Liver Xeno-Repopulation with Human Hepatocytes in *Fah*^{-/-}*Rag2*^{-/-} Mice after Pharmacological Immunosuppression

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Functional human hepatocytes xeno-engrafted in mouse liver can be used as a model system to study hepatitis virus infection and vaccine efficacy. Significant liver xeno-repopulation has been reported in two kinds of genetically modified mice that have both immune deficiency and liver injury-induced donor hepatocyte selection: the *uPA/SCID* mice and *Fah*^{-/-}*Rag2*^{-/-}*Il2rg*^{-/-} mice. The lack of hardy breeding and the overly elaborated technique in these two models may hinder the potential future application of these models to hepatitis virus infection and vaccination studies. Improving the transplantation protocol for liver xeno-repopulation from human hepatocytes will increase the model efficiency and application. In this study, we successfully apply immunosuppressive drug treatments of anti-asialo GM1 and FK506 in *Fah*^{-/-}*Rag2*^{-/-} mice, resulting in significant liver xeno-repopulation from human hepatocytes and human fetal liver cells. This methodology decreases the risk of animal mortality during breeding and surgery. When infected with hepatitis B virus (HBV) sera, *Fah*^{-/-}*Rag2*^{-/-} mice with liver xeno-repopulation from human hepatocytes accumulate significant levels of HBV DNA and HBV proteins. Our new protocol for humanized liver could be applied in the study of human hepatitis virus infection *in vivo*, as well as the pharma-

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Human hepatocytes xeno-engrafted into the liver of immunodeficient mice could be used as a model to study human hepatitis virus infection *in vivo* as well as the efficacy of potential vaccines.^{1–6} Engrafted human hepatocytes can be serially transplanted from primary mice into secondary mice without losing hepatic function.⁷ Mouse recipients of human liver cells must have two capabilities: robust liver repopulation and immune tolerance for human hepatocytes. Liver xeno-repopulation from human hepatocytes was first reported in *uPA/Rag2*^{-/-} mice¹ and *uPA/SCID* mice.^{2–3,8} The levels of liver xeno-repopulation varies in several reports, ranging from 10% to as high as 90%.^{1,8} Humanized livers in *uPA/SCID* mice are susceptible to hepatitis B virus (HBV)^{1,2} and HCV^{3,4} infection. However, *uPA* mice have several disadvantages: i) neonatal death during colony breeding; ii) transplantation of hepatocytes into newborn mice (within the second week of life) is technically difficult due to a bleeding disorder in the mice; iii) there is uncontrollable selection for donor cells; iv) there is autoreversion of endogenous hepato-

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cytes; and v) kidney damage is induced by the human complement system.^{1,2,8,9}

Recently, robust liver xeno-repopulation from human hepatocytes was found in *Fah*^{-/-}*Rag2*^{-/-}*Il2rg*^{-/-} mice, cross-bred from *Fah*^{-/-} mice and *Rag2*^{-/-}*Il2rg*^{-/-} mice.⁷ *Fah*^{-/-}*Rag2*^{-/-}*Il2rg*^{-/-} mice have advantages over previous immunodeficient *uPA* models.⁷ First, *Rag2*^{-/-}*Il2rg*^{-/-} mice lack B, T, and NK cells, rendering more complete immunodeficiency compared with either *Rag2*^{-/-} or *SCID* mice.¹⁰ Second, liver injury in *Fah*^{-/-} mice is controllable by switching on and off 2-(2-nitro-4-trifluoromethyl-benzoyl)-1, 3 cyclohexanedione (NTBC) administration.¹¹ NTBC inhibits accumulation of toxic metabolites in hepatocytes to maintain *Fah*^{-/-} mice in a healthy state. When the NTBC is removed, a powerful selection for fumaryl acetoacetate hydrolase (*Fah*) expressing cells is induced in the liver.¹² However, maintenance of *Fah*^{-/-}*Rag2*^{-/-}*Il2rg*^{-/-} mice during colony breeding, animal growing, and cell transplantation surgery are with high mortality in our experiments. The genotyping of animal offspring is overly elaborate. These concerns have not been discussed in previous publications^{7,13} and may present a barrier to larger scale research projects.

In comparison, *Fah*^{-/-}*Rag2*^{-/-} mice were much more tolerant of breeding and surgery procedures. However, *Fah*^{-/-}*Rag2*^{-/-} mice were thought to have no capacity for liver xeno-repopulation, because their NK cells are intact.⁷ We hypothesized that treatment of *Fah*^{-/-}*Rag2*^{-/-} mice with anti-asialo GM1 could result in complete depletion of NK cells as seen in *Fah*^{-/-}*Rag2*^{-/-}*Il2rg*^{-/-} mice.^{14,15} We further tested the combined treatments of both anti-asialo GM1 and the immunosuppressor tacrolimus (FK506) to *Fah*^{-/-}*Rag2*^{-/-} mice.^{16,17} The results indicated that the combined treatments enabled *Fah*^{-/-}*Rag2*^{-/-} recipients to have a high level of liver xeno-repopulation by human hepatocytes as seen in *Fah*^{-/-}*Rag2*^{-/-}*Il2rg*^{-/-} mice. Our results revealed a new and easily controlled mouse model with humanized liver. Using the same treatments, liver xeno-repopulation with human fetal liver progenitor cells was also achieved in *Fah*^{-/-}*Rag2*^{-/-} mice. Finally, for the first time, we were able to prove that human HBV actively replicated in the humanized *Fah*^{-/-}*Rag2*^{-/-} mice and that viral proteins were released in the serum of humanized *Fah*^{-/-}*Rag2*^{-/-} mice, which showed no significant difference with previous reports of human HBV infection in humanized *uPA/SCID* mice.^{1,2,5}

Materials and Methods

Animal Cross-Breeding and Care

Fah^{-/-} mice were crossed with *Rag2*^{-/-}*Il2rg*^{-/-} mice (Taconic). Strains of both *Fah*^{-/-}*Rag2*^{-/-} mice and *Fah*^{-/-}*Rag2*^{-/-}*Il2rg*^{-/-} mice were obtained by gradual cross-breeding. PCR-based genotyping with primers for *Fah*,¹⁸ *Rag2*, and *Il2rg* genes¹⁰ were used to determine genotypes of offspring. Animals were maintained with drinking water containing NTBC at a concentration of 7.5 μ g/ml.

All mice were housed in individually ventilated cage (IVC) system under special pathogen-free (SPF) facility with barrier conditions, and animal care was in accordance with institutional guidelines.

Treatments with Anti-Asialo GM1

Anti-asialo GM1 (α AsGM1, 50 mg in 200 μ l saline, Wako) was i.p. injected into *Fah*^{-/-}*Rag2*^{-/-} mice 24 hours before cell transplantation and then at 7-day intervals after transplantation. Control mice were treated with nonspecific IgG (eBioscience, San Diego, CA).

Analysis of Immune Cells

Mice were sacrificed after anesthetization, and bone marrow cells from the femur were collected. Bone marrow cells (1×10^6 /tube) were incubated for 10 minutes at 4°C with Fc blocker to prevent nonspecific binding. The cells were then stained with FITC-conjugated anti-mouse CD3 mAb, FITC-conjugated anti-mouse CD19 mAb, PE-conjugated anti-NK1.1, and IgM mAb (all Abs from eBioscience). Percentage of fluorescence-positive cells was analyzed using FACSCalibur (Becton-Dickinson, Franklin Lakes, NJ).

Isolations of Human Hepatocytes and Fetal Liver Cells

Human liver tissue was provided by University of Minnesota Medical Center, Fairview (Minnesota) and Eastern Hepatobiliary Surgery Hospital, Second Military Medical University (Shanghai, China) from donor livers that had been reduced in size for allotransplantation. The procedure for preparation of human hepatocytes for transplantation into mice was performed under institutional guidelines. Human fetal liver tissues were derived from the first- and second-trimester fetuses between 98 and 116 days of gestation from Changhai hospital, Second Military Medical University (Shanghai, China). All human tissues were negative for HBV infection. Patients gave written, informed consent. Experiments were approved by Ethical Committee on Ethics of Biomedicine Research, Second Military Medical University. The approved IRB numbers are 0608M91366 (University of Minnesota) and 2007LL006 (Second Military Medical University).

Human hepatocytes were isolated as described previously.² Human fetal liver tissues were cut and digested by collagenase D (2.5 mg/ml, Roche, Basel, Switzerland) for 20 minutes at 37°C. Digested cells were filtered through a 70 μ m nylon mesh. E-cadherin-positive (E-Cad⁺) cells were enriched as previously.¹⁹ The cells positive for E-cadherin and high SSC were sorted by FACS Vantage (Becton-Dickinson) using Rat anti-human E-cadherin (eBioscience).

Cell Transplantation and Serial Transplantation

Human hepatocytes (3×10^5) or human fetal cells were injected into the spleens of mice recipients. Seven days

before cell transplantation, NTBC in drinking water was reduced to 50% of the original level for three days and further reduced to 25% for two days. Then, NTBC was totally discontinued two days before cell transplantation.

In serial transplantation, the engrafted human hepatocytes in chimeric liver of recipients were isolated after liver perfusion.^{7,12} The recipients were first examined for liver repopulation by biopsy of liver tissue. Harvested liver sections were stained for FAH expression by immunohistochemical analysis. Recipients with the highest level of liver repopulation were selected for collection of donor cells for secondary transplantation. The percentage of FAH-positive hepatocytes was used as a reference to estimate the actual number of human hepatocytes in the prepared cell suspension.

FK506 Treatment

FK506 (Astellas, Dublin, Ireland) dissolved in the drinking water at 7.5 $\mu\text{g}/\text{ml}$ was administered to adult mouse to achieve a dose of 1 $\mu\text{g}/\text{g}$ body weight per day. A separate group of control mice received only sterilized water. FK506 blood concentration was measured by microparticle enzyme immunoassay (MEIA, Abbott Laboratories, Alameda, CA).

Analysis of Human-Specific Hepatic Proteins in Serum

Blood was collected from the retro orbital vein of anesthetized mice. Kits of Human albumin (hAlb) ELISA Quantitation (Bethyl, Montgomery, TX) and Human α 1-Antitrypsin (hAAT) ELISA Quantitation (GenWay, San Diego, CA) were used to measure human proteins according manufacturer's protocols.

For molecular assay, Human Alu sequences in liver of chimeric mice were amplified by PCR as described previously,¹ and real-time PCR of human Alu and AAT gene were conducted as described in previous publication.^{3,20}

Infection and Analysis of HBV

Human serum containing a high HBV DNA content (10^8 IU/ml) was obtained from a HBV chronic carrier. Six weeks after human hepatocyte transplantation, chimeric *Fah*^{-/-}*Rag2*^{-/-} mice were infected with HBV by i.p. injection of 100 μl of above serum. Viral DNA was extracted from HBV-infected chimeric mice sera using the QIAamp Blood Kit (Qiagen, Hilden, Germany), and quantification of HBV-DNA was analyzed using real-time PCR (LightCycler, Basel, Switzerland). Known amounts of cloned HBV DNA were amplified in parallel to establish a standard bar for quantification. HBsAg and HBeAg in sera of HBV-infected chimeric mice were determined by Electrical chemiluminescence immunoassay analysis method (MODULAR ANALYTICS E170, Roche). Associated reagents for HBsAg and HBeAg determination are also manufactured by Roche. HBsAg and HBeAg were inter-

preted using the ratio of the sample signal to the cutoff signal (S/CO), and S/CO ≥ 1.00 was positive.

Immunohistochemical Analysis

Five- μm -thick sections were examined by immunohistochemistry with rabbit anti FAH antibody (AbboMax, San Jose, CA). Human cells within the mice livers were analyzed with a polyclonal antibody against human-specific albumin (Bethyl) and α 1-Antitrypsin (Thermo, Freemont, CA). Expression of HBV proteins was assessed using a polyclonal rabbit antibody against HBcAg (Dako, Copenhagen, Denmark) and a polyclonal goat antibody against HBsAg (Dako). Positive controls from clinical biopsies were stained with these antibodies, and normal liver biopsies as negative controls.

Biochemical Analysis of Liver Metabolic Function

Blood was collected from the retro-orbital sinus of test animals. Plasma was prepared using Microtainer plasma separator tubes (Becton-Dickinson) and stored at -80°C . Biochemical evaluation of liver function was performed as previously described.²¹

Calculations of Liver Repopulation and Statistical Analysis

Calculations of sample size, cell numbers, and percentage of repopulation were performed as previously described.²² Excel was used to calculate average $\pm$ SD. The statistical significance of difference between sample groups was calculated by Student's *t*-test. *P* values < 0.05 were regarded as statistically significant.

Results

*Human Hepatocyte Engraftment in *Fah*^{-/-}*Rag2*^{-/-}*Il2rg*^{-/-} Mice*

We determined the capacities of repopulation from human hepatocytes in *Fah*^{-/-}*Rag2*^{-/-}*Il2rg*^{-/-} mice. Human hepatocytes (3×10^5) were injected into spleen, and total NTBC withdrawal lasted for 12 weeks. Recipients were sacrificed, and liver samples were harvested for detection of human hepatocyte engraftment. *Fah*^{-/-}*Rag2*^{-/-}*Il2rg*^{-/-} recipients on NTBC were as controls. FAH antibody immunostaining indicated that liver samples from 5 of 8 *Fah*^{-/-}*Rag2*^{-/-}*Il2rg*^{-/-} recipients had FAH-positive hepatocytes at levels from 3.1% to 71.3% of total hepatocytes (Figure 1A, Supplemental Table 1 at <http://ajp.amjpathol.org>). However, the *Fah*^{-/-}*Rag2*^{-/-}*Il2rg*^{-/-} mouse breeders have a very small litter size in our colony breeding process (Supplemental Figure 1 at <http://ajp.amjpathol.org>). For this reason, *Fah*^{-/-}*Rag2*^{+/-}*Il2rg*^{+/-} mice could be used as breeders to generate *Fah*^{-/-}*Rag2*^{-/-}*Il2rg*^{-/-} mice. Number of *Fah*^{-/-}*Rag2*^{-/-}

Figure 1. Depletion of NK cells by anti-asialo GM1 enabled liver xeno-repopulation from human hepatocytes in *Fah*^{-/-} *Rag2*^{-/-} mice. Comparison of FAH-positive hepatocytes found in *Fah*^{-/-} *Rag2*^{-/-} *Il2rg*^{-/-} recipients (A; original magnification, ×100), *Fah*^{-/-} *Rag2*^{-/-} recipients (B; original magnification, ×100), and *Fah*^{-/-} *Rag2*^{-/-} recipients with treatment of αAsGM1 (C; original magnification, ×100). *Fah*^{-/-} *Rag2*^{-/-} recipients gained the capacity of liver xeno-repopulation from human hepatocytes after treatment of αAsGM1. D: i.p. injection of αAsGM1 significantly depleted the NK cells (labeled by CD3⁺ NK1.1⁺ in FACS) in *Fah*^{-/-} *Rag2*^{-/-} mice (Fah, *Fah*^{-/-} mice; FRG, *Fah*^{-/-} *Rag2*^{-/-} *Il2rg*^{-/-} mice; FR, *Fah*^{-/-} *Rag2*^{-/-} mice; αAsGM1, anti-asialo GM1).

Il2rg^{-/-} mice was much lower than expected, and only between 1 in 30 and 1 in 20 offspring had the triple homozygous mutant genotype. The pups with genotype of *Fah*^{-/-} *Rag2*^{-/-} *Il2rg*^{-/-} were also less fit than *Fah*^{-/-} and *Fah*^{-/-} *Rag2*^{-/-} littermates. They had about 60% more death during colony breeding and after surgery for cell transplantation (Supplemental Figure 2 at <http://ajp.amjpathol.org>). Because of the significantly low numbers of *Fah*^{-/-} *Rag2*^{-/-} *Il2rg*^{-/-} mice during colony breeding and the significant numbers of *Fah*^{-/-} *Rag2*^{-/-} *Il2rg*^{-/-} recipients lost after cell transplantation, *Fah*^{-/-} *Rag2*^{-/-} *Il2rg*^{-/-} mice could not be used for efficient generation of humanized mice. Thus, we chose the *Fah*^{-/-} *Rag2*^{-/-} mouse to study the capacity for human hepatocyte repopulation after pharmacological treatment to deplete NK cells.

Human Hepatocyte Engraftment in *Fah*^{-/-} *Rag2*^{-/-} Mice after NK Cell Depletion

Using the same experimental protocol and under same conditions, we determined the capacities of repopulation from human hepatocytes in *Fah*^{-/-} *Rag2*^{-/-} mice. *Fah*^{-/-} *Rag2*^{-/-} recipients on NTBC were used as controls that were free of induced liver injury. Three of 17 *Fah*^{-/-} *Rag2*^{-/-} recipients were found with detectable FAH-positive hepatocytes, existing in single cells and small nodules (Figure 1B; Supplemental Table 1 at <http://ajp.amjpathol.org>). Xeno-engraftment of human hepatocytes in *Fah*^{-/-} *Rag2*^{-/-} recipients was not successful in a previous report.⁷ Without treatment of gradual withdrawal of NTBC before cell transplantation, but instead with abrupt removal of NTBC after cells were trans-

planted, none of eight *Fah*^{-/-} *Rag2*^{-/-} *Il2rg*^{-/-} and eight *Fah*^{-/-} *Rag2*^{-/-} recipients had any detectable FAH positive hepatocytes (Supplemental Table 1 at <http://ajp.amjpathol.org>). In addition, eight *Fah*^{-/-} *Rag2*^{-/-} *Il2rg*^{-/-} and eight *Fah*^{-/-} *Rag2*^{-/-} recipients that were always kept on NTBC had no detectable FAH positive hepatocytes (Supplemental Table 1 at <http://ajp.amjpathol.org>).

In comparison with *Fah*^{-/-} *Rag2*^{-/-} *Il2rg*^{-/-} mice, *Fah*^{-/-} *Rag2*^{-/-} mice have significant levels of NK cells (Figure 1D). Anti-asialo GM1 was known from previous reports to deplete NK cells.^{15,16} At 24 hours after i.p. injection of anti-asialo GM1, the level of NK cells labeled by NK1.1⁺CD3⁻ was reduced to 5.6% of the original level in *Fah*^{-/-} *Rag2*^{-/-} mice (Figure 1D). After anti-asialo GM1 treatment, *Fah*^{-/-} *Rag2*^{-/-} mice were transplanted by human hepatocytes (3 × 10⁵) and selection was performed, sample harvest and FAH immuno-assay performed as before. Results indicated that samples from 10 of 18 *Fah*^{-/-} *Rag2*^{-/-} recipients had FAH-positive hepatocytes ranging from 0.1% to 16.2% of total hepatocytes at six weeks and from 3.4% to 31.7% at twelve weeks (Figures 1C and 2, A, B, and E). *Fah*^{-/-} *Rag2*^{-/-} mice after treatment with anti-asialo GM1 gained capacity for liver xeno-repopulation with human hepatocytes. However, the levels of xeno-liver repopulation in *Fah*^{-/-} *Rag2*^{-/-} recipients were still lower than those in *Fah*^{-/-} *Rag2*^{-/-} *Il2rg*^{-/-} recipients.

Enhanced Level of Liver Xeno-Repopulation after Treatment of FK506

FK506 is well known for its effects on immunosuppression.¹⁶ FK506 was also found to induce hepatocyte proliferation and promote liver regeneration in partial hepatectomized rats.¹⁷ We tested the potential effect of FK506 on promoting liver xeno-repopulation in *Fah*^{-/-} *Rag2*^{-/-} recipients, along with treatment of anti-asialo GM1. FK506 was administered to mouse recipients at a dose of 1 μg/g body weight per day. FK506 levels in serum sample of recipients were measured as 7.6 ± 2.3 ng/ml, which was within the expected therapeutic window referenced in clinic orthotopic liver transplantation. FAH immuno-assay indicated that engrafted FAH positive hepatocytes reached as high as 67.2% (ranging from 1.3% to 27.8% at 6 weeks and from 9.8% to 67.2% at 12 weeks) in *Fah*^{-/-} *Rag2*^{-/-} recipients with combined treatments of anti-asialo GM1 and FK506 (Figure 2C-E). In comparison, engrafted FAH positive hepatocytes only reached to 31.7% in *Fah*^{-/-} *Rag2*^{-/-} mice treated with anti-asialo GM1 alone (Figure 2, A, B, and E).

A few engrafted human hepatocyte nodules could be found in *Fah*^{-/-} *Rag2*^{-/-} mouse recipients treated with FK506 alone. These nodules were found to be larger in size than in mice treated with anti-GM1 alone (Supplemental Figure S3 at <http://ajp.amjpathol.org>), which is consistent with previous reports that FK506 treatment promotes hepatocyte proliferation.

Figure 2. Enhanced level of liver xeno-repopulation after treatment of FK506. Liver repopulation of *Fab*^{-/-}*Rag2*^{-/-} mice on 6 w (A; original magnification, ×100) and 12 w (B; original magnification, ×100) after human hepatocyte transplantation without the treatment of FK506; Liver repopulation of FR mice at 6 w (C; original magnification, ×100) and 12 w (D; original magnification, ×100) with the treatment of FK506. E: Comparison of liver repopulation of FR mice at 6 w and 12 w with and without the treatment of FK506.

Human Fetal Liver Cells in Xeno-Engraftment

Based on a previous method¹⁹ for isolation of repopulating fetal liver progenitor cells, we enriched for E-cadherin positive (E-Cad⁺) human fetal liver cells using FACS cell sorting (Figure 3, A and B). E-Cad⁺ cells (3×10^5) were transplanted into liver of *Fab*^{-/-}*Rag2*^{-/-} recipients. Treatments of anti-asialo GM1 and FK506 were performed as before. At 6 and 12 weeks after cell transplantation, we found Fah-positive hepatocytes as individual single cells and nodules together within the harvested liver samples (Figure 3C). The averages of cell replacement by Fah-positive hepatocytes were 2.8% at 6 weeks (6.4% at utmost) and 6.8% at 12 weeks (13.2% at utmost), respectively (Figure 3G). Therefore, human fetal liver cells could xeno-engraft into liver of *Fab*^{-/-}*Rag2*^{-/-} mice for liver xeno-repopulation.

Livers of primary recipients with high level of repopulation (>10% FAH⁺ hepatocyte on sections) were perfused to isolate hepatocytes. The level of FAH⁺ hepatocytes in total isolated hepatocytes was detected by FAH antibody immuno-staining on cytospin slides. Results indicated that two values about FAH⁺ hepatocytes were $12.43 \pm 2.23\%$ and $11.22 \pm 3.12\%$. Based on this information, we transplanted 3×10^5 FAH⁺ hepatocytes into

Figure 3. Human fetal liver cells have capacity for xeno-engraftment. A: Percentage of E-Cad⁺ cells in isolated human fetal liver cells by FACS. B: Percentage of E-Cad⁺ cells after enrichment by FACS; FAH (C; original magnification, ×100) and ALB (D; original magnification, ×100) positive hepatocytes found in *Fab*^{-/-}*Rag2*^{-/-} recipients after primary transplantation of E-Cad⁺ cells. FAH (E; original magnification, ×100) and ALB (F; original magnification, ×100) positive hepatocytes found in *Fab*^{-/-}*Rag2*^{-/-} recipients after second transplantation, complete hepatic differentiation of liver progenitor cells during liver repopulation was strongly supported by serial transplantation. G: Liver repopulation of primary transplantation and second transplantation.

each secondary *Fab*^{-/-}*Rag2*^{-/-} recipients. Treatments of anti-asialo GM1 and FK506 were performed as before. At 6 and 12 weeks, liver samples of recipients were harvested and analyzed. The average levels of FAH⁺ hepatocytes were 8.6% (14.6% at utmost) at 6 weeks and 26.4% (46.7% at utmost) at 12 weeks (Figure 3, E and G). These levels were similar to the levels achieved using adult human hepatocytes. We further confirmed liver xeno-repopulation from human hepatocytes using immunohistochemical analysis with antibody against human albumin. The levels of human albumin-positive hepatocytes were found to be similar to the levels of FAH⁺ hepatocytes in both primary (Figure 3, C and D) and secondary *Fab*^{-/-}*Rag2*^{-/-} recipients (Figure 3, E and F). Thus the FAH⁺ hepatocytes were proven to be human hepatocytes.

Figure 4. Engraftment and hepatic function of human hepatocytes after liver xeno-repopulation. Serial staining of human specific Alb Ab (A; original magnification, $\times 200$), and AAT Ab (B; original magnification, $\times 200$) on liver specimen of chimeric mouse. C: *Fah*^{-/-} *Rag2*^{-/-} *Il2rg*^{-/-} mice (no.252) and *Fah*^{-/-} *Rag2*^{-/-} mice with α AsGM1 and FK506 treatment (no.229) gradually recovered their weight 5 w after human hepatocyte transplantation, whereas *Fah*^{-/-} *Rag2*^{-/-} mice without α AsGM1 and FK506 treatment (no.228) lost weight continuously after NTBC withdrawal. Expression of human albumin (D) and human $\alpha 1$ -anti-trypsin (E) in chimeric mouse sera indicated human hepatocyte functionality after xeno-repopulation (c, negative control).

Hepatic Function of Human Hepatocytes after Liver Xeno-Repopulation

The recovery of hepatic and metabolic function could be reflected by body weight as a parameter of health status.¹¹ We have found that *Fah*^{-/-} *Rag2*^{-/-} recipients with high levels of repopulation gradually gained normal weight by 5 weeks after human hepatocyte transplantation (Figure 4C). By serial staining for hAlb and hAAT with a specific antibody we confirmed the human origin of engrafted hepatocytes and the synthetic function of human hepatocytes (Figure 4, A and B).

Metabolic parameters were used to analyze the recovery of metabolic functions from the replaced human hepatocytes in mouse recipients. Expression of hAlb and hAAT in serum of mouse recipients indicated the synthetic function of human hepatocytes was intact after

xeno-repopulation (Figure 4, D and E). As summarized in Table 1, analysis of the serum of the recipients indicated recovery of metabolic functionality after liver xeno-repopulation.

The presence of human hepatocytes in *Fah*^{-/-} *Rag2*^{-/-} mice was further proven by a molecular assay. We used PCR and real-time PCR assays to detect human *Alu* gene-specific DNA sequences and human *AAT* gene sequences in the chimeric livers (Supplemental Figure S4 at <http://ajp.amjpathol.org>).

HBV Infection of *Fah*^{-/-} *Rag2*^{-/-} Mice after Liver Xeno-Repopulation

HBV infection is one of the most sensitive markers for function of human hepatocytes. The *Fah*^{-/-} *Rag2*^{-/-} re-

Table 1. Biochemical Analysis of Metabolic Function after Human Hepatocyte Transplantation

Liver function parameter	Wild-type mice	<i>Fah</i> ^{-/-} <i>Rag2</i> ^{-/-} mice without liver repopulation	<i>Fah</i> ^{-/-} <i>Rag2</i> ^{-/-} mice with >20% liver repopulation	<i>Fah</i> ^{-/-} <i>Rag2</i> ^{-/-} mice with versus without repopulation
FAH activity	68 $\pm$ 7.6	0.2 $\pm$ 0.15	45.6 $\pm$ 6.3	$P < 0.000001$
ALT	55 $\pm$ 15.1	650 $\pm$ 173	62.3 $\pm$ 5.8	$P < 0.00001$
Total bilirubin	0.116 $\pm$ 0.02	7.65 $\pm$ 1.13	0.12 $\pm$ 0.03	$P < 0.000001$
Creatinine	0.21 $\pm$ 0.08	1.76 $\pm$ 0.28	0.32 $\pm$ 0.06	$P < 0.000001$
Tyrosine	74.5 $\pm$ 36.2	1147 $\pm$ 250	110.4 $\pm$ 42.3	$P < 0.000001$
Phenylalanine	63 $\pm$ 12.8	301 $\pm$ 76	88 $\pm$ 13.5	$P < 0.00001$
Methionine	65 $\pm$ 15	307 $\pm$ 54	93 $\pm$ 16.9	$P < 0.000001$
Glutamine	345 $\pm$ 121	4757 $\pm$ 1483	392 $\pm$ 112	$P < 0.005$
Glycine	245 $\pm$ 113	4333 $\pm$ 2344	282 $\pm$ 128	$P < 0.001$

The values $\pm$ SD are given. The units are μ mol/min per gram for FAH enzyme activity, U/L for ALT (alanine aminotransferase), total bilirubin, and creatinine, μ mol/L for amino acid levels. P values were calculated using the two-tailed t -test.

Figure 5. Expression of HBV proteins and quantification of HBV DNA in transplanted mice. Alb staining of functional human hepatocytes in high liver xeno-repopulation (**A**; original magnification, $\times 40$; **B**; original magnification, $\times 100$); HBS staining of functional human hepatocytes infected with HBV serum (**C**; original magnification, $\times 100$; **D** is an enlargement of **C**); HBC staining of functional human hepatocytes infected with HBV serum (**E**; original magnification, $\times 100$; **F** is an enlargement of **E**). **G**: Quantification of HBV DNA in sera of nontransplanted and transplanted mice at 8 and 14 weeks postinfection. Samples of 1, 2, 5, 6: nontransplanted *Fah*^{-/-}*Rag2*^{-/-} mice (Samples of 1, 2: 8 weeks postinfection; Samples of 5, 6: 14 weeks postinfection) were used as control, showing the negative results of viral DNA existence. Samples of 3, 4, 7, 8: *Fah*^{-/-}*Rag2*^{-/-} mice engrafted with human hepatocytes (Samples of 3, 4: 8 weeks postinfection; Samples of 7, 8: 14 weeks postinfection), showing the significant levels of HBV DNA in sera. **H** and **I**: The significant levels of both HBsAg and HBeAg were found in sera of infected mice recipients engrafted with human hepatocytes (Samples of 1, 2: nontransplanted *Fah*^{-/-}*Rag2*^{-/-} mice; Samples of 3, 4: *Fah*^{-/-}*Rag2*^{-/-} mice engrafted with human hepatocytes; S/CO ≥ 1.00 was positive).

recipients with high levels of xeno-repopulation, as determined by expression of human albumin in liver biopsies (Figure 5, A and B), were infected with HBV serum. Two *Fah*^{-/-}/*Rag2*^{-/-} mice with humanized livers and two

Fah^{-/-}*Rag2*^{-/-} mice littermates without transplantation were injected with HBV-positive human serum. At 8 and 14 weeks postinfection, increasingly positive levels of HBV DNA were detected in the sera of two *Fah*^{-/-}*Rag2*^{-/-} mice with humanized livers (3.0 and 0.9×10^3 IU/ml at eight weeks, and 2.16 and 0.64×10^6 IU/ml at 14 weeks), but no viral DNA was detectable in nontransplantation control mice that were also injected with the HBV-positive serum (Figure 5G). The absence of HBV-DNA in controls demonstrated that the positive result was not due to the original injection and that mice without human hepatocytes are not susceptible to HBV infection. At the same time, immunohistochemistry for HBsAg and HBeAg expression were systematically performed on liver specimens of transplanted and infected mice. HBsAg was distributed mainly in the cytoplasm of human hepatocytes (Figure 5, C and D), and HBeAg was distributed mainly in nuclear and occasionally some cytoplasm (Figure 5, E and F). Furthermore, HBsAg and HBeAg proteins were found in sera of infected chimeric mice (Figure 5, H and I), which suggested that viral proteins in human hepatocytes of *Fah*^{-/-}*Rag2*^{-/-} mice could be released into blood of the chimeric mice.

Discussion

In comparison with other mouse models that have been used to generate humanized livers, *Fah*^{-/-}*Rag2*^{-/-} mice have several advantages including easier genotyping and the low mortality during colony breeding and cell transplantation. Thus, *Fah*^{-/-}*Rag2*^{-/-} mice were chosen by us to test the possibility of liver xeno-repopulations by human hepatocytes. We enhanced immunodeficiency in *Fah*^{-/-}*Rag2*^{-/-} mice by pharmacological treatments to deplete NK cells at the time of cell transplantation, which made robust liver xeno-repopulation possible.

We selected the immunosuppressors anti-asialo GM1 and FK506 based on the spectrum of immunodeficiency reported in previous studies.^{14,15,17,23} We found that pharmacological depletion of NK cells by anti-asialo GM1 treatment was sufficient for liver xeno-repopulation in *Fah*^{-/-}*Rag2*^{-/-} mice. However, the level of xeno-repopulation was not comparable to those found in *Fah*^{-/-}*Rag2*^{-/-}*Il2rg*^{-/-} mice. By using combined treatments of both anti-asialo GM1 and FK506, we successfully obtained robust levels of liver xeno-repopulation in *Fah*^{-/-}*Rag2*^{-/-} mice, which were similar to the levels found in *Fah*^{-/-}*Rag2*^{-/-}*Il2rg*^{-/-} mice. Our results suggested that *Fah*^{-/-}*Rag2*^{-/-} mice with depletion of NK cells by anti-asialo GM1 were still not comparable to *Fah*^{-/-}*Rag2*^{-/-}*Il2rg*^{-/-} mice, and that only with the combined treatments of anti-asialo GM1 and FK506 did *Fah*^{-/-}*Rag2*^{-/-} mice reach a sufficient level of immunodeficiency.

An adenoviral vector carrying uPA is required for liver xeno-repopulation of *Fah*^{-/-}*Rag2*^{-/-}*Il2rg*^{-/-} mice.⁷ The mechanism of action of the adenovirus in encouraging xeno-repopulation was not investigated in the publication. In fact, it made the model more complicated and adds to the disadvantages of the model for the large-scale applications. Adenoviral vector-mediated gene de-

livery might influence the capacity for HBV infection of humanized mice. In our study, the gradual removal of NTBC is required for liver xeno-repopulation with human hepatocytes in both *Fah*^{-/-}*Rag2*^{-/-}*Il2rg*^{-/-} mice and *Fah*^{-/-}*Rag2*^{-/-} mice. In comparison, *Fah*^{-/-}*Rag2*^{-/-}*Il2rg*^{-/-} recipients with immediate total withdrawal of NTBC after cell transplantation had no xeno-repopulation. Liver injury induced by gradual removal of NTBC might mimic that induced by uPA carried adenoviral vector.⁷ Gradual removal of NTBC might induce a mild liver injury before cell transplantation, which may make the liver parenchyma suitable for donor hepatocyte engraftment and cell expansion. This could account for our finding of xeno-repopulation in *Fah*^{-/-}*Rag2*^{-/-} mice.

FK506 is often used during organ or tissue transplantation to inhibit immune-rejection.¹⁶ FK506 reduces macrophage recruitment, attenuates leukocyte accumulation, neutrophil infiltration, and activation of resident immunocompetent cells of hepatic NK cells.^{24,25} Besides immunosuppression, FK506 modulates liver responses by increasing expression of local mitogens such as insulin-like growth factor-I, increasing expression of insulin receptor, and decreasing production of inhibitory cytokines such as interleukin 2, to promote liver regeneration.^{17,26} Without treatment with Asialo-GM antibody, NK cells likely inhibit xeno-engraftment of human hepatocytes. We did find, however, that FK506 treatment alone could promote the proliferation of engrafted hepatocytes in nodules, which were significantly enlarged compared with treatment without FK506. We found the highest levels of liver xeno-repopulation in *Fah*^{-/-}*Rag2*^{-/-} recipients treated with both anti-asialo GM1 and FK506. The mechanism of FK506 in promoting liver repopulation will be analyzed in a future study. We have found that FK506 can also enhance liver xeno-repopulation in *Fah*^{-/-}*Rag2*^{-/-}*Il2rg*^{-/-} mice (data not shown).

Human liver progenitor cells without complete hepatic differentiation could be used directly to generate humanized livers in *Fah*^{-/-}*Rag2*^{-/-} mice. However, the level of liver xeno-repopulation was significantly lower than that from human adult hepatocytes. Our result suggested the possible disadvantage of using human liver progenitor cells in liver xeno-repopulation, which was different from previous findings in partially-hepatectomized mice or rat recipients.^{19,27-29} In partially-hepatectomized recipients, mouse or rat mature hepatocytes had no capacity of liver repopulation, while mouse or rat fetal liver progenitor cells had significant capacity of liver repopulation.^{19,27-29} However, the mechanism of liver injury in *Fah*^{-/-} mice was different from that in partially-hepatectomized mice. The complete hepatic differentiation of human liver progenitor cells happened during liver repopulation in *Fah*^{-/-} mouse recipients. The successful liver xeno-repopulation from human fetal liver progenitor cells in *Fah*^{-/-}*Rag2*^{-/-} recipients supplies us with a useful model to investigate the *in vivo* mechanism of human liver progenitor cells in engraftment, differentiation, and cell expansion.

The generation of humanized *Fah*^{-/-}*Rag2*^{-/-} mice facilitated the study of human hepatotropic viruses, such as HBV. In the present study, actively replicating HBV was

found in liver-humanized *Fah*^{-/-}*Rag2*^{-/-} mice after inoculation with the virus. Because of the advantages of generating humanized liver in *Fah*^{-/-}*Rag2*^{-/-} mice and our results showing successful HBV infection, replication, and release, our new protocol will be useful for the future study of HBV.

In summary, we successfully apply the combined treatments of anti-asialo GM1 and FK506, resulting in significant liver xeno-repopulation from human hepatocytes and human fetal liver cells in *Fah*^{-/-}*Rag2*^{-/-} mice. When infected with HBV serum, HBV DNA and HBV protein could be detected in humanized *Fah*^{-/-}*Rag2*^{-/-} mice. Results indicated that *Fah*^{-/-}*Rag2*^{-/-} mice under new protocols could be used as a more practical humanized liver model in the study of human hepatitis virus infection *in vivo*, as well as for the study of pharmacokinetics and efficacy of potential vaccines.

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