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The reconstituted ‘humanized liver’ in TK-NOG mice is mature and functional

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ABSTRACT

To overcome the limitations of existing models, we developed a novel experimental *in vivo* platform for replacing mouse liver with functioning human liver tissue. To do this, a herpes simplex virus type 1 thymidine kinase (HSVtk) transgene was expressed within the liver of highly immunodeficient NOG mice (TK-NOG). Mouse liver cells expressing this transgene were ablated after a brief exposure to a non-toxic dose of ganciclovir (GCV), and transplanted human liver cells are stably maintained within the liver (humanized TK-NOG) without exogenous drug. The reconstituted liver was shown to be a mature and functioning "human organ" that had zonal position-specific enzyme expression and a global gene expression pattern representative of mature human liver; and could generate a human-specific profile of drug metabolism. The ‘humanized liver’ could be stably maintained in these mice with a high level of synthetic function for a prolonged period (8 months). This novel *in vivo* system provides an optimized platform for studying human liver physiology, including drug metabolism, toxicology, or liver regeneration.

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1. Introduction

We [1] and other groups [2,3] have produced immunocompromised mice with human liver tissue as model system for analysis of drug metabolism and liver regeneration. In several models, human liver cells are transplanted into immunodeficient mice that express a urokinase-type plasminogen activator (uPA) transgene in their liver. Remarkably, human-specific hepatitis viruses can infect these mice [2–4]; their reconstituted livers express enzymes found in human liver cells [5]; and they can generate human-specific metabolites of test substrates, including steroids [6–10]. Although uPA expression facilitates the growth of transplanted human liver cells; it causes continuing and progressive damage to liver parenchymal cells, possibly via activation of plasminogen, which regulates the activity of matrix metalloproteinases that are critical for liver cell growth. Thus, these uPA-dependent models have very significant disadvantages that limit their utility for many applications, including a very poor breeding efficiency, renal disease, and a very narrow time window for transplantation before the mice succumb to their bleeding diathesis. A fumarylacetoacetate hydrolase (*Fah*) knockout mouse [11] has also been utilized for this purpose. In some instances, this model also requires (virus-mediated) uPA expression in liver to facilitate human hepatocyte transplantation, and thus has the same limitations. *Fah* mice also develop liver carcinomas as a consequence of their type I tyrosinemia, and continued or intermittent drug treatment after humanization is required to suppress the development of liver cancer, which enables their long term survival [4]. As a consequence, analyses of drug metabolism or liver regeneration in these models are confounded by the ongoing liver pathology or by the requirement for continued drug treatment. Therefore, we utilized a substantially different approach to overcome these limitations. The targeted expression of the herpes simplex virus type 1 thymidine kinase (HSVtk) in the liver of

Abbreviations: CK8/18, human Cytokeratin (8/18); DEB, debrisoquine; Fah, fumarylacetoacetate hydrolase; GCV, ganciclovir; H&E, hematoxylin and eosin; hAlb, human albumin; HSVtk, herpes simplex virus type 1 thymidine kinase; MIAME, minimum information about a microarray experiment; nHeps, normal human liver cells; RI, replacement index; RT-PCR, reverse transcription-PCR; uPA, urokinase-type plasminogen activator.

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severely immunodeficient NOG mice enabled mouse liver to be stably replaced with mature and functional human liver tissue in the absence of ongoing drug treatment.

2. Materials and methods

2.1. Transgenic mice, human liver cell transplantation and drug biotransformation analysis

The herpes simplex virus type 1 thymidine kinase (*UL23* or HSVtk) gene expression unit was constructed as in Fig. S1A. A vector-free 4.4-kb HSVtk expression fragment was microinjected into fertilized NOD/Shi strain mouse eggs using standard methods. For further information about the creation and breeding of the TK-NOG strain, human liver cell transplantation, and the drug biotransformation studies, see supplementary materials and methods. This study was performed in accordance with institutional guidelines and was approved by the Animal Experimentation Committee of the Central Institute for Experimental Animals.

2.2. Histology and immunohistochemistry

Formalin-fixed and paraffin embedded (5 μm) sections were used for immunohistochemical staining with Cytokeratin (8/18) (h-CK8/18), HLA class I-A, B, C, asialoglycoprotein receptor 1 (ASGR1), albumin, glutamine synthetase (GS) antibodies. To estimate the replacement index (RI), which is the percentage of donor human liver cells in recipient livers, the ratio of the area occupied by h-CK8/18-positive cells to the entire area examined in immunohistochemical sections of three to five lobes was measured.

2.3. Immunoblotting

Human albumin, complement C3 protein, transferrin, and ceruloplasmin secretion was analyzed by Immunoblotting with specific primary antibodies and horseradish peroxidase-labeled secondary antibodies. The general information about the antibodies used for detection of the human protein are provided in the supplementary materials and methods.

2.4. Oligonucleotide array hybridization

Global gene expression was analyzed using the HG-U133A Plus 2 GeneChip array (Affymetrix Inc., Santa Clara, CA). Signal intensity for each transcript (background subtracted and adjusted for noise) and detection call (present, absent, or marginal) were determined using Affymetrix Expression Console Software (Affymetrix Inc.). The signal was normalized by house keeping gene, the human 18S rRNA gene (10098_M_at probe). The MIAME compliant microarray data was deposited in the Center for Information Biology gene EXpression database (CIBEX) at DDBJ (Japan) (CIBEX Accession: CBX102).

2.5. Statistical analyses

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

3. Results

3.1. A reconstituted 'humanized liver' in TK-NOG mice can be stably maintained

Targeted HSVtk expression has previously been used to ablate specific cell types in transgenic mice [12–14]. Therefore, we used an albumin promoter to drive the liver-specific expression of a HSVtk transgene in severely immunodeficient NOG mice [15] to produce TK-NOG mice. Administration of GCV, a drug that is not toxic to human or mouse tissues, induces tissue-specific ablation of transgenic liver parenchymal cells. Since HSVtk catalyzes GCV phosphorylation, which is the rate-limiting step that cannot be performed in mammalian cells lacking this transgene, liver cells expressing the transgene are selectively destroyed. The HSVtk transgene construct, mouse breeding, protocol variables, and the properties of TK-NOG mice are described in the supplementary information and in Fig. S1. We developed an initial protocol that enabled transplanted human liver cells to replace mouse liver. A dose of GCV (0.5–5 mg/kg I.P) that is not toxic to human or mouse tissues was administered on days seven and five prior to transplantation, and 10^6 human liver cells were transplanted via intra-splenic injection. Despite using a non-optimized protocol in the initial pilot studies, a substantial amount of human albumin (hAlb) was detected in the plasma obtained from all 123 TK-NOG recipients after human liver cell transplantation, and the hAlb levels increased steadily to a maximal plasma concentration of 5.9 mg/mL (average 1.5 mg/mL; Table 1; Fig. S2A). The extent of human liver replacement was highly correlated with the measured hAlb levels ($r^2 = 0.9471$; Fig. S2B), and the engrafted human liver cells were incorporated into the existing liver in recipient ('humanized' TK-NOG) mice (Fig. 1A). After optimization of the variables (age of mice at time of transplantation, dose/timing of GCV administration) as described in the supplementary information and Fig. S3, the hAlb concentration (average 3.3 mg/mL) and level of human engraftment (average 43%) in TK-NOG mice was substantially increased (Table 1). It has recently been reported that transplantation of an increased number of human cells increases the level of human liver chimerism in $Fah^{-/-}$ model [4]. However, the average level of human reconstitution (43%) in TK-NOG liver is already at or above that obtained when this modification was used in the $Fah^{-/-}$ mouse. However, it is possible that increasing the number of transplanted human cells could further increase liver chimerism in TK-NOG mice.

Of importance, the 'humanized' TK-NOG livers maintained their synthetic function for a prolonged period. Humanized TK-NOG mice maintained a very high plasma hAlb level over a 8-month period of observation, and did not experience any loss of body weight (Fig. 1B). The functioning human liver was maintained despite the fact these mice did not receive any medication other than the GCV, which was administered prior to transplantation. This prolonged period of human liver survival has not been achieved

Table 1
Engraftment of human liver cells and repopulation rates in chimeric mice using a pilot or optimized transplantation protocol.

Pilot		Optimized		
Mice (*n*)	hAlb (mg/mL)	Mice (*n*)	hAlb (mg/mL)	Chimerism (%)
39	0.2	5	0.8	13.4
26	0.7	14	1.6	22.5
29	1.5	8	3.3	41.9
18	3.2	10	4.5	55.7
8	4.7	1	6.9	83.1
3	5.7	5	7.8	93.4
Total, 123	Average, 1.5	Total, 43	Average, 3.3***	Average, 42.5

The amount of human albumin (hAlb) in plasma and extent of human liver replacement was measured after TK-NOG mice were transplanted with human liver cells using non-optimized pilot or optimized protocols. The extent of human liver chimerism was estimated as a function of the hAlb concentration, which was shown to correlate with the extent of human liver replacement. Protocol optimization (age at time of transplantation and GCV regimen) significantly increased the extent of humanization relative to that obtained in the pilot studies.
*** Mann–Whitney test, difference of $P < 0.0001$.

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