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Xeno-repopulation of *Fah$^{-/-}$Nod/Scid* mice livers by human hepatocytes

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Functional human hepatocytes xenografted into the liver of mice can be used as a model system to study pharmacokinetics, infection of hepatitis viruses, and the efficacy of hepatitis vaccines. Significant levels of liver xeno-repopulation have been reported in *Fah$^{-/-}$Rag2$^{-/-}$Il2rg$^{-/-}$* mice. However, the high mortality and low breeding rate of this model may hinder its application. A new model, termed *Fah$^{-/-}$Nod/Scid* mice, which combines the advantages of liver repopulation in *Fah$^{-/-}$* mice with the ease of xenotransplantation in *Nod/Scid* mice was obtained by gradual cross-breeding. *Fah$^{-/-}$Nod/Scid* mice were easily maintained in breeding colonies and in adult animal care facilities. FK506 treatment combined with gradual withdrawal of NTBC before cell transplantation ensured that *Fah$^{-/-}$Nod/Scid* mice were susceptible to liver xeno-repopulation by human hepatocytes; the proportion of engrafted human hepatocytes reached 33.6%. The function of the expanded human hepatocytes within the chimeric liver was confirmed by weight curve analysis, the expression of characteristic proteins, and the biochemical analysis of liver function. These results show that *Fah$^{-/-}$Nod/Scid* mice are an ideal humanized liver mouse model with many useful applications.

human hepatocyte, humanized liver, cell transplantation, Fah gene knockout mice, Nod/Scid mice

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Functional human hepatocytes xenografted into the liver of mice can be used as a model system to study pharmacokinetics, infection by hepatitis viruses, and the efficacy of hepatitis vaccines [1–9]. Fumarylacetoacetate hydrolase gene knockout mice (*Fah$^{-/-}$* mice) have been established as a model for hereditary tyrosinemia type I (HT1) disease [10]. As one of the most recognized models for liver damage, *Fah$^{-/-}$* mice have been widely used to study liver repopulation by several types of cells [11,12]. For example, 100 adult mouse hepatocytes were able to reconstruct 5% of the cells within a whole liver (about 2×10^5 cells) and rebuild the structure and function of the hepatic lobules after cell transplantation and proliferation [11–13]. Studies show that *Fah$^{-/-}$Rag2$^{-/-}$Il2rg$^{-/-}$* mice lacking B, T and NK cells are susceptible to liver xeno-repopulation by human hepato-

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cytes [7–9]. However, there are some disadvantages to the *Fah*$^{-/-}$*Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mouse model, including high mortality rates during breeding, throughout life and after cell transplantation, which have presented a barrier to its larger scale application [9]. Previously, we successfully treated *Fah*$^{-/-}$*Rag2*$^{-/-}$ mice with the immunosuppressants, anti-asialo GM1 and FK506 to enable significant levels of liver xeno-repopulation by human hepatocytes and human fetal liver cells [9]. When infected with sera containing the hepatitis B virus (HBV), *Fah*$^{-/-}$*Rag2*$^{-/-}$ mice that have undergone liver xeno-repopulation by human hepatocytes develop significant levels of HBV DNA and HBV protein. This reduces the mortality during breeding and surgery. On the basis of the above work, and taking into account the fact that the anti-asialo GM1 antibody needed to inhibit NK cell function is expensive (and needs to be injected every 7 d), we committed to the further optimization of the humanized liver mouse model. Non-obese diabetic-SCID mice (Nod/Scid) have many advantages, such as T and B lymphocyte combined immune deficiency, low NK cell activity, a lack of circulating complement and macrophages, and antigen presenting cell dysfunction. They also breed normally [14]. Therefore, as *Nod/Scid* mice have both innate and acquired immune deficiency, are less prone to immune escape, and have higher rates of xeno-transplantation than *Scid* mice, they were considered to be the best model for xeno-transplantation.

Combining the advantages of high levels of liver repopulation seen in *Fah*$^{-/-}$ mice with the ease of xenotransplantation in *Nod/Scid* mice, we produced *Fah*$^{-/-}$*Nod/Scid* mice by a process of gradual cross-breeding. *Fah*$^{-/-}$*Nod/Scid* mice were easily maintained in breeding colonies and in adult animal care facilities and, as such, could provide a large number of homozygous mice for humanized mouse liver studies. FK506 treatment combined with gradual withdrawal of NTBC before cell transplantation enabled *Fah*$^{-/-}$*Nod/Scid* mice susceptible to liver xeno-repopulation by human hepatocytes (by up to 30%). The cellular function of the expanded human hepatocytes within the chimeric liver was confirmed by analysis of weight curves, the expression of characteristic proteins, and by biochemical analysis of liver function. The results show, for the first time, that *Fah*$^{-/-}$*Nod/Scid* mice are an ideal humanized liver model with many potential applications.

1 Materials and methods

1.1 Establishment of *Fah*$^{-/-}$*Nod/Scid* mice

Nod/Scid, *Fah*$^{-/-}$ (25–30 g, 129S4 strain) and 129S4 wild-type mice were raised in an individual ventilated cage system in the specific pathogen free grade animal room in the animal center of the Second Military Medical University. Conditions were circadian rhythm (7:00–19:00), free food and acidic water (pH 3.0). *Fah*$^{-/-}$ mice were given drinking water containing NTBC (7.5 μg mL^{-1}).

Fah$^{-/-}$ mice were crossed with *Nod/Scid* mice to establish the *Fah*$^{-/-}$*Nod/Scid* mouse strain. PCR-based genotyping for *Fah* and *Scid* was used to determine the genotypes of the offspring. The method for *Fah* genotyping has been described previously [10]. *Scid* genotyping was done as follows: primers (5′-GGAAAAGAATTGGTATCCAC-3′, 5′-AGTTATAACAGCTGGGTTGGC-3′). PCR conditions: 94°C for 30 s, 53°C for 30 s, 72°C for 30 s. The PCR products were digested overnight with *Alu* I, separated by electrophoresis on 20% polyacrylamide gels, dyed with 0.5 g mL^{-1} ethidium bromide solution and photographed. Digested wild-type products were 68 and 11 bp (the 11 bp band is not visible in the photograph), and the mutant products were 38, 28 and 11 bp (the 11 bp band is also not visible).

1.2 Analysis of immune cells

Four mice from each strain (*Fah*$^{-/-}$, *Fah*$^{-/-}$*Nod/Scid* and *Fah*$^{-/-}$*Rag2*$^{-/-}$*Il2rg*$^{-/-}$) were sacrificed under anesthesia. Bone marrow cells from the femurs were collected and the concentration adjusted to 1×10^6 cells/tube. After conventional treatment, the cells were incubated with anti-CD3, anti-CD19 and anti-NK1.1 primary antibodies (eBioscience) and expression detected by flow cytometry using the appropriate secondary antibodies (FITC-conjugated anti-mice CD3 mAb, FITC-conjugated anti-mice CD19 mAb, or PE-conjugated anti-NK1.1; eBioscience). Data from the *Fah*$^{-/-}$ mice were normalized to one.

1.3 Isolation of human hepatocytes

Human liver tissue was provided by Eastern Hepatobiliary Surgery Hospital, Second Military Medical University (Shanghai, China) from donor livers that had been reduced in size for allotransplantation. Clinical test results confirmed that markers for HBV, HCV, HIV and *Treponema pallidum* were all negative. Biopsy confirmed that these were normal liver tissues. The method used to separate human hepatocytes has been described previously [12]. Cell viability was determined by trypan blue staining.

1.4 Cell transplantation and FK506 treatment

Twenty *Fah*$^{-/-}$*Nod/Scid* mice (six mice in the control group) were used for cell transplantation. Seven days before transplantation, the level of NTBC in the drinking water was reduced to 50% for 3 d, and further reduced to 25% for 3 d. NTBC was discontinued 2 d before transplantation. This treatment induced liver injury ahead of schedule, and promoted the engraftment and proliferation of the transplanted cells.

FK506 (Astellas Ireland Co.; 7.5 μg mL^{-1}) was dissolved in the drinking water and administered to adult mice to achieve a dose of 1 μg g^{-1} body weight per day.

1.5 Molecular biology

For the molecular assays, the human *Alu* sequence within the chimeric liver was amplified by PCR as described previously [2]. To further determine whether the expanded human hepatocytes had normal cell function, Western blotting was performed to detect the expression of the Fah protein in the chimeric liver. The livers of 129S4 wild-type mice, *Fah*$^{-/-}$ mice and chimeric mice were collected and the liver proteins extracted and quantified. Total protein (50 μg) was separated on acrylamide gels, and transferred onto a nitrocellulose membrane. Blots were blocked with blotto (5% nonfat milk powder in PBS plus 0.1% Tween 20) for 1 h at room temperature. Fah primary antibody (1∶3000; Affinitein) was added for 1 h at room temperature. Blots were washed three times with PBS/0.1% Tween 20 before incubation with the secondary antibody (HRP conjugated goat anti-rabbit IgG, 1∶10000; Sigma) for 1 h. Blots were then washed briefly and proteins detected using an ECL kit (Abcam).

1.6 Hematoxylin & eosin (H & E) staining and immunohistochemical analysis

Transplanted mice underwent partial hepatectomy either 3 d or six weeks after cell transplantation, and were sacrificed after 12 weeks. Sections (4 μm thick) were examined by immunohistochemistry with antibodies to Fah, Alb, AAT, and by H & E staining. Briefly, liver samples were fixed in 4% paraformaldehyde and embedded in paraffin. Sections were dewaxed with xylene, rehydrated, incubated with primary antibodies (Fah, Affinitein, 1∶3000; hAlb, Bethyl, 1∶200; Alb, DAKO, 1∶200; or hAAT, Therm, 1∶1000) and then incubated with HRP-conjugated goat anti-rabbit IgG secondary antibodies, followed by detection with DAB (DAKO) and counterstaining with Mayer's hematoxylin solution (Sigma). For immunofluorescence, a fluorescence labeled secondary antibody (Donkey α Goat, 1∶800 or Donkey α Rabbit, 1∶800; Jackson) was added before mounting with DAPI mounting medium (Dapi-Fluoromount-G; SouthernBiotech). Sections were then observed under a fluorescence microscope.

1.7 Weight curve and biochemical analysis of liver metabolic function

Body weights before treatment were normalized to one, and a weight curve drawn 1–8 weeks after transplantation of human hepatocytes. Serum was collected from the retro orbital vein of anesthetized mice. Biochemical analysis of liver metabolic function was done as previously described [15]. Human albumin (hAlb) ELISA kits (Bethyl) were used to measure hAlb protein levels in accordance with the manufacturer's instructions.

1.8 Statistical analysis

The level of liver repopulation was evaluated using Adobe Photoshop CS3 software. Data were expressed as mean±SD. Comparison of data was done using the Student's *t*-test and statistical software (SPSS 10.0; SPSS Inc.). A *P*-value of <0.05 was considered statistically significant.

2 Results

2.1 Establishment of *Fah*$^{-/-}$*Nod/Scid* mice and analysis of immune cells

The results of the genotyping analysis of *Fah*$^{-/-}$*Nod/Scid* mice are shown in Figure 1A and B. *Fah*$^{+/-}$ mice had both the wild-type band (~180 bp) and the mutant band (~240 bp), while *Fah*$^{-/-}$ mice only had the mutant band (Figure 1A). The PCR products from the *Nod/Scid* gene were digested and electrophoresed through vertical strips. The wild-type product was 68 bp and the mutant products were 38 and 28 bp (Figure 1B).

Flow cytometry was used to detect the number of T cells ($CD3^+$), B cells ($CD19^+$) and NK cells ($CD3^-NK1.1^+$) in the *Fah*$^{-/-}$, *Fah*$^{-/-}$*Nod/Scid*, and *Fah*$^{-/-}$*Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice. Compared with the *Fah*$^{-/-}$ mice, *Fah*$^{-/-}$*Nod/Scid* mice lacked T cells, B cells and NK cells, signifying complete immune deficiency. The extent of immunodeficiency in *Fah*$^{-/-}$*Nod/Scid* mice was equal to that in the *Fah*$^{-/-}$*Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice (Figure 1C).

2.2 Liver xeno-repopulation in *Fah*$^{-/-}$*Nod/Scid* mice

Human hepatocytes (1×10^6) were injected into the spleens of 14 *Fah*$^{-/-}$*Nod/Scid* mice. Six *Fah*$^{-/-}$*Nod/Scid* mice were injected normal saline as the control group. Gradual withdrawal of NTBC one week before transplantation caused liver injury in *Fah*$^{-/-}$*Nod/Scid* mice and induced the engraftment of transplanted human hepatocytes. At the same time, mice were given FK506 to promote the proliferation of human hepatocytes. The Fah enzyme was detected in transplanted cells using immunohistochemistry to determine the level of liver repopulation in the *Fah*$^{-/-}$ mice.

No Fah-positive hepatocytes were found in the *Fah*$^{-/-}$*Nod/Scid* control group (Figure 2A). A few engrafted human hepatocytes were seen in the recipients in the experimental groups 3 d after transplantation. Fah-positive hepatocytes were observed as individual cells or nodules containing 2–4 cells (Figure 2B). A Fah immunoassay conducted six weeks after transplantation showed up to 8.2% engrafted Fah-

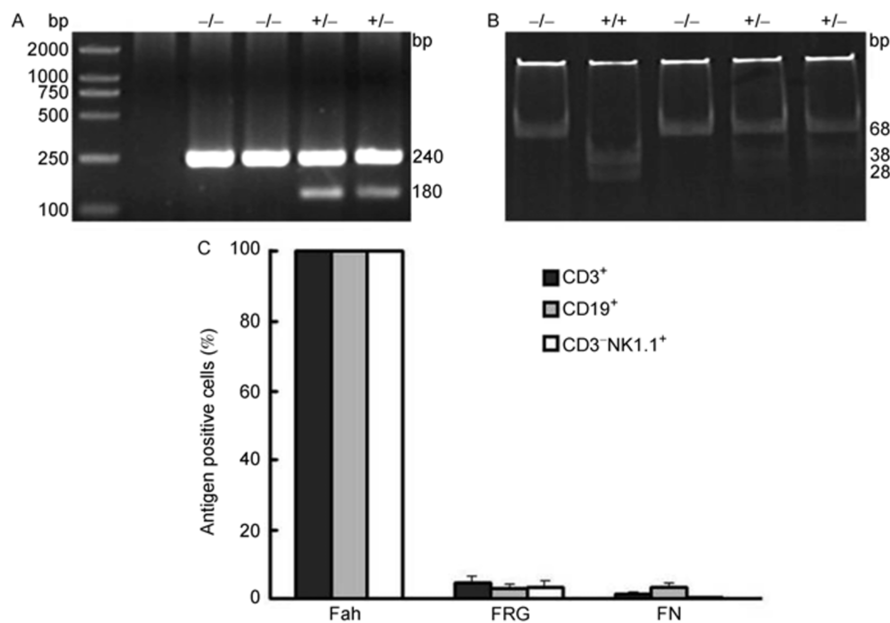

Figure 1 Establishment of *Fah*$^{-/-}$*Nod*/*Scid* mice strain and analysis of immune cells. A, *Fah* genotyping analysis. B, *Nod/Scid* genotyping analysis. C, Analysis of immune cells: *Fah*$^{-/-}$*Nod*/*Scid* mice lacked T cells, B cells and NK cells. Fah, *Fah*$^{-/-}$ mice; FRG, *Fah*$^{-/-}$*Rag2*$^{-/-}$*Il2rg*$^{-/-}$ mice; FN, *Fah*$^{-/-}$*Nod*/*Scid* mice.

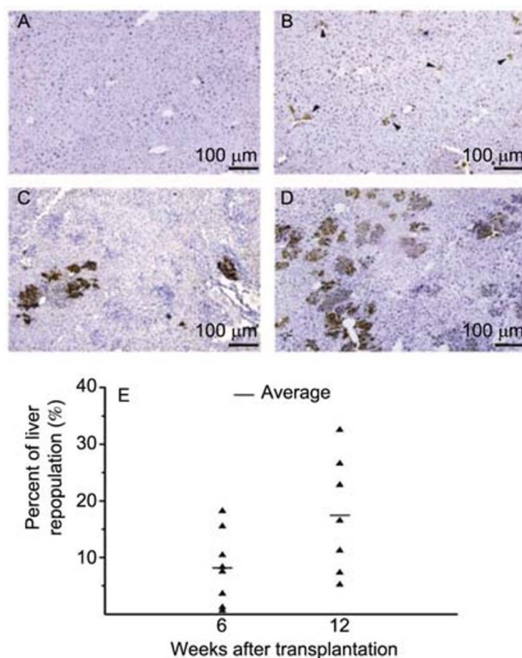

Figure 2 Liver xeno-repopulation in *Fah*$^{-/-}$*Nod*/*Scid* mice by human hepatocytes. A, No Fah-positive hepatocytes were found in the control group without cell transplantation. B, A few engrafted human hepatocytes were seen in the experimental groups 3 d after transplantation (arrows). C, Typical nodules formed by human hepatocytes were seen after six weeks. D, Liver xeno-repopulation in *Fah*$^{-/-}$*Nod/Scid* mice by human hepatocytes after 12 weeks. E, Comparison of liver repopulation in *Fah*$^{-/-}$*Nod*/*Scid* mice after six and 12 weeks.

positive hepatocytes (range, 0.6%–18.5%) (Figure 2C and E). This increased to 17.4% (range, 5.2%–33.6%) after 12 weeks (Figure 2D and E). Overall, chimerism was successfully induced in 57.1% (8/14) of the mice.

2.3 Assay of human hepatocytes within chimeric livers

A PCR assay was used to detect human *Alu* gene-specific DNA sequences. The *Alu* sequence (440 bp) was amplified from the chimeric livers, confirming the presence of human hepatocytes (Figure 3A). The Fah protein was confirmed in chimeric livers by Western blotting. No Fah protein was detected in the livers of *Fah*$^{-/-}$*Nod*/*Scid* mice that did not undergo hepatocyte transplantation. This shows that the Fah protein is expressed in engrafted human hepatocytes, which proliferated within the chimeric livers of *Fah*$^{-/-}$*Nod*/*Scid* mice (Figure 3B).

2.4 Human hepatocytes within chimeric livers are functional

Immunohistochemical staining for functional proteins expressed by human hepatocytes was carried out. A non-specific albumin (Alb) antibody (reacts with albumin in hepatocytes from both mice and humans), and human-specific (no cross reaction with murine hepatocytes) Alb and α-antitrypsin (AAT) antibodies were used. The results showed that human hepatocytes were successfully engrafted, and were proliferating and expressing functional proteins in the livers of *Fah*$^{-/-}$*Nod*/*Scid* mice (Figure 4A–C). This was further confirmed by serial staining using hAlb and hAAT antibodies (Figure 4D and E). H & E staining indicated that human hepatocytes contributed to the formation of the hepatic plate

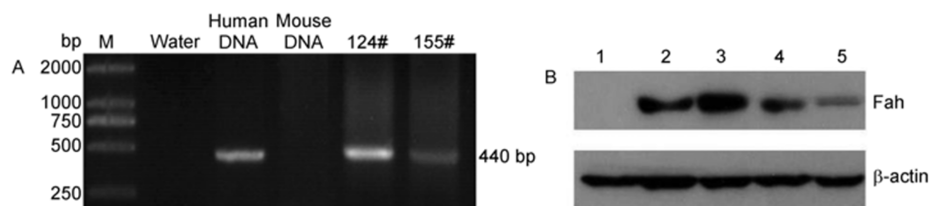

Figure 3 Assay of human hepatocytes in chimeric livers. A, Human *Alu* gene-specific DNA sequences (440 bp) were only amplified from chimeric livers (124#, 155#). B, Fah protein was identified in chimeric livers by Western blotting. Lane 1, $Fah^{-/-}$ mice; Lane 2, 129S4 wt mice; Lanes 3–5, chimeric liver mice with human hepatocytes (3, 124#; 4, 155#; 5, 163#).

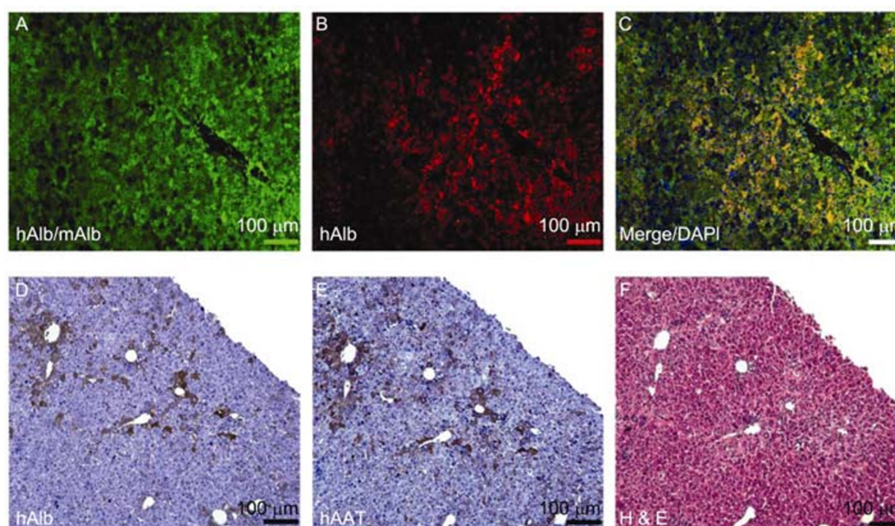

Figure 4 Albumin and α-antitrypsin are expressed in human hepatocytes. A, Non-specific immunofluorescence staining of albumin in mouse and human hepatocytes (green). B, Immunofluorescence staining of human-specific albumin in human hepatocytes (red). C, Merged picture of A and B and DAPI nuclear staining. Mice hepatocytes are green and human hepatocytes are yellow in the overlay. D and E, Serial staining of human specific albumin and α-antitrypsin in chimeric livers. F, H & E staining showed that the proliferated human hepatocytes participated in the composition of the hepatic plate and maintained the integrity of the liver lobules.

and maintained the integrity of the liver lobules (Figure 4F). As Fah contributes to the metabolism of tyrosine, its expression confirmed that the proliferated human hepatocytes had normal metabolic function (Figure 2B–D).

2.5 Liver function of humanized liver mice

$Fah^{-/-}$ mice were healthy and reproduced normally while treated with NTBC. Once NTBC was withdrawn, $Fah^{-/-}$ mice lost weight gradually and died of subacute liver failure after 3–6 weeks. The recovery of liver metabolic function in $Fah^{-/-}$ mice is reflected by a change in body weight [7,9]. Because the liver has a strong compensatory ability, liver function in $Fah^{-/-}$ mice returns to normal when the percentage of functional human hepatocytes in the chimeric liver exceeds 20% [9]. In the present study, we also found that chimeric mice with high levels of liver repopulation gradually gained weight by four weeks after human hepatocyte transplantation, indicating recovery of liver function in these mice (Figure 5A).

Metabolic parameters were used to analyze the recovery of metabolic function in the human hepatocytes. Expression of hAlb in the serum of the recipients indicated that the synthetic function of the human hepatocytes was intact after liver repopulation; serum hAlb levels of up to 3.12 mg mL^{-1} were measured (Figure 5B). As summarized in Table 1, analysis of recipient serum indicated the recovery of metabolic function after liver xeno-repopulation. These results show that human hepatocytes can proliferate in the chimeric liver have normal function.

3 Discussion

Functional human hepatocytes xenografted into the livers of mice can be used as a model system to study pharmacokinetics, hepatitis infection and the efficacy of hepatitis vaccines [1–9]. There are many studies of humanized mice based on *uPA* transgenic [1–6] and *Fah* knockout mouse models [7–9] and levels of liver xeno-repopulation range from 10% to 90%. However, *uPA* mice have several disadvantages [1,2,16,17]: (i) high rates of neonatal death during

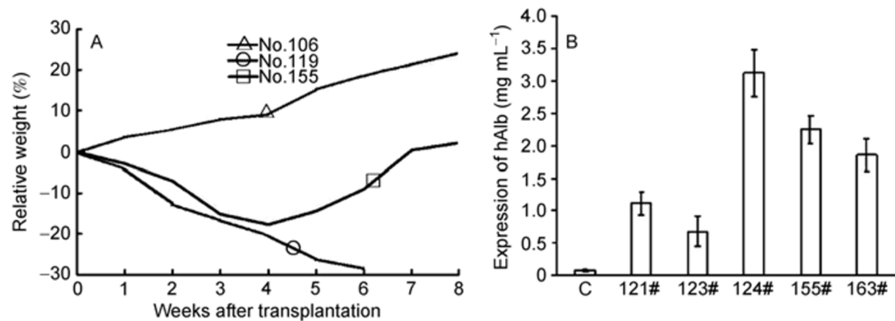

Figure 5 Weight curve and detection of human serum albumin. A, Weight curve. The weight of No. 155 (chimeric mice) stopped declining, but gradually increased four weeks after human hepatocyte transplantation. The weight of No. 106 ($Fah^{-/-}$*Nod*/*Scid* mice on NTBC) increased steadily, while the weight of No. 119 ($Fah^{-/-}$*Nod*/*Scid* mice, off NTBC and without human hepatocytes transplantation) declined gradually and the mice died after six weeks. B, Detection of serum albumin in chimeric mice secreted by human hepatocytes.

Table 1 Biochemical analysis of metabolic function after human hepatocyte transplantation

Mouse	ALT (U L^{-1})	AST (U L^{-1})	Total bilirubin (μmol L^{-1})	Fah activity (μmol min^{-1} g^{-1})	Tyrosine (μmol L^{-1})
129S4 wt	18.5±3.3	88.1±19.9	0.5±0.4	72.5±10.2	81.5±28.6
$Fah^{-/-}$*Nod*/*Scid*[a)]	267.6±78.5	735.5±412.4	62.8±23.7	0.4±0.3	812.4±156.8
121#	86.7	187.4	18.5	20.8	222.7
123#	112.5	179.6	26.7	15.6	258.5
124#	54.6	125.5	3.9	40.2	162.3
155#	68.2	154.6	4.8	31.5	185.6
163#	70.8	165.7	10.6	25.3	194.8

a) Bodyweight fell by over 20% after withdrawal of NTBC.

colony breeding; (ii) transplantation of hepatocytes into newborn (within the 2nd week of life) is technically difficult due to a bleeding disorder in these mice; (iii) there is uncontrollable selection for donor cells; (iv) there is auto-reversion of endogenous hepatocytes; and (v) kidney damage is induced by human complement. These deficiencies make it difficult to establish a stable and reliable animal model. Recently, robust liver xeno-repopulation from human hepatocytes was seen in $Fah^{-/-}Rag2^{-/-}Il2rg^{-/-}$ mice [7–9]. However, maintenance of $Fah^{-/-}Rag2^{-/-}Il2rg^{-/-}$ mice during colony breeding, growing and cell transplantation is difficult, with high mortality rates seen in our previous experiments [9]. Also, the genotyping of the offspring is overly elaborate. Overall, there are still problems with the large-scale application of $Fah^{-/-}Rag2^{-/-}Il2rg^{-/-}$ mice.

Azuma *et al.* [7] reported that human hepatocytes could be engrafted into $Fah^{-/-}$*Nod*/*Scid* mice without any significant proliferation, which hinted at the fact that $Fah^{-/-}$*Nod*/*Scid* mice may be a possible model for humanized mice. In the present study, we combined the advantages of liver repopulation seen in $Fah^{-/-}$ mice with the ease of xenotransplantation seen in *Nod/Scid* mice to produce $Fah^{-/-}$*Nod*/*Scid* mice by a gradual process of cross-breeding. Analysis of immune cells showed that the $Fah^{-/-}$*Nod*/*Scid* mice lacked T cells, B cells and NK cells, signifying complete immune deficiency. The extent of this immunodeficiency was the same as that seen in $Fah^{-/-}Rag2^{-/-}Il2rg^{-/-}$ mice. The $Fah^{-/-}$*Nod*/*Scid* homozygous mice also grew healthily and reproduced normally. These advantages ensured a large number of recipient mice for use as a chimeric liver model.

To promote engraftment and proliferation of the transplanted human hepatocytes, pretreatment of the recipient mice is required. For example, an adenoviral vector carrying uPA is required for liver xeno-repopulation in $Fah^{-/-}Rag2^{-/-}Il2rg^{-/-}$ mice. This induces liver damage and promotes the proliferation of transplanted hepatocytes [7]. Our previous study established a simple method of inducing liver injury in $Fah^{-/-}$*Nod*/*Scid* mice without significantly increasing mortality [9]. Seven days before transplantation, the NTBC in the drinking water of $Fah^{-/-}$*Nod*/*Scid* was reduced to 4.0 mg L^{-1} (from 7.5 μg mL^{-1}) for 3 d, and further reduced to 2.0 mg L^{-1} for 2 d. NTBC was then withdrawn 2 d before cell transplantation. This treatment induced liver injury in $Fah^{-/-}$*Nod*/*Scid* mice before transplantation and promoted engraftment and proliferation of the transplanted human hepatocytes. Without this treatment (and, instead, abruptly removing NTBC after cell transplantation) single human hepatocytes only occasionally engrafted into the livers of $Fah^{-/-}$*Nod*/*Scid* mice (data not shown).

Gradual removal of NTBC prior to transplantation induced the engraftment and proliferation of human hepatocytes, but the extent of liver xeno-repopulation was still

very low. The use of FK506 played an important role by improving the efficiency of liver xeno-repopulation in *Fah$^{-/-}$Nod/Scid* mice. In addition to immunosuppression, FK506 modulates liver responses by increasing the expression of local mitogens, such as insulin-like growth factor-I, and insulin receptors while, at the same time, decreasing the production of inhibitory cytokines such as interleukin 2, to promote liver regeneration [18–20]. FK506 reduces macrophage recruitment and attenuates leukocyte accumulation, neutrophil infiltration and the activation of resident immunocompetent hepatic NK cells [21,22]. In the present study, *Fah$^{-/-}$Nod/Scid* mice showed significantly enhanced levels of liver repopulation after treatment with FK506. Fah immunoassays showed that the level of engrafted Fah-positive hepatocytes reached 33.6%. The function of expanded human hepatocytes was confirmed using weight curve analysis, the expression of characteristic proteins and by biochemical analysis of liver function. The results showed that the repopulated hepatocytes displayed the normal functions of liver cells. These results show, for the first time, that *Fah$^{-/-}$Nod/Scid* mice are an ideal humanized liver model with many applications. Our new method also simplifies the protocols for generating *Fah$^{-/-}$* mice for use as a humanized liver model.

In practice, we could have chosen chimeric mice with complete liver xeno-repopulation according to the weight curves and Fah/albumin staining after partial hepatectomy. Mice showing >20% chimerism could then be selected for further study. Studies show that human hepatocytes can participate in the composition of the liver plate in immunodeficient *Fah$^{-/-}$* mice, and are functional for a lifetime [7–9]. In our previous study, chimeric mice were inoculated with HBV virus. The virus titer increased from 10^3 IU mL^{-1} at eight weeks to 10^6 IU mL^{-1} at 14 weeks, confirming that the human hepatocytes proliferated in these mice and had normal function [9].

Azuma *et al.* [7] reported that liver xeno-repopulation in *Fah$^{-/-}$Rag2$^{-/-}$Il2rg$^{-/-}$* mice reached up to 90%. In our previous study, human hepatocytes achieved up to 67.2% repopulation in *Fah$^{-/-}$Rag2$^{-/-}$* mice after pharmacological immunosuppression [9]. Tateno *et al.* [15] reported that chimeric mice died of renal atrophy and necrosis when the level of liver repopulation reached more than 50% because complement produced by the human hepatocytes caused damage to the *uPA/SCID* mouse kidneys via deposition of hC3 (human C3) and hMAC (human membrane attack complex) in the renal basilar membrane. This problem was solved by inhibiting the complement reaction using the proteinase inhibitor, nafamostat mesilate (Futhan). After Futhan treatment, liver repopulation by human hepatocytes reached more than 90% and the chimeric mice were healthy. Bissig *et al.* [8] used Futhan pretreatment along with intravenous injection of liposomes containing clodronate prior to human hepatocyte transplantation. The latter inhibited the function of the Kupffer cells in the liver and further reduced the immune or other factors which could affect repopulation by human hepatocytes and increase the levels of liver repopulation. According to the analysis of immune cells, the extent of immunodeficiency in the *Fah$^{-/-}$Nod/Scid* mice was equal to that in *Fah$^{-/-}$Rag2$^{-/-}$Il2rg$^{-/-}$* mice. However, the level of liver repopulation by human hepatocytes in the *Fah$^{-/-}$Nod/Scid* mice was lower than that in *Fah$^{-/-}$Rag2$^{-/-}$Il2rg$^{-/-}$* mice. This might be related to differences in the complement response and Kupffer cell function between the two strains of mice. Future studies will involve the analysis and comparison of these differences, leading to the optimization of the experiment protocols to improve the level of liver repopulation in *Fah$^{-/-}$Nod/Scid* mice.

Humanized liver mice are an excellent model for studying liver cell biology and for basic and clinical research into liver disease. They can be used to study the mechanisms underlying liver regeneration after injury and as a platform for evaluating drug metabolism. Humanized mice overcome the shortcomings of cultured cells *in vitro*, such as poor stability, short life span, and the lack of an *in vivo* environment. They can also be used as a new model for the prevention and cure of viral hepatitis. Humanized liver mice could mimic the biological behavior of the human body after infection by hepatotropic viruses, such as HBV and HCV. They are already used as model systems to study the infection and pathogenesis of hepatitis viruses, antiviral therapy, and vaccine efficacy. Finally, they could be used as a research model for other hepatotropic pathogens, such as malaria. Therefore, this model is an important innovation with many useful applications.

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