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Establishment of a humanized model of liver using NOD/Shi-*scid* IL2Rg^{null} mice

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ABSTRACT

Severely immunodeficient NOD/Shi-*scid* IL2Rg^{null} (NOG) mice are used as recipients for human tissue transplantation, which produces chimeric mice with various types of human tissue. NOG mice expressing transgenic urokinase-type plasminogen activator in the liver (uPA-NOG) were produced. Human hepatocytes injected into uPA-NOG mice repopulated the recipient livers with human cells. The uPA-NOG model has several advantages over previously produced chimeric mouse models of human liver: (1) the severely immunodeficient NOG background enables higher xenogeneic cell engraftment; (2) the absence of neonatal lethality enables mating of homozygotes, which increased the efficacy of homozygote production; and (3) donor xenogeneic human hepatocytes could be readily transplanted into young uPA-NOG mice, which provide easier surgical manipulation and improved recipient survival.

The metabolism of xenobiotic compounds, including medicinal drugs, predominantly occurs in the liver. Due to the significant inter-species differences in many liver enzymes that mediate drug metabolism, the metabolism of candidate pharmaceuticals is usually initially evaluated *in vitro* using human cells or cell extracts [1,2] or *in vivo* in rodent models. Although *in vivo* pharmacokinetic studies are routinely performed in experimental animal models, significant inter-species differences in drug metabolism limit their ability to predict human drug metabolism. There are many examples where the findings from rodent models do not extrapolate to man. *In vitro* studies using transformed human hepatic cells, human hepatocytes or human hepatocyte extracts have also been used to predict human drug metabolism. Although they utilize human cells, *in vitro* systems have a very limited ability to predict human drug metabolism *in vivo*. Drug-induced alterations of the level of expression of drug metabolizing enzymes, which is a common problem in clinical medicine, does not occur in the *in vitro* extracts. In addition, the pattern of expression of drug metabolizing enzymes is altered within a very short time period after hepatocytes are cultured *in vitro* (reviewed in [2]). Human drug metabolizing enzymes have been expressed as transgenes in mice. For example, a mouse expressing a human CYP2D6 transgene in liver had a human-specific profile of metabolism of a test substrate [3]. However, transgenic expression of one or a few human enzymes in mouse liver does not represent the biotransformation capabilities of human liver.

To overcome the limitations associated with currently used *in vitro* and *in vivo* experimental methods for studying drug metabolism and toxicity, investigators have utilized several different methods for producing chimeric mice with artificial human livers. Historically, chimeric mice have been generated by introducing human tissues or cells into immune deficient SCID mice, which have an inactivating mutation in the catalytic subunit of the DNA-dependent protein kinase (Prkdc^{scid}, known as the *scid* mutation). Ralph Brinster and colleagues demonstrated that transgenic expression of urokinase-type plasminogen activator (AL-uPA) within mouse liver directed by an albumin promoter caused progressive damage to liver parenchymal cells [4,5]. The mice usually died within weeks due to progressive liver failure. Although the mechanism of hepatic cell destruction has not fully characterized in detail; uPA regulates the activation of plasminogen, which controls the activation of matrix metalloproteinases that are critical for liver cell growth (reviewed in [6]). In addition, the livers in surviving mice are replaced at 8–12 weeks of age by nodular growths that have deleted the transgene. In an elegant series of experi-

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ments, investigators showed that the AL-uPA mice could be rescued from liver failure by transplant of congenic or xenogenic hepatocytes [7–10]. Other investigators subsequently demonstrated that immunocompromised (recombinant activation gene-2; RAG-2 deficient or SCID mutation) mice expressing this transgene could be partially repopulated (~15%) with human hepatocytes [9,10].

Recently, we developed NOD/Shi-*scid* IL2Rg^{null} (NOG) mice by crossing IL-2 receptor gamma chain-deficient (IL2Rg^{null}) mice [11] with NOD/Shi-*scid* mice [12]. NOG mice lack T and B lymphocytes and natural killer (NK) cells, and they have impaired dendritic cell function [13–15]. Because of their severe immunodeficient state, NOG mice have been used as an *in vivo* model to study human hematopoietic stem cell [13,14] and endometrial tissue [16,17] engraftment. Here, we used NOG mice to develop an uPA/NOG-transgenic model that allows the engraftment of human hepatocytes into a damaged liver. This model could provide a useful tool for characterization of hepatic drug metabolism *in vivo* within a humanized liver.

Materials and methods

Transgenic mice. The mouse albumin promoter-driven urokinase-type plasminogen activator (*Plau*, known as uPA) gene expression unit (GenBank Accession No. AB453180) was constructed as follows. The 2345-bp *Not*I–*Bam*HI fragment of the mouse albumin enhancer/promoter from plasmid p2335A-1 [18], the 304-bp *Hin*dIII–*Nhe*I fragment of the chimeric intron from pCI plasmid (Promega Corp., WI, USA), the 1367-bp *Nhe*I–*Sal*I fragment of the PCR-amplified mouse uPA cDNA (MuPA-Nhe1-F, 5′-GCTA GCGGCACTACCATGAAAGTC-3′; MuPA-Sal1-R, 5′-AATTAAGTCGACA ACAAGTGACCC-3′), and the 624-bp *Sma*I–*Eco*RI fragment of the PCR-amplified human growth hormone (*GH1*) 3′-flanking region (hGHF, 5′-GCTCTACTGCTTCAGGAAGGACAT-3′; hGHR, 5′-GAATTCA ACAGGCATCTACTGA-3′) were cloned into the pBlueScript II vector (Promega) with appropriate polylinkers (5′-GATCCAAGCTTATGC AGTCGACCCGGGCATGCGAATTCTCGA-3′). A vector-free, 4.6-kb uPA expression fragment was prepared by *Not*I and *Kpn*I digestion and microinjected into fertilized NOD/Shi strain mouse eggs using standard methods. Transgenic founders were mated with NOD·Cg-*Prkdc*^{scid} *Il2rg*^{tm1Sug}/ShiJic (NOG) mice to confer the *scid* and IL2Rg^{null} mutations. The *scid* and IL2Rg^{null} mutations were genotyped by PCR as described previously [13,19]. The mice were assigned the following genetic designation: NOG-*Tg* (*Alb-Plau)11-4*/ShiJic [formally, NOD·Cg-*Prkdc*^{scid} *Il2rg*^{tm1Sug} *Tg(Alb-Plau)11-4*/ShiJic; abridged name: uPA-NOG]. The present study was performed in accordance with institutional guidelines and was approved by the Animal Experimentation Committee of the Central Institute for Experimental Animals.

Detection of uPA transcripts by RT-PCR. Total cellular RNA samples from the liver, kidney, and spleen were obtained from 29-week-old uPA-NOG mice using the RNeasy Mini kit (QIAGEN, Tokyo, Japan). Reverse transcription-polymerase chain reaction (RT-PCR) was performed using the High Capacity cDNA Reverse Transcription kit (Applera Corporation, CA, USA). The spMuPAF forward primer (5′-GAGGCACTGGGCAGGTGTCC-3′) and MuPAR reverse primer (5′-AGGGCCGACCTTTGGTATCAGTG-3′) were used to detect the spliced form of the external mouse uPA transcript (365-bp band). The 479-bp glyceraldehyde-3-phosphate dehydrogenase (*Gapdh*) fragment was amplified using the G3PDHF forward primer (5′-TCACCATCTTCCAGGAGCGAGA-3′) and the G3PDHR reverse primer (5′-GAAGGCCATGCCAGTGAGCTT-3′) and used as an internal control.

Spontaneous liver damage. The degree of liver damage was examined by determining the serum levels of alanine aminotransferase (ALT). Clinical biochemical analyses of serum samples were carried out with the Fuji DRI-CHEM 7000 (FUJIFILM Corporation, Tokyo, Japan).

Southern blot analyses. Genomic DNA samples from liver and kidney were obtained from 8-week-old uPA-NOG mice and non-transgenic mice by overnight incubation with proteinase K and subsequent extraction with phenol: chloroform: ethanol according to the standard protocol. *Xba*I, *Xho*I, *Bgl*II, and *Bam*HI restriction enzyme-digested genomic DNA was electrophoresed on a 0.6% agarose gel and transferred to a positively charged nylon membrane (F. Hoffmann-La Roche Ltd., Basel, Switzerland). Hybridization was carried out with a DIG-labeled probe that was prepared using the PCR DIG Probe Synthesis Kit (F. Hoffmann-La Roche Ltd.) using forward (5′-GTTCTCCAGCTTGGGATCGACCTG-3′) and reverse (5′-TTGATAGGAAAGGTGATCTGTGTGCAG-3′) primers according to the manufacturer's instructions.

Transplantation of human hepatocytes. Commercially available cryopreserved hepatocytes (Lonza Walkersville Inc., MD, USA) were used as donor cells. Young (6-week-old) uPA-NOG hemizygotes and homozygotes were used as recipients. Cell number and viability were determined by trypan blue exclusion in a hemocytometer. One million viable hepatocytes in 40 μl of Hank's Balanced Salt Solution (HBSS) were injected intrasplenically via a Hamilton syringe with a 26 G needle.

Human albumin measurements. Small volumes of blood were collected biweekly from the retro-orbital venousplexus with a plastic capillary. After a 500- to 30,000-fold dilution with Tris-buffered saline that contained 1% bovine serum albumin and 0.05% Tween-20, human albumin concentrations were measured with the Human Albumin ELISA Quantitation Kit (Bethyl Laboratories Inc., TX, USA) according to the manufacturer's protocol.

Immunoblotting. Diluted serum samples (2000× dilution) were solubilized in SDS sample buffer with 5% β-mercapthoethanol. Proteins were subjected to SDS–PAGE and transferred to Hybond-ECL membranes (GE Healthcare Bio-Sciences Corp., NJ, USA). The membranes were incubated for 90 min with biotinylated polyclonal goat anti-mouse albumin antibodies (A90-234A; Bethyl Laboratories) and biotinylated polyclonal goat anti-human albumin antibodies (A80-229A; Bethyl Laboratories), washed, and incubated with a streptavidin–horseradish peroxidase conjugate (GE Healthcare Bio-Sciences) for 60 min. The biotinylated antibodies were prepared using the FluoReporter Mini-Biotin-XX Protein Labeling Kit (Invitrogen Corp., CA, USA). The immunoblots were developed using the ECL Western Detection System and Hyperfilm ECL (GE Healthcare Bio-Sciences).

Histology and immunohistochemistry. The tissues were fixed with 4% (v/v) phosphate-buffered formalin, and paraffin-embedded sections were stained with hematoxylin and eosin (H&E). Polyclonal goat anti-human albumin antibodies (Bethyl Laboratories) were used as the primary antibodies. The antibodies were visualized using amino acid polymer/peroxidase complex-labeled anti-goat Ig antibody (Histofine Simple Stain Mouse MAX PO (G); Nichirei Bioscience Inc., Tokyo, Japan) and diamino-benzidine (DAB; Dojindo Laboratories, Kumamoto, Japan) substrate [0.2 mg/ml 3,3′-diaminobenzidinetetrahydrochloride, 0.05 M Tris–HCl (pH 7.6), and 0.005% H_2O_2]. Sections were counter-stained with hematoxylin.

Statistical analyses. Statistical analyses were performed with StatView ver. 5.0 (SAS Institute, Tokyo, Japan) and the Prism 5 software (GraphPad Software, CA, USA).

Results and discussion

Previously prepared AL-uPA transgenic mice [*Tg(Alb-1,Plau)-Bril44* lines 1353–8 and lines 1944–6] possess five or 10 copies,

respectively, of the transgene joined head-to-tail in a tandem array [20]. In both lineages, half of the transgenic offspring bled extensively and died as neonates. Neonatal lethality was correlated with the level of expression of the uPA transgene, which is determined, at least in part, by the copy number. Therefore, we predicted that mice with fewer copies of the uPA transgene would have less liver damage, which would eliminate the uPA-mediated neonatal lethality. We produced uPA-transgenic mice in severely immunodeficient NOG mice [NOG-*Tg(Alb-Plau)11-4*/ShiJic, referred to as uPA-NOG] with the uPA gene expression unit (Fig. 1A). RT-PCR analysis

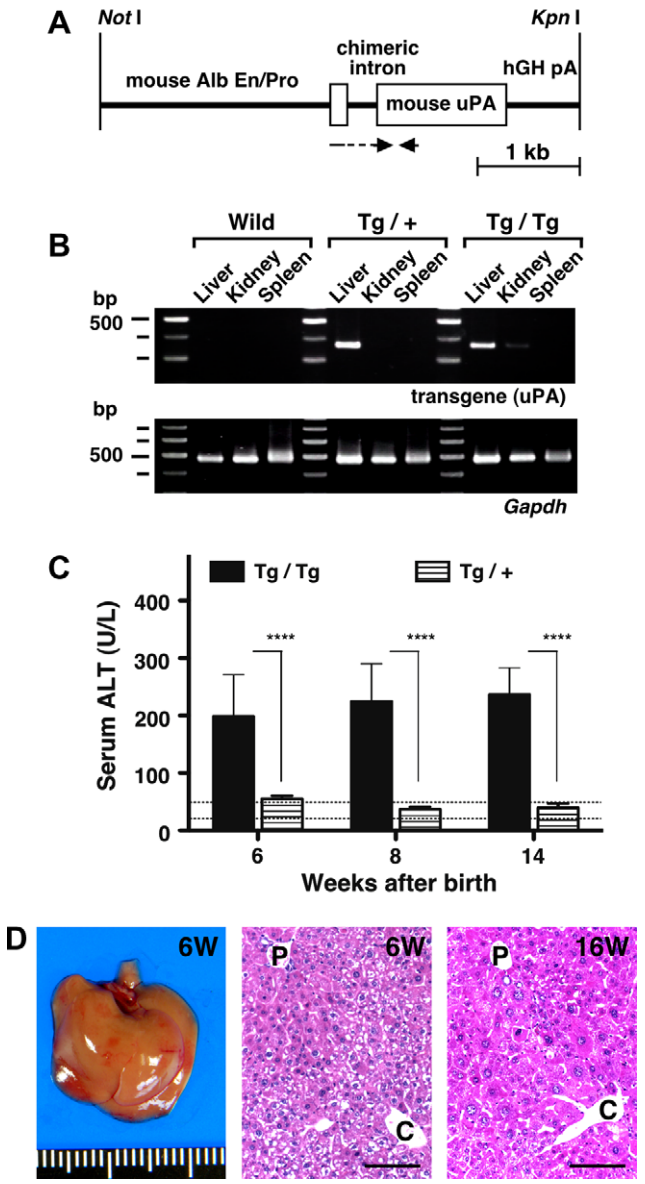

Fig. 1. Establishment of the uPA-NOG mouse as a model of spontaneous hepatic injury. (A) The uPA expression unit contains the mouse albumin enhancer/promoter (Alb En/Pro), the chimeric intron, mouse uPA cDNA, and the 3′-UTR of the human growth hormone gene with polyadenylation (pA) signal. Arrowheads depict the positions and directions of the RT-PCR primers. (B) RT-PCR analyses of uPA transgene expression. Wild, non-transgenic NOG; Tg/+, hemizygous; and Tg/Tg, homozygous uPA-NOG mice. *Gapdh* was used as an internal control. (C) ALT activities in uPA-NOG mice. All the values for the homozygous uPA-NOG (Tg/Tg) are significantly higher than those for the hemizygote (Tg/+) ($P < 0.0001$, unpaired *t*-test). Dashed lines indicate the two standard deviation ranges for the values for the non-transgenic NOG mice ($n = 7$). Each of the points for the hemizygous and homozygous uPA-NOG mice represent the mean ± SD of four to seven samples. (D) Gross appearance and histopathology of a homozygous uPA-NOG mouse liver. Scale bar, 100 μm; P, portal tract; C, central vein.

of uPA transgene expression indicated that the chimeric introns were appropriately spliced to produce the uPA expression unit. Although the transgene was expressed in the livers of hemizygous (Tg/+) and homozygous (Tg/Tg) uPA-NOG mice; it was not expressed in the kidneys, spleens or any other tissues obtained from non-transgenic NOG mice (Fig. 1B). uPA-NOG hemizygotes were indistinguishable from non-transgenic littermates; they have never exhibited spontaneous intestinal or intra-abdominal bleeding that was typically seen in previously produced AL-uPA transgenic mice [21]. The level of expression of the transgene in hemizygous uPA-NOG mice was below that required to cause liver disease. The serum ALT activity in the hemizygote, an indicator of hepatocyte damage, did not increase in mice at any age and was identical to that of a non-transgenic NOG mouse (Fig. 1C).

Since transgene zygosity affects the phenotype of transgenic mice via a gene dosage effect; we produced a homozygous line of uPA-NOG mice that carries two copies of the transgene array that stably reinforces transgene expression. To do this, female and male homozygotes were bred to produce homozygous uPA-NOG mice, which no longer needed progeny testing or genotyping for zygosity. At birth, the homozygotes, whose zygosity was confirmed by progeny testing, were slightly smaller than their hemizygous littermates. Quantitative RT-PCR revealed that transgene expression in the livers of homozygous uPA-NOG mice was 2.4- to 3.6-fold higher than hemizygotes (data not shown). However, perinatal hemorrhaging, embryonic lethality or neonatal lethality did not occur in homozygous uPA-NOG mice. The serum ALT level (75.0 ± 9.1, $n = 4$) in the homozygote at 4 weeks of age was slightly above that of the hemizygote, but this trend did not reach statistical significance (data not shown). However, the ALT levels began to increase by 6 weeks of age in the uPA-NOG homozygotes, and remained elevated through 14 weeks of age (Fig. 1C). The livers of 6-week-old uPA-NOG homozygotes had evidence of modest hepatic injury. The hepatocytes focally showed degeneration foci and a few eosinophilic bodies predominantly around the central veins (Fig. 1D, center). The livers of 16-week-old uPA-homozygous mice revealed megalocytosis with increased eosinophilic bodies (Fig. 1D, right).

The phenotype and liver histology of uPA-NOG mice was significantly different from that of previously produced AL-uPA transgenic mice. uPA-NOG mice did not develop the severe edema that was observed AL-uPA homozygote mice at 3–6 weeks of age [4]. The color of the liver parenchyma was also significantly different. The livers of 3- to 5-week-old AL-uPA-transgenic homozygotes were smooth and pale to nearly white in color (known as "white liver"), and the livers of the hemizygotes were also profoundly abnormal in appearance, with multiple reddish nodules in addition to the white parenchyma [4]. However, the liver of 6-week-old homozygous uPA-NOG mouse did not show any "white liver" changes, regardless of uPA transgene zygosity (Fig. 1D, left). AL-uPA transgenic mice have an age-dependent decrease in uPA expression that is caused by deletion of the integrated transgene [4]. However, Southern blot analysis indicated that the transgene array was stably integrated at a single locus in the uPA-NOG homozygote (Fig 2A). The fact that there were no differences in hybridization banding patterns in the liver and kidney DNA samples in 8-week-old uPA-NOG mice indicate that there is a relatively low frequency of physical loss of the transgene from uPA-NOG mice. Furthermore, stable level of expression of the uPA transgene (Fig. 1B) and the persistence of high levels of the hepatic injury marker (ALT) also indicate the transgene is stably expressed (data not shown). Further analysis of the banding pattern after *Xho*I digestion indicate that uPA-NOG mice have at least three copies of the 4.6-kb monomeric transgene. The low copy number of the integrated transgene in uPA-NOG mice may prevent neonatal lethality. The architecture of the integrated transgene in uPA-NOG mice was further analyzed by southern blot analyses with additional restric-

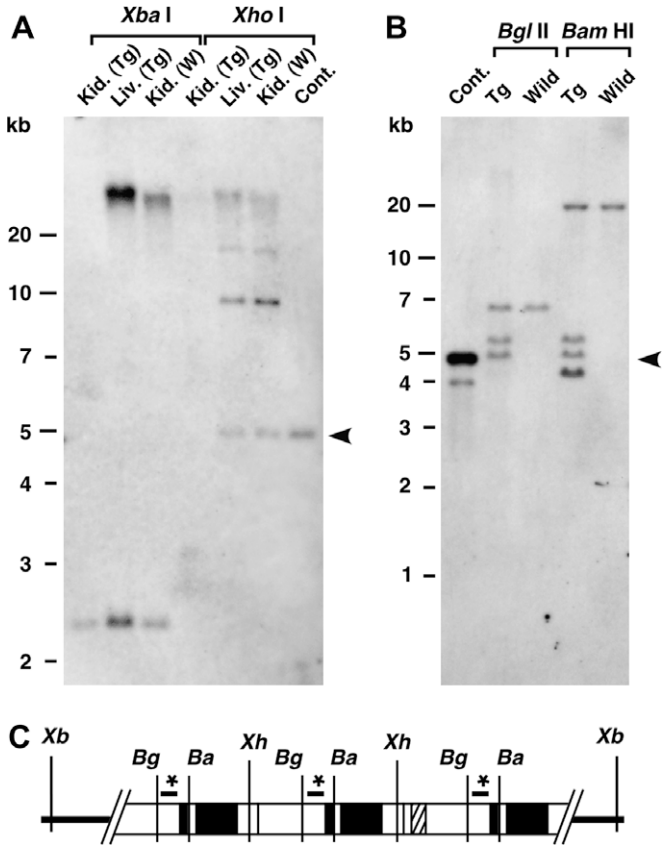

Fig. 2. Molecular analyses of the integrated transgene in uPA-NOG mice. (A) DNA samples from non-transgenic NOG mouse kidney (Kid.; W) and uPA-NOG hemizygote kidney (Kid.; Tg) and liver (Liv.; Tg) were digested with XbaI and XhoI for Southern blot analysis. (B) DNA samples from non-transgenic NOG mouse kidney (Wild) and uPA-NOG hemizygote kidney (Tg) was digested with BglII and BamHI. The uPA expression unit (4.6-kb fragment) was used as a positive control (arrowhead). (C) Restriction map and structure of the transgene in uPA-NOG mice. Solid boxes indicate transcribed segments. Asterisks indicate the positions recognized by DIG-labeled probes. The shaded box indicates the rearranged region. Restriction enzymes: Xb, XbaI; Xh, XhoI; Bg, BglII; Ba, BamHI.

tion enzymes (Fig 2B) to produce the restriction map of the integrated transgene array (Fig. 2C). This array consists of at least three copies of the uPA gene expression unit joined head-to-tail in a tandem array. One of the uPA gene expression units that located at downstream position had a small rearrangement (Fig. 2C). However, the rearrangement was likely not an acquired characteristic, because the rearrangement was observed in the founder mice from this line (data not shown).

To determine whether uPA-NOG mice could support repopulation by transplanted hepatocytes, commercially available cryopreserved human hepatocytes were transplanted into 6-week-old uPA-NOG hemizygous and homozygous recipients. The hepatocytes were successfully transplanted only into the homozygous uPA-NOG mice. Engraftment was demonstrated by different methods, including histology, human serum albumin measurements (ELISA), immunoblotting, and immunohistochemical staining (Fig. 3). At the time of transplantation, the recipient mice were clinically healthy and indistinguishable from non-transgenic uPA-NOG mice. Growth of transplanted human hepatocytes was not detectable in either the hemizygous or homozygous uPA-NOG mouse livers by gross inspection (Fig. 3A) because of the lack of obvious differences, such as color [8]. Periodic measurements of human albumin levels in the blood of each recipient were performed, to estimate repopulation of the mouse livers with human hepatocytes. Hemizygous recipients transplanted with human

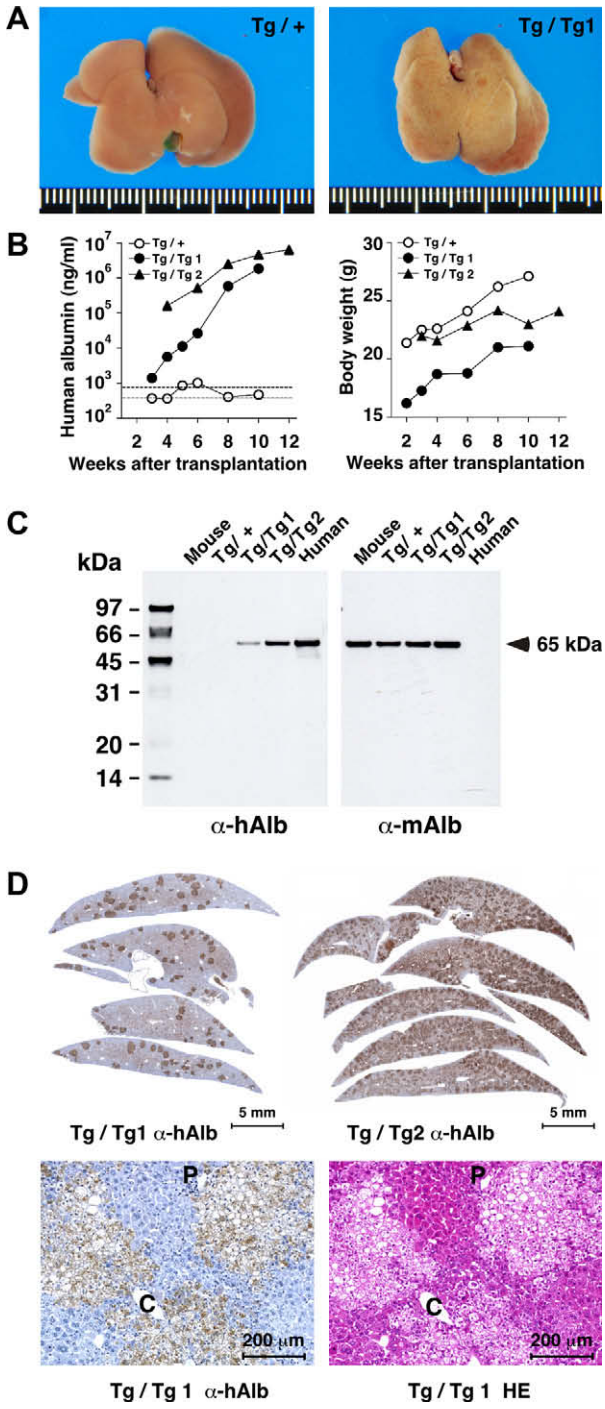

Fig. 3. Engraftment and repopulation of human hepatocytes in uPA-NOG mice. (A) Gross appearance of the uPA-NOG mouse liver 10 weeks after human hepatocyte transplantation. Tg/+, hemizygous; Tg/Tg, uPA-NOG homozygote. (B) Blood albumin concentrations in human cell recipients were assayed by ELISA. Dashed lines indicate the two standard deviation ranges for the values for the untransplanted uPA-NOG mice ($n = 6$). Body-weight changes after human hepatocyte transplantation are shown. (C) Immunoblot analysis shows human albumin (α-hAlb) and mouse albumin (α-mAlb) production in hemizygous (Tg/+) or homozygous (Tg/Tg) uPA-NOG transplant recipient mice. (D) Histology and immunohistochemistry of livers from uPA-NOG mice that were repopulated with human hepatocytes. Immunohistochemical staining for human albumin (top and lower left) and H&E staining (lower right). P, portal tract; C, central vein.

hepatocytes did not produce levels of human serum albumin that were indicative of a successful transplant (Fig. 3B, left); human albumin was not detected by immunoblotting (Fig. 3C, left) or

immunohistochemical staining (data not shown). Conversely, both homozygous recipients repopulated after transplantation of the human hepatocytes. These mice produced more than 1 mg/ml human albumin, and immunohistochemical staining suggested that the livers were comprised of at least 10–15% human hepatocytes (Fig. 3B, left). In one mouse, the circulating human serum albumin level continued to increase to 6.5 mg/ml. Although uPA-NOG hemizygotes and homozygotes both gained weight after transplantation (Fig. 3B, right), immunoblot analyses showed successful engraftment of the transplanted human hepatocytes in only the uPA-NOG homozygotes (Fig. 3C). Furthermore, human albumin was detected in the sera from both homozygotes (Tg/Tg1 and Tg/Tg2) with the anti-human albumin antibody; immunohistochemical staining for albumin further confirmed the human origin of these hepatocytes. Sera obtained from hemizygous uPA-NOG mice transplanted with human hepatocytes did not contain detectable amounts of human albumin. However, mouse albumin was detected in the sera from all of the mice.

Numerous mature human hepatocytes were identified in the livers of the transplanted uPA-NOG homozygotes (Fig. 3D, top). The number of human hepatocyte foci and the repopulation index, which was calculated based on the human hepatocyte/total liver cell number, appears to correspond to the measured blood levels of human albumin. In the Tg/Tg1 uPA-NOG homozygote, the blood human albumin levels reached 1.8 mg/ml, and the repopulation index was estimated to be 20% (Fig. 3D, upper and lower left). As mentioned previously, the human albumin levels reached 6.5 mg/ml in one mouse, a Tg/Tg2 uPA-NOG homozygote. In this mouse, almost 80% of the hepatocytes showed positive staining for human albumin, which suggests that the recipient liver was replaced by the transplanted human hepatocytes (Fig. 3D, top right). The human hepatocytes were fairly swollen and had a rarefied cytoplasm, such that these cells were easily discriminated from their murine counterparts (Fig. 3D, lower right). Periodic acid-Schiff (PAS) reaction showed that the empty spaces in these cells corresponded to areas of glycogen storage [22,23]. Furthermore, wisps and small clumps were evident in the cytoplasm, indicating that the organelles were displaced by excessive glycogen accumulation.

We anticipate that these humanized uPA-NOG mice will be useful for studying drug metabolism, general metabolic effects after hepatic engraftment, and the differentiation of stem cells for hepatocytes.

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