is P450. One of the major isoforms, CYP3A4, has been reported to be responsible for the metabolism of more than 50% of clinical drugs (Pelkonen et al., 1998). In chimeric mice, human CYP3A4 mRNA and human CYP3A4 protein could be detected in an hAlb concentration-dependent manner by real-time reverse-transcription polymerase chain reaction (RT-PCR) and Western blotting, respectively, which detected human CYP3A4 but not murine Cyp3a isoforms (Katoh et al., 2004). As shown in Table 1, dexamethasone 6-hydroxylase activity (DEXOH) catalyzed by human CYP3A4 was also significantly correlated with the hAlb concentrations. Dexamethasone is primarily metabolized to 6-hydroxydexamethasone

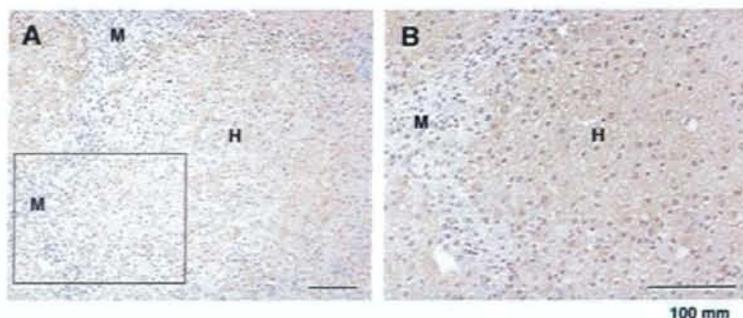


Fig. 2. Demonstration of mouse liver chimerism. (A) A uPA^{+/+}SCID mouse was transplanted with 7.5×10^5 hepatocytes from 9-month-old Caucasian male, and killed at 68 days post-transplantation. Frozen liver sections were prepared from the chimeric mouse, and stained with anti-hCK8/18 antibodies. Positive (brown colored) and negative (blue colored) regions showed human (H) and mouse (M) hepatocyte-occupied areas, respectively. RI determined as described in the text was 93%. (B) The region enclosed by the square in A is magnified and shown in B. Bar, 100 μ m. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

in humans but to 6-hydroxy-9 α -fluoro-androsta-14-diene-11 β -hydroxy-16 α -methyl-3,17-dione in mice (Tomlinson et al., 1997). DEXOH in liver microsomes from the donor A chimeric mouse with an RI of 90% showed almost the same activity as those from the donor A (Katoh et al., 2004) indicating that the liver of the chimeric mice possessed CYP3A4 capacity similar to that of the donor.

CYP2C9 is responsible for the hydroxylation of many non-steroidal anti-inflammatory drugs such as diclofenac. CYP2C8 and CYP2D6 are involved in the hydroxylation of the anticancer drug paclitaxel and many antipsychotropic drugs, respectively. In the chimeric mice, human specific activities such as diclofenac 4'-hydroxylase activity (DICOH) by CYP2C9, paclitaxel 6 α -hydroxylase activity (PTXOH) by CYP2C8, and debrisoquine 4'-hydroxylase activity (DBOH) by CYP2D6 could be detected (Katoh et al., 2004). DICOH, PTXOH, and DBOH as well as DEXOH correlated with the hAlb concentrations (Table 1) and the chimeric mouse with the highest hAlb concentration exhibited similar enzyme activities to those of the donor. Therefore, the liver of the chimeric mice could reflect the phenotype of the donor. In our previous report (Katoh et al., 2004), the other four human P450 isoforms, CYP1A2, CYP2A6, CYP2C19, and CYP3A5, could be detected at the mRNA, protein and/or enzyme activity level. In the report by Nishimura et al. (2005b), the mRNA of 20 human P450 isoforms could be detected in the liver of the chimeric mice. It is surmised that all P450 isoforms expressed in the donor can be expressed in the liver of the chimeric mice. The chimeric mice have much fewer activities of mouse P450s and high activities of human P450s because uPA^{+/+}/SCID mice causes severe liver failure in mouse hepatocytes and the proliferation of human hepatocytes is needed for survival. That is one of the advantages as compared to transgenic mice with human P450s, which usually express only one human P450 isoform with many mouse P450s retaining normal activities.

3.2. Other phase I enzymes

Nishimura et al. (2005b) investigated the hepatic mRNA expression of human phase I enzymes in chimeric mice by TaqMan real-time RT-PCR. The mRNA of 35 phase I enzymes except for P450s was determined. Carboxylesterase, epoxide hydrolase, and alcohol dehydrogenase could be detected at the mRNA level. Based on the results from P450s, if the mRNA is expressed, human drug metabolizing enzymes would exhibit almost the same potency in the chimeric mouse liver. It was assumed that all of the phase I enzymes expressed in the donor could be expressed with their activities intact in the livers of the chimeric mice.

3.3. Phase II enzymes

The major hepatic phase II enzymes in humans are UDP-glucuronosyltransferase (UGT), sulfotransferase (SULT), *N*-acetyltransferase (NAT), and glutathione-S-transferase (GST). The contribution of phase II conjugation to the clearance of a drug was estimated to be approximately 30% or higher in

humans (Bjornsson et al., 2003). In recent drug development, the relative involvement of phase II enzymes in the metabolic clearance of drugs is greater due to preselection concerning P450 metabolism in the early preclinical stage (Fisher et al., 2001). Taken together, attention needs to be paid to the interactions via UGT. Hyperbilirubinemia caused by the inhibition of UGT1A1 has been reported (Zucker et al., 2001), since bilirubin glucuronosyltransferase activity is catalyzed by UGT1A1.

The mRNA and protein of major UGT isoforms could be detected in the liver of chimeric mice (Katoh et al., 2005b). A specific human UGT2B7 activity, morphine 6-glucuronosyltransferase activity, correlated with the hAlb concentration as shown in Table 1. As in the case of phase I enzymes, human UGTs are also expressed in the chimeric mice and possess enzyme activities. Generally, GST plays an important role in the detoxification of various xenobiotics and reactive metabolites. For applying the chimeric mice to toxicological studies, the expression of human GST needed to be evaluated. The mRNA of human GSTs could be detected in the chimeric mice indicating that human GST may also have the potency of conjugation. In terms of SULT and NAT, since mRNA, protein, and/or enzyme activities could be detected (Katoh et al., 2005b), human phase II enzyme can also be expressed in the chimeric mice. That the livers of the chimeric mice expressed both human phase I and phase II enzymes is of great value because a series of human drug metabolisms in the liver can be investigated.

3.4. In vivo drug metabolism

In vivo experiments of chimeric mice would be more appropriate for the evaluation of human pharmacokinetics and toxicity. Therefore, we examined the in vivo drug metabolism catalyzed by human CYP2D6 in the chimeric mice. CYP2D6 function has been reported to exhibit large species differences, but is responsible for the metabolism of one quarter of the known clinical drugs (Ingelman-Sundberg, 2005). In addition, genetic polymorphism of human CYP2D6 is known to exist and many mutant alleles have been reported (<http://www.cypalleles.ki.se/cyp2d6.htm>). The prediction of the human pharmacokinetics of a CYP2D6 substrate is important. A typical CYP2D6 substrate, debrisoquine, is mainly metabolized to 4'-hydroxydebrisoquine (4-OH debrisoquine) in humans but only slightly in mice (Masubuchi et al., 1997). In our previous study (Katoh et al., 2007), the donor B chimeric mice were used for investigating the in vivo pharmacokinetics of debrisoquine. After oral administration of debrisoquine, the C_{max} and AUC values of 4-OH debrisoquine in the chimeric mice were significantly higher than those in the control uPA^{-/-}/SCID mice (Table 2, Katoh et al., 2007). The in vitro K_m value of DBOH in the chimeric mice was similar to that in humans (62–107 μ M, Nakajima et al., 2002) but was different from that in uPA^{-/-}/SCID mice (213 μ M) suggesting that the chimeric mice could reliably shown to exhibit a humanized profile of drug metabolism in both in vivo and in vitro studies. Thus the chimeric mice could become a useful model for evaluating the biotransformation of drugs by human drug metabolizing enzymes.

Table 2
Pharmacokinetic and kinetic parameters of 4-OH debrisoquine in chimeric mice with humanized liver and uPA^{-/-}/SCID mice

	Pharmacokinetic parameter		Kinetic parameter
	C _{max} (nM)	AUC ₀₋₈ (nM·h)	K _m (μM)
Chimeric mouse	297.1 ± 37.7***	887 ± 90***	72 ± 8***
uPA ^{-/-} /SCID mouse	61.4 ± 9.0	279 ± 32	213 ± 12

In vivo study for pharmacokinetic analysis, debrisoquine was orally administered at 2.0 mg/kg. Data represent the mean ± S.E. (n = 8). The donor B chimeric mice with high hAlb concentrations (>5.0 mg/ml and RI > 70) were used. ***p < 0.001, compared with uPA^{-/-}/SCID mice.

3.5. Genetic polymorphism

The interindividual variability of the pharmacokinetics and toxicity is partly related to the genetic polymorphism of drug metabolizing enzymes (Eichelbaum et al., 2006; Nagar and Remmel, 2006). Pharmacogenetics has become important in the developing concept of personalized medicine. FDA has approved genetic testing for mutations in the CYP2C19, CYP2D6, and UGT1A1 genes. In the case of CYP2D6, the CYP2D6 polymorphism might affect 3,4-methylenedioxymethamphetamine cytotoxicity (Carmo et al., 2006) and is associated with the occurrence of adverse effects of some antidepressants (Rau et al., 2004). The necessary dose adjustments of antidepressants according to the CYP2D6 genotype have been summarized (Kirchheiner et al., 2004) and will facilitate the selection of a drug and its dosage. However, the necessary dose adjustment and the effects on drug safety by many variants of other drug metabolizing enzymes remain unclear.

Genetic polymorphism of the CYP2A6 gene has been suggested to be related to the risk of lung cancer because CYP2A6 has a major impact on nicotine clearance (Nakajima and Yokoi, 2005). Bosch et al. (2007) hypothesized that the lethal toxicity of an anticancer drug, UFT (uracil/tegafur), is probably caused by a decrease in tegafur metabolism due to CYP2A6 polymorphism. The donor A was homozygous for CYP2A6*4 alleles, in which the whole CYP2A6 gene is deleted (Katoh et al., 2004). Human CYP2A6 protein and coumarin 7-hydroxylase activity catalyzed by CYP2A6 could not be detected in the liver of the donor A chimeric mouse with a high hAlb concentration (13.7 mg/ml; RI = 90), whereas they could be detected in that of the donor B chimeric mouse with a high hAlb concentration (7.7 mg/ml; RI = 90). The donor A chimeric mice were genotyped as CYP2A6*4/CYP2A6*4 using genomic DNA extracted from the liver suggesting that the chimeric mice have the same genotype and phenotype as the donor. With regard to other P450 isoforms, CYP2C19 and CYP3A5, the genotype of the chimeric mouse was the same as the donor.

NAT2 carries out the acetylation of arylamines and arylhydrazines including environmental carcinogens. According to the isoniazid metabolism, individuals have been classified as slow and rapid acetylator phenotypes. NAT2 genetic polymorphism is known to be involved in such variations (Meyer and Zanger, 1997). The frequency of the main adverse reaction of isoniazid, peripheral neuropathy, in the slow acetylator phenotype was higher than that in the rapid acetylator phenotype (Hughes et al., 1954). The donor B and the donor B chimeric mice were genotyped as NAT2*6/NAT2*13, which was classified

as slow acetylator (Cascorbi et al., 1995). Sulfamethazine N-acetyltransferase activity is a human specific activity catalyzed by human NAT2. Such activity in the donor B chimeric mice was lower than that in the donor A chimeric mice (Katoh et al., 2005b), which was consistent with the result of the genotyping.

Troglitazone is a thiazolidinedione antidiabetic agent that was withdrawn from the market in 2000 due to its association with idiosyncratic hepatotoxicity. A strong correlation between transaminase elevation and the combined GSTM1 and GSTT1 null genotype was observed (Watanabe et al., 2003). The susceptibility to tacrine hepatotoxicity was increased in individuals with these null genotypes (Simon et al., 2000). GSTM1 and GSTT1 are expressed in the liver in humans. Human GSTM1 mRNA in the liver of the donor B chimeric mice could not be detected by TaqMan real-time RT-PCR suggesting that the GSTM1 gene was deleted in donor B. Chimeric mice generated by a donor with GSTM1 and GSTT1 null genotypes may become a useful tool for predicting such hepatotoxicity.

If a donor with a poor metabolizer phenotype is used for generating the chimeric mice, the influence of genetic polymorphism on the pharmacokinetics and toxicity could be investigated. It is a great advantage that the chimeric mice can retain both the genotype and phenotype of the donor for personalized medicine and toxicological studies.

4. Induction of P450 in chimeric mice with humanized liver

Enzyme induction of a drug-metabolizing enzyme is a long-term consequence of chemical exposure and leads to an elevation of the enzyme activity (Lin and Lu, 2001). Drug interactions caused by the induction of P450, especially CYP3A4, are sometimes a serious problem because the induction may result in changes in the efficacy and toxicity of a drug (Pascucci et al., 2003; Niemi et al., 2003). Many CYP3A4 inducers are used as drugs in clinical practice and they can induce CYP3A4 at clinically used doses (Pascucci et al., 2003). By the coadministration of a typical CYP3A4 inducer, rifampicin, the pharmacokinetic of other drugs is sometimes changed significantly (Niemi et al., 2003). Moreover, P450 induction by herbal medicines including St John's wort could become a serious problem depending on the increased use of herbal medicines (Henderson et al., 2002). Therefore, the screening of drug candidates for induction potencies regarding drug-metabolizing enzymes is important in preclinical studies.

Using a primary culture of cryopreserved chimeric mouse hepatocytes, Nishimura et al. (2005a) investigated the induc-

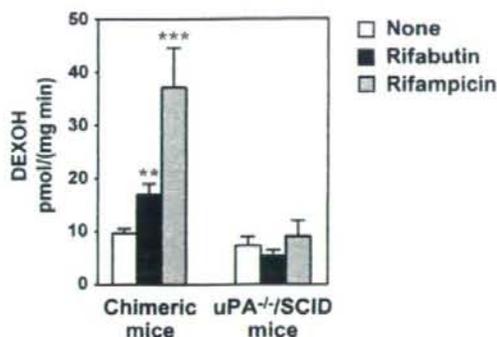


Fig. 3. Induction of DEXOH in rifabutin- or rifampicin-treated donor B chimeric mice with humanized liver. Rifabutin and rifampicin were intraperitoneally administered daily for 4 days at 50 mg/(kg day). Data represent the mean \pm S.D. ($n \geq 3$). ** $p < 0.01$, *** $p < 0.001$ compared with none.

tion of human CYP3A4 mRNA by TaqMan real-time RT-PCR. They reported that CYP3A4 mRNA was significantly increased 2.2–8.4-fold by the treatment with 50 μ M rifampicin for 24 h, which was a similar increase to that shown in fresh and cryopreserved human hepatocytes. After intraperitoneal treatment with rifampicin at 50 mg/(kg day) for 4 days, DEXOH had significantly increased by 4.1-fold on average in liver microsomes from the chimeric mice (Fig. 3, Katoh et al., 2005a). As mentioned above, since DEXOH is a human specific activity for CYP3A4, the increase of DEXOH would indicate that human CYP3A4 in the chimeric mice has in vivo induction potencies. Iwasaki et al. (2005) also determined the changed excretion of 6-hydroxydexamethasone after treatment with rifampicin in chimeric mice. After 4-day treatment with rifampicin (50 mg/kg, intraperitoneally), dexamethasone at 10 mg/kg was administered subcutaneously in chimeric mice and then the urinary excretion of 6-hydroxydexamethasone was measured. The excretion of 6-hydroxydexamethasone was increased 1.9–3.3-fold suggesting that the chimeric mice could be applied for in vivo and in vitro induction studies.

Rifabutin, which is a specific inducer of human CYP3A4 but not mouse Cyp3a, was used to evaluate the induction of human CYP3A4 in chimeric mice (Katoh et al., 2005c). DEXOH was increased in the liver microsomes from rifabutin-treated chimeric mice (Fig. 3, Katoh et al., 2005c). The induction potencies of rifampicin have been reported to be stronger than those of rifabutin (Perucca et al., 1988; Li et al., 1997), which is consistent with our result. The degree of induction can be evaluated using the chimeric mice.

Other inducible P450 isoforms, CYP1A1 and CYP1A2, were also examined for the induction potency in the chimeric mice. CYP1A1 and CYP1A2 play critical roles in the metabolic activation of many carcinogens. CYP1A induction has long been studied concerning its association with the toxicity of carcinogens and the risk of cancer (Ma and Lu, 2007). In the chimeric mice, the mRNA and protein of both CYP1A1 and CYP1A2 were induced by the treatment with typical inducers, β -naphthoflavone or 3-methylcholanthrene, in vitro and in vivo studies (Katoh et al., 2005a; Nishimura et al., 2005b; Yoshitsugu

et al., 2006). Although the human specific CYP1A1/CYP1A2 activities could not be evaluated, these activities in the chimeric mice might be induced as in the case of human CYP3A4.

For induction studies, it is believed that human hepatocytes are better than any other models, although human hepatocytes lose the enzyme activity very quickly. Because this chimeric mouse line could overcome such difficulties, it could become a better tool for induction studies in humans.

5. Inhibition of P450 in chimeric mice with humanized liver

Enzyme inhibition is an acute decrease of metabolism by a co-administered drug or a time-dependent decrease in the amount of an enzyme by several factors (Pelkonen et al., 1998). Drug interactions are often caused by the inhibition of P450 activities (Dresser et al., 2000). The prediction of human pharmacokinetic parameters from in vitro technologies has progressed (Houston and Galetin, 2003) but quantitative extrapolations from in vitro to human in vivo and from experimental animals to humans are still difficult. It would be a great advantage if drug interactions in humans could be predicted using this chimeric mouse line.

The P450 inhibition by a coadministered drug is thought to be very critical when a patient requires long-term treatment with it. Some CYP2D6 inhibitors such as antidepressants are prescribed for long periods of time. The inhibition of CYP2D6 enzyme activity by quinidine and selective serotonin reuptake inhibitors is well known in clinical practice (Caporaso and Shaw, 1991; Hemeryck and Belpaire, 2002). Therefore, we focused on CYP2D6 inhibition and the drug interactions between debrisoquine and quinidine were investigated in chimeric mice in vivo (Katoh et al., 2007). Quinidine, a frequently prescribed antiarrhythmic agent, is a specific inhibitor of human CYP2D6 but not mouse Cyp2d indicating that the specific inhibition of human CYP2D6 could be determined in this experiment. After 3-day treatment with quinidine, the AUC and C_{max} values of 4-OH debrisoquine were significantly decreased in the chimeric mice, but such values did not change in the control uPA^{-/-}/SCID mice (Fig. 4, Katoh et al., 2007).

An in vitro inhibition study using liver microsomes also demonstrated the human specific inhibition. The K_i value of quinidine on DBOH in the chimeric mice (0.049 μ M) was significantly lower than that in the control uPA^{-/-}/SCID mice (29 μ M, $p < 0.001$ compared with the chimeric mice, Katoh et al., 2007). Therefore, the specific inhibition of human CYP2D6 highly expressed in the chimeric mice suggested that human drug interactions could occur in the chimeric mice. Given the possibility of using chimeric mice for induction and inhibition studies, they could be a useful in vivo tool to predict drug interactions and toxicity.

6. In vivo excretion in chimeric mice with humanized liver

A drug is mostly eliminated by biliary and urinary excretion. To elucidate the excretion of a drug as well as the metabolism is essential for understanding the pharmacokinetics and toxic-

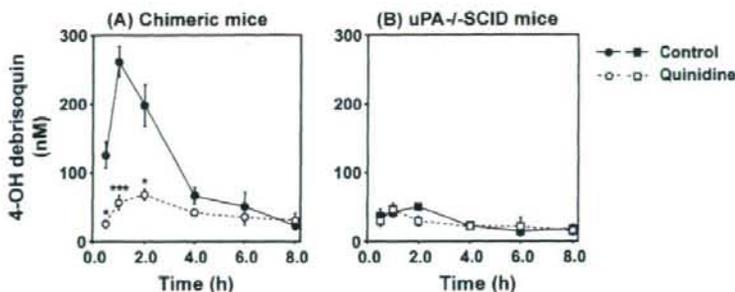


Fig. 4. Effect of quinidine treatment on serum concentration of 4-OH debrisoquin in donor B chimeric mice (A) and the control uPA^{-/-}/SCID mice (B). After treatment with quinidine (100 mg/kg day) for 3 days, debrisoquin was orally administered at 2.0 mg/kg. Data represent the mean \pm S.E. ($n=4$). Open and closed symbols represent the values with or without quinidine treatment, respectively. * $p < 0.05$, *** $p < 0.001$ compared with control.

Table 3
Cefmetazole excretion in chimeric mice with humanized liver

	<i>n</i>	Excretion (% of the dose)	
		Urine	Feces
Chimeric mouse	5	81.0 \pm 9.5***	5.9 \pm 4.7***
uPA ^{-/-} /SCID mouse	7	23.7 \pm 8.8	59.4 \pm 11.0

Cefmetazole was intraperitoneally administered at 25.0 mg/kg. Urine and feces samples were collected during 24 h after the cefmetazole administration. Data represent the mean \pm S.D. *** $p < 0.001$, compared with uPA^{-/-}/SCID mice.

ity. Species differences in the excretory pathway may make the extrapolation from experimental animals to humans difficult. In the case of an antibiotic agent, cefmetazole was mainly excreted in urine in humans (Ko et al., 1989) but in feces in rats and mice in an unchanged form (Murakawa et al., 1980; Okumura et al., 2007). Thus, cefmetazole was a better probe to investigate the humanization of the excretion pathway in chimeric mice. Following intraperitoneal administration, the 24-h cumulative recovery of cefmetazole in urine and feces was quantified in the chimeric mice (Okumura et al., 2007). As shown in Table 3, the main pathway was urinary excretion in chimeric mice whereas it was fecal excretion in control uPA^{-/-}/SCID mice indicating that the excretory profile was humanized by the replacement with human hepatocytes.

Many drug transporters have been clarified to be responsible for drug excretion. Species differences of drug transporters have been clarified (Katoh et al., 2006; Suzuyama et al., 2007). As shown by Nishimura et al. (2005b) and us (Katoh et al., 2005c; Okumura et al., 2007), human drug transporters were expressed in the liver of the chimeric mice. The humanization of drug transporters may be also one of the determinants of the alteration of the cefmetazole excretory pathway. Although further study is needed, this chimeric mouse line might be a suitable model for predicting the humanized type of excretion. Since genetic polymorphism of drug transporters exists, the changed hepatic uptake and excretion can also be studied using the chimeric mice.

7. Conclusions

The chimeric mice have been shown to exhibit a humanized profile of drug metabolism, induction and inhibition of drug

metabolizing enzymes, and excretion in *in vivo* studies. This chimeric mouse line should be a promising model for evaluating the *in vivo* pharmacokinetics in humans. In recent drug development, adverse pharmacokinetic and bioavailability have reduced as a cause of attrition although toxicology has increased (Kola and Landis, 2004). Drug-induced hepatotoxicity is one of the major problems in drug development and clinical practice. Recently, Yamamoto et al. (2007) demonstrated the usefulness of the chimeric mice in a toxicological study that evaluated acetaminophen. This chimeric mouse model could therefore contribute to estimate human specific toxicity.

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References

- Bjornsson, T.D., Callaghan, J.T., Einolf, H.J., Fischer, V., Gan, L., Grimm, S., Kao, J., King, S.P., Miwa, G., Ni, L., Kumar, G., McLeod, J., Obach, R.S., Roberts, S., Roe, A., Shah, A., Snikeris, F., Sullivan, J.T., Tweedie, D., Vega, M., Walsh, J., Wrighton, S.A., 2003. The conduct of *in vitro* and *in vivo* drug-drug interaction studies: a Pharmaceutical Research and Manufacturers of America (PhRMA) perspective. *Drug Metab. Dispos.* 31, 815–832.
- Bosch, T.M., Meijerman, I., Beijnen, J.H., van Thiel, S.W., Vlasveld, L.T., 2007. Lethal toxicity of uracil/tegafur in the treatment of sigmoid carcinoma. *Ann. Oncol.* 18, 607–608.
- Caporaso, N.E., Shaw, G.L., 1991. Clinical implications of the competitive inhibition of the debrisoquin-metabolizing isozyme by quinidine. *Arch. Intern. Med.* 151, 1985–1992.
- Carmo, H., Brulport, M., Hermes, M., Oesch, F., Silva, R., Ferreira, L.M., Branco, P.S., Boer, D., Remião, F., Carvalho, F., Schön, M.R., Krebsfaenger, N., Doehmer, J., Bastos, Mde, L., Hengstler, J.G., 2006. Influence of CYP2D6 polymorphism on 3,4-methylenedioxymethamphetamine ('Ecstasy') cytotoxicity. *Pharmacogenet. Genomics* 16, 789–799.
- Cascorbi, I., Drakoulis, N., Brockmöller, J., Maurer, A., Sperling, K., Roots, L., 1995. Arylamine-N-acetyltransferase (NAT2) mutations and their allelic linkage in unrelated Caucasian individuals: correlation with phenotypic activity. *Am. J. Hum. Genet.* 57, 581–592.
- Dandri, M., Burda, M.R., Torok, E., Pollok, J.M., Iwanska, A., Sommer, G., Rogiers, X., Rogler, C.E., Gupta, S., Will, H., Greten, H., Petersen, J., 2001.

- Repopulation of mouse liver with human hepatocytes and in vivo infection with hepatitis B virus. *Hepatology* 33, 981–988.
- Dresser, G.K., Spence, J.D., Bailey, D.G., 2000. Pharmacokinetic–pharmacodynamic consequences and clinical relevance of cytochrome P450 3A4 inhibition. *Clin. Pharmacokinet.* 38, 41–57.
- Eichelbaum, M., Ingelman-Sundberg, M., Evans, W.E., 2006. Pharmacogenomics and individualized drug therapy. *Annu. Rev. Med.* 57, 119–137.
- Fisher, M.B., Paine, M.F., Strelevitz, T.J., Wrighton, S.A., 2001. The role of hepatic and extrahepatic UDP-glucuronosyltransferases in human drug metabolism. *Drug Metab. Rev.* 33, 273–297.
- Gomez-Lechon, M.J., Donato, M.T., Castell, J.V., Jover, R., 2003. Human hepatocytes as a tool for studying toxicity and drug metabolism. *Curr. Drug Metab.* 4, 292–312.
- Hemeryck, A., Belpaire, F.M., 2002. Selective serotonin reuptake inhibitors and cytochrome P-450 mediated drug–drug interactions: an update. *Curr. Drug Metab.* 3, 13–37.
- Henderson, L., Yue, Q.Y., Bergquist, C., Gerden, B., Arlett, P., 2002. St John's wort (*Hypericum perforatum*): drug interactions and clinical outcomes. *Br. J. Clin. Pharmacol.* 54, 349–356.
- Houston, J.B., Galetin, A., 2003. Progress towards prediction of human pharmacokinetic parameters from in vitro technologies. *Drug Metab. Rev.* 35, 393–415.
- Hughes, H.B., Biehl, J.P., Jones, A.P., Schmidt, L.H., 1954. Metabolism of isoniazid in man as related to the occurrence of peripheral neuritis. *Am. Rev. Tuberc.* 70, 266–273.
- Ingelman-Sundberg, M., 2002. Polymorphism of cytochrome P450 and xenobiotic toxicity. *Toxicology* 181/182, 447–452.
- Ingelman-Sundberg, M., 2005. Genetic polymorphisms of cytochrome P450 2D6 (CYP2D6): clinical consequences, evolutionary aspects and functional diversity. *Pharmacogenomics* 5, 6–13.
- Iwasaki, K., Yamato, Y., Emoto, C., Kuroda, M., Soeno, Y., Katoh, M., Horie, T., Yokoi, T., Yoshizato, K., 2005. Induction of cytochrome P450 3A4/5 by rifamycins in chimeric mice with humanized liver. *Drug Metab. Rev.* 37 (Suppl. 2), 143.
- Katoh, M., Matsui, T., Nakajima, M., Tateno, C., Kataoka, M., Soeno, Y., Horie, T., Iwasaki, K., Yoshizato, K., Yokoi, T., 2004. Expression of human cytochromes P450 in chimeric mice with humanized liver. *Drug Metab. Dispos.* 32, 1402–1410.
- Katoh, M., Matsui, T., Nakajima, M., Tateno, C., Soeno, Y., Horie, T., Iwasaki, K., Yoshizato, K., Yokoi, T., 2005a. In vivo induction of human cytochrome P450 enzymes expressed in chimeric mice with humanized liver. *Drug Metab. Dispos.* 33, 754–763.
- Katoh, M., Matsui, T., Okumura, H., Nakajima, M., Nishimura, M., Naito, S., Tateno, C., Yoshizato, K., Yokoi, T., 2005b. Expression of human phase II enzymes in chimeric mice with humanized liver. *Drug Metab. Dispos.* 33, 1333–1340.
- Katoh, M., Watanabe, M., Tabata, T., Sato, Y., Nakajima, M., Nishimura, M., Naito, S., Tateno, C., Iwasaki, K., Yoshizato, K., Yokoi, T., 2005c. In vivo induction of human cytochrome P450 3A4 by rifabutin in chimeric mice with humanized liver. *Xenobiotica* 35, 863–875.
- Katoh, M., Suzuyama, N., Takeuchi, T., Yoshitomi, S., Asahi, S., Yokoi, T., 2006. Kinetic analyses for species differences in P-glycoprotein-mediated drug transport. *J. Pharm. Sci.* 95, 2673–2683.
- Katoh, M., Sawada, T., Soeno, Y., Nakajima, M., Tateno, C., Yoshizato, K., Yokoi, T., 2007. In vivo drug metabolism model for human cytochrome P450 enzyme using chimeric mice with humanized liver. *J. Pharm. Sci.* 96, 428–437.
- Kirchheiner, J., Nickchen, K., Bauer, M., Wong, M.L., Licinio, J., Roots, I., Brockmoller, J., 2004. Pharmacogenetics of antidepressants and antipsychotics: the contribution of allelic variations to the phenotype of drug response. *Mol. Psychiatry* 9, 442–473.
- Ko, H., Novak, E., Peters, G.R., Bothwell, W.M., Hosley, J.D., Closson, S.K., Adams, W.J., 1989. Pharmacokinetics of single-dose cefmetazole following intramuscular administration of cefmetazole sodium to healthy male volunteers. *Antimicrob. Agents Chemother.* 33, 508–512.
- Kola, I., Landis, J., 2004. Can the pharmaceutical industry reduce attrition rates? *Nat. Rev. Drug Discov.* 3, 711–715.
- Li, A.P., Reith, M.K., Rasmussen, A., Gorski, J.C., Hall, S.D., Xu, L., Kaminski, D.L., Cheng, L.K., 1997. Primary human hepatocytes as a tool for the evaluation of structure–activity relationship in cytochrome P450 induction potential of xenobiotics: evaluation of rifampin, rifapentine and rifabutin. *Chem. Biol. Interact.* 107, 17–30.
- Lin, J.H., Lu, A.Y., 2001. Interindividual variability in inhibition and induction of cytochrome P450 enzymes. *Annu. Rev. Pharmacol. Toxicol.* 41, 535–567.
- Ma, Q., Lu, A.Y., 2007. CYP1A induction and human risk assessment: an evolving tale of in vitro and in vivo studies. *Drug Metab. Dispos.* 35, 1009–1016.
- Masubuchi, Y., Iwasa, T., Hosokawa, S., Suzuki, T., Horie, T., Imaoka, S., Funae, Y., Narimatsu, S., 1997. Selective deficiency of debrisoquine 4-hydroxylase activity in mouse liver microsomes. *J. Pharmacol. Exp. Ther.* 282, 1435–1441.
- Mercer, D.F., Schiller, D.E., Elliott, J.F., Douglas, D.N., Hao, C., Rinfret, A., Addison, W.R., Fischer, K.P., Churchill, T.A., Lakey, J.R., Tyrrell, D.L., Kneteman, N.M., 2001. Hepatitis C virus replication in mice with chimeric human livers. *Nat. Med.* 7, 27–933.
- Meyer, U.A., Zanger, U.M., 1997. Molecular mechanisms of genetic polymorphisms of drug metabolism. *Annu. Rev. Pharmacol. Toxicol.* 37, 269–296.
- Miners, J.O., McKinnon, R.A., Mackenzie, P.I., 2002. Genetic polymorphisms of UDP-glucuronosyltransferases and their functional significance. *Toxicology* 181/182, 453–456.
- Murakawa, T., Sakamoto, H., Fukada, S., Nakamoto, S., Hirose, T., Itoh, N., Nishida, M., 1980. Pharmacokinetics of ceftiozime in animals after parenteral dosing. *Antimicrob. Agents Chemother.* 17, 157–164.
- Nagar, S., Rimmel, R.P., 2006. Uridine diphosphoglucuronosyltransferase pharmacogenetics and cancer. *Oncogene* 25, 1659–1672.
- Nakajima, M., Yokoi, T., 2005. Interindividual variability in nicotine metabolism: C-oxidation and glucuronidation. *Drug Metab. Pharmacokinet.* 20, 227–235.
- Nakajima, M., Tane, K., Nakamura, S., Shimada, N., Yamazaki, H., Yokoi, T., 2002. Evaluation of approach to predict the contribution of multiple cytochrome P450s in drug metabolism using relative activity factor: effects of the differences in expression levels of NADPH-cytochrome P450 reductase and cytochrome b₅ in the expression system and the differences in the marker activities. *J. Pharm. Sci.* 91, 952–963.
- Nelson, D.R., Koymans, L., Kamataki, T., Stegeman, J.J., Feyereisen, R., Waxman, D.J., Waterman, M.R., Gotoh, O., Coon, M.J., Estabrook, R.W., Gunsalus, I.C., Nebert, D.W., 1996. P450 superfamily: update on new sequences, gene mapping, accession numbers and nomenclature. *Pharmacogenetics* 6, 1–42.
- Niemi, M., Backman, J.T., Fromm, M.F., Neuvonen, P.J., Kivisto, K.T., 2003. Pharmacokinetic interactions with rifampicin: clinical relevance. *Clin. Pharmacokinet.* 42, 819–850.
- Nishimura, M., Yokoi, T., Tateno, C., Kataoka, M., Takahashi, E., Horie, T., Yoshizato, K., Naito, S., 2005a. Induction of human CYP1A2 and CYP3A4 in primary culture of hepatocytes from chimeric mice with humanized liver. *Drug Metab. Pharmacokinet.* 20, 121–126.
- Nishimura, M., Yoshitsugu, H., Yokoi, T., Tateno, C., Kataoka, M., Horie, T., Yoshizato, K., Naito, S., 2005b. Evaluation of mRNA expression of human drug-metabolizing enzymes and transporters in chimeric mouse with humanized liver. *Xenobiotica* 35, 877–890.
- Okumura, H., Katoh, M., Sawada, T., Nakajima, M., Soeno, Y., Yabuuchi, H., Ikeda, T., Tateno, C., Yoshizato, K., Yokoi, T., 2007. Humanization of excretory pathway in chimeric mice with humanized liver. *Toxicol. Sci.* 97, 533–538.
- Pascucci, J.M., Gerbal-Chaloin, S., Daujat, M., Drocourt, L., Pichard-Garcia, L., Vilarem, M.J., Maurel, P., Klieber, S., Torrelles, F., Bourrie, M., Guillou, F., Fabre, G., 2003. CYP gene induction by xenobiotics and drugs. In: Lee, J.S., Obach, R.S., Fisher, M.B. (Eds.), *Drug Metabolizing Enzymes*. Marcel Dekker, New York, pp. 337–374.
- Pelkonen, O., Mäenpää, J., Taavitsainen, P., Rautio, A., Raunio, H., 1998. Inhibition and induction of human cytochrome P450 (CYP) enzymes. *Xenobiotica* 28, 1203–1253.
- Perucca, E., Grimaldi, R., Frigo, G.M., Sardi, A., Mönig, H., Ohnhaus, E.E., 1988. Comparative effects of rifabutin and rifampicin on hepatic microsomal enzyme activity in normal subjects. *Eur. J. Clin. Pharmacol.* 34, 595–599.

- Rau, T., Wohlleben, G., Wuttke, H., Thuerauf, N., Lunkenheimer, J., Lanczik, M., Eschenhagen, T., 2004. CYP2D6 genotype: impact on adverse effects and nonresponse during treatment with antidepressants—a pilot study. *Clin. Pharmacol. Ther.* 5, 386–393.
- Rodrigues, A.D., Rushmore, T.H., 2002. Cytochrome P450 pharmacogenetics in drug development: in vitro studies and clinical consequences. *Curr. Drug Metab.* 3, 289–309.
- Simon, T., Becquemont, L., Mary-Krause, M., de Waziers, I., Beaune, P., Funck-Brentano, C., Jaillon, P., 2000. Combined glutathione-S-transferase M1 and T1 genetic polymorphism and tacrine hepatotoxicity. *Clin. Pharmacol. Ther.* 67, 432–437.
- Suzuyama, N., Katoh, M., Takeuchi, T., Yoshitomi, S., Higuchi, T., Asashi, S., Yokoi, T., 2007. Species differences of inhibitory effects on P-glycoprotein-mediated drug transport. *J. Pharm. Sci.* 96, 1609–1618.
- Tateno, C., Yoshizane, Y., Saito, N., Kataoka, M., Utoh, R., Yamasaki, C., Tachibana, A., Soeno, Y., Asahina, K., Hino, H., Asahara, T., Yokoi, T., Furukawa, T., Yoshizato, K., 2004. Near completely humanized liver in mice shows human-type metabolic responses to drugs. *Am. J. Pathol.* 165, 901–912.
- Tomlinson, E.S., Maggs, J.L., Park, B.K., Back, D.J., 1997. Dexamethasone metabolism in vitro: species differences. *J. Steroid. Biochem. Mol. Biol.* 62, 345–352.
- Watanabe, I., Tomita, A., Shimizu, M., Sugawara, M., Yasuno, H., Koishi, R., Takahashi, T., Miyoshi, K., Nakamura, K., Izumi, T., Matsushita, Y., Furukawa, H., Haruyama, H., Koga, T., 2003. A study to survey susceptible genetic factors responsible for troglitazone-associated hepatotoxicity in Japanese patients with type 2 diabetes mellitus. *Clin. Pharmacol. Ther.* 73, 435–455.
- Yamamoto, T., Tomizawa, K., Fujikawa, M., Sato, Y., Yamada, H., Hori, I., 2007. Evaluation of human hepatocyte chimeric mice as a model for toxicological investigation using panomic approaches—effect of acetaminophen on the expression profiles of proteins and endogenous metabolites in liver, plasma and urine. *J. Toxicol. Sci.* 32, 205–215.
- Yoshitsugu, H., Nishimura, M., Tateno, C., Kataoka, M., Takahashi, E., Soeno, Y., Yoshizato, K., Yokoi, T., Naito, S., 2006. Evaluation of human CYP1A2 and CYP3A4 mRNA expression in hepatocytes from chimeric mice with humanized liver. *Drug Metab. Pharmacokinet.* 21, 465–474.
- Zucker, S.D., Qin, X., Rouster, S.D., Yu, F., Green, R.M., Keshavan, P., Feinberg, J., Sherman, K.E., 2001. Mechanism of indinavir-induced hyperbilirubinemia. *Proc. Natl. Acad. Sci. U.S.A.* 98, 12671–12676.

Susceptibility of Chimeric Mice with Livers Repopulated by Serially Subcultured Human Hepatocytes to Hepatitis B Virus

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We previously identified a small population of replicative hepatocytes in long-term cultures of human adult parenchymal hepatocytes (PHs) at a frequency of 0.01%–0.09%. These hepatocytes were able to grow continuously through serial subcultures as colony-forming parenchymal hepatocytes (CFPHs). In the present study, we generated gene expression profiles for cultured CFPHs and found that they expressed cytokeratin 19, CD90 (Thy-1), and CD44, but not mature hepatocyte markers such as tryptophan-2,3-dioxygenase (TO) and glucose-6-phosphatase (G6P), confirming that these cells are hepatic progenitor-like cells. The cultured CFPHs were resistant to infection with human hepatitis B virus (HBV). To examine the growth and differentiation capacity of the cells *in vivo*, serially subcultured CFPHs were transplanted into the progeny of a cross between albumin promoter/enhancer-driven urokinase plasminogen activator-transgenic mice and severe combined immunodeficient (SCID) mice. The cells were grafted into the liver and were able to grow for at least 10 weeks, ultimately reaching a maximum occupancy rate of 27%. The CFPHs in the host liver expressed differentiation markers such as TO, G6P, and cytochrome P450 subtypes and could be infected with HBV. CFPH-chimeric mice with a relatively high replacement rate exhibited viremia and had high serum levels of hepatitis B surface antigen. **Conclusion:** Serially subcultured human hepatic progenitor-like cells from postnatal livers successfully repopulated injured livers and exhibited several phenotypes of mature hepatocytes, including susceptibility to HBV. *In vitro*-expanded CFPHs can be used to characterize the differentiation state of human hepatic progenitor-like cells. (HEPATOLOGY 2008;47:000-000.)

Abbreviations: 9MM, 9-month-old Caucasian male; 10YF, 10-year-old Caucasian female; 12YM, 12-year-old Asian male; 16YF, 16-year-old Asian female; AAT, α 1-antitrypsin; AFP, α -fetoprotein; ALB, albumin; BGP, biliary glycoprotein; BrdU, 5-bromo-2'-deoxyuridine; CFPH, colony-forming parenchymal hepatocyte; CK, cytokeratin; G6P, glucose-6-phosphatase; h, human; HBsAg, hepatitis B surface antigen; HBV, hepatitis B virus; CYP, cytochrome P450; m, mouse; MDR, multidrug resistance protein; MRP, multidrug resistance-associated protein; PH, parenchymal hepatocyte; RI, replacement index; RT-PCR, reverse-transcription polymerase chain reaction; SH, small hepatocyte; TO, tryptophan-2,3-dioxygenase; uPA, urokinase plasminogen activator.

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Studies using rodents with damaged livers have shown that parenchymal hepatocytes (PHs) have great growth potential. When mouse (*m*) hepatocytes were transplanted into the livers of albumin promoter/enhancer-driven urokinase plasminogen activator (uPA)-transgenic mice,¹ they engrafted and repopulated the host liver. Serial transplantation experiments using *m*-hepatocytes in mice with tyrosinemia showed their enormous growth capacity.² The replicative potential of rat hepatocytes has also been demonstrated by transplanting them into the partially hepatectomized liver of a retorsine-treated rat,³ and uPA-transgenic mice crossed with severely immunodeficient mice, such as severe combined immunodeficient (SCID)/beige mice,⁴ SCID mice,^{5,6} or recombination activation gene 2 knockout mice⁷ have been used to show the growth potential of human (*h*)-hepatocytes. When transplanted into uPA/SCID mice, PHs from a human juvenile male grew in the host liver to a level at which the proportion (replacement index) of the area of repopulated *h*-hepatocytes to the total number (host and donor) of hepatocytes reached 96% at 64 days posttransplantation.⁵ Such *h*-hepatocyte-chimeric mice have been used to study the pharmacological responses of *h*-hepatocytes⁵ and to investigate *h*-hepatitis viral infections.^{4,6-8}

In contrast, normal hepatocytes have limited replicative capacity *in vitro* and acquire an abnormal phenotype if they are cultured for extended periods.^{9,10} Studies on hepatocytes cultured in a newly devised medium (hepatocyte clonal growth medium^{11,12}) revealed a subpopulation of highly replicative PHs, known as small hepatocytes (SHs), in both rats¹² and humans.¹³ Their occupancy rate in *h*-liver ranged from 0.01% to 0.09% and was dependent on donor age.¹³ The *h*-SHs formed colonies and grew continuously through several subcultures, which led us to name them colony-forming PHs (CFPHs).¹³ Replication of the CFPHs was donor age-dependent up to passage 7 ($p = 7$),¹³ and the cells did not exhibit a normal hepatocytic phenotype. Instead, they exhibited the traits of hepatocytes or biliary cells depending on the culture conditions. In addition, the CFPHs were not susceptible to infection with hepatitis B virus (HBV) (unpublished data).

In this study, we generated gene expression profiles of CFPHs and transplanted serially subcultured CFPHs into homozygous uPA/SCID mice to examine their growth and differentiation capacity. Our results indicate that the cells were engrafted onto the liver parenchyma and repopulated the tissue, ultimately differentiating into mature hepatocytes. Importantly, the *in vitro*-propagated CFPHs became susceptible to infection with HBV. This study supports our previous suggestion that CFPHs from

h-postnatal liver are hepatic progenitor-like cells with the potential to assume a normal hepatocytic phenotype.¹³

Materials and Methods

***h*-Hepatocytes.** This study was performed with the approval of the Hiroshima Prefectural Institute of Industrial Science and Technology Ethics Board. PHs were isolated as described^{13,14} from livers donated by a 12-year-old Asian male (12YM) and a 16-year-old Asian female (16YF) according to the guidelines of the 1975 Declaration of Helsinki. Cryopreserved PHs from a 9-month-old Caucasian male (9MM) and a 10-year-old Caucasian female (10YF) were obtained from In Vitro Technologies (Baltimore, MD) and BD Biosciences (San Jose, CA), respectively.

Culture of CFPHs. Cryopreserved PHs from the 9MM, 12YM, and 16YF were thawed⁵ and serially subcultured to obtain *in vitro*-expanded CFPHs.¹³ Commercial 9MM PHs and freshly isolated 12YM and 16YF PHs were each subcultured to $p = 3$. The expanded cells were then cryopreserved, thawed upon use, and cultured on collagen-coated plates for 14-20 days as described.¹³

Flow Cytometry. We detached 12YM CFPHs ($p = 4$ or 5) from culture plates by treatment with 0.25% Trypsin-EDTA (Invitrogen, Carlsbad, CA), suspended, incubated on ice for 30 minutes with *m*-monoclonal antibodies against *h*Thy-1 (clone F15-42-1; Chemicon, Temecula, CA), and incubated with antibodies against *m*-immunoglobulin G Alexa-488 (Molecular Probes, Eugene, OR). We used *m*-immunoglobulin G₁ as a negative control. The cells were then analyzed and separated using a fluorescence-activated cell sorter (Becton Dickinson, Franklin Lakes, NJ) as reported.¹²

Transplantation of PHs and CFPHs. We detached 9MM and 12YM CFPHs ($p = 4$) from their culture plates and treated for 1 hour with DMEM containing 10% fetal bovine serum and 3 $\mu\text{g}/\text{mL}$ anti-*h*-integrin $\alpha 1$ monoclonal antibodies (clone FB12, Chemicon).¹⁵ This procedure improved engraftment of the CFPHs in uPA/SCID *m*-liver and reduced host mortality.

Transplantation of PHs and CFPHs was performed as described previously.⁵ Homozygous uPA/SCID mice were injected with 0.75×10^6 9MM and 12YM PHs or $0.75\text{-}1.0 \times 10^6$ *in vitro*-expanded 9MM and 12YM CFPHs into the inferior splenic pole. When necessary, 10 mM 5-bromo-2'-deoxyuridine (BrdU) (Sigma, St. Louis, MO) and 1.2 mM 5-fluoro-2'-deoxyuridine (Wako, Osaka, Japan) in saline were injected intraperitoneally into the mice at 10 $\mu\text{L}/\text{g}$ body weight 1 hour prior to death. The animals were treated according to the guidelines of our local committee on animal experiments.

Table 1. Summary of CFPH and PH Transplantation Experiments in uPA/SCID Mice

Group	Donor Cells	Time of Sacrifice (Weeks After Transplantation)	No. of Transplanted Mice	No. of Mice with Engraftment* [RE (%)]	RI† [Mean ± SD (n)]
A	12YM CFPHs (p = 4)	3	9	3 (33)	0.06-0.19% [0.14 ± 0.07% (n = 3)]
B	9MM CFPHs (p = 4)	3	6	4 (67)	0.03-0.05% [0.04 ± 0.01% (n = 4)]
C	9MM PHs	3	3	3 (100)	5.1-19.4% [6.4 ± 2.9% (n = 3)]
D	12YM CFPHs (p = 4)	9-10	27	14 (52)	0.2-27.0% [6.6 ± 8.3% (n = 14)]
E	9MM PHs	10-11	23‡	23 (100)‡	32.6-82.2% [57.4% (n = 2)]
F	12YM PHs	10	6	4 (67)	31.0-77.0% [62.3 ± 23.8% (n = 4)]
G§	12YM CFPHs (p = 4)	17-20	4	ND	ND

Abbreviation: ND, not determined.

*Number of mice whose livers were engrafted with transplanted PHs or CFPHs. The RE was determined via *h*ALB immunohistochemistry on sections prepared from 5 lobes of a liver.

†Ranges of RI of chimeric mice used in each group.

‡Data from Tateno et al.⁵

§Mice from group G were used for HBV infection studies.

We transplanted 9MM and 12YM CFPHs into 6 and 40 uPA/SCID mice, respectively. The mice were then killed 3, 9, or 10 weeks later, depending on the experimental purpose. In a previous report, we used 9MM and 12YM PHs as donor cells.⁵ In this study, we used some of the preserved livers from these mice for histological examinations and as sources of RNA for reverse-transcription polymerase chain reaction (RT-PCR) analysis. The mice used in our transplantation experiments were separated into 7 groups (A-G) as shown in Table 1, which includes the rates of engraftment and replacement indices (RIs) of the chimeric mice.

Blood samples (5 μ L) were collected periodically after transplantation from the tail veins of the hosts, and the level of *h*-albumin (ALB) in each was determined using a Human Albumin ELISA Quantitation Kit (Bethyl Laboratories, Montgomery, TX) to monitor the growth of the transplanted CFPHs.

RT-PCR. An RNeasy Tissue Kit (Qiagen, Valencia, CA) was used to isolate total RNA from freeze-thawed 9MM and 10YF PHs, cells of the *h*-hepatoma cell line HepG2, and 12YM and 16YF CFPHs (p = 4). RNA was also isolated with Isogen (Nippon Gene, Tokyo, Japan) from the livers of homozygous uPA/SCID mice and mice chimeric for 12YM PHs or 12YM CFPHs. Each RNA sample was treated with deoxyribonuclease (Takara Bio, Kyoto, Japan) and used as the template for RT-PCR. The RNA (1 μ g) was reverse-transcribed with random hexamers using PowerScript Reverse Transcriptase (Clontech, Kyoto, Japan). All reactions were performed with Ex Taq (Takara Bio). Semiquantitative PCR was performed to allow linear amplification of the targets. The following *h*-specific or *m* and *h* cross-reactive genes were subjected to RT-PCR under the conditions shown in Supplementary Table 1: ALB, α 1-antitrypsin (AAT), tryptophan-2,3-dioxygenase (TO), glucose-6-phosphatase (G6P),

α -fetoprotein (AFP), cytokeratin 19 (CK19), biliary glycoprotein (BGP), Thy-1, CD44, multidrug resistance protein 1 (MDR1), multidrug resistance-associated protein 1 (MRP1), MRP2, and glyceraldehyde-3-phosphate dehydrogenase.

In Situ Hybridization. Cryosections (7 μ m thick) were fixed with 4% paraformaldehyde, then incubated with 100 ng/mL proteinase K for 10 minutes at 37°C. The sections were then treated at 90°C for 6 minutes and hybridized for 2 hours at 37°C with biotinylated *h*-DNA probes (Dako, Glostrup, Denmark). The sections were also used to detect whole *h*-genomic DNA using the Gen-Point System (Dako) according to the manufacturer's instructions. Finally, they were stained with hematoxylin-eosin.

Immunohistochemistry and Histochemistry. Formalin-fixed livers were embedded in paraffin and sectioned 5 μ m thick. The sections were heated in a microwave oven for 5 minutes in Target Retrieval Solution (Dako), then placed at room temperature for 20 minutes. The livers used to generate frozen sections were embedded in OCT compound (Sakura Finechemicals, Tokyo, Japan), frozen in liquid nitrogen, and sectioned 5 μ m thick. The cultured cells were fixed in cold ethanol for 10 minutes. The primary antibodies and conditions used for immunohistochemistry are listed in Supplementary Table 2. For bright-field immunohistochemistry, the antibodies were visualized using a Vectastain ABC Kit (Vector Laboratories, Burlingame, CA) using DAB substrates. Fluorescence immunohistochemistry was performed using Alexa 488-conjugated or Alexa 594-conjugated secondary antibodies (Molecular Probes). The nuclei were stained with Hoechst 33258. Glycogens were visualized using a periodic acid-Schiff (PAS) staining kit (Muto Pure Chemicals, Tokyo, Japan). RIs were determined using

*h*ALB-immunostained sections of chimeric *m*-livers as reported previously.⁵

HBV Infection. We obtained *h*-serum containing high-titer HBV DNA (8.1 log₁₀ genome equivalents/mL serum) from an HBV genotype C carrier after obtaining informed consent. The serum was kept at -80°C until use. Four CFPH-chimeric mice were intravenously injected with 100 μL of the HBV-positive serum 9-12 weeks after transplantation.

HBV Marker Analysis. Hepatitis B surface antigen (HBsAg) was measured using an Architect Analyzer (Abbott, Osaka, Japan). Serum DNA was extracted using a SMITEST EX-R&D Nucleic Acid Extraction Kit (Genome Science Laboratories, Fukushima, Japan). Small amounts of HBV DNA (<300 copies/mL) were detected via nested PCR.⁸ If HBV DNA was detected during the initial round of PCR, the copy number was determined via real-time PCR as reported.⁸

Results

Phenotypes of CFPHs In Vitro. We seeded 9MM and 12YM PHs on culture dishes and confirmed that the CFPHs from the 2 donors were similar in morphology and replicative capacity. A small number of the CFPHs (0.01%-0.09% of the seeded PHs) began to replicate after 5 days, and the number of replicating cells gradually increased until colonies appeared at 17 days (Fig. 1A); after 21 days, the cells covered the surface of the dish (Fig. 1B). Most of the seeded PHs were not replicative, and they gradually flattened, acquiring a senescent morphology within 20 days of seeding (Fig. 1A). The CFPHs showed an epithelial cell-like morphology with scant cytoplasm (Fig. 1B), and they retained this appearance during subculture (Fig. 1C). The population doubling time (PDT) of the CFPHs gradually increased as the passage number increased. Up to *p* = 4, the CFPHs from the young donors replicated with a population doubling time of 170-220 hours; subsequently, the population doubling time increased until the cells finally became senescent.¹³

The expression of several marker genes was compared among PHs, HepG2 cells, and CFPHs (Fig. 1D). In our experience, no significant differences exist in the marker gene expression profiles of PHs among different donors, and the same trend applies to subcultured CFPHs.¹³ At *p* = 4, the CFPHs expressed less ALB and AAT messenger RNA compared with the PHs. The PHs expressed TO and G6P, both of which are markers of mature hepatocytes, whereas the CFPHs did not. CK19, a hepatic progenitor/biliary cell marker, was expressed in both the CFPHs and HepG2 cells, but not in the PHs. BGP, a cell-cell adhesion molecule in epithelium, endothelium,

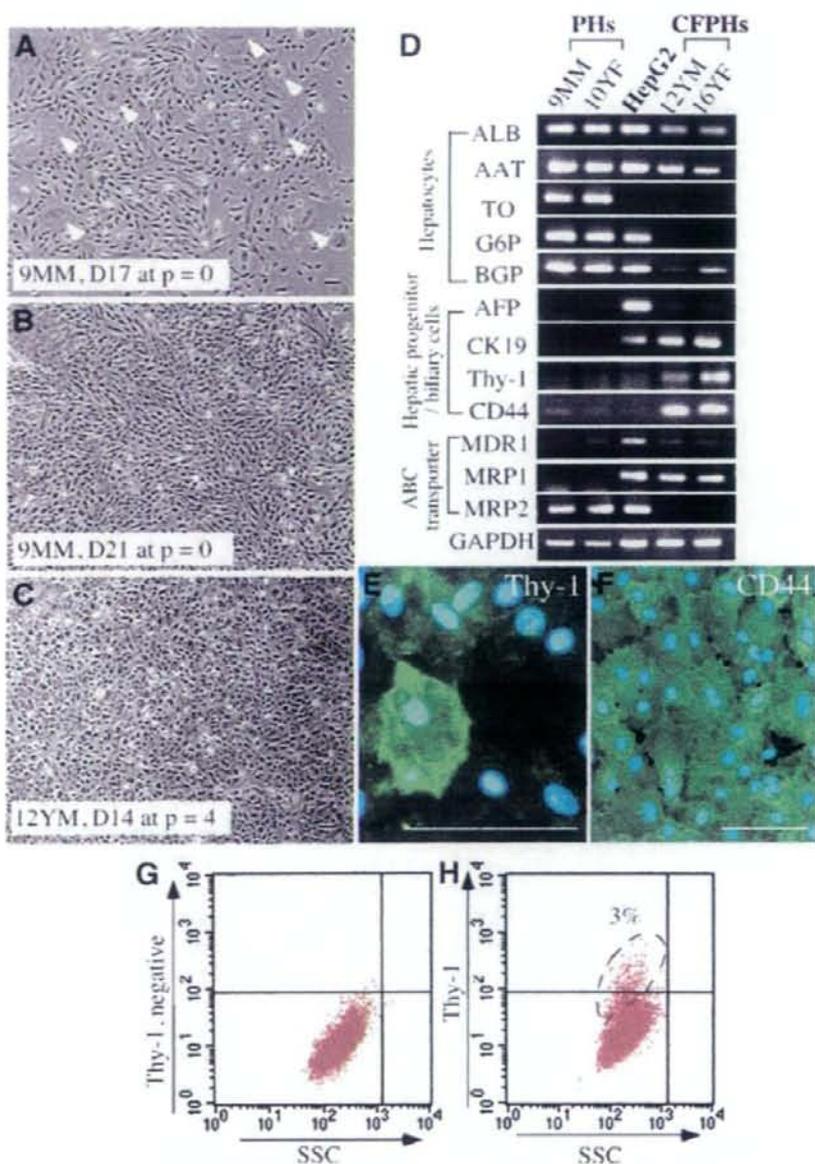
and myeloid cells,¹⁶ was expressed in the PHs and HepG2 cells, but only faintly in the CFPHs. The CFPHs, but not the PHs or HepG2 cells, expressed Thy-1, a hematopoietic/hepatic progenitor cell marker. AFP, a hepatic progenitor/carcinoma cell marker, was only detectable in HepG2 cells. CD44, an SH¹⁷ or oval cell marker,¹⁸ was strongly expressed in CFPHs, but only faintly in PHs and HepG2 cells. PHs and CFPHs faintly expressed MDRI. PHs expressed MRP2, but not MRP1. In contrast, CFPHs expressed MRP1, but not MRP2. A change from MRP2 to MRP1 expression during culture has been reported in rat hepatocytes.¹⁹

Thy-1 and CD44 expression in CFPHs was assessed via immunocytochemistry (Fig. 1E-F). A few CFPHs were positive for Thy-1 (Fig. 1E), whereas the majority was strongly positive for CD44 (Fig. 1F). Fluorescence-activated cell sorting indicated that a minor population of the CFPHs expressed Thy-1 (Fig. 1G-H), with an occupancy rate of 1%-3% (Fig. 1H). The CFPHs expressed CK7, CK8, CK18, and CK19 in the pre-confluent state and became CK7- and CK19-negative in condensed regions postconfluence (data not shown), which is in agreement with our previous findings.¹³ Other hepatic stem cell markers such as CD34 and *c-kit* were undetectable in our CFPHs (data not shown).

Repopulation of CFPHs in uPA/SCID Mouse Liver. We transplanted 12YM CFPHs (*p* = 4) into 27 homozygous uPA/SCID mice. The serum concentration of *h*ALB was monitored posttransplantation as a measure of the RI of CFPHs (Fig. 2A). Approximately half of the hosts had no or only a small increase in the level of *h*ALB throughout the experimental period. The remaining mice showed a continuous increase in the concentration of *h*ALB, which reached >10 μg/mL after 9 to 10 weeks. Animal 27 showed the greatest increase, reaching 0.7 mg/mL after 10 weeks. The RI of each of the 14 mice in which blood *h*ALB concentration was >8 μg/mL after 9 to 10 weeks was determined by dividing the *h*ALB-positive areas by the entire area measured,⁵ and the data were plotted against the corresponding blood *h*ALB concentrations (Fig. 2B). RIs between 0.2% and 27.0% were well correlated with blood *h*ALB concentrations in the 9-728 μg/mL range.

Livers of mice engrafted with the CFPHs were subjected to immunohistochemical staining for *h*ALB (Fig. 3A-D,H) and *in situ* hybridization using *h*-genomic DNA probes (Fig. 3I). *h*ALB-positive cells were visible within 3 weeks posttransplantation as single cells or small clusters consisting of up to 25 cells (Fig. 3A-B). Larger clusters containing 20-450 *h*ALB-positive cells appeared after 9 to 10 weeks (Fig. 3C for animal 2 and Fig. 3D for animals 17 and 27). To detect replicating CFPHs, the mice were

Fig. 1. CFPH growth and gene expression. (A-C) CFPH colony formation. We seeded 9MM PHs at 8×10^3 cells/cm² and cocultured with mitomycin C-treated Swiss 3T3 cells in *h*-hepatocyte clonal growth medium. A few CFPHs proliferated and formed colonies. CFPHs were cultured for (A) 17 and (B) 21 days. PHs were nonreplicative and were gradually expelled by replicative CFPHs. Arrowheads indicate the remaining flattened PHs, whose size increased. (C) Cryopreserved 12YM CFPHs ($p = 3$) were thawed and cultured in *h*-hepatocyte clonal growth medium with Swiss 3T3 cells for 14 days. (D) CFPH messenger RNA expression profiles. RNA was extracted from 9MM and 10YF PHs, HepG2 cells, and 12YM and 16YF CFPHs ($p = 4$). Semiquantitative RT-PCR was performed for ALB, AAT, TO, G6P, BGP, AFP, CK19, Thy-1, CD44, and the ABC transporters MDR1, MRP1, and MRP2. Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as an internal control. (E,F) Immunohistochemistry of Thy-1 and CD44. 12YM CFPHs ($p = 4$) were cultured for 14 days and stained for (E) Thy-1 and (F) CD44. The nuclei were stained with Hoechst 33258. Scale bar: 100 μ m. (G,H) Flow cytometric analysis of CFPHs for Thy-1. Cells were suspended in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum with (G) *m*-immunoglobulin G₁ as a negative control or (H) anti-*h*Thy-1 antibodies. Living cells were analyzed via fluorescence-activated cell sorting. A small fraction (3% in this case) of the CFPHs was Thy-1⁺. Three independent analyses were performed with similar results.



given BrdU after 9 weeks. BrdU-positive CFPHs were observed at the edges of the colonies (Fig. 3E-G). Serial liver sections were prepared from CFPH-chimeric mice 9 to 10 weeks after transplantation for *h*ALB immunohistochemistry (Fig. 3H) and for *in situ* hybridization with an *h*-DNA probe (Fig. 3I). The regions identified as containing *h*-hepatocytes by the 2 methods were identical.

Comparison of Repopulation by CFPHs and PHs. PHs and CFPHs ($p = 4$) were prepared from the livers of 9MM and 12YM donors and transplanted into uPA/

SCID mice, and the mice were killed 3 and 10 weeks posttransplantation. The transplanted cells were identified as *h*ALB-positive from histological sections. The number of PH- and CFPH-derived clusters was 125.0 ± 28.2 ($n = 3$) and 3.3 ± 7.5 ($n = 7$), respectively, per cross-section of the left lobe of the livers 3 weeks after transplantation, suggesting that the rate of engraftment of the CFPHs was much lower than that of the PHs.

The CFPHs were smaller in size compared with the PHs after 3 weeks (Fig. 4A-B). The cytoplasm of the

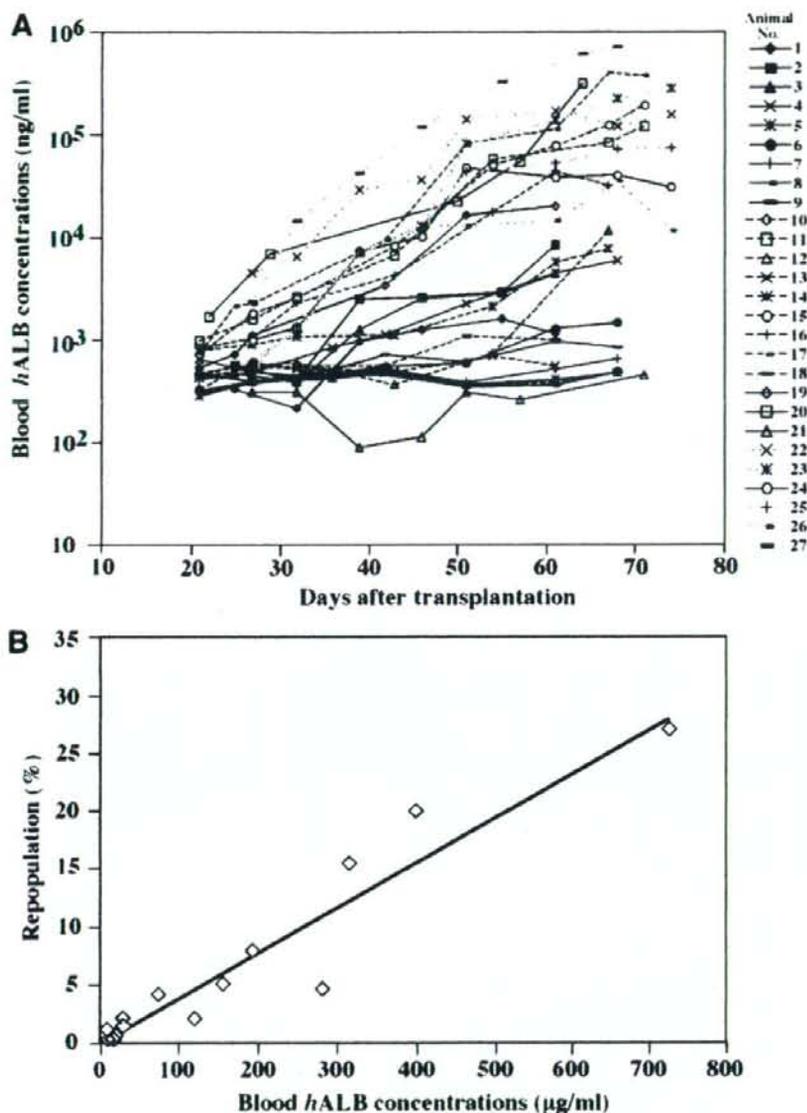


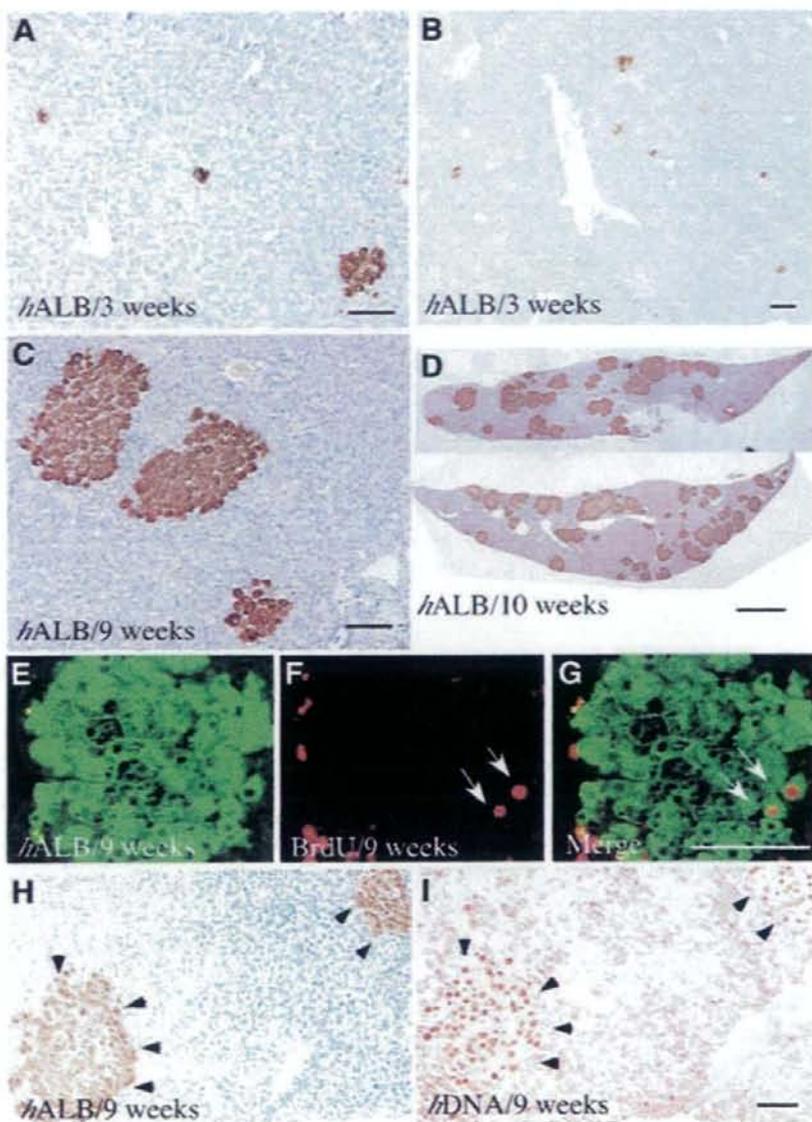
Fig. 2. Transplantation of CFPHs into uPA/SCID mice. The chimeric mice in this experiment are included in group D in Table 1. (A) We transplanted 12YM CFPHs ($p = 4$) into 27 mice and the serum level of hALB was monitored individually. Ten hosts (animals 1, 5, 6, 7, 8, 9, 10, 13, 18, and 21) did not show significantly elevated hALB levels during the experimental period. Four hosts (2, 3, 4, and 14) showed slight elevation. The hALB concentration of 13 mice (11, 12, 15, 16, 17, 19, 20, 22, 23, 24, 25, 26, and 27) reached $>10 \mu\text{g/mL}$ at 9 to 10 weeks after transplantation. (B) Correlation between the blood hALB level and RI. Fourteen CFPH-chimeric mice (animals 2, 11, 12, 15, 16, 17, 19, 20, 22, 23, 24, 25, 26, and 27) were selected from the mice shown in panel A for RI determination. Their liver sections were immunostained for hALB. RIs were determined for each animal and plotted against the hALB concentration. The correlation coefficient (r^2) between the 2 parameters was 0.91.

former was less abundant and more strongly stained for hALB than that of the latter. We observed hCD44 in the plasma membrane of the CFPH-derived cells (Fig. 4E), but not in that of the PH-derived cells (data not shown). At 10 weeks posttransplantation, the CFPHs had increased in size to match those of the PHs, whose sizes were unchanged (Fig. 4C-D), and hCD44 expression disappeared from the CFPH-derived cells (Fig. 4F). The diameter of each CFPH and PH was quantified as follows: $18.3 \pm 5.1 \mu\text{m}$ (mean \pm SD, $n = 65$) versus $25.8 \pm 6.4 \mu\text{m}$ ($n = 124$) at 3 weeks and $27.0 \pm 5.5 \mu\text{m}$ ($n = 185$) versus $25.8 \pm 4.8 \mu\text{m}$ ($n = 187$) at 10 weeks. We found

no significant differences in this parameter between the 12YM and 9MM samples. Thus, it appears that the CFPHs replicated without changing their original small size until 3 weeks posttransplantation, when they became larger.

Liver sections from the chimeric mice were stained with hematoxylin-eosin to compare the morphological features of PHs and CFPHs at 10 weeks. The repopulated CFPHs (Fig. 4G) showed no significant difference in morphology compared with the repopulated PHs (Fig. 4H). As reported previously,^{5,6} the PHs in the chimeric livers were enlarged and had less eosinophilic cytoplasm

Fig. 3. Engraftment and repopulation of CFPHs in chimeric mouse liver. The chimeric mice in this experiment are included in groups A and D in Table 1. We performed *h*ALB immunohistochemistry using liver sections from CFPH-chimeric mice (A,B) 3, (C) 9, and (D) 10 weeks after transplantation. (A,B) Small clusters composed of 1-25 cells were scattered throughout the liver at 3 weeks in 3 of 9 mice. (C,D) The clusters became larger at 9 to 10 weeks. The liver sections in panel C were prepared from animal 2 in Fig. 2A (RI = 1.1%). The liver sections in panel D were prepared from animals 17 (RI = 20.0%; upper section) and 27 (RI = 27.0%; lower section). Three mice were randomly selected for the BrdU incorporation experiments (animals 2, 19, and 20 in Fig. 2A). They were given BrdU 1 hour before death at 9 weeks post-transplantation. Serial liver sections were subjected to (E) *h*ALB- and (F) BrdU immunohistochemical staining. The image in panel G is panel E and panel F merged. Similar results were obtained from these experiments, and the result from animal 19 (RI = 0.6%) is shown in panels E-G. Serial liver sections were prepared from CFPH-chimeric mice (animals 2, 15, and 17 in Fig. 2A) 9 to 10 weeks after transplantation for *h*ALB immunohistochemistry (H) and for *in situ* hybridization with an *h*-genomic probe (I). Similar results were obtained from the 3 mice. The results shown in panels H and I were obtained from animal 2 (positive cells are indicated by arrowheads). Scale bars in panels A-C, G, and I: 100 μ m. Scale bar in panel D: 1 cm.



than the PHs in *h*-livers. The livers of the mice that had low *h*ALB levels at 10 weeks posttransplantation were mostly occupied by red nodules, which have been reported to be formed by the transgene-deleted hepatocytes of the host.²⁰

Gene and Protein Expression Profiles of CFPHs in Chimeric Mice Compared with Those of PHs. Three 12YM CFPH-chimeric mice (11, 15, and 17) were randomly selected from the mice in Fig. 2A and killed 10 weeks after transplantation. RNA was extracted from each liver to generate gene expression profiles via RT-PCR.

RT-PCR was also performed on 2 12YM PH-chimeric mice that were included in a previous study.⁵ The CFPH livers expressed *h*ALB, *h*AAT, *h*TO, *h*G6P, and *h*MRP2, but not *h*CK19, *h*Thy-1, or *h*MRP1, just as in the PH-livers (Fig. 5). Previously, we showed that the PHs in chimeric mice expressed various *h*-cytochrome P450 (*h*CYP) subtypes in a manner similar to the donor liver.⁵ In this study, we found that the expression of *h*CYPs 1A2, 2C8, 2C9, 2D6, and 2E1, but not 3A4, in the CFPH-chimeric mice was similar to that in the PH-chimeric mice (data not shown). Expression of *h*CYP3A4 was very

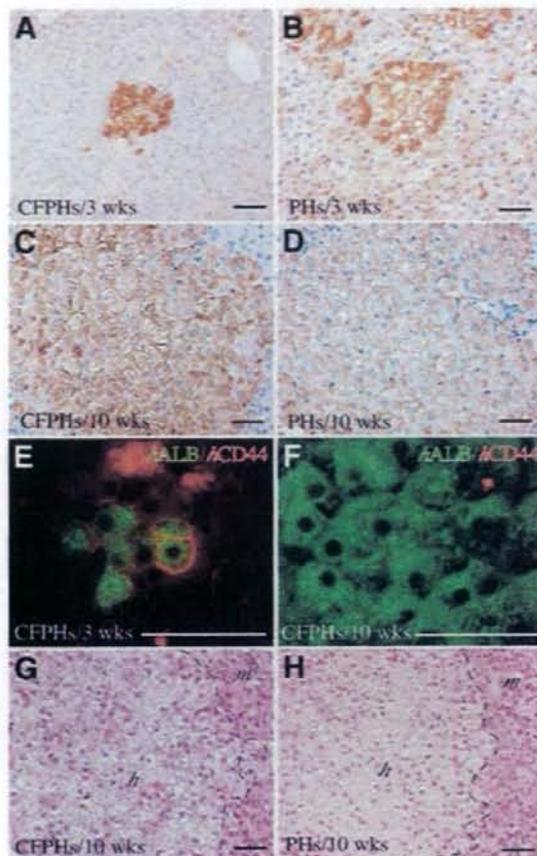


Fig. 4. Immunohistochemical staining for CFPFHs and PHs in chimeric mice. Immunohistochemical analysis with antibodies against (A-D) *hALB* and (E-F) *hCD44*. We produced 3 12YM CFPFH-chimeric mice and 4 9MM CFPFH-chimeric mice [(A) and (E), included in groups A and B in Table 1] and 3 9MM PH-chimeric mice [(B), group C], which were killed at 3 weeks posttransplantation. At 10 weeks posttransplantation, 3 12YM CFPFH-chimeric mice that were randomly selected from the mice shown in Fig. 2A (15, 16, and 17) were killed [(C) and (F), group D], as were 9MM and 12YM PH-chimeric mice, 2 mice each [(D), groups E and F]. (A-D) Representative images of liver sections prepared from the animals and stained with anti-*hALB* antibodies. The diameters of the *hALB*-positive cells were measured in 10-15 randomly selected fields. (E,F) Double-fluorescence immunostaining. Green and red stains depict *hALB* and *hCD44*, respectively. (G,H) Hematoxylin-eosin staining. (G) Eight CFPFH mice were randomly selected from the mice shown in Fig. 2A and killed at 10 weeks posttransplantation. Their liver tissues were then subjected to hematoxylin-eosin staining. (H) Three 12YM PH-chimeric mice were killed at 10 weeks posttransplantation for hematoxylin-eosin staining as above. Similar results were obtained for the 8 CFPFH-chimeric mice and 3 PH-chimeric mice. (E-F) Sections from (E) a CFPFH-chimeric mouse (RI = 20.0%) and (F) a PH-chimeric mouse (RI = 57%). *h*, *h*-hepatocyte region; *m*, *m*-hepatocyte region. Dashed lines show the boundary between the 2 regions. Scale bars: 50 μ m.

low (less than one-fifth) in CFPFHs compared with that in PHs.

Protein expression was investigated immunohistochemically for the CFPFH-chimeric livers at 3, 9, and 10 weeks posttransplantation. All of the examined CFPFHs were Thy-1-negative, CK7-negative, CK19-negative, and AFP-negative (data not shown). The *hALB*-positive cells were coincident with the *hCK18*-positive cells at both 3 (data not shown) and 9 weeks posttransplantation (Fig. 6A-C). MRP2-positive signals were present on the bile canalicular membranes of the transplanted CFPFHs at 10 weeks (Fig. 6D-F). CYP3A4-expressing CFPFHs were localized in the pericentral zone (Fig. 6G-I) as reported previously,²¹ but their distributions were unique. Although some of the CFPFHs were positive for CYP3A4, approximately 70% of them were negative. In contrast, all of the CFPFHs in the pericentral zone strongly expressed CYP1A2 (Fig. 6J-L), which is known to be expressed in postnatal liver.²² The CFPFHs in the chimeric mice were strongly PAS-positive (Fig. 6N), whereas the *in vitro* CFPFHs were faintly PAS-positive (data not shown). From

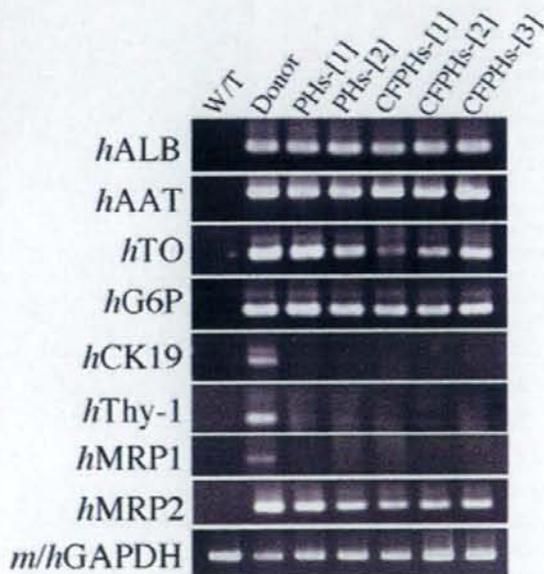
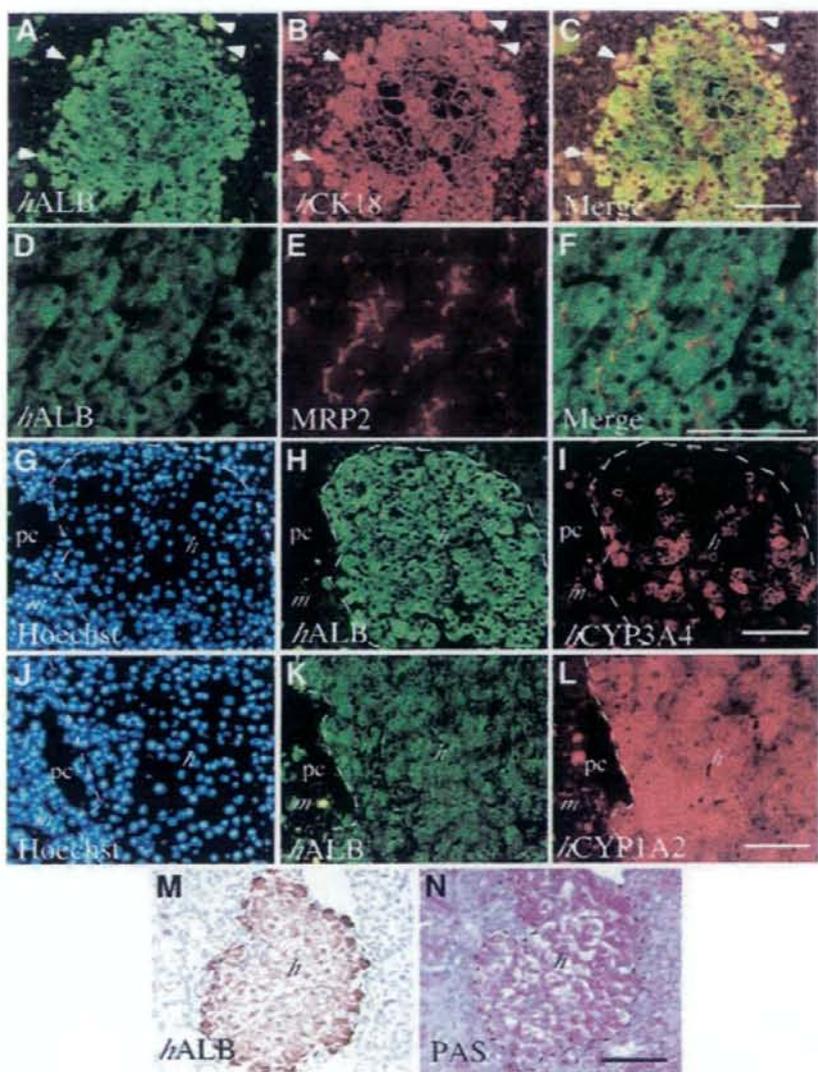


Fig. 5. Gene expression profiles of CFPFHs in chimeric mice. Two uPA/SCID mice were transplanted with 12YM PHs ([1] and [2]); 3 uPA/SCID mice were transplanted with 12YM CFPFHs ([1], [2], and [3]). The chimeric mice in this experiment are included in groups D and F in Table 1. After 10 weeks, the livers were removed for RT-PCR analysis. At the time of death, the PH-[1]-, PH-[2]-, CFPH-[1]-, CFPH-[2]-, and CFPH-[3]-chimeric mice had RIs of 41.0%, 57.0%, 2.1%, 7.9%, and 20.0%, respectively. The analysis was repeated using liver tissues from donor and uPA/SCID mice without transplantation (W/T). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) amplification was used as an internal control.

Fig. 6. Protein expression profiles of the CFPBs in chimeric livers. Mice were transplanted with 12YM CFPBs, and their livers were removed 9 to 10 weeks after transplantation for immunohistochemical analysis of (A,D,H,K) *h*ALB, (B) *h*CK18, (E) MRP2, (I) CYP3A4, and (L) CYP1A2. The chimeric mice in this experiment are included in group D in Table 1. Representative images are shown. (A-F) Double-fluorescence immunostaining. (A,D) *h*ALB is stained green. (B) *h*CK18 and (E) *h*MRP2 are stained red. Panels A and B were merged to create panel C; panels D and E were merged to create panel F. The arrowheads in panels A-C show macrophages engulfing such wastes as lipids. Serial sections of liver tissues subjected to 2 series of immunohistochemical examinations, one for (G-I) *h*CYP3A4 and the other for (J-L) *h*CYP1A2. The sections were stained with (G,J) Hoechst 33258, and for (H,K) *h*ALB, (I) *h*CYP3A4, and (L) *h*CYP1A2. Serial sections of liver tissues at 9 weeks posttransplantation were subjected to *h*ALB-immunostaining (M) and PAS staining (N). The positive cells appear brown in (M) and red in (N). *h*, *h*-hepatocyte region; *m*, *m*-hepatocyte region; *pc*, pericentral zone. Dashed lines show the boundary between the *h*-hepatocyte and *m*-hepatocyte regions. Scale bars: 100 μ m.



these results, we conclude that the transplanted CFPBs differentiated into functionally mature hepatocytes. No *b*-cell tumors were formed during any of our experiments in the uPA/SCID mice.

Infection of CFPB-Chimeric Mice with HBV. To further examine whether CFPBs had exhibited normal differentiated phenotypes in chimeric mice, we tested their susceptibility to HBV infection. Four CFPB-chimeric mice with various serum *h*ALB levels (0.2, 1.6, 7.3, and 222.0 μ g/mL) were inoculated with 100 μ L of HBV-positive *h*-serum at 9–12 weeks posttransplantation. The animals were then tested every 2 weeks for HBV viremia and serum *h*ALB levels (Fig. 7A). The amount of HBV

DNA in the animals increased between 2 and 8 weeks after inoculation, and all 4 mice developed measurable viremia within 8 weeks. However, a correlation was observed between the HBV DNA and/or HBsAg level and the *h*ALB level: the former appeared to be high when the latter was high (Fig. 7A). HBsAg was detectable in the serum of the chimeric mice when they showed elevated virus titers: the HBsAg levels of chimeric mice with HBV DNA levels of 2×10^3 , 5.2×10^5 , 5.9×10^7 , and 7.7×10^8 copies/mL 8 weeks after inoculation were <0.05 , <0.05 , 3.2, and 124.0 IU/mL, respectively. HBV was infectious to CFPB-chimeric mice with very low levels of *h*ALB ($<10^4$ ng/mL), and all mice showed quantitatively

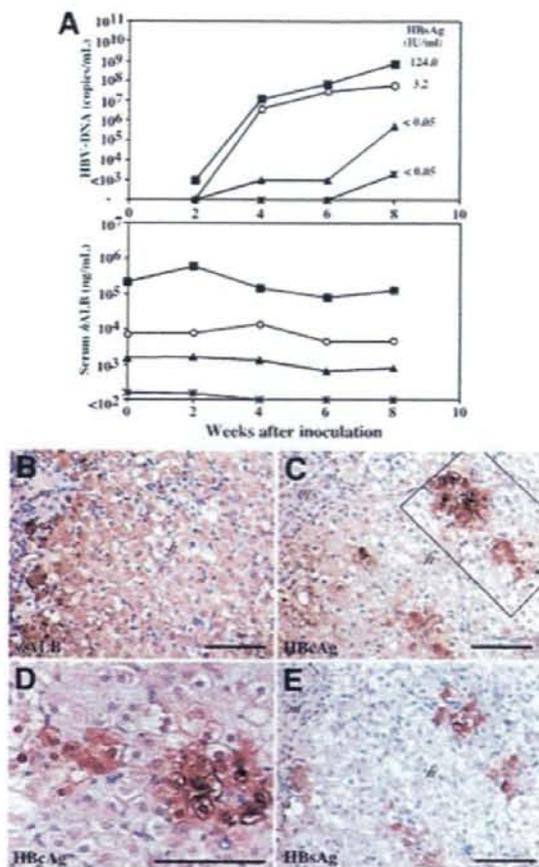


Fig. 7. Susceptibility of chimeric mice to infection with HBV. The chimeric mice in this experiment are included in group G in Table 1. uPA/SCID mice were transplanted with 12YM CFPH ($p = 4$). (A) The serum hALB concentration of each mouse was determined 9–12 weeks posttransplantation just before the mouse was intravenously injected with 100 μ L of HBV-positive *h*-serum (0.2 μ g/mL at 12 weeks, 1.6 μ g/mL at 10 weeks, 7.3 μ g/mL at 11 weeks, and 222.0 μ g/mL at 9 weeks). The animals were examined every 2 weeks for HBV viremia and serum hALB level. The upper and lower graphs show the HBV DNA levels (copies/mL) and serum hALB concentrations (ng/mL), respectively. The amount of HBV DNA ($<10^3$ copies/mL) was semiquantitatively measured via nested PCR. The values in the upper graph represent the HBsAg levels at 8 weeks. (B–E) Immunohistochemical analysis of chimeric livers infected with HBV. Serial sections of liver tissues at 8 weeks after inoculation were stained for (B) hALB, (C,D) hepatitis B core antigen, and (E) HBsAg. The region enclosed by a square in panel C is magnified in panel E. Scale bars: 100 μ m.

measurable viremia ($>10^3$ copies/mL) up to 8 weeks after inoculation. In contrast, most PH-chimeric mice with $<10^4$ ng/mL hALB did not show quantitatively measurable levels of viremia up to 12 weeks after inoculation (data not shown) as reported previously.⁸ In this study, we confirmed that CFPHs were not susceptible to infection

with HBV prior to transplantation. The presence of hepatitis B core antigen and HBsAg in the CFPHs from HBV-infected chimeric livers was examined immunohistochemically (Fig. 7C,E). CFPHs were positive for both antigens that were sporadically distributed in the same regions among the CFPH colonies. Hepatitis B core antigen-positive cells accounted for $18.7 \pm 8.3\%$ of the total number of CFPHs ($n = 3$; total cell count = 1,215) (Fig. 7C), and both the nucleus and cytoplasm of the cells showed signals (Fig. 7D).

Discussion

This study supports our previous conclusion that CFPHs are *h*-hepatic progenitor-like cells.¹³ Cultured CFPHs expressed such hepatic progenitor cell markers as CK19, Thy-1, and CD44, but not mature hepatocyte markers such as TO and G6P. We also found that *in vitro*-expanded CFPHs in uPA/SCID mice were able to repopulate the parenchyma, in which they differentiated into mature hepatocytes. FISH (fluorescence *in situ* hybridization) using mouse X chromosome probes showed that the engrafted and propagated CFPHs did not fuse to the mouse cells (data not shown). Thus, replicative CFPHs isolated from postnatal liver are normal, functional hepatocyte progenitor-like cells.

The existence of stem/progenitor cells in the adult liver is controversial.^{23–25} In the present study, we showed that the CFPHs expressed CK19, Thy-1, and CD44, but not AFP, in serial culture. Thy-1 antigens are expressed in *h*-hepatic progenitor cells in fetal liver²⁶ and in rat oval cells,²⁷ but not in normal adult hepatocytes. We showed that Thy-1-expressing cells were present among the CFPHs at an occupancy of 1%–3%. SHs show greater growth potential than PHs in rats.¹² Other studies have reported that CD44 is a specific marker for rat SHs *in vitro* and *in vivo*, and that its expression level decreases with SH maturation *in vitro*.¹⁷ Moreover, a recent study demonstrated that CD44 was strongly expressed by oval cells in a 2-acetylaminofluorene/partial hepatectomy, a D-galactosamine, and a retrorsine/partial hepatectomy rat model, but not by small hepatocyte-like progenitor cells (SHPCs)¹⁸ that appeared in a retrorsine/partial hepatectomy model.²⁸ We detected CD44 expression in CFPHs at the plasma membrane. These results suggest that Thy-1 and CD44 may be common markers for both rat and *h*-hepatic progenitor cells.

Mouse embryonic liver stem cell lines differentiate into both hepatocytes and bile ducts in uPA/SCID mice.²⁹ Like PHs, our CFPHs differentiated into mature hepatocytes, but not into biliary epithelial cells, in uPA/SCID mice. CFPHs are considered to be hepatic progenitor-like cells, like rat SHs^{12,30–33} and SHPCs.^{28,34} SHPCs are